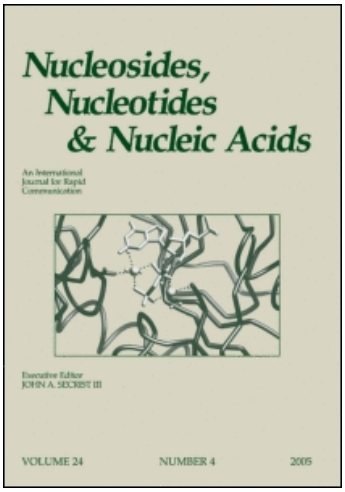

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The Behaviour of 2'-Deoxy-2'-Fluorouridine Incorporated into Oligonucleotides by the Phosphoramidite Approach

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THE BEHAVIOUR OF 2′-DEOXY-2′-FLUOROURIDINE INCORPORATED INTO OLIGONUCLEOTIDES BY THE PHOSPHORAMIDITE APPROACH

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ABSTRACT 2′-Deoxy-2′-fluorouridine has been chemically incorporated into an oligodeoxynucleotide of the structure 5′ACGGAX 3′ (X=U(2′-F)) using the phosphoramidite method and the behaviour of the product has been studied. 5′-O-Monomethoxytrityl-2′-deoxy-2′-fluorouridine was fixed on silica gel at the 3′-end and the chain elongated on a DNA-synthesizer using nucleoside methoxyphosphoramidites. After alkaline work-up two products were observed. One was found to be the desired fluoro containing hexamer, whereas the other corresponds to an araU-hexamer (X=arabinofuranosyluridine). The latter compound is supposed to be a product of alkaline hydrolysis of the C-2′-F-bond. The oligomers containing 2′-fluoro- and ara-U at their 3′-end were chemically sequenced by a solid phase method on CCS-paper which confirmed the right primary structure.

INTRODUCTION

Less attention has been paid in the last years to the synthesis of oligonucleotides containing fluorosubstituted carbohydrates. Recently, we have studied the introduction of 3′-deoxy-3′-fluorothymidine at the 3′- and 5′-end of shorter and also some longer DNA-fragments[1-3]. Furthermore, we have synthesized a tetramer containing 2′-deoxy-2′-fluorouridine at its 5′-end[1]. The synthesis of some 2′-fluorosubstituted shorter oligonucleotides and poly-2′-FU has recently been described by Ikehara [4-7].

In contrast to all former studies the present work is directed towards the incorporation of 2′-deoxy-2′-fluoro-

uridine into synthetic oligonucleotides at their 3′-end. Further attention has been paid to the question of the stability of the C-2′-F-bond under the conditions of the phosphoramidite synthesis. This was tested by synthesizing the hexamer d(ACGGAU(2′-F)) with 2′-deoxy-2′-fluorouridine at its 3′-end. Furthermore, the above hexamer was designed to serve as a model compound for studying the method of chemical ligation of DNA-duplexes in the presence of carbodiimides or imidazolides developed by Shabarova et al [8]. Thereby the influence of the conformation of some DNA-fragments of type I-III (X=T, Tp, U) on the efficiency of ligation has already been studied in detail [9].

```
          I                  II
5′  A C G G A X  C C A G G A G T G A C  3′
3′    G C C T A  G G T C C T C A C      5′
                 III
```

In the light of these results the hexamer reported here should be used to study the influence of fluoro-atoms in the fragment I on the chemical ligation.

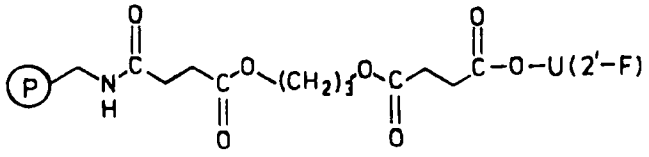
MATERIALS AND METHODS

Oligonucleotide synthesis

The synthesis of 2′-deoxy-2′-fluorouridine was performed by cleavage of O-2,2′-anhydrouridine with HF/dioxan. The product was purified by column chromatography on silica gel [10].

