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SYNTHESIS OF SITE-SPECIFIC OLIGONUCLEOTIDE-POLYAMINE CONJUGATES†

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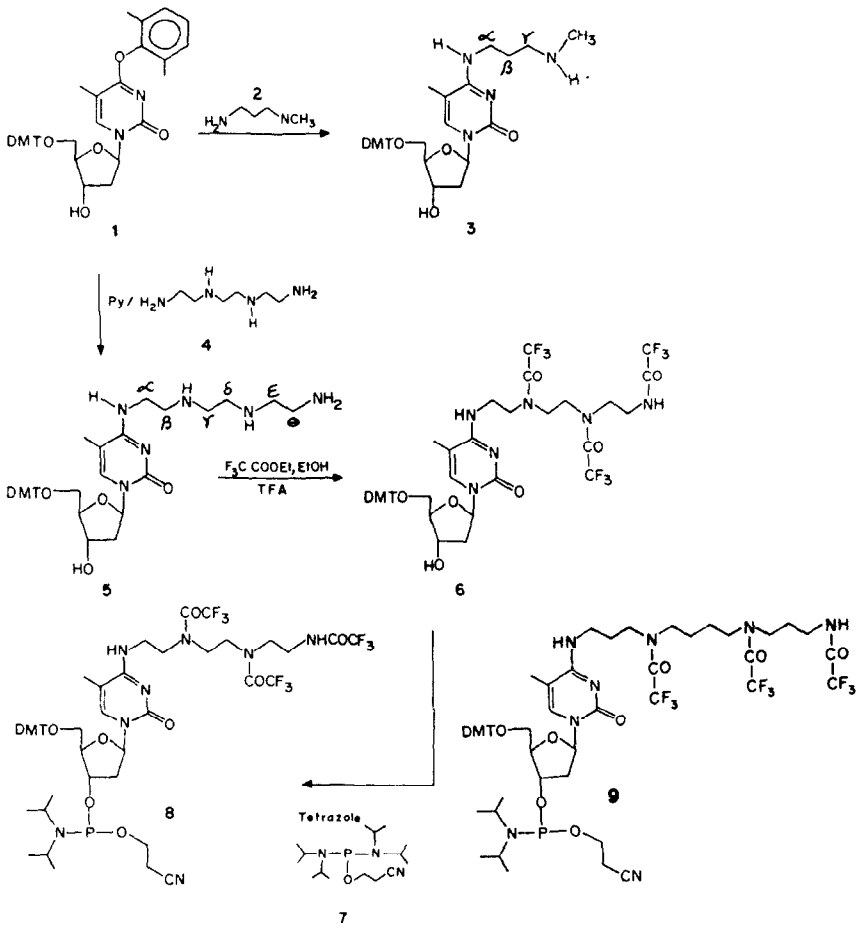
Abstract: Synthesis of 5-Me-dC-amidite regiospecifically tethered at C4, with triethylenetetramine (**8**) and spermine (**9**) and their site specific incorporation into DNA to obtain oligonucleotide-polyamine conjugates (**10-15**) is reported.

Polyamines and their bio-conjugates have emerged as wide ranging biological effector molecules with bright prospects for medicinal developments.[1] The naturally occurring polyamines like spermine and spermidine are largely protonated at physiological pH and exhibit net positive charge close to +4 and +3 respectively. Their inherent positive charge and chain conformational mobility allow electrostatic interaction with anionic phosphate groups of polynucleotides and induce specific structural changes in DNA.[2] Covalent attachment of natural/un-natural polycationic polyamines to oligonucleotides may prove to be useful for a systematic study of such induced effects. Photoaffinity analogues of spermine have been used to study the binding sites on naked double stranded DNA[3], which suggested a role for polyamines in regulating the structure of chromatin *in vivo*. Additionally, the net diminished charge on polyamine linked oligonucleotides may enhance the nuclease resistance as well as membrane permeability, effectively making them useful antisense drugs that are currently of great importance.[4] Continuing our interest on amine tethered oligonucleotides,[5] we report in this communication a strategy for covalent attachment of polyamines at N^4 of 5-MedC and site-specific incorporation of these base modified nucleosides into oligonucleotides on solid support using the well established phosphoramidite chemistry.[6]

For site-specific conjugation of polyamines to oligonucleotides, the convertible nucleoside approach[7] was used with the difference that the modification was effected before the oligonucleotide assembly. A convertible nucleoside such as 5'-O-DMT-4-O-(2,4-dimethyl phenyl)-2'-deoxythymidine upon treatment with an amine (RNH_2) is known to undergo nucleophilic substitution to yield N^4-alkyl-5-methyl-dC-nucleoside.[7] This reaction proceeds smoothly with primary amines but with polyamines (e.g. $H_2N(CH_2)_nNH(CH_2)_nNH(CH_2)_nNH_2$), we envisioned problems due to side reactions, particularly due to the higher nucleophilicity of secondary amines. This would lead to a mixture of oligonucleotides tethered by polyamines either at primary or secondary nitrogens, which

