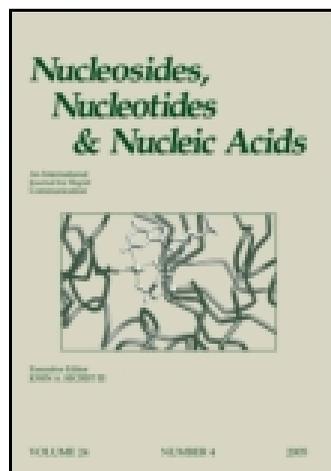


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Synthesis of a new Photo-Cross-Linking Nucleoside Analogue Containing an Aryl(Trifluoromethyl)Diazirine Group: Application for Eco RII and Mva I Restriction-Modification Enzymes

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SYNTHESIS OF A NEW PHOTO-CROSS-LINKING NUCLEOSIDE
ANALOGUE CONTAINING AN ARYL(TRIFLUOROMETHYL) DIAZIRINE
GROUP: APPLICATION FOR *EcoRII* AND *MvaI* RESTRICTION-
MODIFICATION ENZYMES

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Abstract: A new photo-cross-linking dU analog, 5-[4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl]-2'-deoxyuridine, was synthesized and incorporated into the recognition site of *EcoRII* and *MvaI* restriction-modification enzymes. The resulting base-modified 14-mer substrate was tested for cross-linking to these enzymes. Cross-linking is effected by irradiation of the enzyme-substrate complexes at 366 nm.

INTRODUCTION

Photochemical site-specific cross-linking methods employing photoactive derivatives of nucleotides find a broad range of applications in the elucidation of nucleic acid structure and in the analysis of protein-nucleic acid complexes¹⁻³. A great number of photoactive nucleotide analogues have been synthesized that have different cross-linking groups, excitation maxima, the cross-linker position on the nucleotide (base, sugar or phosphate) and length of the cross-linker⁴. Among the derivatives of uridine, the more attractive analogues contain photoactive groups at the C-5 position of the pyrimidine ring. This position is not involved in hydrogen bonding and is exposed into the major groove of DNA double-helix. This allows significant steric tolerance, and makes the C-5 position ideal for attachment of any reporter groups, with little effect on hybridization.

Previously, bromo- and azido-derivatives were predominantly used as pyrimidine derivatives carrying photoreactive groups at position 5⁵. Azido-

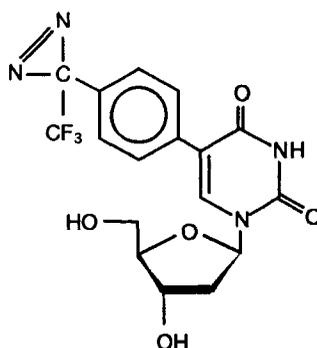
derivatives are more reactive than halogen-containing analogues and continue to play a role as the most commonly used class of reagents. However, they have some disadvantages characteristic of nitrene-generating compounds.

Aryl(trifluoromethyl)diazirines (Atfmd) are known to represent a promising class of photolabelling reagents. They are remarkably stable in a variety of chemical conditions and are efficiently photolysed at 350 nm. This wavelength is far from the usual absorbance maxima of proteins and nucleic acids. Much progress has been made in the development of synthetic methods and approaches to introduce Atfmd-photoreactive groups into peptides, proteins and hydrophobic components of membranes ⁶. Analogues of nucleotides containing Atfmd-function at the nucleic base have not yet been described to date. There was a publication on the synthesis of unnatural 2'3'-dideoxyuridylate analogs bearing Atfmd group connected to the C-5 position of the uracyl ring through a vinyl linker ⁷.

We report here a multi-step synthesis and characterization of a new photoactive reagent - 5-[4-(3-(trifluoromethyl)-3H-diazirin 3-yl)phenyl]-2'-deoxyuridine (I) (FIG.1), its incorporation into the synthetic oligodeoxynucleotide and the use in photoaffinity labelling of *Eco*RII and *Mva*I restriction-modification (R-M) enzymes.