5′-O-Monomethoxytrityl-2′-deoxy-2′-fluorouridine as starting nucleoside is fixed at the 3′-end on silica gel (Silichrom C80, USSR) using a long spacer shown in FIG. 1. The loading proceeds troubleless in pyridine in the presence of TPS/MeIm yielding approximately 60 µmol nucleoside/g support. According to the phosphoramidite approach the elongation was carried out on the soviet DNA-synthesizer "Victoria 4M" (Academic plants, Novosibirsk). The elongation cycle using blocks 2 which were prepared according to standard procedures [11] is shown in TABLE 1.

The oligomer was deprotected by treatment with thiophenol/dioxan/triethylamine = 2:1:1 (v/v) and removed



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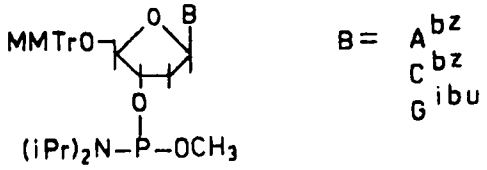

2

FIG. 1 Phosphoramidite synthons

from the solid support with conc. ammonia at 50 °C overnight. Thus, 130 O.D. could be isolated at the end of the synthesis related to 49 mg of the support. This is equivalent to an overall yield of 82 % and corresponds to a yield of 96 % for each step.

Purification

After cleavage from the solid support and removing all protecting groups the product was primarily purified by anion-exchange chromatography (column 4.6 x 250 mm, Polisil CA (5K), T= 45 °C, linear gradient of dihydrogenphosphate 0-0.3 M pH 6.5 in 30 % methanol . The eluated product was rechromatographed by RP-18 HPLC on a HPLC equipment of TRACOR (USA) using ultraspher-TM-octadecyl columns (4.6 x 250 mm). Products were eluated with linear gradient of 0-35 % methanol in 0.1 M ammonium acetate at 40 °C. The HPLC- profiles are shown in FIG.2, where the first peak at 4.5 min corresponds to hexamer I and the second one at 5.5 min to II.

Tab.1 Outline of Chain Elongation Cycle

number	operation	reagents and solvents [a)]	time (s)
1	washing	acetonitrile, dichloromethane	50
2	detritylation	1 % trifluoroacetic acid in dichloromethane	50
3	washing	dichloromethane acetonitrile	50
4	condensation	0.2 M amidite and 0.5 M tetrazole in acetonitrile [b)]	190
5	washing	acetonitrile	50
6	oxidation	0.2 M iodine in pyridine:acetic acid=9:1	60
7	washing	acetonitrile, dichloromethane	60
8	capping	acetic anhydride:N-methyl-imidazol:acetonitrile: diisopropylethylamine = 4.5:1:30:4.5 (v/v)	180
9	washing	acetonitrile	30

a) flow rate 2 ml/min

b) total amount of amidite used per cycle 30 µmol

Cleavage experiments

Both hexamers isolated were enzymatically cleaved by snake venom phosphodiesterase and alkaline phosphatase from E.coli. 4-5 O.D. of the appropriate hexamer were dissolved in 100 µl of 40 mM Tris-HCl pH 8.5, 0.4 M $MgCl_2$ and 50 µl of a phosphodiesterase solution (1 mg/ml) and 100 µl of a phosphatase solution (0.1 units/ml) added. The hexamer was incubated at 37 °C for 3 hours. The cleavage products were analyzed by HPLC using RP-18 columns and a linear gradient of acetonitrile 0-35 % in 0.1 M ammonium acetate.

