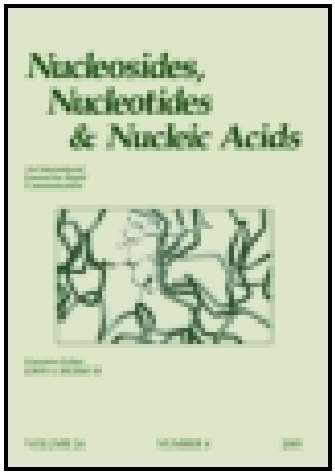

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Synthesis and Mass Spectrometry Analysis of Oligonucleotides Bearing 5-Formyl-2′-Deoxyurldlne in Their Structure

T. Berthod [a] , Y. Pétillot [b] , A. Guy [a] , J. Cadet [a] , E. Forest [b] & D. Molkot [a]

[a] Département de Recherche Fondamentale sur la Matière condensée /S. C. I. B./ Lésions des Acides Nucléiques , CEA / Grenoble, 17, Rue des Martyrs, 38054, Grenoble, Cedex 9, France

[b] Laboratoire de Spectrométrie de Masse des Protéines , lnstitut de Biologie Structurale (CEA/CNRS) , 41 Rue des Martyrs, 38027, Grenoble, Cedex 1, France

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SYNTHESIS AND MASS SPECTROMETRY ANALYSIS OF OLIGONUCLEOTIDES BEARING 5-FORMYL-2'-DEOXYURIDINE IN THEIR STRUCTURE

T. Berthod†, Y. Pétillot‡, A. Guy†, J. Cadet†, E. Forest‡ and D. Molko†*

† Département de Recherche Fondamentale sur la Matière Condensée / S.C.I.B. / Lésions des Acides Nucléiques, CEA / Grenoble, 17, Rue des Martyrs, 38054 Grenoble Cedex 9, France

‡ Laboratoire de Spectrométrie de Masse des Protéines, Institut de Biologie Structurale (CEA/CNRS), 41 Rue des Martyrs, 38027 Grenoble Cedex 1, France.

Abstract : Two oligonucleotides containing FdU (**1**) have been synthesized. The use of the "Pac-amidites" for the natural nucleosides has allowed the incorporation of the oxidized thymine residue without protection of the aldehydic function. The oligonucleotide composition was confirmed by enzymatic digestion and electrospray mass spectrometry.

5-Formyl-2'-deoxyuridine (FdU ; Compound **1** ; FIG. 1) is an oxidized nucleoside resulting from either the action of OH radical[1] or the photo-oxidation[2] of the exocyclic methyl group of thymidine. When administrated to cells in culture, FdU (**1**) is mutagenic[1], presumably due to its incorporation into cellular DNA. Furthermore, it can react with lysine under physiological conditions to form FdU-lysine adducts[1] or Schiff bases with amines[3]. Consequently, it is plausible that the formation of (**1**) in DNA may lead to the generation of DNA protein cross-links with histones.

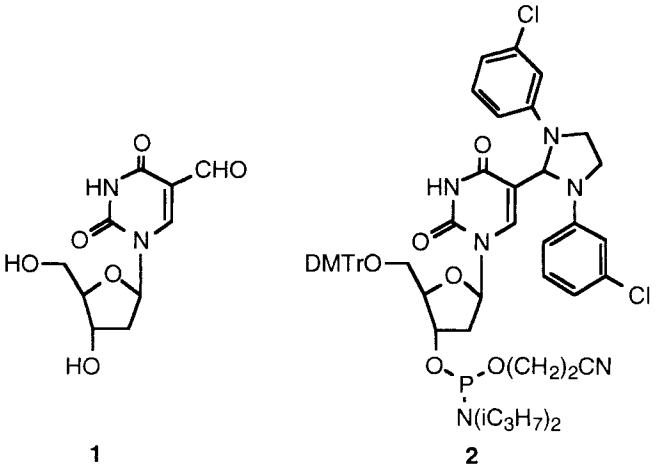


Figure. 1 : Structure of 5-formyl-2'-deoxyuridine (**1**) and its phosphoramidite synthon (**2**).

Attempts to further investigate biochemical and conformational changes associated with the presence of FdU (**1**) in DNA have been hampered by the absence of suitable methods for the preparation of FdU-containing oligonucleotides. In particular, the polymerase method[4] is not suitable for the preparation of large quantities of oligonucleotides containing FdU in selected sites that would be required for physical studies. In addition, the latter approach is less adaptable for the choice of appropriate sequences.

Recently, Matsuda *et al.*[5] reported the incorporation of 5-formyl-2'-deoxyuridine (**1**) into a (poly-T) oligonucleotide. For this purpose a derivative of 1,3-diphenylimidazolidine was used as a specific protecting group for the exocyclic aldehyde function. This gave rise to compound **2** which may be deprotected in 80 % aqueous acetic acid.

We report herein an alternative and simple method for the solid-phase site specific incorporation of FdU (**1**) in oligonucleotides. It should be added that the present method does not require the protection of the modified base.

RESULTS and DISCUSSION

1. Study of the stability of FdU (1) under the conditions of oligonucleotide synthesis :

During the course of the synthesis of oligonucleotides with phosphoramidite chemistry, iodine is used after each elongation step to oxidize the internucleotidic phosphite. Since it was reported that aldehyde functions can be oxidized under alkaline conditions in the presence of iodine[6], we tested the stability of FdU (**1**) under the conditions of oxidation used during the solid phase oligonucleotide synthesis. For this purpose, aliquots of the reaction mixture of FdU (**1**) with a commercially available solution of iodine were collected and analyzed by Reversed Phase Liquid Chromatography (RPLC). Using thymidine as an internal standard, no detectable degradation of FdU (**1**) was observed, even after the reaction mixture was left for 24 h at room temperature.