The attachment of Atfmd-substituent directly to the C-5 carbon atom of the heterocycle without any linker allows one to reduce the size of photoactive group; its distance from heterocyclic ring is equal to about 7Å. This deoxyuridine analogue may be considered as a thymidine analogue in which 5-methyl group is replaced by Atfmd group. The compound I (FIG.1) might be a promising photoactive probe for investigation of nucleic acid-protein and nucleic acid-nucleic acid interactions.

Using phosphoramidite approach nucleoside I was incorporated into oligonucleotide 5'-GCCAACCCdU*GGCTCT (II) to yield a substrate analog of *Eco*RII restriction endonuclease (*R.Eco*RII) and DNA methyltransferases (*M.Eco*RII and *M.Mva*I). These enzymes recognize a 5'-CCT/AGG-3' sequence in DNA. For enzyme activity, cofactors are needed: S-adenosyl-L-methionine, which is converted to S-adenosyl-L-homocysteine (AdoHcy) for the methylases and Mg²⁺ ions for the endonucleases. We aimed to construct a 14-mer DNA



I

FIG.1. The structure of 5-[4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl]- 2'-deoxyuridine (dU*).

duplex III:

5'GCCAACCdU*GGCTCT
3'CGGTTGG-A-CCGAGA (III).

In this duplex, the dT residue of the center of the *Eco*RII (*Mva*I) recognition site is replaced by modified dU* for photoaffinity labelling of the enzymes.

RESULTS AND DISCUSSION

The synthetic route used in the present study to prepare the title compound is depicted in FIG.2 and FIG.3.

The key compound, 4'-(3-(trifluoromethyl)-3H-diazirin-3-yl)iodobenzene (IX), bearing photoaffinity label was prepared starting from 1,4-diiodobenzene (IV) in five steps (FIG.2). The latter was converted to 4'-iodo-2,2,2-trifluoroacetophenone (V) by reaction with BuLi, followed by ethyl trifluoroacetate treatment as described⁸. The subsequent steps were carried out as in the procedures described with minor modifications⁹. The structures of the compounds obtained were confirmed by mass spectrometry.

Starting from deoxyuridine, 5-chloromercuro-2'-deoxyuridine (X) was obtained by the technique reported by Chang et al.¹⁰ and then was coupled with