Sequencing

After labeling the hexanucleotides at the 5′-end by γ-^{32}P-ATP· (Centrale Institute of Nuclear Research, Academy of Sciences, GDR) and polynucleotide kinase (Boehringer, Mannheim), they were sequenced by solid phase chemical degradation using CCS-paper according to Rosenthal et al[12,13]. In addition to the products of the four usual solid-phase modification reactions G, A+G, T>Pu and C, respectively, the starting material as well as the product of the reaction of the hexamer with 10 % piperidine (2 h at 90 °C, lyophilization) have been electrophoresed.

RESULTS AND DISCUSSIONS

In relation to usual observations on the elongation of DNA-fragments containing natural bases by phosphoramidite procedures the foregoing isolation of two main products with nearly equal yields at the end of synthesis (see FIG.2, HPLC-profiles) is undoubtedly surprising.

Thus, it is anticipated that the presence of a sugar modified nucleoside as starting compound is responsible for the formation of products I and II. Therefore, the structure of both products were analyzed by enzyme digestion. It was clearly shown that the digestion products of I contain 1-(β - D - arabinofuranosyl)uracil instead of 2′-deoxy-2′-fluorouridine. On the other hand the analysis of the hydrolysate of product II confirms the desired composition of a hexamer containing 2′-deoxy-2′-fluorouridine. Accordingly, the composition of the products isolated by HPLC corresponds to 5′ ACGGAaraU 3′ for profile I and 5′ ACGGAU(2′-F) 3′ for profile II, respectively. It has to be assumed that the formation of a hexamer containing araU is due to the hydrolysis of the starting nucleoside U(2′-F) under the mainly alkaline conditions of the DNA synthesis transforming the desired product II partly to product I. The transformation of 2′-deoxy-2′-fluorouridine into araU presumably takes place during removal of the N-acyl protecting groups which requires strong alkaline conditions (conc. ammonia, 50 °C overnight).

But, it seems to be possible that also other bases used in the amidite procedure are able to attack C-2′, thus hydrolyzing the C-F-bond. As shown in FIG.3, the preferred