Similar control experiments were made in 80% aqueous acetic acid, under the detritylation conditions of the 5'-hydroxyl group. It was observed that FdU (**1**) is also stable under the latter acidic conditions.

The final deprotection step of the oligonucleotide synthesis involves an alkaline treatment with concentrated aqueous ammonia. This is aimed at cleaving the DNA fragments from its solid support and removing the protecting groups from both the internucleotidic phosphates and the amino functions of the bases. Armstrong *et al.* reported that 5-formyluridine was unstable under aqueous alkaline conditions and suffered anomerization[7]. Therefore it was particulary relevant to check for the stability of FdU (**1**) in concentrated aqueous ammonia for 24 h at room temperature. RPLC analysis showed the presence of 2 additional products (FIG. 2) which were further characterized by ^{1}H-NMR and positive mode FAB mass spectrometry (FAB-MS).

The first eluted compound from RPLC was identified as 5-formyluracil by comparison of its FAB-MS and ^{1}H-NMR features with those of the authentic sample. The FAB-MS spectra of the second compound exhibits a notable protonated molecule at m/z 257 ($[MH]^+$). Two other fragments at m/z 141 and 117 were observed which correspond to the base moiety ($[BH_2]^+$) and the deoxyribose ($[dR]^+$), respectively. This is indicative of a molecular structure similar to that of FdU (**1**), in fact an isomer resulting from a modification of the sugar moiety.This received further confirmation from the inspection of the ^{1}H-NMR spectrum obtained in D_2O (TABLE I).

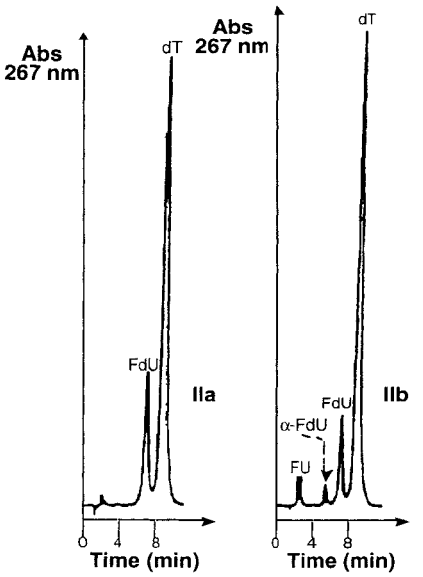


Figure. 2 : RPLC analysis (system A) of the reaction of FdU (**1**) in 29% NH_4OH at room temperature in 4 h (IIa) and in 24 h (IIb)-

These data reveal the presence in the low field region of two singlets at 8.70 and 9.74 ppm corresponding to the pyrimidine H_6 and the aldehyde proton, respectively. These signals confirm that the base moiety is intact.

Therefore, significant differences in the ^{1}H-NMR features of the sugar moiety are noticeable by comparison with those of FdU (**1**). The structure of the sugar moiety was elucidated by considering the coupling constants involving the protons of the sugar ring. Three forms could be expected ; they are the α- and β-pyranoses with respectively a $^1C_4$ and a $^4C_1$ preferential conformation[8] in addition to the α-furanose structure.

In the case of the β-pyranose form, the preferential $^4C_1$ conformation should imply the presence of two pairs of diaxial protons in the ring which are $H_{1'}$, $H_{2'}$ and $H_{4'}$, $H_{5''}$. Strong couplings (> 10 Hz) are typical of such a structure. These values are not observed in the spectrum. Likewise, the preferential $^1C_4$ conformation for the α-pyranose form should imply the presence of two pairs of diaxial protons $H_{1'}$, $H_{2''}$ and $H_{2''}$, $H_{3'}$. Again, this possibility was ruled out.

Table I : ^{1}H-NMR (400 MHz) chemical shifts (ppm) and coupling constants (Hz) of FdU (**1**) and α-FdU (**3**) obtained in D_2O as inferred from computer iterative analysis (LAOCOON III). * : an additional long range $^4J_{i-j}$ coupling due to a planar structure is observed.

δ (ppm)	1'	2'	2"	3'	4'	5'	5"	6	CHO
FdU	6.33	2.46	2.56	4.53	4.18	3.92	3.82	8.87	9.73
α-FdU	6.27	2.80	2.36	4.52	4.64	3.80	3.72	8.70	9.74

J_{i-j} (Hz)	1'-2'	1'-2"	2'-2"	2'-3'	2"-3'	2"-4' *	3'-4'	4'-5'	4'-5"	5'-5"
FdU	5.6	6.5	-14.3	6.7	5.0	—	3.9	3.2	4.7	-12.2
α-FdU	6.8	1.5	-15.1	5.5	1.5	0.5	1.5	3.9	5.5	-12.3

The last remaining structure to be considered is the α-furanose isomer. The resonance signal of the anomeric proton appears as a doublet of doublet at 6.27 ppm. This is due to the small coupling constant between $H_{1'}$, $H_{2"}$ ($^3J_{H,H}$=1.5 Hz) corresponding to a *trans* geometry with the protons in preferential equatorial orientation. Two other similar small coupling constants are observed between the protons $H_{2"}$, $H_{3'}$ ($^3J_{H,H}$=1.5 Hz) and $H_{3'}$, $H_{4'}$ ($^3J_{H,H}$=1.5 Hz). These may be rationalyzed in terms of torsion angles $\phi_{H,H}$ close to 90°. These data are compatible with an epimerisation of the $C_{1'}$, leading to an α-furanosyl structure with a preferential $C_{2'}$ endo conformation (82%)[9]. Thus, the latter nucleoside was identified as the α–anomer of FdU (α-FdU, Compound **3**).

