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5-Fluoro-4-thiouridine phosphoramidite: New synthon for introducing photoaffinity label into oligodeoxynucleotides

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ABSTRACT

The synthesis of phosphoramidite of 5-fluoro-4-thio-2′-*O*-methyluridine is described. An appropriate set of protecting groups was optimized including the 4-thio function introduced via 4-triazolyl as the 4-(2-cyanoethyl)thio derivative, and the *t*-butyldimethyl silyl for 2′ and 3′ hydroxyl protection, enabling efficient synthesis of the phosphoramidite. These protecting groups prevented unwanted side reactions during oligonucleotide synthesis. The utility of the proposed synthetic route was proven by the preparation of several oligonucleotides via automated synthesis. Photochemical experiments confirmed the utility of the synthon.

1. Introduction

Photocrosslinking has been shown to be an extremely useful and versatile technique for structural studies in relation to nucleic acids and protein interactions,[1] thus becoming a routine laboratory tool.[1f,1g] Macromolecules can form associates due to different interactions such as hydrogen bonds, charge transfer, and electrostatic attraction. Such weakly and reversibly bound associates, when irradiated with light of a proper wavelength, can form covalent bonds between reactive sites of the macromolecules. Occurrence of this phenomenon is based on the ability of the reactive sites to absorb the energy of the applied light and use it to form the covalent bond. The applied wavelength has to be specific for the reacting parts of the macromolecule and different from the absorption region of the bulk of chromophores present in the molecule. Among the different candidates for the photoprobe moiety, 4-thio-5-halogenopyrimidines appear to be especially suitable.[2] Sulfur substitution causes a bathochromic shift of the main absorption band[3] and increases yield of the first excited triplet state. Halogen substituents enhance the reactivity because of their lability in the excited state.[4] We have observed[2c] that these beneficial effects culminate in the case of 4-thio-5-fluoro-uracil (FSU) nucleoside **1a** (Fig. 1).

FSU efficiently produces a photodimer with a thymidine upon irradiation, and the product exhibits pronounced fluorescent properties.[5] The relatively small steric modification of the pyrimidine base shown in Figure 1 should not interfere with base pairing. We hypothesize, that if this modified nucleoside (FSU) were introduced into an oligonucleotide, and the duplex of this oligonucleotide with its complementary chain were irradiated with UV light of λ >300 nm, efficient cross-links would occur that will engage FSU and thymine residue located in close proximity to the complementary strand. Preliminary experiments suggest that a thymidine moiety next to a complementary adenosine unit would be involved.[5]

2. Results and discussion

For more detailed studies of the photocrosslinking process on the oligomer level, we needed simple and efficient synthetic access to FSU phosphoramidite. Our initial studies with a ribo-FSU **1a** unit

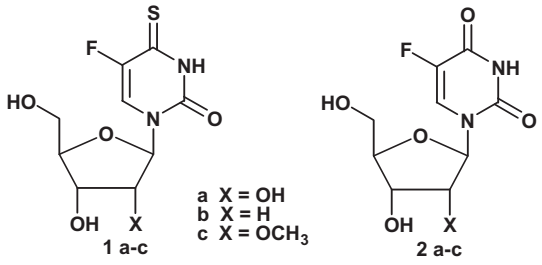

Figure 1. Structures of the modified pyrimidine nucleosides.

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indicated that it has considerable drawbacks due to the necessity of the 2′-hydroxyl protection and low reactivity. From two other candidates, 2′-deoxy-5-fluoro-4-thio-uridine **1b** and 2′-*O*-methyl-5-fluoro-4-thio-uridine **1c**, the 2′-deoxy analog was excluded due to instability in the conditions applied during synthesis.