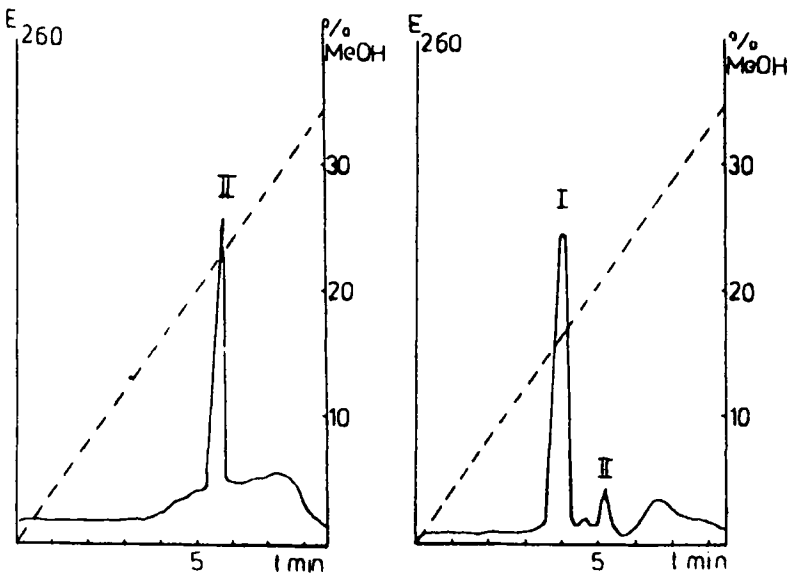

FIG. 2 HPLC-profiles of isolated products

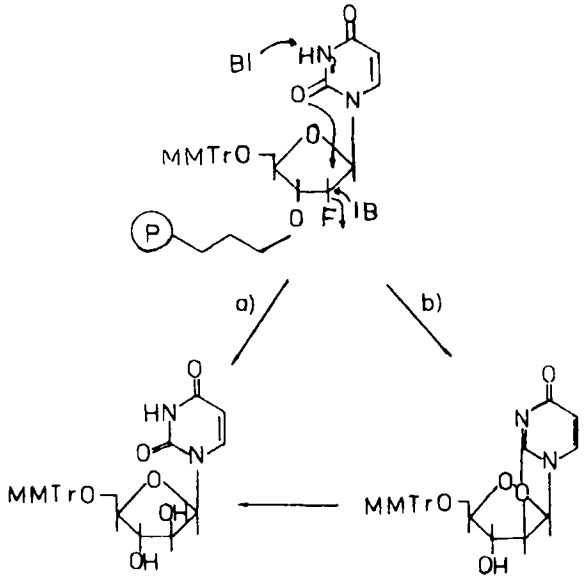

FIG. 3 Hydrolysis of fluorouridine

pathway for that hydrolysis is an alkaline catalyzed formation of an intermediate product O-2,2'-anhydrouridine and its stereospecific cleavage to the ara-compound (path b). It seems unlikely that the hydrolysis occurs via direct nucleophilic attack at C-2' (path a).

In order to proof the hypothesis whether hydrolysis of the C-2'-fluoro bond in the intact hexamer occurs, we tried to hydrolyse the polymer bound 2'-deoxy-2'-fluorouridine 1 as well as 2'-deoxy-2'-fluorouridine with ammonia. It was found that treatment of 1 or 2'-deoxy-2'-fluorouridine with NH_3 at 50 °C for 24 hrs. gave ara-uridine in about 50 % yield. These observations are in contrast to the stability of the compound in 0.2 N NaOH [13] and are in agreement with the observed instability of other 2'-deoxy-2'-fluoro-nucleosides. 2'-deoxy-2'-fluorothymidine and 2'-deoxy-2'-fluorocytidine are hydrolyzed under the same conditions. [15] Moreover it was shown by us and other authors, that ara-derivatives are formed during the procedure of cleavage of anhydrocompounds by HF or F^- [14] Up to now, there is no evidence whether the compound is formed, by splitting of anhydrobond or by hydrolysis of the C-F-bond. This indicates again the lability of the C-2'-fluoro bond.

For final analysis, both hexamers containing 2'-deoxy-2'-fluorouridine or ara-uridine were chemically sequenced using a solid phase method on CCS-paper. In both cases the correct primary structures were confirmed (FIG.4). In addition, special degradation patterns for the sugar modified uridines were established confirming the correct structure and position of these monomers within the oligonucleotide chain. The latter result is due to the fact that 2'-deoxy-2'-fluorouridine and ara-uridine are chemically degraded in different manner allowing the identification of both compounds in the sequencing pattern. Obviously, different elimination procedures during the treatment of both modified hexamers with piperidine are responsible for these differences in the degradation behaviour due to different substitutions at C-2'. The oligonucleotide containing ara-uridine shows a normal band spacing in the sequencing pattern (FIG.4, right). The five deoxynucleosides dA, dC, dG, dG, and dA, respectively, can easily be identified. The 3'-terminal ara-uridine is characterized at position 6 by three strong bands in the