The observed anomerisation of FdU (**1**) clearly shows that the use of the standard amino-protecting groups must be precluded for the synthesis of oligonucleotides bearing FdU. However, kinetic data showed that no degradation was detectable after a 4 h treatment. It should be added that only 4 % of FdU (**1**) was decomposed after a period of 8 h (FIG. 2). The relative good stability of FdU (**1**) in concentrated ammonia at room temperature should be compatible with the use of more labile protecting groups for the exocyclic amine functions of normal nucleosides. "Pac-amidites" appeared as alternative protecting agents[10] which permit the deprotection of the oligonucleotides in 4 h at room temperature. Therefore, their use should allow the synthesis of DNA fragments containing-FdU without protection of its aldehydic function.

*2. Preparation of the phosphoramidite synthon of 5-formyl-2'-deoxyuridine (**5**):*

5-Formyl-2'-deoxyuridine (FIG. 3, Compound **1**) was prepared by menadione mediated photosensitization[2] of a 5 mM aqueous solution of thymidine upon exposure to UVA radiation (λ_m= 350 nm). Under these conditions, FdU (**1**) is the major photooxidation product which results from the deprotonation of the initially generated pyrimidinic radical cation, followed by the addition of molecular oxygen[2]. FdU (**1**) is easily purified by preparative RPLC in a 9 % yield.

The 5'-hydroxyl group of FdU (**1**) was protected by dimethoxytrilation in pyridine at room temperature. Moreover, we may note the formation, in about 10% yield, of the 3',5'-bis-dimethoxytrityl-5-formyl-2'-deoxyuridine as a by-product. The phosphoramidite synthon of FdU (compound **5**) was obtained by using the well established procedure[11,12] in a 58 % yield. Its purity was confirmed by ^{31}P-NMR in dichloromethane, which showed a singlet at 149,5 ppm. The FAB-MS analysis confirmed the structure of the desired product (MH^+= 759.2 ± 0.1 Da , MNa^+= 781.2 ± 0.1 Da).

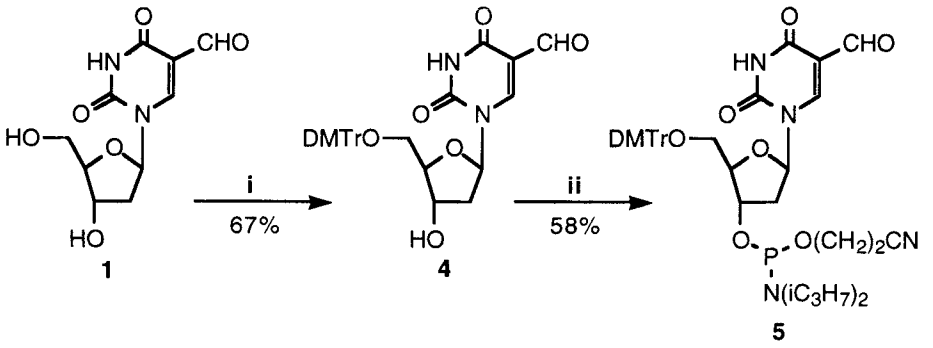
3. Solid-phase synthesis of oligonucleotides containing-FdU :

The synthesis of a pentamer (**6**) and a decamer (**7**) in which FdU (**1**) was site specific incorporated was achieved by using "Pac-amidites" for the natural nucleosides[10]. A similar approach was used to prepare the decamer (**8**) including thymidine, the normal nucleoside, in place of FdU (**1** ; see TABLE II). The usual synthesis cycle was performed for the incorporation of FdU (**1**) in DNA sequences. A coupling yield of 88-90% was obtained with this synthon. Several deprotection and purification methods were used with the aim of selecting the conditions which minimize the decomposition of FdU (**1**). They are summarized in TABLE II.

4. Deprotection and analysis of the modified oligonucleotides :

In a first approach, the decamer (**7**), d[CCT CTA C**F**A T], was deprotected in 29% ammonia during a period of 4 h and purified by RPLC.

The nucleosides released through an enzymatic digestion were analyzed by RPLC (FIG. 4). The chromatogram shows the presence of dA, dC, dT and 5-formyl-2'-deoxyuridine (**1**). No trace of the α-anomer of FdU (**3**) was detected at its expected retention time. Therefore, considering the detection limits of the assay, we can conclude that the anomerisation of the nucleoside, if it occurs, was less than 1%.



i : DMT-Cl, pyridine ; **ii** : N,N'-diiisopropylammonium tetrazolate, cyano-2-ethoxy tetra-N,N,N',N'-diisopropyl phosphorodiamidite, CH_2Cl_2, argon.

Figure. 3 : Synthetic reactions used for the preparation of the phosphoramidite synthon of FdU (**1**).

Table II : Deprotection and purification conditions used for the synthesized oligonucleotides

	Sequence	Deprotection	Purification
Pentamer **6** (containing-FdU)	5' CG**F** AT 3'	NH_4OH	C_{18}
Decamer **7** (containing-FdU)	5' CCT CTA C**F**A T 3'	NH_4OH, NH_4OH/MeOH and NaOH/MeOH	anion-exchange (SAX) and C_{18}
Decamer **8** (containing-T)	5' CCT CTA C**T**A T 3'	NH_4OH, NH_4OH/MeOH and NaOH/MeOH	anion-exchange (SAX) and C_{18}

The pentamer (**6**) (d[CG**F** AT]) was synthesized to confirm that FdU (**1**) may be incorporated in an oligonucleotide containing the four natural nucleosides. It was deprotected and purified under the same conditions used for the preparation of the decamer (**7**). The electrospray-ionization-mass spectrometry (ESI-MS) analysis of compound (**6**) gave the expected pseudomolecular ions : $[M-H]^-$ at m/z= 1491 and $[M-2H]^{2-}$ at m/z= 745[13].