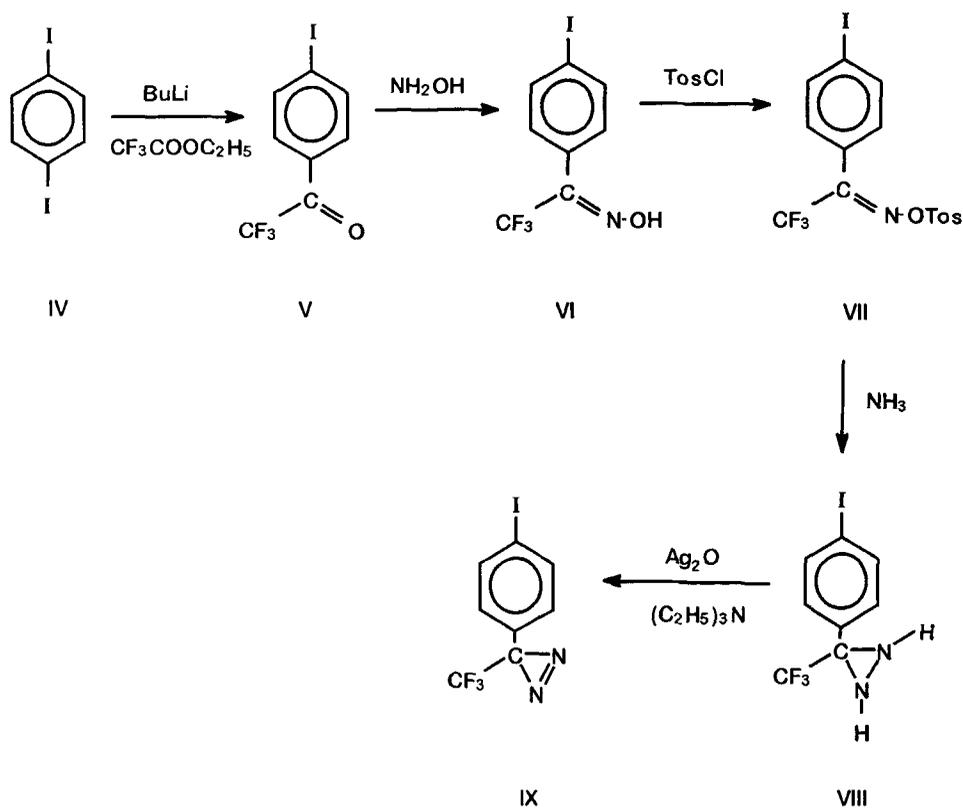


FIG. 2. Synthetic scheme for 4'-(3-(trifluoromethyl)-3H-diazirin-3-yl)iodobenzene (IX).

IX in the presence of a palladium catalyst (FIG.3). Final compound I was obtained in a 10 % yield. This low yield can be explained by uncomplete reaction between IX and X: some amounts of unreacted X were found in the reaction mixture.

The presence of Atfmd-group at the 5 position of uracil residue in I was confirmed by UV, ^1H NMR spectra and MS data. Treatment of I with dimethoxytrityl chloride in dry pyridine and purification of the product on a silica gel column using a chloroform-methanol mixture resulted in 5'-O-dimethoxytrityl-5-[4-(3-(trifluoromethyl)-3H-diazirin-3-yl) phenyl]-2'-deoxyuridine (XI) as a chromatographically pure compound in a 93 % yield. The corresponding 5'-O-dimethoxytrityl-5-[4-(3-(trifluoromethyl)-3H-diazirin-3-yl) phenyl]-2'-

