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# A Convenient Synthesis of 5-Hydroxy-2'-Deoxycytidine Phosphoramidite and its Incorporation into Oligonucleotides

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***Abstract:*** Oligonucleotides containing 5-hydroxy-2'-deoxycytidine (**2**) have been synthesized using the phosphoramidite chemistry. The presence and the integrity of the modified nucleoside in the synthetic oligomers were confirmed by electrospray mass spectrometry and chromatoghraphic analysis of enzymatic digestion coupled with UV detection. © 1997 Elsevier Science Ltd.

5-Hydroxy-2'-deoxycytidine (5-OHdC) **2** is one of the major stable $^{\bullet}OH$ radicals and one-electron mediated oxidation products of 2'-deoxycytidine **1**. Different studies have shown that the level of **2** increases substantially when DNA is exposed to UV, ionizing radiation or oxidizing agents[1]. In order to study the structural and the biological role of 5-OHdC **2** in DNA, it is necessary to use oligonucleotides containing the oxidative lesion **2** at defined sites. Due to the relative instability of the modified nucleoside **2** and the necessity to protect the additional hydroxyl group in position 5 of the base, this lesion to our knowledge has not been yet incorporated in oligonucleotides by chemical approaches[2].

We report herein the first site-specific incorporation of **2** into oligonucleotides using the phosphoramidite chemistry. Prior to the preparation of the phosphoramidite synthon **8**, the stability of 5-OHdC **2** was checked at room temperature under the three main conditions used during solid support synthesis : 30% aqueous ammonia, 80% acetic acid and a commercial oxidizing solution of iodine[3]. No detectable degradation of **2** was observed after 24h of incubation. Furthermore, the $N^4,O^5$-diprotected nucleoside **6** was quantitatively converted into the parent compound **2** upon treatment with aqueous ammonia for 2h at room temperature. The kinetic and stability studies showed the compatibility of **2** with the "Pac phosphoramidite" chemistry[4].

The synthesis of the target phosphoramidite **8** was achieved in 6 steps as shown in Scheme 1. First, 5-OHdC **2** was prepared by bromination of 2'-deoxycytidine **1** in an aqueous medium followed by 2,4,6-collidine treatment as described in the literature[5]. Compound **2** was then converted into the $N^4,O^5$-diprotected intermediate **6** via the TIPDS ether **3**. This was accomplished by treating **2** with $TIPDSCl_2$ in DMF[6] in the presence of imidazole and $NEt_3$. The reaction gives the 3',5'-O-TIPDS derivative **3** and the 5,5'-O-TIPDS

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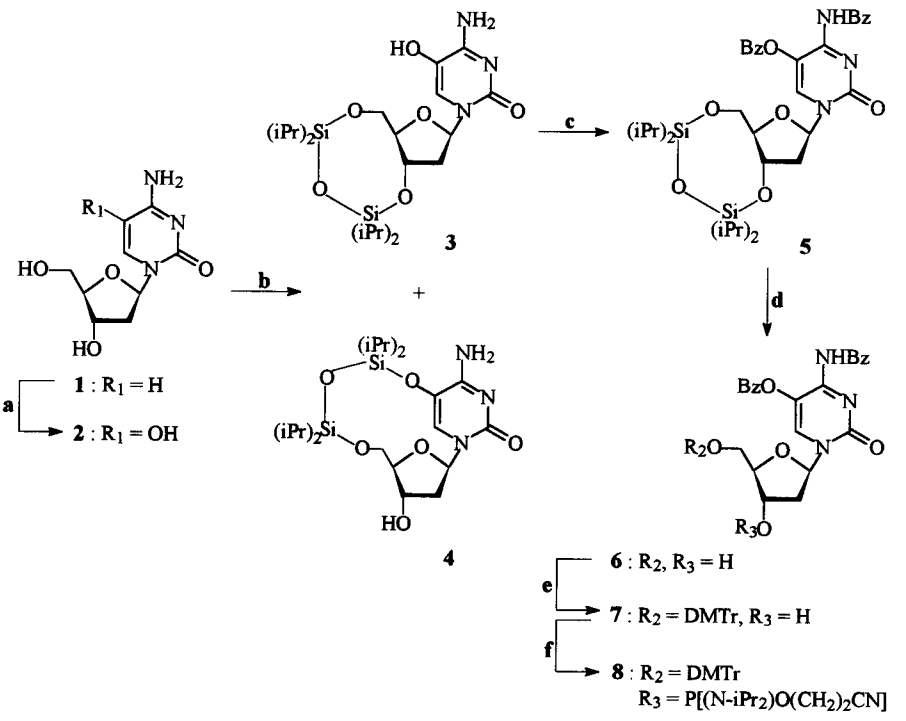
derivative **4** in 25% and 30% yields respectively[7]. Benzoylation of **3** in $CH_2Cl_2$ with pyridine provided the corresponding protected nucleoside **5**. The 3',5'-O-TIPDS group was selectively removed by treating **5** with $NEt_3.3HF$ in acetonitrile[8], yielding the 3',5'-dihydroxy derivative **6**. Standard dimethoxytritylation and phosphitylation of **6** gave phosphoramidite **8**.[9]