Consequently, the purity of the previous decamer (**7**) was investigated by electrospray-ionization-mass spectrometry (ESI-MS). The spectrum shows the presence of the major product at M= 2951.3 ± 0.3 Da (FIG. 5). Unfortunately, the mass

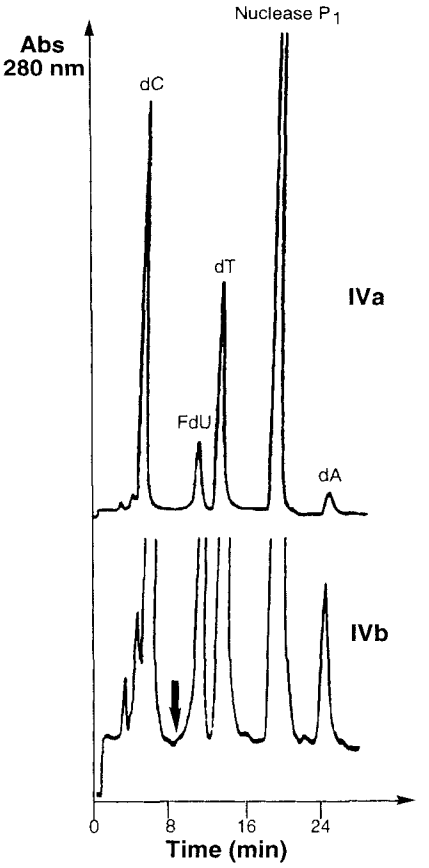


Figure. 4: RPLC analysis (system D) of the enzymatic digestion of the decamer (**7**) (5'-CCTCTA C**F**AT-3'), attenuation 0.032 (a) ; attenuation 0.004 (b). The arrow indicates the position of α-FdU (**3**).

spectrum also indicates the presence of three other compounds with a molecular mass of 2968.0 ± 0.3, 3021 ± 0.3 and 3085.5 ± 0.3 Da. These appear to correspond to addition products with a molecule of ammonia, an isobutyryl function and a phenoxyacetyl group respectively.

This result was surprising since the product was homogeneous by reversed phase chromatography. Several possibilities may be considered to explain the presence of such adducts in the oligonucleotide. One could be related to an incomplete deprotection. Another is the presence of NH_3 as a counter ion at the level of an

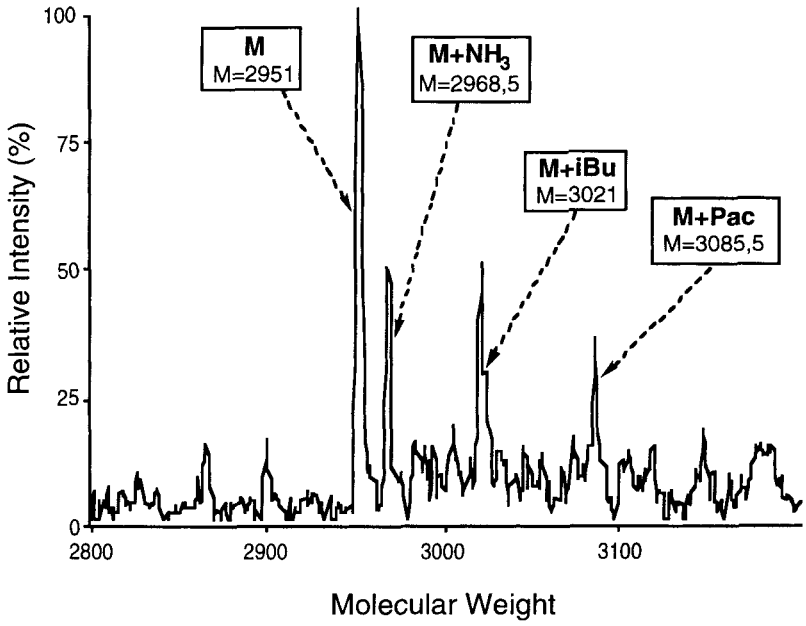


Figure. 5 : ESI-MS of the decamer (**7**) deprotected in 29% NH_4OH and purified by RPLC (System C)

internucleotidic phosphate. We may also suspect a 1,4-Michael addition of nucleophilic species present during the deprotection step. Thus, we decided to compare the deprotection of the modified decamer with that of the parent product in which FdU (**1**) is replaced by dT.

5. Comparative study of the deprotection of the two decamers :

Since RPLC, in the ion suppression mode, was unefficient to separate the different species present in the modified decamer, strong anion-exchange chromatography was used (FIG. 6). The latter analysis showed the presence of four compounds which were collected and analysed by ESI-MS after the salts were removed by RPLC with a gradient of acetonitrile of 3 to 12.5% in 25 mM triethylammonium acetate (TEAA) buffer (System C). It was found that they exhibit similar features than the " heavier "products previously observed.

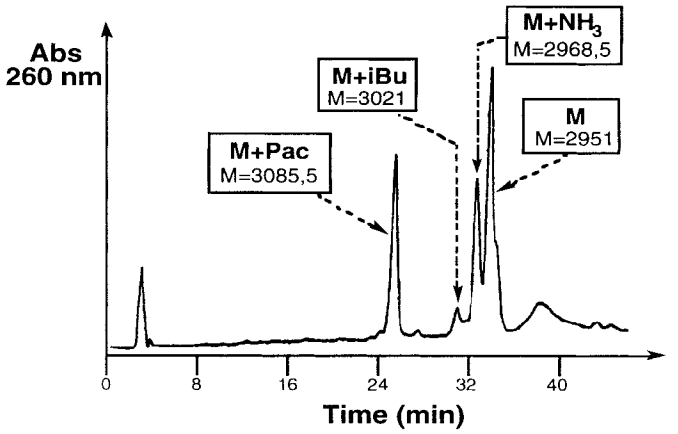


Figure. 6 : HPLC analysis on an anion-exchange (system E) of the decamer (**7**) fraction analyzed by ESI-MS.

