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Preparation and Properties of Oligodeoxynucleotides containing 4-O-Butylthymine, 2-Fluorohypoxanthine and 5-Azacytosine[1]

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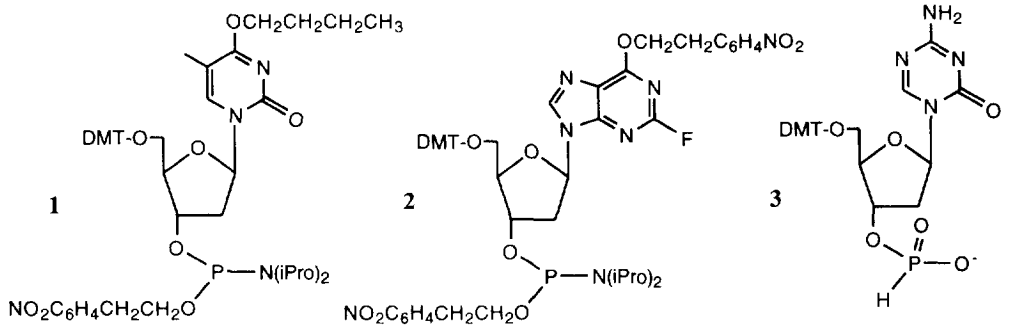
Abstract : Oligonucleotides carrying the ammonia sensitive bases 4-O-butylthymine, 2-fluorohypoxanthine and 5-azacytosine have been prepared for the first time using a special protocol that avoids the use of nucleophiles during the final deprotection.

The search for mild deprotection methods in oligonucleotide synthesis has led to the development of several protective groups for the nucleobases such as phenoxyacetyl (Pac),[2] *tert*-butylphenoxyacetyl (tBPA, Expedite),[3] formamidine (FOD),[4] 2-(acetoxymethyl)benzoyl (Amb),[5] allyloxycarbonyl,[6] fluorenyloxycarbonyl (Fmoc)[7] and *p*-nitrophenylethyloxycarbonyl (Npeoc).[8] Alternatively, a recent method for the preparation of oligonucleotides without using nucleophiles during the final deprotection has been described.[9] This method is based on the use of the (*p*-nitrophenyl)ethyl (Npe and Npeoc) protecting groups developped by Pfleiderer *et al*.[8] These groups are removed under non-hydrolytic conditions using strong non-nucleophilic bases (usually 1,8-diazabicyclo[5.4.0]undec-7-ene, DBU) in aprotic solvents, thus allowing the possibility of introducing ammonia-sensitive molecules into synthetic oligonucleotides. In the present communication we describe the preparation and properties of oligonucleotides carrying three non-natural bases of biological importance that are inherently sensitive to ammonia. Until now, their instability towards ammonia has prevented their successful use in oligonucleotide synthesis.

The formation of O^4-alkylthymidines in DNA resulting from the action of alkylating agents on the biopolymer has been associated with the mutagenic events attributed to the alkylating agents.[10] O^4-Alkylthymines are typical among the unstable bases which under standard conditions used in DNA synthesis would react with ammonia during the deprotection step giving 5-methylcytosine.[11a] This problem has been solved for some O^4-alkylthymidines such as O^4-methyl, O^4-ethyl, O^4-propyl, O^4-isopropyl and O^4-benzyl, by using more labile phosphoramidites which will be deprotected after the synthesis of the oligomer with the appropriate alcohol in the presence of DBU to give the desired O^4-alkylthymidine oligonucleotides.[11] However, when the size of the alkyl group increases, the corresponding alkoxide is less reactive and deprotection is slower. Specifically in the case of O^4-*n*-butylthymidine, we could not obtain the desired O^4-*n*-butylthymidine-modified oligonucleotide after using a DBU solution in butanol (data not shown). As a way to

circumvent this problem, the 4-nitrophenylethyl (Npe) phosphoramidite of 5-DMT-O4-*n*-butylthymidine (compound **1**) was prepared using previously described protocols.[9a, 11d] Oligonucleotide sequences A : 5' AGCT^{bu}TA 3' and B: 5' CTACAT^{bu}CTGGA 3' were assembled on an automatic DNA synthesizer (1 µmol scale) using the appropriate Npe, Npeoc-protected nucleoside Npe-phosphoramidites[9a] and the solid supports with the *o*-nitrophenylethyl linkage.[9b] Oligonucleotides were deprotected from their solid supports using a 0.5 M DBU solution in dry pyridine containing 5 mg of thymine as a scavenger for the olefins generated during deprotection.[9a] After Sephadex G-10 chromatography, the desired oligonucleotides were isolated by HPLC using the standard protocols for oligonucleotides with the DMT group on.[9a] Oligonucleotides A and B were obtained in 13 and 10% overall yield (including synthesis and purification) and the presence of the intact T^{bu} was confirmed by HPLC analysis after enzyme digestion[9b].