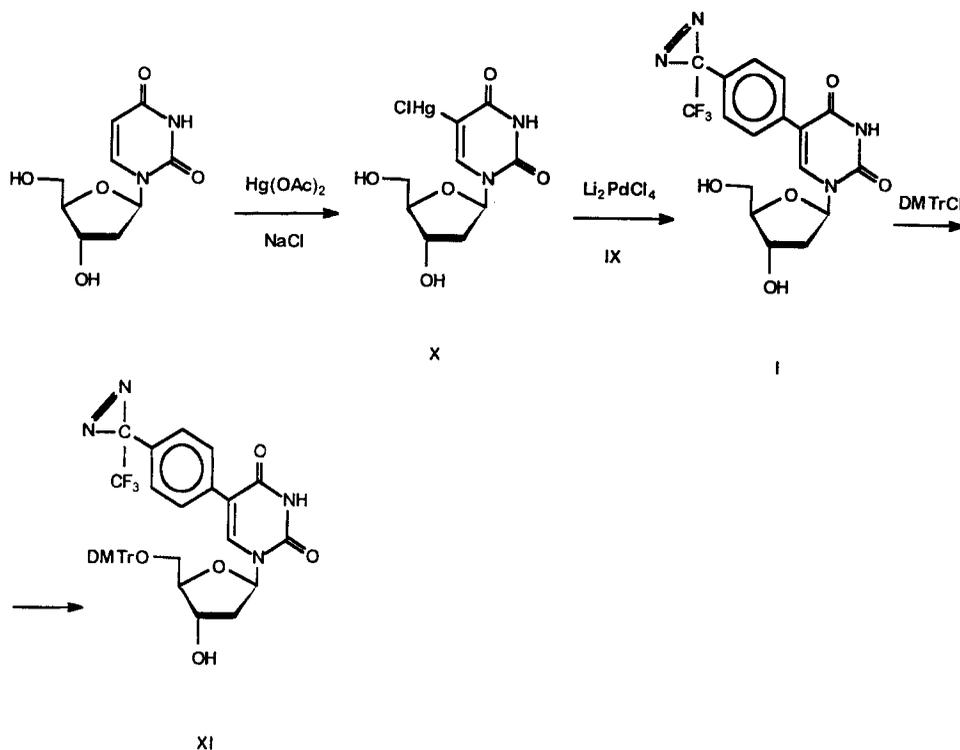


FIG. 3. Synthetic scheme for 5'-dimethoxytrityl-[4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl]-2'-deoxyuridine (XI).

deoxyuridine-3'-O-(2-cyanoethyl)-N,N'-diisopropylphosphoramidite was synthesized by a standard procedure using chloro-(2-cyanoethoxy)-diisopropylaminophosphine¹¹. The modified oligonucleotide II was prepared on an automated synthesizer and purified by reverse phase high-performance liquid chromatography. The low coupling yield of the modified phosphoramidite (about 40%) was observed. After HPLC purification we obtained pure product (FIG. 4) (retention time: 21.39 min).

Sequence analysis of II by a Maxam and Gilbert method confirms the structure of the oligonucleotide. The modified dU* residue behaves similar to dT residues in reaction with KMnO_4 .

To determine the influence of the photomodified nucleoside dU* on the stability of the duplex III, we have measured T_m of III in comparison with the

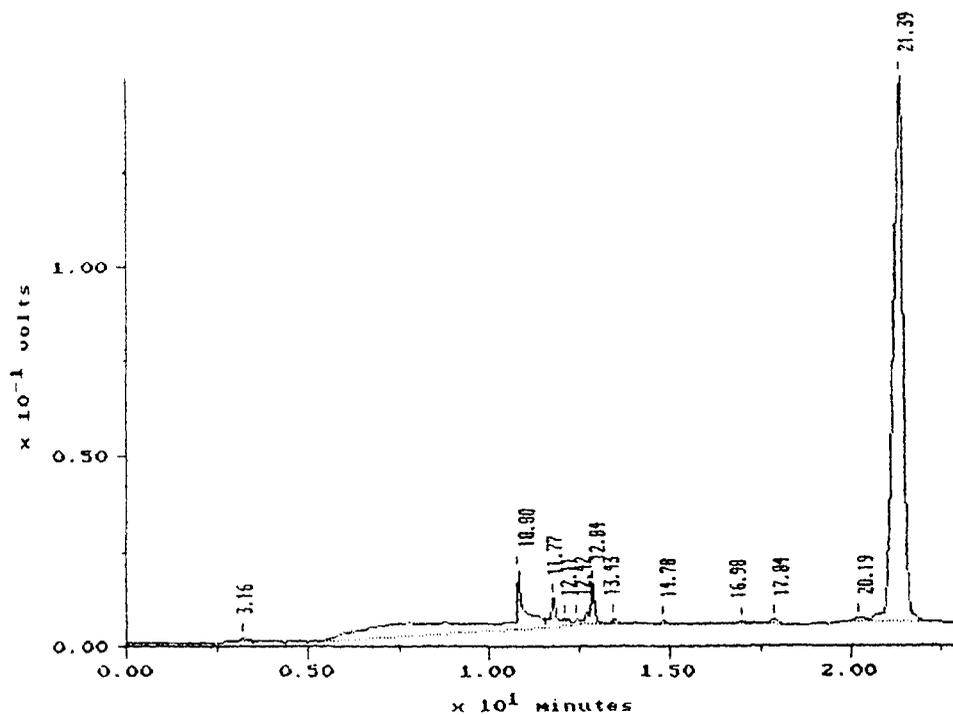


FIG. 4. HPLC analysis of the purified product (oligonucleotide II). Conditions see in "MATERIALS AND METHODS".

corresponding regular DNA duplex:

5'GCCAACCTGGCTCT
3'CGGTTGGACCGAGA.