The analysis of the same sequence (decamer **8** containing dT in place of FdU **1**) showed a complete deprotection under the same conditions. So we concluded that in the case of the oligonucleotide bearing FdU (**1**), the addition probably occurred at position 6 of the base by a 1,4-Micheal mechanism. This was supported by the fact that position 6 is far less electronegative than the aldehydic function. This property has been calculated by *ABINITIO* LOCAL SPIN THEORY using DGAUSS Program of UNICHEM.2.3.1.PACKAGE from the cristallographic data of FdU (**1**)[13].

As the ammonia deprotection was suspected to induce these side reactions, other deprotection conditions were checked : 29% NH_4OH : MeOH (1:1) and 0.2N NaOH : MeOH (1:1), at room temperature. The mixtures were analyzed by SAX HPLC. The most significant HPLC chromatograms are presented in FIGURE. 7.

From these experiments we can conclude that the use of NaOH does not improve the deprotection profile. Indeed, we observed more degradation of the modified oligonucleotides (**7**, **8**) that when the deprotection was achieved with 29% NH_4OH.

On the contrary, the deprotection of decamer (**7**) in 29% NH_4OH/MeOH (1:1) permitted to obtain a better chromatographic profile of the mixture. Under these conditions, the purification of the decamer (**7**), the major product present, was easier. The purity of the decamer (**7**) was reinvestigated by ESI-MS (FIG. 8), after the salts were removed by two successive RPLC separations with a gradient of acetonitrile of 3 to 12.5% in 25mM TEAA buffer (System C).

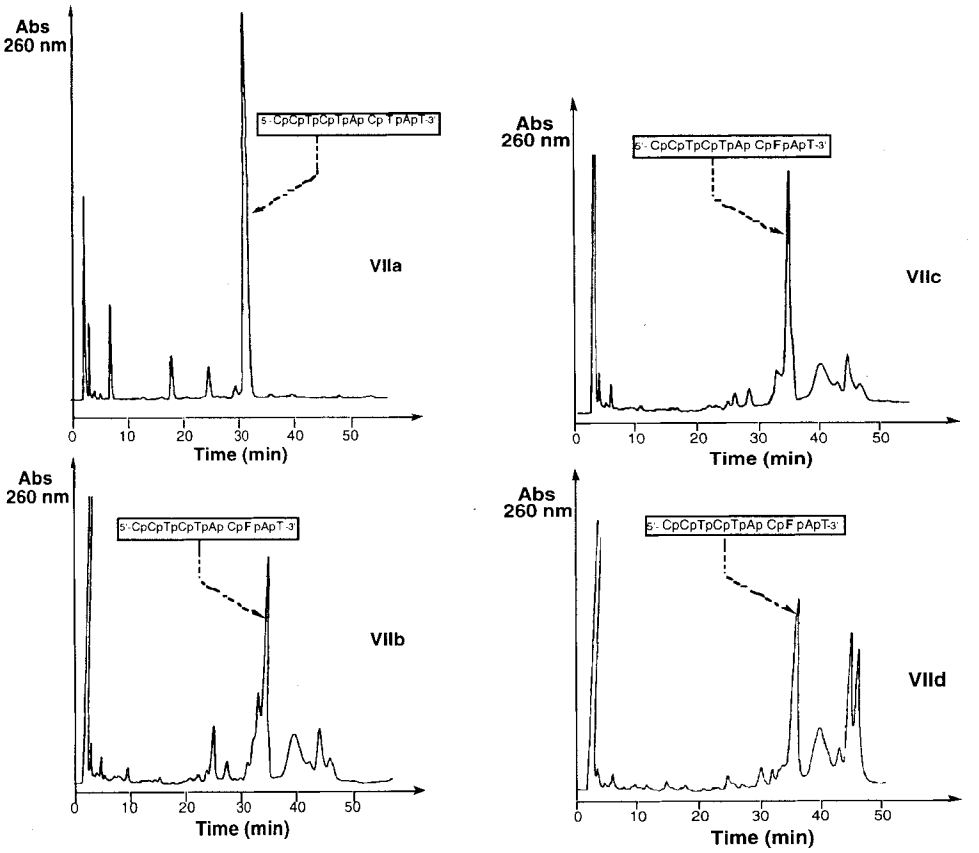


Figure. 7 : H.P.L.C. analysis (system E) of the deprotection in 29% NH_4OH for 4 h of the decamer **8** (a) and of the decamer **7** (b) ; of the decamer **7** in 29% NH_4OH/MeOH (1:1, v/v) for 8 h (c) ; and of the decamer **7** in 0.2N NaOH/MeOH (1 : 1; v/v) for 8 h (d)

The ESI-MS analysis of the decamer (**7**) gave the expected peak at M=2951.0 ± 0.3 Da, and it revealed that it was not contaminated by the different adducts previously observed (FIG. 8). In addition, the ESI-MS spectrum indicated the presence of a fragment at M= 2810 ± 0.3 Da. This corresponds to the loss of the 5-formyluracil (FU) residue.

It should be noted that this compound was not observed by ESI-MS during the previous analysis of the decamer (**7**). Since, no depurination was found in the