SCHEME 1



10, d(CGC*GAATTCGCG)

11, d(CAC*TGCTAAGCT

12, d(CACTGC*TAAGCT)

13, d(TCTTTTTTCTTTTTTC*TT)

14, d(TC*TTTTTTCTTTTTTC*TT)

15, d(TC*TTTTTTC*TTTTTC*TT)

16, d(AGAAAAAAGAAAAAAGAA)

17, d(TTCTTTTTTCTTTTTTCT)

C* = 5-methyl-4-N-(triethylenetriamine)-2'-deoxycytidine.

C# = 5-methyl-4-N-(spermine)-2'-deoxycytidine.

would be intractable for analysis. Modification at nucleoside level prior to assembly gives an opportunity for unambiguous characterization of the regiospecific presence of the tether in modified nucleobase.

To establish the reactivity of C4 in **1** towards primary and secondary amine nucleophiles present simultaneously in the same molecule, compound **1** was reacted in pyridine (16 h, 60 °C) with excess (15 eq.) of N-methyl-1,3-diaminopropane **2**. The reaction gave a single amine tethered nucleoside **3** (yield, 90%) whose structure was unambiguously established by ^{13}C NMR[8] which gave a peak at 33.3 ppm[9] assigned for aliphatic N-CH_3 (confirmed by INEPT experiment) rather than that due to NCH_3 linked to nucleobase with an expected chemical shift around 48 ppm. ^1H NMR supported this structure in which N-CH_3 appeared as a peak at 2.65 ppm as against 2.9-3.0 ppm expected for dC-N^4-CH_3. The relative reactivity of primary and secondary amines towards hindered electrophiles is governed by their nucleophilicity as well as steric effects. The formation of a single product **3** in this case indicates the dominance of steric reasons over higher nucleophilicity of secondary amines.

The reaction of triethylene tetramine **4** with **1** yielded exclusively the 5-methyl-dC derivative **5** (yield, 85%) in which the polyamine is linked through the ω-primary amino function. This is evident from the ^{1}HNMR of **5** which showed a characteristic peak at 3.6 ppm, integrating to 2H and ascribed to dC-NH^4-CH_2-. This is similar to that seen for N^4-CH_2- in **3**. A reaction at secondary amine would have generated two sets of such protons (dC-N4$<^{CH2}_{CH2}$). These results were corroborated by ^{13}C NMR signals seen for N^4-CH_2 in two compounds (**3**, 53.7 and **5**, 56.1). The primary and secondary amino functions of **5** were protected by trifluoroacetyl group to yield **6**. The TFA group has been previously shown to be suitable for protecting aliphatic amino groups in oligonucleotide synthesis by phosphoramidite chemistry.[5b,c] The structure of **6** was supported by FAB MS (984, M^++Na^+) which indicated the presence of 3 trifluoroacetyl groups. The TFA protected polyamine-nucleoside conjugate **6** was converted into the corresponding β-cyanoethyl phosphoramidite **8** (Scheme 1) by using standard methods.[6] No N-phosphonylation was observed during this reaction, as seen by ^{31}P NMR, which contained only 2 signals at 149.5 and 149.1 ppm characteristic of O-amidite. Starting from **1** and spermine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$] following a similar sequence of reactions, the spermine conjugated phosphoramidite **9** was synthesised.

The amidite monomers **8** and **9** were individually incorporated into various oligonucleotide sequences (**10-15**) at specific positions on an automated DNA synthesiser (Pharmacia, GA Plus). The coupling efficiency of polyamine tethered amidites **8** and **9** were similar to commercial phosphoramidites of normal nucleosides. After completion of synthesis, final on-column detritylation was followed by aqueous NH_3 treatment (60°C, 18h) to yield the fully deprotected oligonucleotides **10-15**. These were desalted and purity checked by reversed phase HPLC[10] (Figure 1).

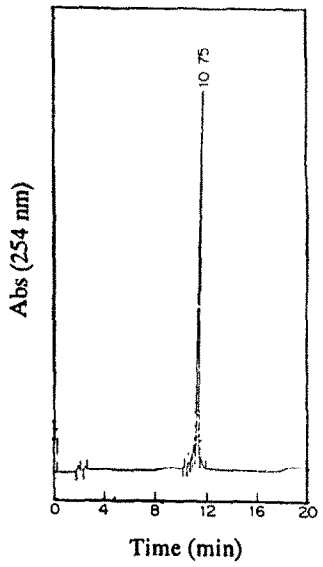


Figure 1: Reversed phase HPLC of **12**. For conditions see ref. 10.