The base-pairing properties of T^{bu} were assessed by melting experiments on duplexes containing T^{bu}•G and T^{bu}•A base pairs. As seen in table 1, the presence of T^{bu} produced an important drop in the melting temperature with the T^{bu}•G base pair showing increased stability over T^{bu}•A. This behaviour is in agreement with earlier observations with other O-4-alkylthymidines[11] and with the observed trend of melting temperatures decreasing as a function of the size of the alkyl group.

Table 1 : Melting temperatures of O4-*n*-butylthymine base pairs. Duplexes formed by mixing 5'CTACAXCTTGA 3' and 3' GATGTYGAACT 5' in 0.1 M NaCl, 50 mM Tris.HCl pH 8.0. Duplex concentration was 4 µM.

X : T^{bu}, Y : A	Tm 18.6 °C	X : T, Y : A	Tm 45.5 °C
X : T^{bu}, Y : G	Tm 22.5 °C	X : T, Y : G	Tm 33.4 °C

2-Fluoro-2'-deoxyinosine and its protected derivatives have been used as a source of 2-aminosubstituted guanine derivatives through nucleophilic displacement with the appropriate amine.[12] Usually, this displacement reaction is performed at the nucleoside level, but has also been described in oligonucleotides.[12b,f,g] For that purpose, the 5'-O-DMT-O6-Npe-2-fluoro-2'-deoxyinosine 3'-O-(2-cyanoethyl) phosphoramidite has been prepared and the displacement reaction performed during or before ammonia deprotection[12b,f,g]. However, the incorporation of the intact base is precluded due to its sensitivity to ammonia when the standard protecting groups are used.[12g] Moreover, the preparation of poly(2-

fluoroinosinic) acid using an enzymatic polymerization reaction gave very poor yields owing to the anomalous low pKa of 2-fluoroinosine (pKa 4.1-4.6).[13]

To overcome this deficiency, the preparation of oligonucleotides containing 2-fluoro-2'-deoxyinosine was undertaken with the preparation of the Npe phosphoramidite of 2-fluoro-2'-deoxyinosine (compound **2**) synthesized using a previously described protocol.[9a, 12g] Oligonucleotide sequences C: 5' AAI^{F}CAC 3' D: 5' ATI^{F}ATA 3' and E: 5'TTACAI^{F}CTGGA 3' were prepared on an automatic DNA synthesizer (1 μmol scale) using the Npe, Npeoc-protected Npe phosphoramidites and the corresponding solid supports.[9] Oligonucleotides were deprotected by treatment with a 0.5 M DBU solution in pyridine (1.5 ml) containing thymine (5 mg) as described.[9b] The oligonucleotide products were obtained in 13% (C), 9% (D and E) after HLPC purification, and were further characterized by enzyme digestion and mass spectrometry.[14]

The base pairing properties of 2-fluorohypoxanthine with the natural bases were determined at two different pH (8.0 and 4.9). From table 2, it can be seen that at pH 8.0 melting temperatures of the duplexes containing this modified base are very low compared with base pairs of duplexes containing G. This indicates that 2-fluorohypoxanthine is not forming a strong base pair with the natural bases due to the presence of the negative charge formed in the same manner as with xanthine base pairs.[15] At pH 4.9, near the pKa of 2-fluorohypoxanthine, the I^F•A base pair becomes more stable as well as the I^F•G base pair, but still the melting temperatures are low compared to duplexes containing G.