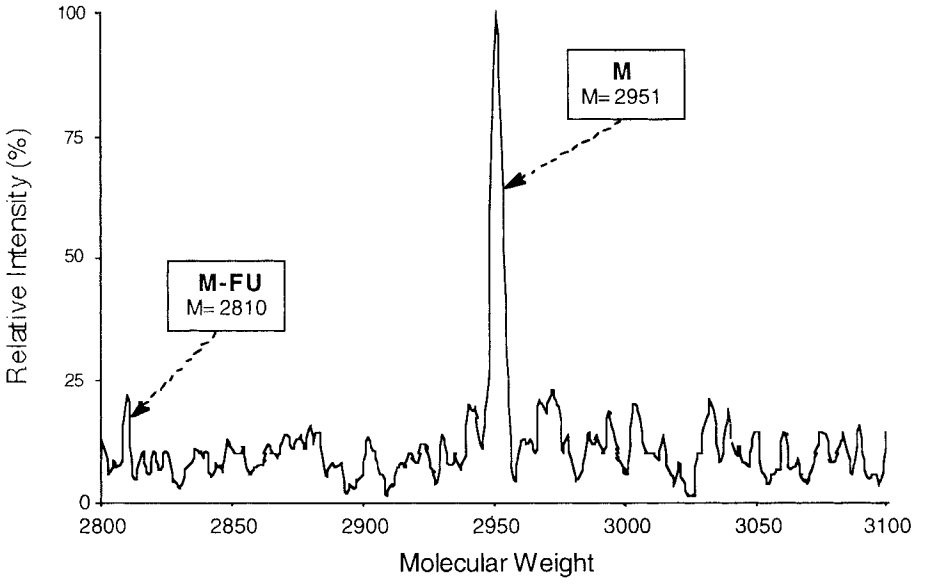


Figure. 8 : ESI-MS of the decamer (**7**) deprotected in 29% NH_4OH/MeOH (1:1, v/v) and purified by anion-exchange HPLC (system E)

previous enzymatically digested 10-mer (no trace of 5-formyluracil was detected), we suspected that this phenomenon occured in the source of the mass spectrometer. This was confirmed by the analysis of the mass spectrum of the pentamer (**6**) in which this fragmentation was also observed[13]. These results are in agreement with the observations of Reddy and Iden[15], and McLuckey and Habibi-Goudarze[16]. Furthermore, our experiments on FdU (**1**) showed an increase in the yield of fragmentation of the N-glycosidic bond of FdU (**1**) concomittant with the increase in the voltage of the source. Therefore, we may conclude that the loss of the base was due to a fragmentation occuring in the spectrometer source.

CONCLUSION

It is possible to incorporate 5-formyl-2'-deoxyuridine (**1**) in synthetic oligonucleotides without protection of the aldehyde function. The phosphoramidite synthon is easy to obtain in good yields. The use of Pac-amidites allows a short

deprotection time without detectable anomerisation and/or loss of the pyrimidine residue. Nevertheless, 1,4-Michael additions to the base have been observed, lowering the overall yield of the synthesis. These additions can be reduced by using a 29 % aqueous ammonia solution in methanol (1 : 1). In all cases, the various products obtained were easily separated by SAX chromatography, providing pure modified oligonucleotides.

MATERIALS and METHODS

High-Performance Liquid Chromatography Separations :

Differents chromatographic systems were used for the different purification steps. The HPLC approaches consisted of a gradient pump Spectra System P2000 (Spectra Physics, Fremont, CA, USA) equipped with a Rheodyne 7125 (Berkeley, CA, USA) injector loop. The detection was achieved with a Spectra System UV2000 and the data were recorded on a Spectra System Chromjet Integrator. Several chromatographic systems were used. System **A** : column, Merck LiChrocart LiChrospher 100RP-8 (endcapped, 5µm, 125x4mm) ; eluent, 25 mM TEAA/acetonitrile (96:4, v:v) at a flow-rate of 1 mL/min. System **B** : column : Merck LiChrosorb RP-18 (10µm, 250x4 mm) ; eluent, a gradient of 6 to 10% of acetonitrile in 25mM TEAA. System **C** : column : Merck LiChrosorb RP-18 (10µm, 250x4 mm) ; eluent, a gradient of 3 to 12,5% of acetonitrile in 25mM TEAA. System **D** : column : Waters Nova Pack C_{18} (4µm, 150x3.9 mm) ; eluent, 25mM TEAA/acetonitrile (96:4, v:v). System **E** : column : anion-exchange Interchrom Partisil P10 SAX 25F (5µm, 250x4 mm) ; eluent gradient from 14 to 49% of a 0,2M $NaHPO_4$ buffer containing 30% acetonitrile in a mixture of H_2O/ acetonitrile (70:30, v:v).

For the purification of FdU and α-FdU, the HPLC system (System **F**) used consisted of a gradient pump Waters 600E System Controller (Waters Associate, Milford, USA) equipped with a Rheodyne 7125 injector loop. The UV detection was achieved with a Delsi SM25 Instrument (Delsy-Nermag, Argenteuil, France). Samples were injected onto a semi-preparative column Waters Delta Pack C_{18} 100 Å (15 µm, spherical, 300x19 mm) and the eluent was a mixture of water/methanol (87:13 ; v:v) at a flow-rate of 6mL/min. k' represented the capacity factor of the differents products in the conditions described previously.

Thin-layer chromatography analysis :

Thin-layer chromatography separations were performed using Merck 60 F_{254} silica gel plates (Merck, Darmstadt, Germany ; 0.2 mm, thickness).All compounds were located on the plates by fluorescence quenching at 254 nm. Another possibility to reveal 2'-deoxuribonucleoside was to spray the plates with 10% acid, followed by heating at 110°C.

Spectrometric measurements :

Fast atom bombardement (FAB) mass spectrometry analyses were carried out on a model ZAB 2-SEQ spectrometer (Fisons-V.G, Manchester, United kingdom) in the positive mode detection. The modified nucleosides were dissolved either in a glycerol or in a thioglycerol matrix.

The electrospray-mass spectrometric measurements of the synthetic oligonucleotides (**6**, **7** and **8**) were carried out on a Perkin-Elmer Sciex API III+ triple quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray (ionspray) source. The instrument was tuned and calibrated in the positive mode and polarities were inverted in the negative-mode operations for the detection of anions[17,18]. Experiments were performed at a declustering potential (orifice voltage) of - 60 V which proved to be the optimum value: lower values (down to 45 V) increased the number and intensity of the ammonium cluster ions while higher values (up to 80 V) decreased the intensity of deprotonated oligonucleotides. Samples in ammonium acetate solution, were infused at a flow rate of 3 µL/min into the source using a 50 µm I.D. fused silica capillary connected to a Harvard 22 syringe pump and a Valco C6W injector equipped with a 1µL internal loop. To promote deprotonation of the oligonucleotides in the negative mode, the solvent contained either methanol or acetonitrile (20-40%) and ammonia or triethylamine (0.1-1%) in water. Mass spectra were the average of five to ten scans obtained with a dwell time of 2 ms per 0.5 m/z step in a m/z range from 500 to 1800. Fragmentations of FdU (**1**) were studied by increasing the orifice voltage of the mass spectrometer from -50 to -80 V in a m/z range from 100 to 300.

