PREPARATION OF DNA DUPLEXES CONTAINING 2'-FLUORO-2'-DEOXYURIDINE AND ARABINOSYLURACIL

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The synthesis of two 14-membered duplexes containing 2'-fluoro-2'-deoxyuridine and 1-β-D-arabinosyluracil, respectively., has been effected by chemical ligation (CL). During the synthesis of the first of them, it was observed that the ammonolysis of the completely blocked ACGGAU$^{2'F}$ was accompanied by the transformation of the modified unit into arabinouridine with the formation of 45% of ACGGAU^{ara}. It has been established that the introduction of an electron-accepting substituent adjacent to the reacting 3'-OH group has no influence on a characteristic feature established previously: CL is considerably more effective if the reacting phosphate group is present at the 3'-end, and not the 5'-end, of the oligonucleotide.

One of the possible methods for the direct introduction of modifications into double helical DNAs is chemical ligation (CL). This method consists in the condensation on a complementary template under the action of chemical reagnets of oligonucleotides one of which bears a modified unit at the 3'- or the 5'-end of the chain. The CL method is particularly convenient when it is necessary to obtain a series of duplexes differing by single substitutions in one of the chains, since on direct chemical synthesis the introduction of each modification presupposes the development of an individual scheme of synthesis.

However, the CL reaction has its own specific nature, owing to the complementation interactions of the reacting oligomers within the duplexes. We began a study of the general laws of the CL reaction on a series of short DNA duplexes differing by the structure of the units adjacent to the break [1]. It was found that one of the factors affecting the efficacy of template reactions is the nature and orientation of the functional groups in the reaction center. Thus, the replacement in the reaction center of 3'-terminal thymidine by uridine leads to a sharp fall in the yield of desired product [2]. This was explained by conformational differences between ribo- and deoxyribonucleosides in the double helix.

Developing these investigations in order to elucidate the role of substituents in position 2' of the phosphate-accepting nucleoside, we have constructed duplexes containing 2'-fluoro-2'-deoxyuridine (duplexes (Ia and b)) and 1-β-D-arabinosyluracil (duplex (Ic)) and have studied the influence of these nucleosides on the CL reaction.

(5′—3′) ACGGAX ↓ YCAGGAGTCAG
(3′—5′) GCCTA GGTCCTCAG

Duplex I

(the prefix d has been omitted, and the arrow shows the position of the single-strand break)

a: $X=U^{2'F}$	$Y=pC$	d: $X=T$,	$Y=pC$
b: $X=U_p^{2'F}$	$Y=C$	e: $X=U$,	$Y=pC$
c: $X=U^{ara}$	$Y=pC$		

For comparison, in parallel with duplexes (Ia-c), CL was performed in duplexes (Id and e) containing thymidine and uridine, respectively, as acceptors.

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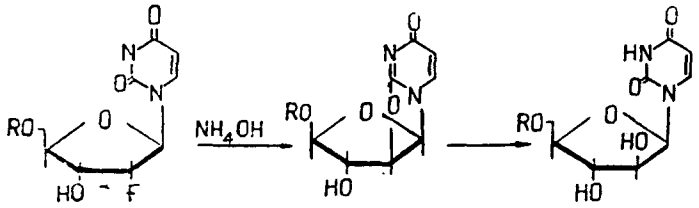
Duplexes (Ia-e) were constructed on a common principle: they all contained a 14-membered template and, complementary to it, an 11-membered oligomer and a hexanucleotide, the latter differing by the structure of the units adjacent to the break. It must be mentioned that the duplexes investigated contained a section of recognition for the restriction endonuclease RcoRII, and in duplexes (Ia-c) the modified nucleoside was immediately adjacent to this section. It was therefore obvious that these duplexes are of interest as analogs of substrates for the endonuclease EcoRII.

As the condensing reagent in CL we used cyanogen bromide, the advantages of which in comparison with a water-soluble carbodiimide we have described previously [3]. The efficiency of CL under the action of cyanogen bromide depends to a considerable degree on the position (3' or 5') of the phosphate group in the reaction center: on the use of 3'-phosphorylated oligonucleotides the yield of desired product is twice as great as in the case of 5'-phosphorylated derivatives [3]. In order to determine whether this relationship was retained when the reaction was performed with the participation of 2'-fluoro-2'-deoxyuridine (a nucleoside containing a strongly electronegative substituent in the 2'-position of the furanose, it appeared desirable to synthesize two hexanucleotides) ACGGAU$^{2'F}$ and ACGGAU$^{2'F}$p. The latter was obtained by a method described previously from ACGGAU$^{2'F}$U by periodate oxidation and the subsequent β-elimination of the 3'-terminal uridine [4].