Table 2. Melting temperatures of duplexes containing 2-fluorohypoxanthine base pairs. Duplexes were prepared by mixing 5' TTACAXCTGGA 3' and 3' AATGTYGACCT 5' in A) 0.15 M NaCl, 50 mM tris.HCl pH 8.0 and B) 0.15 M NaCl, 50 mM sodium phosphate pH 4.9. Duplex concentration was 4 μM.

pH = 8.0				pH = 4.9			
X : Y	Tm(°C)	X : Y	Tm(°C)	X : Y	Tm(°C)	X : Y	Tm (°C)
I^F:A	22.9	G : A	30.4	I^F: A	34.4	G : A	30.6
I^F:C	23.3	G : C	45.3	I^F:C	24.6	G : C	45.8
I^F:G	16.3	G : G	34.1	I^F:G	21.7	G : G	32.5
I^F:T	15.1	G : T	30.8	I^F:T	17.1	G : T	31.3

Finally, as an additional test to this methodology, the preparation of oligonucleotides containing 5-aza-2'-deoxycytidine was attempted. Oligonucleotides containing this modified base at specific sites are expected to serve as probes to define more clearly the role of this base on the inhibition of DNA methyltransferases.[16] The hydrolytic instability of the triazine ring in 5-azacytosine nucleosides is well documented[17] and such an instability precludes its utilization with the standard phosphoramidite protocol, particularly during the treatment with ammonia. The preparation of oligonucleotides containing the reduced base 5,6-dehydro-5-azacytosine has been described,[18] but conversion of the reduced base to the desired 5-azacytosine was not efficient enough to yield the desired oligonucleotides in good yields.[18] In order to overcome this deficiency, the use of the Npe protection was attempted.

5-Aza-2'-deoxycytidine was synthesized as described[19]. Protection of the 5'-hydroxy with DMT was performed according to the standard protocol with DMT-Cl in dry pyridine. Cognizant of the low

reactivity of the exocyclic amino group towards acylating reagents[18] protection at this position was not deemed necessary. In consideration to the special lability of the 5-azacytosine ring to base, H-phosphonate chemistry was selected over phosphoramidite chemistry for the synthesis of oligonucleotides. This was based on our concern for the stability of this base under the oxidation conditions of the phosphite-triester with an iodine solution containing water and base, and which are repeated after each coupling. Although a similar solution is used in H-phosphonate chemistry, it consists of a single treatment at the end of the synthesis rather than after each nucleotide addition. The H-phosphonate derivative **3** was prepared using 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one[20a,b] in good yield.[21] Attempts to use the $POCl_3$/ 1,2,4-triazole method[20b,c] did not yield the desired H-phosphonate but, instead, produced a mixture of two products. These products were identified as the H-phosphonate diester and the phosphite-triester derivatives based on the chemical shifts determined by ^{31}P-NMR[22].

Oligonucleotide sequence F : 5' TTC^{Az}TA 3' was prepared on commercially available DMT-A^{bz}-succinyl-CPG (0.5 μmol) using the H-phosphonate derivatives of T and 5-aza-2'-deoxycytidine. Oxidation was performed using a 0.1 M iodine solution in tetrahydrofurane, N-methylmorpholine, water (18:1:1) for 2 min at room temperature. At the end of the synthesis, treatment of the solid support with a 0.5 M DBU solution in pyridine, followed by Sephadex G-10 chromatography and HPLC, produce the protected oligonucleotide DMT-TTC^{Az}TA^{bz} in 3% yield that was characterised by mass spectrometry (figure 1)[23] indicating that 5-azacytidine is not affected by the DBU deprotection conditions. The lower yield obtained in this case is probably due to the decrease efficiency of cleavage of the commercially available succinyl linkage with DBU.[24] Afterwards, oligonucleotide sequence G : 5' TTC^{Az}TC^{Az}TC^{Az}TA 3' was prepared using the same protocol described for oligonucleotide F except that this time DMT-A^{Npeoc}-(*o*-nitrophenyl)ethyl-CPG (0.5 μmol) was used. After standard HPLC purification (DMT on, 80% AcOH treatment, DMT off) the fully deprotected oligonucleotide was obtained in 10% overall (synthesis and purification) yield. Mass spectrometry confirmed the presence of the three intact molecules of 5-azacytidine in the fully deprotected oligonucleotide.[23] These results indicate that even the extremely labile 5-azacytosine ring endures the deprotection conditions used for the removal of the Npe groups. The biological properties of these and other oligonucleotides containing 5-aza-2'-deoxycytidine are in progress and it will be reported elsewhere.