Scheme 1




Scheme 1 : a) $Br_2/H_2O$, 2,4,6-collidine (4.4 eq), 2h, 50% ; b) $TIPDSCl_2$ (1.1 eq), imidazole (4.4 eq), $NEt_3$ (2 eq), DMF, 1h, 25% (**3**), 30% (**4**) ; c) Benzoyl chloride (8 eq), pyridine (10 eq), $CH_2Cl_2$, 14 h, 88% ; d) $NEt_3.3HF$ (16.5 eq), $CH_3CN$, 3h, 92% ; e) DMTrCl (2 eq), DMAP (0.2 eq), pyridine, 22h, 60% ; f) $P(N\text{-}iPr_2)_2O(CH_2)_2CN$ (1.1 eq), N,N'-diisopropylammonium tetrazolate (0.6 eq), $CH_2Cl_2$, argon, 1h, 50%.

Two oligodeoxynucleotides, namely [5'-d(ATC GTG ACT GAT CT)-3'] **9** and [5'-d(ATX GTG ACT GAT CT)-3' ; where X = 5-OHdC **2**] **10** were then prepared by phosphoramidite solid-phase synthesis on a 1μmole scale, using phenoxyacetyl protective group for dA and dG and isobutyryl protective group for dC respectively. After standard deprotection with aqueous ammonia at room temperature for 4h, the 5'-DMTr-oligomers were purified and deprotected on line by reverse phase HPLC[10]. The purity and homogeneity of collected fractions were controlled by HPLC and gel electrophoresis. Respectively, 20 $AU_{260nm}$ and 15 $AU_{260nm}$ of purified oligomers **9** and **10** were obtained.

The determination of the molecular weight of the oligonucleotide **10** was accomplished by electrospray mass spectrometry in the negative mode (Calculated : 4269.80 ; Found : 4268.43) which confirmed (a) the incorporation of 5-OHdC **2** and (b) that no side reactions have occured. Besides, the difference with the molecular weight of the non-modified oligonucleotide **9** (Calculated : 4253.80 ; Found 4252.36) was equal to 16.07 ; this value corresponds to the expected increase in molecular weight associated with the insertion of **2** in place of **1**. Furthermore, enzymatic digestion of **10** and analysis of the resulting mixture of nucleosides by reverse phase HPLC (Figure 1) provided 5-OHdC, dC, dG, dT and dA in a 1:2:3:5:3 ratio confirming the structure. The identification of 5-OHdC **2** was achieved by comparaison of the retention time ($t_R$ = 9.2 min) with a standard and by electrospray mass measurement of the collected peak.