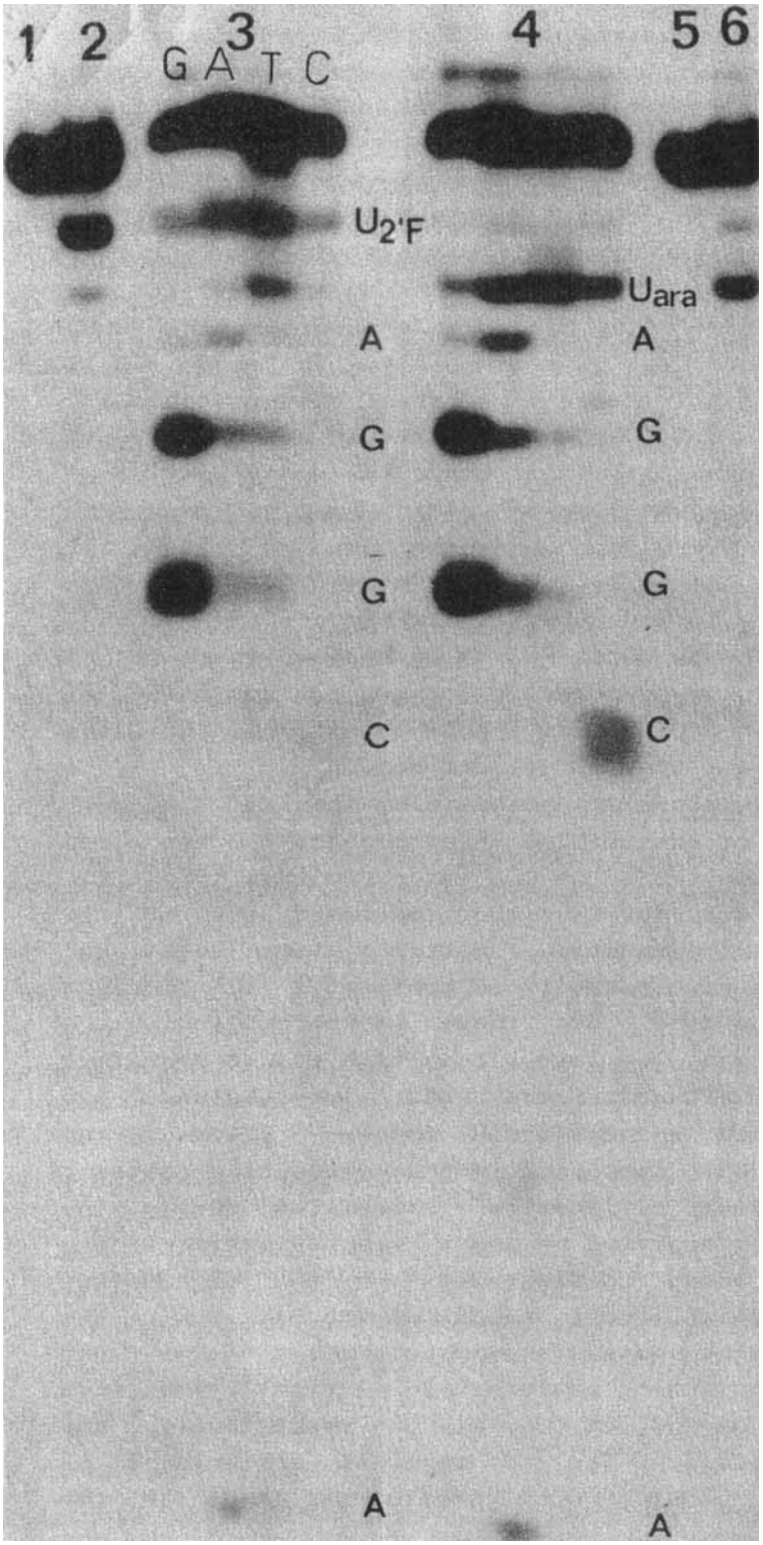

Fig. 4
Lane 1 purified d(³²pACGGAU(2′-F))
Lane 2 d(³²pACGGAU(2′-F)) after heating in 10 % piperidine
Lane 3 d(³²pACGGAU(2′-F)) after chemical degradation from left to right: G, A+G, T>Pu and C specific reactions
Lane 4 d(³²pACGGAU(ara)) after chemical degradation from left to right: G, A+G, T and C specific reactions
Lane 5 purified d(³²pACGGAU(ara))
Lane 6 d(³²pACGGAU(ara)) after heating in 10 % piperidine

A+G-, T>Pu- and C-reaction as well as by a slight band in the G-reaction. This indicates that ara-uridine is stronger modified by $KMnO_4$, NH_2OH and HCOOH, respectively. The normal band spacing between the 5th and 6th band indicates a complete β-elimination of the carbohydrate moiety which leads to the desired product 32-pdApdCpdGpdGpdAp. In contrast, the oligonucleotide containing 2'-deoxy-2'-fluoro-uridine shows a sequencing pattern with an irregular band spacing at the 3'-end. Thus, 2'-deoxy-2'-fluorouridine is characterized by two strong bands in the A+G- and T>Pu-reaction and two slight bands in the G- and C-reaction.