T_m of III is 60°C, which is four degrees less than T_m of the regular duplex. One may conclude that the incorporation of a photoreactive Atfmd group results on a small decrease of the stability of the double-helical structure.

The ternary complexes of *EcoRII* and *MvaI* methylases were formed with radiolabelled reagent III in the presence of the cofactor analog AdoHcy by incubating the reaction mixtures for 5 min at room temperature and for 15 min at 0°C. After formation of complexes, UV irradiation (366 nm) was used to create a DNA-protein cross-link (FIG.5). The cross-linking of the reagent III to *EcoRII* restriction endonuclease in the presence and in the absence of Mg^{2+} ions has been performed as in the case of the methylases (FIG.5).

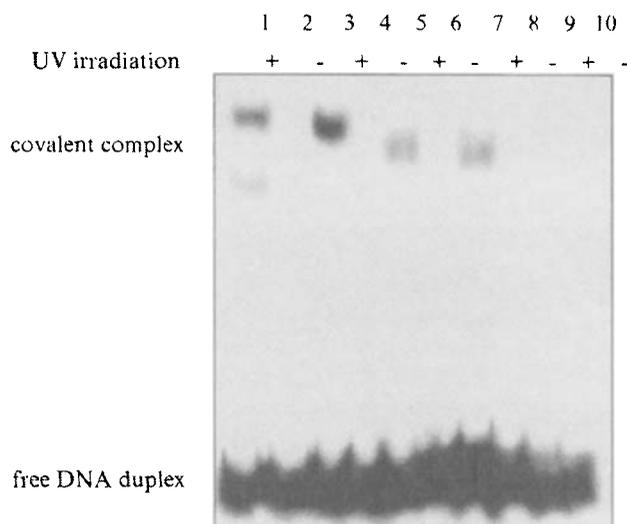


FIG.5. Cross-linking of Atfmd-containing DNA duplex III to *M.EcoRII* (lanes 1, 2); *M.MvaI* (lanes 3, 4); *R.EcoRII* in the presence (lanes 5, 6) and in the absence (lanes 7, 8) of Mg^{2+} . Lanes 9, 10: duplex III in the buffer B. UV irradiation: 366 nm for 30 min. Electrophoresis: 8% polyacrylamide gel containing 0.1% SDS.

To destroy the non-covalent complexes, the reaction mixtures were treated with SDS. SDS-PAGE/autoradiography revealed formation of cross-linked reagent III-protein complexes for all three enzymes. No cross-linking takes place without irradiation. Also, the control experiments were done in the absence of R-M enzymes and in the presence of albumin - containing buffer (FIG.5). It was shown that regular DNA-substrates do not crosslink to the *EcoRII* and *MvaI* R-M enzymes upon irradiation at 300 nm (data not shown).

The yields of the cross-linked products were 9, 13 and 6% for *M.EcoRII*, *M. MvaI* and *R.EcoRII*, respectively. No difference in the cross-linking of *R.EcoRII* to III in the presence and absence of Mg^{2+} ions was observed. Thus, the DNA duplex with photoactive Atfmd group incorporated in the *EcoRII* (*MvaI*) recognition site is capable of producing high-yield photo-cross-linking to *EcoRII* and *MvaI* restriction - modification enzymes. It is noteworthy, that cross-linking occurs at soft irradiation conditions (366 nm). The identification of nucleotide - amino acid point contacts in the photo-cross-linked DNA-enzymes complexes is in progress

now. The data described above show that DNA duplexes containing photoactivatable Atfmd-group in the recognition site of DNA-methyltransferases or restriction endonucleases are effective and promising reagents for affinity labelling of the enzymes. The UV-cross-linking procedure developed may be recommended for affinity labelling of other DNA - binding enzymes.