In conclusion, we have shown that Npe protection, which allows the preparation of oligonucleotides under non-hydrolytic conditions, opens the possibility of accessing modified oligonucleotides containing important non-natural bases that are sensitive to ammonia. When this method is compared to the standard amide protection method, it is less efficient and, in addition, it requires the preparation of the Npe-protected monomers which are not commercially available. The lower yields are probably related to an incomplete release of the oligonucleotide from the solid support. Preliminary studies on the release of DMT-nucleotides linked to the solid support through a *o*-nitrophenylethyl linkage showed that they are quantitatively released from the support by a 15 minute treatment with a 0.5 M DBU solution.[25] Similar experiments have been performed with DMT-oligonucleotide supports showing that yields depends on the nucleotide sequence and in some instances only 50-60 % cleavage efficiencies could be obtained.[26] Despite these inconveniences, this method delivers modified oligonucleotides that cannot be obtained through standard protocols. Additional applications of our methodology can be envisaged for the synthesis of modified oligonucleotides containing

other base-sensitive components such as fatty acid esters[27] or alkylating moieties.[28]

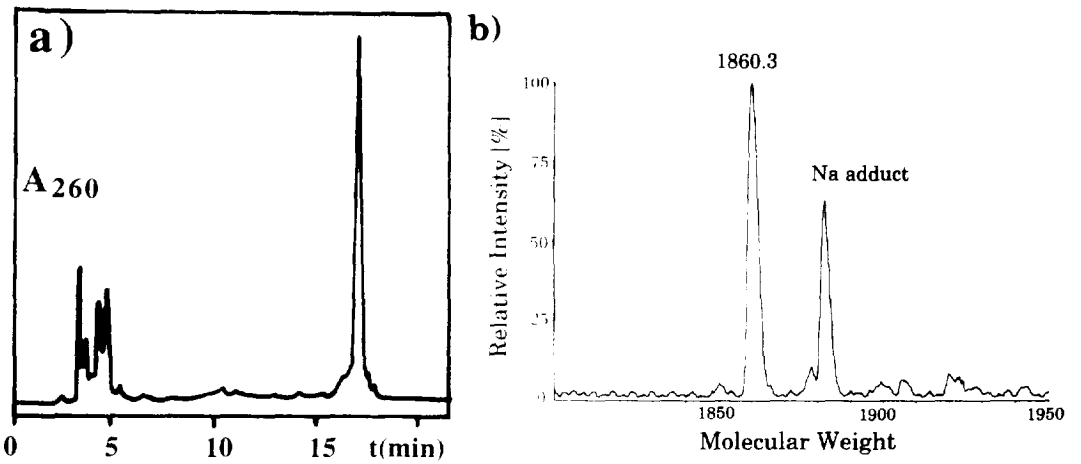


Figure 1 : a) Analytical HPLC of oligonucleotide sequence F (DMT-TTC^{Az}TTAbz) obtained after DBU treatment and Sephadex G-10 chromatography. b) Computer deconvolution of the peaks obtained from the electrospray mass spectrum of DMT-TTC^{Az}TTA^{bz} obtained from HPLC purification.

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References and Notes

1. Abbreviations used are as follows: bz : benzoyl; DBU : 1,8-diazabicyclo[5.4.0]undec-7-eno; DCM : dichloromethane; C^{Az} : 5-azacytosine; DMT: dimethoxytrityl; EtOH : ethanol; I^{F} : 2-fluoro-2'-deoxyinosine; Npe : *p*-nitrophenylethyl; Npeoc : *p*-nitrophenylethoxycarbonyl; T^{bu} : O^{4}-*n*-butylthymidine; Tris : tris(hydroxymethyl)aminoethane.

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14. Mass spectra (electrospray). Oligonucleotide C : M 1787.9, expected 1787. Oligonucleotide D : M 1817.4, expected 1818.

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21. Yield after silica gel purification : 51%. TLC (10% EtOH/ DCM) Rf: 0.05 . ^{31}P-NMR ($CHCl_3$, external reference, 101.27 Hz): -2.8 ppm ($J_{P\text{-}H}$ = 614 Hz).

22. ^{31}P-RMN ($CHCl_3$, external reference, 101.27 MHz) : Side product I (TLC 10% EtOH/ DCM, Rf =0.66) 136.04 ppm. Side product II (TLC 10% EtOH/ DCM Rf 0.83) 4.61 ppm ($J_{P\text{-}H}$ = 713 Hz).

23. Mass spectra (electrospray) : 5' DMT-TTC^{Az}TA^{bz} 3', M : 1860.2, expected 1860. Oligonucleotide G: 5'TTC^{Az}TC^{Az}TC^{Az}TA M : 2642.4, expected 2643.

24. See note 11 in reference 9b

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