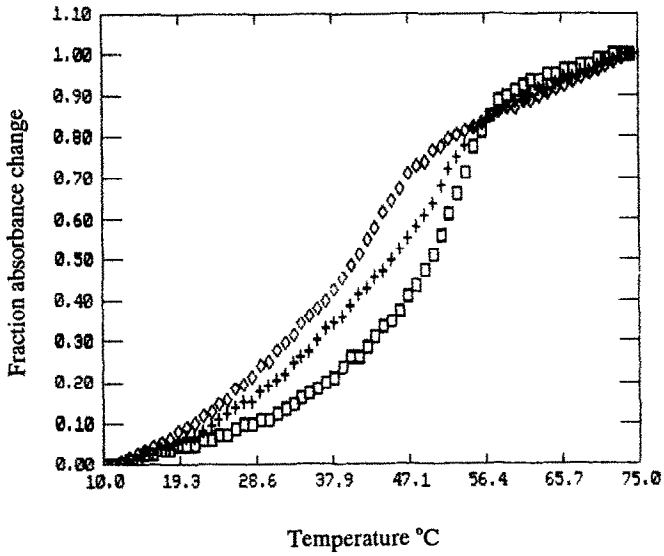


Figure 2: Melting curves for duplexes **16:17** (□ , T_m 51°C), **16:13** (+, T_m 46°C), 16:14 (◊, T_m 42°C); in 25 mM Tris, 100 mM NaCl, 20 mM $MgCl_2$, pH 7.0.

To ensure that polyamine conjugated nucleobases have survived the synthetic chemistry of oligonucleotide assembly by phosphoramidite approach and the subsequent step of ammoniacal deprotection, enzymatic hydrolysis of **13-15** were done using snake venom phosphodiesterase and alkaline phosphatase.[11] RP-HPLC analysis of the hydrolysate indicated the presence of the modified nucleoside (C*) in addition to the normal deoxynucleosides. In order to check for their ability to bind to DNA, the spermine conjugated oligonucleotides (**13- 15**) were individually hybridised with the complementary strand **16** and T_m of the duplexes were determined[12] (Figure 2). It is seen that the polyamine oligonuclotide conjugates form duplexes, with slightly lower T_m compared to the unmodified duplex (**16:17**).

In summary, this communication reports a strategy for synthesis of oligonucleotides with site-specific conjugation of polyamines, including spermine. Further studies are in progress to assess the sequential effect of modifications on DNA duplex and triplex stabilities. Since polyamines can also form metal complexes, capable of cleaving DNA[13], the compounds such as those synthesised in this communication may have utility in site directed cleavage of DNA.

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8. **General procedure (3 and 5)**: **1** (0.33 g, 0.5 mmol) in dry pyridine (1.5 ml was treated with either N-methyl-1,3-diaminopropane (0.5 ml) or triethylenetetramine (4 ml) at 60 °C for 10-12 hr. The reaction mixture was concentrated and usual work-up gave products (**3** or **5**) which was purified by column chromatography over silica gel.

Selected data:

Compound 3: TLC (silica gel) R_f = 0.4 (15 % MeOH-CH_2Cl_2). **^{1}H NMR** ($CDCl_3$) δ7.6 (s, 1H, H6), 6.35 (t, J = 6.3Hz, 1H, H1'), 4.5 (brs, 1H, H3'), 4.1 (brs, 1H, H4'), 3.75 (s, 6H, 2xOCH_3), 3.6 (m, 2H, αH), 3 (m, 2H, γH), 3.38 (m, 2H,H5' and H5''), 2.65 (s, 3H, N-$C\underline{H}_3$), 2.45 (m, 1H, H2'), 2.13 (m, 3H, βH, H2''), 1.53 (s, 3H, 5-CH_3), **^{13}C NMR** ($CDCl_3$), 163.7 (C4), 156.4 (C2), 137 (C6), 103.7 (C5), 86, 85.6 (C1', C4'), 71.9 (C3'), 63.7 (C5'), 41.7 (C2'), 53.6 (Cα), 37.6 (Cβ), 46.7 (Cγ), 33.4 (N-$\underline{C}H_3$), 12.8 (5-$\underline{C}H_3$).