Figure 1

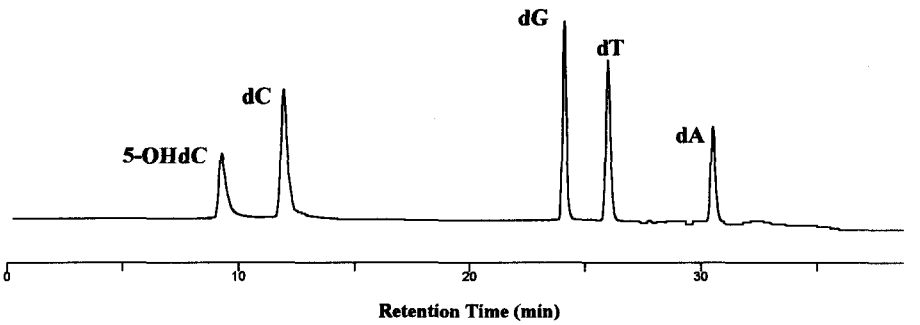



**Figure 1.** HPLC profile of enzymatic digestion mixture ; enzymatic digestion of **10** with Nuclease $P_1$ (*Penicilium citrinum*) and calf intestine alkaline phosphatase (AP). The mixture (50 µl) was analyzed by HPLC on a Hypersil 5µ $C_{18}$ column (4.6x250 mm) ; elution with TEAA (25mM, pH = 7) and acetonitrile {100% TEAA (10 min), linear gradient from 0 to 10% of acetonitrile (30 min)} at a flow rate of 1.0 ml/min. The detection was achieved at 230 nm, which is one of the absorption maxima for 5-OHdC ($AU_{230nm/260nm}$ = 3.4).

In conclusion, the synthesis reported herein provides a facile method for the preparation of oligonucleotides containing 5-OHdC **2** at specific positions[11]. These modified DNA fragments are suitable for further studies aimed at determining both the biochemical (mutagenesis, repair) and conformational features of 5-OHdC **2** into DNA fragments.

**Acknowledgments.**

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   Purmal, A.A. ; Wah Kow, Y. ; Wallace, S.S. *Nucleic Acids Res.* 1994, 22, 72-78.
3. Aliquots of the reaction mixture were taken up at increasing periods of time and analyzed by reverse phase HPLC (Interchrom Hypersil 5µ $C_{18}$ column, 4.6x250 mm) with aq. triethylammonium acetate (TEAA, 25mM, pH = 7) as the eluent.
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7. The mixture of **3** and **4** was prepurified by flash column chromatography ($SiO_2$, 0-10% MeOH-$CHCl_3$). Then, these two compounds were separated by semi-preparative reverse phase HPLC (Macherey-Nagel Nucleosil $C_{18}$ column, 21x250mm) with methanol and water as the eluents (isocratic, MeOH-$H_2O$ 70%-30%). $^1H$ NMR (200 MHz, methanol-$d_4$) δ ppm : 7.21 (s, $H_6$, **3**), 8.46 (s, $H_6$, **4**). FAB MASS m/e 486.3 $(M+H)^+$.
8. Gasparutto, D. ; Livache, T. ; Bazin, H. ; Duplaa, A. M. ; Guy, A. ; Khorlin, A. ; Molko, D. ; Roget, A. ; Téoule, R., *Nucleic Acids Res.* **1992**, 20, 5159-5166.
9. **8** : $^1H$ NMR (200 MHz, acetone-$d_6$) two diastereoisomers δ ppm : 0.97-1.47 (m, 12 H, $CH(C\underline{H}_3)_2$), 2.81 (m, 4 H, $OCH_2C\underline{H}_2CN$, $H_{2'}$, $H_{2''}$), 3.45-3.85 (m, 6 H, $C\underline{H}(CH_3)_2$, $OC\underline{H}_2CH_2CN$, $H_{5'}$, $H_{5''}$), 3.85 (s, 6 H, $OCH_3$), 4.44 (m, 1 H, $H_{4'}$), 5.01 (m, 1 H, $H_{3'}$), 6.49 (t, J=6 Hz, 0.6 H, $H_{1'}$), 6.50 (t, J=6 Hz, 0.4 H, $H_{1'}$), 6.89-8.12 , (m, 23 H, benzoyl, phenyl, methoxyphenyl), 8.43 (s, 0.4 H, $H_6$), 8.46 (s, 0.6 H, $H_6$). $^{31}P$ NMR (101 MHz, acetone-$d_6$) two diastereoisomers δ ppm : 149.79, 149.90. FAB MASS m/e 954.3 $(M+H)^+$.
10. The crude 5'-DMTr-oligomers were purified by reverse phase HPLC (Hamilton PRP3 column (polymeric phase) 10µ , 7x305 mm) with acetonitrile and TEAA as eluents using a non-linear gradient {100% TEAA, 25mM, pH = 7, (5 min), then isocratic TEAA/acetonitrile 92/8 (10 min)} ; in order to remove the DMTr group, the oligomers were treated with a solution of trifluoroacetic acid and water (TFA 1%) {isocratic 100% TFA(1%) (6 min)}. The resulting deprotected oligomers were again purified using the same eluents {gradient from 2 to 10% of acetonitrile (24 min)}.
11. Several 5-OHdC-containing oligonucleotides including a 22 mer are in preparation..


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