^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR spectra were recorded on AM400, WM250 and AC200 Brüker spectrometers operating in the Fourier transform mode (Brüker, Wissemburg, France). The ^{1}H and ^{13}C chemical shifts are expressed in p.p.m with reference to 3-(trimethylsilyl)-propionate-2,2,3,3-d_4 used as the internal standard. On the other hand, 85% H_3PO_4 was used as an external standard for ^{31}P-NMR spectra.

Assignment of ^{1}H and ^{13}C signals was achieved by specific homo- and heteronuclear decoupling experiments and two dimensional ^{1}H-^{13}C heteronuclear correlated NMR experiments (XHCORRC). The ^{1}H-NMR spectra of FdU (**1**) and α-FdU in D_2O were computer simulated by using the iterative LAOCOON III and PANIC Brücker programs. The simulated values of the chemical shifts and the coupling constants were obtained with a quadratic error < 0.05 and gave further confirmation of the assignments of the proton signals.

1-(2-deoxy-α-D-erythro-pentofuranosyl)-5-formyluracil (**3**) :

100 mg (0.39 mmole) of FdU and 120 mg (0.49 mmole) of thymidine were dissolved in 40 mL of 29% NH_4OH from Applied Biosystems. The mixture was stirred at room temperature. Aliquots were taken (40µL) after 0, 4, 8, 16 and 24 h, neutralized with 1N acetic acid and evaporated to dryness. The fractions (k'= 3.59) were analyzed by HPLC system A.

After 120 h, the mixture was neutralized with a solution of 1N acetic acid and evaporated to dryness. The residue obtained was purified by HPLC system F. The appropriate fractions (k'=1.34) were collected and then lyophilised. 9 mg of α-FdU (componud **3** ; 35 µmoles) were obtained. (Yield : 9%).

FAB-MS positive mode : MH^+= 257 ± 0.1 Da ; $BH2^+$=147.1 ± 0.1 Da ;
dR^+=117 ± 0.1 Da.

^{1}H-NMR (400.13 MHz ; D_2O) : see Table I.

^{13}C-NMR (100.62 MHz ; D_2O) δ : 189.36 (CHO) ; 163.93 (C-4) ; 151.51 (C-6) ; 150.98 (C-2) ; 110.71 (C-5) ; 90.05 (C-4') ; 89.05 (C-1') ; 70.79 (C-3') ; 61.58 (C-5') ; 39.88 (C-2').

5'-O-(4,4'-dimethoxytrityl)-5-formyl-2'-deoxyuridine (**4**) :

578.6 mg (2.26 mmoles) of 5-formyl-2'-deoxyuridine (**1**) were dried by evaporating 2x20 mL of dry pyridine. The resulting oily residue was dissolved in 15 mL of dry pyridine, and 4,4'-dimethoxytrityl chloride (2.49 mmoles) was added. The mixture was left overnight at 5°C and then the reaction was checked for completion by TLC. The solution was cooled in an ice bath and methanol (3 mL) was added. After 10 min, the mixture was dissolved in a solution of ethanol/toluene (2:1, v/v) and evaporated to near dryness. Two other coevaporations with this solution were performed and finally the residue was again coevaporated with dichloromethane. The residue was taken up in 100 mL of dichloromethane and washed by 3x75 mL of 5% $NaHCO_3$ and 2x75 mL

of water. The organic layer was dried over sodium sulfate and then evaporated to dryness. The residue was then purified by flash-chromatography on a Merck Kieselgel silica gel column PF_{254} (60 g), the mobile phase was a step gradient of 0 to 5% methanol in chloroform. The appropriate fractions were concentrated to dryness. 844 mg (1.51 mmoles) of 5'-O-(4,4'-dimethoxytrityl)-5-formyl-2'-deoxyuridine (**4**) were obtained. (Yield : 67%).

FAB-MS positive mode : MH^+= 559 ± 0.1 Da, MNa^+= 581 ± 0.1 Da.

^{1}H-NMR (250.13 MHz ; Acetone d_6) δ : 9.85 (s, 1H, CHO) ; 8.56 (s, 1H, H-6) ; 7.61-6.94 (m, 13 H, H Ar) ; 6.31 (t, $J_{1',2'}$=5.41 Hz and $J_{1',2''}$=7.52 Hz, 1H, H-1') ; 4.67 (d, $J_{OH\text{-}3'}$=3.82 Hz, 1H, 3'-OH) ; 4.62 (m, $J_{3'\text{-}2'}$=3.57 Hz, $J_{3'\text{-}2''}$=6.45 Hz and $J_{3'\text{-}4'}$=3.51 Hz, 1H, H-3') ; 4.27 (m, $J_{4'\text{-}5'}$=4.72 Hz and $J_{4'\text{-}5''}$=3.49 Hz, 1H, H-4') ; 3.89 (s, 6H, CH_3O) ; 3.49- 3.44 (m, $J_{5'\text{-}5''}$=-10.55 Hz, 2H; H-5' et H-5") ; 2.62-2.51 (m, $J_{2'\text{-}2''}$=-14.12 Hz, 2H, H-2' et H-2").