Hexa- and heptanucleotides containing 2'-fluoro-12'-deoxyuridine residues were synthesized on a Viktoriya 4M automatic synthesizer by the phosphoramidite method. For this purpose, in the preparation of ACGGAU$^{2'F}$ MeOTrU$^{2'F}$ was added to the support by the procedure described in [5], and in the preparation of ACGAU$^{2'F}$U MeOTr-uridine was added similarly. After the deblocking of the resulting hexanucleotide by successive treatment with concentrated NH_3 (50°C, 12 h) and 80% CH_3COOH solution, two oligonucleotides in approximately equal amounts were detected in the reaction mixture by reversed-phase HPLC. After the hydrolysis of the nucleotide material of both peaks with a mixture opf PDE and PME from snake venom and comparison with control mixtures, it was found that the hydrolysates differed only by the fact that the first contained U$^{2'F}$ and the second U^{ara}. The possibility of the partial conversion of 2'-fluoro-2'-deoxyuridine into arabinosyluracil on ammonolysis had been detected previously [6]. The mechanism of this transformation can be represented by the following scheme:



R—N-blocked oligonucleotide

Thus, as the result of the synthesis performed it was possible to obtain simultaneously two hexanucleotides one of which contained 2'-fluoro-2'-deoxyuridine and the other arabinosyluracil at the 3'-end. When a blocked heptanucleotide in which 2'-fluoro-2'-deoxyuridine was not the terminal unit was treated with ammonia, no by-products were formed.

The synthesis of the 14- and 11-membered oligomers entering into the composition of the duplexes under investigation has been described previously [6]. Chemical ligation was carried out by a procedure developed previously [3]. Table 1 gives the oligonucleotide composition of the duplexes and the yields of ligation products, which were determined from the radioactivities of the corresponding zones on electrophoresis in 20% polyacrylamide gel.

It can be seen from the figures given in Table 1 that chemical ligation in the duplexes studied took place with different efficiencies. In duplexes (Ia) and (Ib), containing U$^{2'F}$ in the reaction center, the dependence of the yield of desired product on the position of the phosphate groups that had been detected previously for unmodified duplexes was preserved: the yield was considerably greater when the phsophate group was present at the 3'-end of the hexanucleotide. This is explained both by the greater reactivity of the 5'-OH groups of nucleosides than of the 3'-OH groups and also by the more rigid fixation of the phosphate group in the 3'-position of the furanose moiety as compared with the 5'-position.

The yields of the products of CL in duplexes (Ia) and (Ib) were close to one another, amounting to only 16-17%. Consequently, the electron-accepting nature of the substituent adjacent to the reacting 3'-OH group has no influence on the yield of CL.

TABLE 1. Oligonucleotide Compositions of Duplexes (Ia-e) and Yields of Products of BrCN-Induced Condensation

Number of the duplex	Oligonucleotide composition*	Yield, %
Ia	$6U^{2'F}$+*p11+14	16
Ib	*p$6U^{2'F}$p+11+14	91
Ic	$6U^{ara}$+*p11+14	5
Id	6T+*p11+14	41
Ie	6U+*p11+14	17

*The figures denote the lengths of the oligonucleotide chains, and in the case of the hexanucleotide the 3'-terminal nucleoside is shown; for example, 6U represents ACGGAU, 6T – ACGGAT, etc.; *p – ^{32}P-labeled phosphate.

The efficiency of the reaction was apparently determined mainly by conformational factors. As in uridine, in 2'-fluoro-2'-deoxyuridine the carbohydrate fragment exists in the N conformation (3'-endo-) that is characteristic for the A form of nucleic acids. Since the neighboring section of the duplex formed by the hexanucleotide was present in the B form, the adjacency of the two different conformation apparently created a situation unfavorable for CL. The low yield of the products of CL in duplex (Ic) was not surprising, since the U^{ara} fragment has the N conformation with the unsuitable, remote from the reaction center, equatorial position of the acceptor 3'-OH group.