MATERIALS AND METHODS

2'-Deoxyuridine, dimethoxytrityl chloride (Sigma), the other reagents (Russia) were used. All chemicals and solvents were purified and distilled before use. Reagents for automatic DNA synthesis were from Eurogentec (Belgium). Melting points were determined on a PHMK apparatus (VEB Wagetechnik Rapido). Thin-layer chromatography (TLC) was performed on precoated silica gel 60 F₂₅₄ plates (Merck). Visualization was done with UV light and I₂. Diaziridines were detected by spraying with ninhydrine. The following solvents were used: (A) chloroform; (B) dichloromethane/petroleum ether (3:1); (C) petroleum ether/chloroform (1:2); (D) diethyl ether/methanol (20:1); (E) chloroform/methanol (95:5); (F) chloroform/methanol (90:10). Column chromatography was performed on silica gel L 40/100 (Chemapol). Fast atom bombardment mass spectrometry (FAB MS) was done in the Mass Spectrometry Laboratory of the Institute of Bioorganic Chemistry (Moscow). UV-spectra and thermal melting curves were obtained using a Hitachi 150-20 spectrophotometer (Japan). ¹H NMR-spectra were obtained with a VXR-400 spectrometer.

4'-Iodo-2,2,2-trifluoroacetophenone (V) was obtained by the technique described⁸.

Oxime 4'-iodo-2,2,2-trifluoroacetophenone (VI)

Solution of V (19 g, 63.3 mmol) in 20 ml of dry pyridine was slowly added to a solution of hydroxylamine hydrochloride (4.9 g, 69.5 mol) in the mixture of 40 ml of dry pyridine and 20 ml of abs. ethanol with stirring. After being stirred for 4 hours at 60°C, the mixture was concentrated under reduced pressure. The resulting oil was dissolved in 100 ml of diethyl ether and organic solution was extracted with 0.1 M HCl (2x100 ml), H₂O, brine and dried (Na₂SO₄). Diethyl ether was evaporated to give product as white needles. This material was used for the next step without further purification. The small amounts of VI were purified by

column chromatography on silica gel using chloroform as eluent. Yield 91%, m.p.96°C, Rf (A) 0.27, Rf (B) 0.36. MS: m/z (%) 315 (100) [M+], 299 (35.5), 265 (1.2), 253 (4.1), 246 (5.7), 230 (48.0), 229 (64.2), 204 (8.3), 203 (22.7).

O-p-Tosyloxime 4'-iodo-2,2,2-trifluoroacetophenone (VII)

A solution of VI (20.2 g, 63.3 mmol) in abs. pyridine (50 ml) was added to a solution of p-toluenesulfonylchloride (15.09 g, 79.13 mmol) in 200 ml of abs. pyridine, placed in two-necked, round bottomed flask, equipped with a magnetic stirrer, a reflux condenser with a CaCl₂ drying tube. The solution was refluxed for 2 hours. Analysis of the reaction mixture was carried out by TLC in chloroform. After evaporation of pyridine, the residue was dissolved in ether (250 ml), the resulting solution was washed with H₂O (250 ml x 2) and dried (Na₂SO₄). Ether was removed, the residue was purified by flash chromatography (silica gel, chloroform) to give VII (22.3 g, 47.5 mmol) as a colorless solid. The yield 75.1%, m.p. 131-133 °C, Rf (A) 0.82, Rf (C) 0.49. MS: m/z (%) 469 (1.6) [M+], 299 (93.3), 230 (100), 229 (37.9), 203 (5.5), 155 (31.3), 127 (8.6), 115 (10.9), 103 (55.1), 102 (36.3).