In contrast to the β-elimination products of ara-uridine, the cleavage products of 2'-deoxy-2'-fluoro-uridine move slower through the PAA gel. This is due to their higher weight. To explain the unusual degradation pattern of 2'-deoxy-2'-fluorouridine it is necessary to assume that the β-elimination of 2'-deoxy-2'-fluorouridine proceeds not to completion. As a result an elimination product of the following typ 32-pdApdCpdGpdGpdAp-R (R=alkyl) with higher molecular weight is formed. Probably, there is no butadiene derivative formed from the carbo-hydrate moiety during the elimination process of 2'-deoxy-2'-fluorouridine.

The degradation of the carbohydrate takes place at another position yielding an alkyl substituted 3'-phosphate residue as the elimination product. In addition, 2'-deoxy-2'-fluorouridine shows a further band in the T>Pu-reaction moving at the same position like ara-uridine in the PAA gel. Likely, a small fraction of 2'-deoxy-2'-fluorouridine is partly transformed into ara-uridine during the piperidine reaction. In order to confirm this assumption, the reaction of 2'-deoxy-2'-fluorouridine with piperidine has been studied. After short treatment of 2'-deoxy-2'-fluorouridine with hot piperidine remarkable traces of ara-uridine were identified by t.l.c..

The reaction products of both hexamers with boiling piperidine are shown in lane 2 and 6 (FIG.4). Compounds I and II were dissolved in 10 % piperidine, 2 h boiled, lyophilyzed and subjected to the gel. It is clearly shown from the band pattern of these lanes that 10 % piperidine is able to induce a substantial hydrolysis (β-elimination) of

2′-deoxy-2′-fluorouridine and ara-uridine without any former chemical modification reactions of the heterocyclic base uracil. It has to be noticed that the above mentioned slight bands in the G- and C-reaction for 2′-deoxy-2′-fluorouridine and in the G-reaction for ara-uridine are due to this observed instability of both derivatives.

The results presented here demonstrate that the C-2′-fluoro linkage of 2′-deoxy-2′-fluorouridine is unstable under alkaline conditions such as ammonia or piperidine if this monomer is incorporated at the 3′-end of the oligomer chain. In contrast there is no evidence for any hydrolysis of the C-2′-F-linkage if 2′-deoxy-2′-fluorouridine is incorporated at other positions of the chain, where the 3′-function is substituted by the phosphate group. This has been confirmed by synthesizing and sequencing a heptanucleotide whereby the 2′-deoxy-2′-fluorouridine residue was incorporated at the 6th position from the 5′-end (data not shown).

CONCLUSION

Oligonucleotides containing 2′-deoxy-2′-fluorouridine at their 3′-end show some instability under special alkaline conditions. Thus, 2′-deoxy-2′-fluorouridine is likely transformed to ara-uridine under the conditions of oligonucleotide synthesis especially during the removal of the N-acyl protecting groups. This reduces either the yield of the final product and makes it difficult to isolate the desired oligonucleotide. The mechanism of transformation of 2′-deoxy-2′-fluorouridine to ara-uridine in the intact oligonucleotide is not definitively established yet. It seems to be likely that a base catalyzed formation of O-2,2′-anhydrouridine and its splitting under alkaline conditions could be responsible for this transformation.

Uridine derivatives containing 2′-substituted carbohydrate moities selectively react under the conditions of chemical degradation in the sequencing analysis and, therefore, can easily be identified in sequencing pattern due to their different behaviour of elimination.

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15. unpublished results

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