^{13}C-NMR (100.62 MHz ; CD_2Cl_2) δ : 185.47 (CHO) ; 161.98 (C-4) ; 158.88 (C-4, -4' of DMT) ; 149.62 (C-2) ; 146.22 (C-6) ; 144.81 (C-1" of DMT) ; 135.66 and 135.51 (C-1, C-1' of DMT) ; 130.21 (C-2, C-2', C-6, C-6' of DMT) ; 128.09 (C-2", C- 3", C-5", C-6" of DMT) ; 127.09 (C-4" of DMT) ; 113.37 (C-3, C-3', C-5, C-5' of DMT) ; 111.31 (C-5) ; 87.13 (C-1') ; 87.04 ((MeOØ)$_2$ØCO) ; 87.04 (C-4') ; 72.25 (C-3') ; 63.60 (C-5') ; 55.37 (CH_3O) ; 41.60 (C-2').

5-formyl-2'-deoxyuridine phosphoramidite derivative (**5**) :

5'-O-(4,4'-dimethoxytrityl)-5-formyl-2'-deoxyuridine (*compound* **4** ; 0.79 *mmole*) and diisopropyl-ammonium tetrazolate (0.39 mmole) was dried by coevaporation with dry dichloromethane. Then, the solid residue was dissolved in dry dichloromethane (4 mL) under argon. Subsequently, 440 µL of cyanoethyl-bisdiisopropylamine-phosphine (0.87 mmole) were added with a syringe through a septum. The reaction was stirred during 35 min. The formation of the desired product. was checked by TLC. After dilution in 40 mL of ethyl acetate, the mixture was concentrated to dryness. The subsequent residue was taken up in 40 mL AcOEt and washed with 2x40 mL of 5% $NaHCO_3$ and 2x40 mL of a saturated solution of NaCl. The organic layer was evaporated to dryness. The dry residue was purified by flash-chromatography on a Merck Kieselgel silica gel column PF254 (50 g), the mobile phase was a step gradient of 0 to 3% AcOEt in dichloromethane/triethylamine (99:1, v/v). The appropriate fractions were pooled and concentrated to dryness. The product was dissolved in 5 mL dry dichloromethane and then precipitated with hexane (70 mL) at -78°C. Filtration gave a

white powder corresponding to the phosphoramidite synthon of FdU which was dried under reduced pressure in a dessicator, and then stored under dry argon. Under these conditions, 350 mg (0.46 mmole) of (**5**) were obtained. Yield : 58%.

FAB-MS positive mode : MH^+= 759.2 ± 0.1 Da ; MNa^+= 781.2 ± 0.1 Da.

In addition, the exact mass measurement of the pseudomolecular ion of $[M+H]^+$ (m/z=758.2665) as obtained from a high resolution FAB-MS analysis is indicative of an empirical formula of $C_{40}H_{47}N_4O_9P_1$.

^{1}H-NMR (200.15 MHz ; CD_2Cl_2) two diastereomers δ : 9.59 and 9.58 (s, 1H, CHO) ; 8.43 and 8.39 (s, 1H, H-6) ; 7.41-6.80 (m, 13 H, H Ar) ; 6.13 (t, 1H, H-1') ; 4.52 (m, 1H, H-3') ; 4.23 (m, 1H, H-4') ; 3.76 (s, 6H, CH_3O) ; 3.75-3.48 (m, 3H, CH-iPr et CH_2OP) ; 3.32 (m, 2H; H-5' and H-5") ; 2.60 (m, 1H, H-2') ; 2.45 (t, 2H, -CH_2CN) ; 2.37 (m, 1H, H-2") ; 1.44-1.16 (m, 12H, CH_3-iPr).

^{31}P-NMR (101.21 MHz; CD_2Cl_2) δ : 149.5 (m; 1P).

-General coupling procedures for the assembly of oligonucleotides :

Syntheses were performed on a Applied Biosystems Mod. 381A DNA synthesizer using a 1 µmole protected nucleoside functionnalised on a chemical modified CPG polymer support. For optimal coupling efficiency, the flow-rate delivery of reagents was fixed at 2.3 mL/min. The synthesized amidite (**5**) and the "Pac-amidites"[10] were used in 0,08 M dry acetonitrile solution. Standard 0.2 µmole scale synthesis cycles were used. The coupling yield of the FdU unit (**5**) was 90% estimated by quantitation of the dimethoxytrityl cation before and after the FdU cycle.

-Removal of the oligomer from the CPG-beads, deprotection and purification :

The oligonucleotides bound to the support were treatred with several alkali solutions for different periods of time at room temperature. For the deprotection using 29% NH_4OH and 29% NH_4OH /MeOH (1:1, v/v) solutions, the collected fractions were then evaporated to dryness. For the 0.2N NaOH/MeOH (1:1, v/v) deprotection, the collected fractions were neutralized with an aqueous solution of 1M acetic acid and evaporated to dryness.

The pentamer (**6**) and the decamer (**7**) were further purified by HPLC/system B and system C, respectively. The decamers (**7**) and (**8**) were purified by HPLC/system E. The salts were removed from the purified products using HPLC/system C.

-Enzymatic digestion of decamer (**7**) :

0.1 $AUFS^{260nm}$ of purified decamer (**7**) was taken up in 30 µL of 0.3 M sodium acetate buffer. 10 µL Nuclease P_1 (1 µg) were added and the mixture was incubated at 37°C for 1 h. After the addition of 33 µL of Tris-HCl-$MgCl_2$ buffer (pH=9). Alkaline Phosphatase (20 U) was added and the resulting mixture was incubated for 2 h at 37°C. After lyophilisation, the residue obtained was taken up with 1 mL of water, centrifuged (12000 rpm) and the supernatant was analysed by HPLC/system D. The UV detection was carried out at 254 and 280 nm.

k' (dC)=	1.74	k' (dT)=	6.18
k' (α-FdU)=	3.31	k' (Nuclease P_1)=	9.13
k' (FdU)=	4.75	k' (dA)=	13.07

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