Compound 5: λ = 276 nm (MeOH, ε, 11 x 10^4), M. P. = 114 °C, **^{1}H NMR** ($CDCl_3$), δ 7.65 (s, 1H, H6), 6.46 (t, J = 6.98 Hz, 1H, H1'), 4.55 (brs, 1H, H3'), 4.1 (brs, 1H, H4'), 3.8 (s, 6H, 2 X OCH3), 3.6 (brs, 2H, α H), 3.45 (m, 1H, H5'), 3.3 (m, 1H, H5"), 2.05-2.95 (m, 12H,α, β, γ, δ, θ H, H2' and H2"), 1.5 (s,

3H, 5-CH3), **^{13}C NMR** ($CDCl_3$) δ 162.9 (C4), 156.1 (C2), 136.6 (C6), 102.1 (C5), 85.7 (C1'), 85.4 (C4'), 71 (C3'), 63.4 (C5'), 39.3 (C2'), 12.2 (5-CH_3), 56.1, 51.6, 48.4, 47.8, 41.5, 41.1 (Cα), Cβ, Cγ, Cδ, Cε, Cθ FAB MS 696 (M^++Na^+)

Compound 6: R_f ($MeOH:CH_2Cl_2$ 1:9) = 0.52, λ = 276.5 nm (MeOH, ε, 11 x 10^4), **^{1}H NMR** ($CDCl_3$) δ 7.75 (s, 1H, H6), 6.45 (t, J = 6.5 Hz, 1H, H1'), 5.55 (brs, 1H, NH), 4.35 (brs, 1H, H3'), 4.1 (brs, 1H, H4'), 3.8 (s, 6H , 2 X OCH_3), 3.6 (brs, 2H, α)H, 3.15-3.7 (m, 12H, α), β, γ, δ, ε, θ H, H5' & H5"), 2.8 (m, 2H,θH), 2.43 (m, 1H, H2'), 2.2 (m, 1H, H2"), 1.45 (s, 3H, 5-CH_3), FAB MS 984 (M^++Na^+)

Compound 8: Rf ($EtOAc:CH_2Cl_2$, 1:1, 0.5% TEA) = 0.32 (2 spots, distereoisomers), **1H NMR** ($CDCl_3$) δ 7.45 (s, 1H, H6), 6.45 (m, 1H, H1'), 5.10 (brs, 1H, NH), 4.2 (m, 4H, H3', H4', $POCH_2$), 3.80 (s, 6H, 2xOCH_3), 3.3-3.73 (m, 12H, H5',H5", α, β, γ, δ, ε, θ H), 2.75 (m, 4H, 2xN<u>CH</u>Me_2, CH_2CN), 2.43 (m, 1H, H2"), 2.2 (m, 1H, H2'), 1.40 (s, 3H, 5-CH_3), 1.25 (m, 12H, NCH(<u>CH_3</u>)$_2$)

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10. Column: Novapak C18, <u>Buffer A</u>: 5 % CH_3CN in 0.1 M triethyl ammonium acetate, Buffer B: 30 % CH_3CN in 0.1 M triethylammonium acetate, <u>Gradient:</u> A to B 20 min., Flow rate 2 ml/min.

11. Oligonucleotides **13-15**, (0.5 OD_{254} Units) were dissolved in 10 mM KH_2PO_4 (100 μl, pH 7), containing $MgCl_2$ (10 mM) and treated with Snake Venom phosphodiesterase (10 μl, 1 mg/0.5 ml) and alkaline phosphatase (10 μl, 1 unit/μl) at 37°C for 12 hr. This hydrolysate (2 μl) was analysed on analytical C18 RP-column and eluted with 0.1 M triethyl ammonium acetate, pH 6.5, 1ml/min. The peaks were detected using photodiode array detector. Standard nucleosides: dC (1.2 min), dG (2.3 min), dT (2.7 min) and dA (4.4 min). Enzymatic hydrolysate: dC (1.2 min, λ_{max} 271 nM), dC* (1.4 min, λ_{max} 280 nM) and dT (2.7 min, λ_{max} 269 nM). The enzymic hydrolysate of **15** which has only dC* showed absence of dC and presence of dC* and dT.

12. DNA melting experiments were done in Tris buffer (25 mM, pH 7.0) containing NaCl (100 mM) and $MgCl_2$ (20 mM). Appropriate complementary strands (0.5 μM each based on UV absorbance calculated using molar extinction coefficients at 260 nM, dA = 15.4, dC = 7.3, dG = 11.7, T = 8.8 $cm^2/\mu mol$) were mixed and the resulting duplexes were heated at 80°C for 5 min and annealed to room temperature. The melting experiments were carried out by heating the samples from 10°C to 75°C at the rate of 0.5°C/min; dry nitrogen gas was flushed in the spectrophotometer chamber to prevent moisture condensation.

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