4'-Iodo-3-(trifluoromethyl)diaziridine (VIII)

VII (21 g, 44.78 mmol) and abs. ether (200 ml) were placed into a 700 ml steel bomb, which was cooled to -65°C by mixture chloroform-liquid N₂. At this temperature liquid ammonia (38 ml, 1.63 mol) was added and the bomb was hermetically closed. The reaction mixture was slowly allowed to warm to room temperature and was shaken for 12 hours. Then bomb was again cooled by mixture chloroform-liquid N₂ and its content was poured into a 600 ml glass to remove an excess of ammonia. The residue was partitioned between ether and H₂O (each 200 ml), ether phase was washed by H₂O (200 ml), dried (Na₂SO₄) and evaporated. Purification of VIII was done on silica gel column with chloroform as eluent. The yield of VIII was 12.8g (91%), m.p.<65, Rf (A) 0.46. VIII gives specific red color with ninhydrine. MS: m/z (%) 314 (44.5) [M+], 299 (23.3), 293 (23.8), 245 (12.5), 230 (30.6), 203 (4.8), 187 (47.2), 167 (17.5), 166 (14.4) 138 (12.0).

4'-(3-(Trifluoromethyl)-3H-diazirin-3-yl)iodobenzene (IX)

To a solution of VIII (0.75 g, 2.39 mmol) in ether (24 ml), freshly prepared Ag₂O (1.125 g, 4.85 mmol) was added by small portions under stirring. A suspension was stirred for 20 min, the solid was filtered off, washed by ether (15

ml x 3), the filtrate was evaporated in vacuum. IX (0.679 g, 2.17 mmol) was obtained as a colorless liquid. The yield 91.1 %. R_f (A) 0.90. MS: m/z (%) 312 (1.5) [M⁺], 284 (100), 265 (3.9), 231 (4.8), 203 (2.7), 157 (52.8), 137 (34.8), 107 (18.7), 85 (28.1). All procedures with IX were carried out in flasks covered by aluminium foil.

5-Chloromercurio-2'-deoxyuridine (X) was obtained according to Chang et al.⁹ starting from deoxyuridine in the presence of mercuric acetate. Yield 85,7%.

2'-Deoxy 5-[4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl]-uridine (I)

To a suspension of X (370 mg, 0.80 mmol) in methanol (5ml) lithium tetrachloropalladate (223 mg, 0.85 mmol) was added under argon at a room temperature. After 15 min stirring IX (250 mg, 0.80 mmol) was added to the reaction mixture and stirring was continued for 3 days. The resulting suspension was purged with an excess H₂S. The black deposit was filtered off, washed by methanol, the filtrate was evaporated under reduced pressure. The purification of the residual solid by chromatography on a silica gel column with mixture of ether-methanol (20:1) as eluent yielded I (33.3 mg, 0.081 mmol) as a white solid. The yield 10.1 %. R_f (D) 0.39, R_f (ethylacetate) 0.48. UV: max 249, 286, 358; MS: m/z 412. ¹H NMR (MeOD) using tetramethylsilylmethane as an internal standard; δ: 2,32 (m, 2H-2'); 3,78 (qd (AB-part ABX system, J_{A-B} = 11,9 Hz, J_{A-X} = 3,0 Hz), 2H-5'); 3,95 (q (dt, with J_{4-5'} = J_{4-3'} = 3,0 Hz; X-part ABX system), H-4'); 4,44 (q (nonideal) (dt, with J_{3-2'} ≈ J_{3-4'} = 3,0 Hz), H-3'); 6,33 (t, H-1'); 7,23 (d (J = 8,5 Hz), 2h-Ph); 7,70 (d (J = 8,5 Hz), 2H-Ph); 8,43 (s, H-6). 210 mg (0,67 mmol) of unreacted X was isolated from the reaction mixture.

2'-Deoxy (5'-O-dimethoxytrityl)-5-[4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl]-uridine (XI)

I (25mg, 60.7 μmol) (dried over P₂O₅) was coevaporated few times with abs. toluene, then was dissolved in anhydrous pyridine (600 μl). Dimethoxytrityl chloride (90 mg, 270 μmol) was added and the reaction mixture was stirred at room temperature for 4 hours until the starting material (I) completely disappeared (TLC-analysis in petroleum ether-chloroform, 1:1). The solution was evaporated to dryness, coevaporated twice with abs. toluene and was chromatographed on a silica gel column with chloroform-methanol, 10:1 as an eluent to give XI (40.3 mg, 56.4 μmol), the yield 93 %. R_f 0.24 (E), R_f 0.39 (F).