EXPERIMENTAL

The following materials were used: domestic nucleosides (Omutninsk); 2'-fluoro-2'-deoxyuridnie from Humboldt University (Germany); MeOTrCl, tetrazole, 1-MeIm, 2,4,6-triisopropylbenzenesulfony chloride (TPS), 2-morpholinoethanesulfonate (MES), 2-mercaptoethanol, Tris, and BrCN from Merck (Germany); N,N'-methylenebisacrylamide, acrylamide, ammonium persulfate, and ehylenediamine from Reanal (Hungary); spermidine from Fluka (Switzerland); ATP, Bromophenol Blue, Xylene Cyanol, and N,N,N',N'-tetramethylenediamine from Serva (Germany); and [γ-^{32}P]ATP with a specific radioactivity of 1000 Ci/mmole from Izotop (USSR). The following buffer solutions were used: 1) 0.05 M Tris borate buffer, pH 8.3, 0.001 M EDTA; 2) 1 M potassium acetate, pH 8.0; 3) 0.05 M Tris-HCl, pH 9.0, 0.01 M $MgCl_2$, 0.005 M dithiothreitol, 0.002 M spermidine; 4) 0.25 M MES, pH 7.5, 0.02 M $MgCl_2$; 5) 0.05 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.001 M EDTA, 0.01 M 2-mercaptoethanol; and 6) 0.2 M Tris-HCl, pH 8.5.

Electrophoresis was conducted in 20% polyacrylamide gel 0.3 mm thick in the presence of 7 M urea in buffer 1 at a constant voltage of 1000 V. The yield of CL product was determined from the ratio of its radioactivity to the total radioactivity of the initial [5'-^{32}P]-hexa- or undecanucleotide and the product. The oligonucleotides were eluted from the gel with buffer 2.

The 5'-^{32}P-phosphorylation of the oligonulceotides was performed in 10 µl of buffer 3 containing 0.1 mM oligonucleotide, 10 µCi of [γ-^{32}P]ATP, and 1 activity unit of T4 polynucleotide kinase at 37°C for 30 min. The 5'-^{32}P-labeled oligonucleotides were isolated by electrophoresis in 20% PAAG.

Chemical ligation in duplexes (Ia-e) was carried out in buffer 4 using freshly distilled BrCN by a procedure described previously [3].

2'-Fluoro-5'-O-monomethoxytrityl-2'-deoxyuridine 3'-(Methyl N,N-Biisopropylphosphoramidite). A mixture of 1 g (1.9 mmole) of $MeOTrU^{2'F}$ obtained by a standard procedure and 0.171 g (0.95 mmole) of diisopropylammoniotetrazolide was dried in vacuum for 12 h, and was then dissolved in 10 ml of methylene chloride, and 0.65 ml of methyl bis-N,N-diisopropylphosphoram-

idite was added. The reaction was monitored in TLC in the chloroform–ethanol (9:1) system with the addition of 2% of triethylamine. After 1 h, the reaction mixture was poured into a saturated solution of $NaHCO_3$ and was washed with a saturated solution of NaCl. The organic layer was dried with Na_2So_4 and was evaporated in a rotary evaporator. Yield 90%.

Hydrolysis of ACGGAU$^{2'F}$ and ACGGAU^{ara} with a Mixture PME and PDE from Snake Venom. A solution of 0.7 OU (10 mmole) of one of the oligonucleotides in 30 μl of water was treated with 15 μl of buffer 6 and then with 15 μl each of solutions of PME and PDE (the concentration of each enzyme in the reaction mixture was 0.1 mg/ml). The mixture was kept at 37°C for 3 h, and then the enzymes were extracted with chloroform–isoamyl alcohol (24:1). The hydrolysate was analyzed by HPLC on a Tracor chromatograph (Netherlands) with a 0.46 × 25 cm column containing the support Ultrasphere octyl. Standard conditions: linear gradient (3-35%) of methanol in 0.1 M ammonium acetate; rate of elution 1 ml/min; temperature 40°C. The enzymatic hydrolysate was compared with a control mixture containing nucleosides in a ratio corresponding to the nucleoside composition of the oligomer under investigation.

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FUSARENON X AND 7-DEOXYNIVALENOL IN CULTURES OF *Fusarium graminearum* ISOLATES

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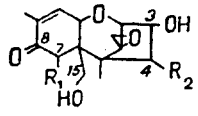
It has been established that the biomass of isolates of *Fusarium graminearum* Schw. that form nivalenol on grain contain two of its structural analogs — 7-deoxynivalenol and 4-acetylnivalenol (fusarenon X). The substances were identified by a combination of chromatographic characteristics and the chemical-ionization mass spectra of their complete trimethylsilyl ethers. 7-Deoxynivalenol is a new, not previously described, metabolite of fusariogenic nature.

We have previously reported the presence of nivalenol (3,4,7,15-tetrahydroxy-12,13-epoxytrichothec-9-en-8-one) (I) in the biomass of an isolate of *Fusarium graminearum* Schw. 15/2 VNIIVS [1]. We have now identified nivalenol (I) in the biomasses of five other isolates of the same species of fungus from feed grain attacked by fusarial wilt.



I. $R_1=R_2=OH$
II. $R_1=OH$, $R_2=OCOCH_3$
III. $R_1=H$, $R_2=OH$

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