Synthesis of the oligodeoxynucleotides

The O-(β -cyanoethyl)-N,N-diisopropyl-phosphoramidite derivative of compound XI was prepared according to standard procedures ¹¹. The oligonucleotides were synthesized on an automated synthesizer ABI 394B (Applied Biosystems, USA) using CPG as a support and commercial β -cyanoethyl phosphoramidites. Oligomers were prepared on a 1.0 μ mol scale employing the standard synthesis protocols ¹². Modified base phosphoramidite was dried in vacuo and dissolved in anhydrous acetonitrile at the concentration of 0.1M. The solution was introduced at the n-th base position on the machine. Removal of the oligonucleotides from the solid support and deprotection was carried out at 55°C in conc. aqueous ammonia for 3 hours. The oligonucleotides were purified by reversed phase HPLC with dimethoxytrityl "ON" using a RPC18 column (4.6x250mm), eluted with a linear gradient from 15 to 45% B in 45 min (eluent A: 50mM triethylammonium acetate pH 7; eluent B: 50 mM triethylammonium acetate pH 7 in 70% acetonitrile). The purified product was analyzed using equidistant reversed phase HPLC in the ion-pair mode¹³ on Waters chromatograph (Millipore, USA), sorbent Diasorb C-16_T (7.5 mm) of ALSICO (Russia), column size 4x250 mm, column temperature 45°C, in logarithmic 5-40% gradient of acetonitrile concentration in 48 mM potassium phosphate buffer (pH 7.0) containing 2 mM tetrabutylammonium dihydrogen phosphate, flow rate 1 ml/min.

Hybridization studies

Complex formation was monitored by thermal denaturation (T_m) analysis using changes in absorbance at 260 nm as a function of temperature. The experiments were carried out in 40 mM Tris-HCl buffer, pH 7.6, 50 mM NaCl, 5 mM MgCl₂. DNA-duplex concentration was 2×10^{-6} M.

Photoaffinity labeling of EcoRII and MvaI methylases and EcoRII endonuclease

Buffers: **A**, 40 mM Tris-HCl, pH 7.9, 5 mM dithiothreitol (DTT), 1 mM EDTA, 0.1mM AdoHcy; **B**, 50 mM Tris-HCl, pH 9.0, 20 mM NaCl, 1 mM DTT, 100 μ g/ml BSA, 0.1mM AdoHcy; **C**, 40 mM Tris-HCl, pH 7.6, 50 mM NaCl, 7 mM DTT; **D**, 40 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM MgCl₂, 7 mM DTT. Cross-linking of *M.EcoRII* (3.16 μ M) and *M.MvaI* (2.47 μ M) to ³²P-labeled DNA duplex III (0.17 μ M) was performed in 10 μ l of buffers A and B respectively.

Cross-linking of R.*Eco*RII (2.9 μ M) to 32 P-labeled DNA duplex III (0.17 μ M) was carried out in 10 μ l of buffers C and D. The samples were incubated at 25 $^{\circ}$ C for 5 min and at 0 $^{\circ}$ C for 15 min. For cross-linking reactions, the samples were placed on parafilm and UV irradiated (366 nm) for 30 min on ice using a high intensity UV lamp (model UVGL-58). Reactions were followed by 0.1% SDS-8% PAGE after heating the samples in 0.1% SDS at 95 $^{\circ}$ C. The gels were analyzed by autoradiography. The cross-linking yields were determined as the ratio of the covalent conjugate radioactivity to the total radioactivity of the conjugate and unbound DNA.

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