We turned our attention to 2′-*O*-methyl-FSU nucleoside **1c**. It appeared to be the compound of choice for several reasons. Methylation of the 2′-hydroxyl while masking the hydroxyl reactivity[6] does not affect ability of the oligonucleotide to form duplexes with DNA or RNA[7] nor does it change considerably the conformation of the ribose ring.[8] Oligonucleotides methylated at 2′ position are resistant to base or nuclease hydrolysis[9] and are much less prone to glycosidic bond cleavage under acidic conditions.

As the starting material, we have chosen 5-fluorouridine **2a**. It is easily accessible commercially or synthetically.[10] In the view of literature reports on FSU reactivity,[11] the sulfur atom in the position 4 had to be protected in order to avoid undesired modification during the oligonucleotide synthesis.

From the available protecting groups, we have tested pivaloyloxymethyl[12] and 2-cyanoethyl,[13] which were successfully applied in 4-thiopyrimidines and 6-thiopurines syntheses (Fig. 2). The pivaloyloxymethyl group turned out to be difficult to introduce into the FSU system using the reported procedure.[12] Only a very modest yield of the desired product **4a** was achieved, and considerable decomposition occurred.

Initial attempts to introduce 2-cyanoethyl group via the reaction of **3** with 2-cyanoethyl bromide[13b] did not lead to the desired product **4b**.

Another way was to use 2-cyanoethane thiol[14] to displace the good leaving group[15] in position 4 of the 5-fluoropyrimidine-2-one ring. Approach through displacement of 4-methoxy group[15] in several reaction conditions tested (K_2CO_3, DIPEA or TEA as a base, dioxane, DMF or neat thiol as solvents, elevated temperature, microwave irradiation). Contrary to this, 1,2,4-triazolyl leaving group,[16] in the derivative **6**, was smoothly substituted by thiol in the presence of excess DIPEA, leading to the desired 5-fluoro-4-(2-cyanoethylthio)uridine **4b** (Fig. 3).

Introduction of the 2′-*O*-methyl group posed another problem. Fluorouridine **2c** methylated at 2′ position is commercially available, but is also prohibitively expensive. Direct methylation of 5-fluorouridine can lead to formation of undesired 3-methyl derivative[17] in considerable amounts. Having possession of the 4-*O*-methyl 5-fluorouridine, we tried the methylation of its 5′-*O*-dimethoxytrityl derivative **7a** with the known diazomethane–$SnCl_2$ procedure[18] (Fig. 4). The methylation proceeded easily and with rather strong selectivity towards the desired 2′-*O*-methyl nucleoside product **8a** (**8a**:**9a** isomer ratio equal to 87:13 by ^{1}H NMR). Unfortunately, as was explained earlier, this good result could not be exploited further due to the resistance by the 4-methoxy group to substitution by a sulfur nucleophile.

Fortunately the ease of methylation and selectivity was preserved in the case of 5-fluoro-4-(2-cyanoethylthio)uridine **7b**. The 5′-*O*-DMTr derivative gave the desired 2′-O-methylated product **8b** with excellent 81% isolated yield together with an 8% yield of the 3′-O-derivative **9b**. Both isomers could be efficiently separated by flash chromatography.

To achieve good overall yield of the synthesis, proper arrangement of protecting groups was required. After testing three sets of protections for the starting 5-fluorouridine: 2′,3′,5′-tri-*O*-acetyl, 5′-*O*-dimethoxytrityl-2′,3′-di-*O*-phenoxyacetyl[19] and 5′-*O*-dimethoxytrityl-2′,3′-di-*O*-*t*-butyldimethylsilyl, we found the latter as the most efficient and straightforward, giving good yield of the desired product without excessive by-products formation. Removal of the silyl protection required careful adjustment of the fluoride applied for deprotection. A commonly used solution of TBAF in THF[20] caused almost complete removal of the cyanoethyl protection from sulfur. Milder fluorides such as TEBA–HF hydrate or TEA–HF hydrate[21] were not efficient in removing silyl groups. A popular reagent used for deprotection of silylated oligoribonucleotides, TEA–3HF complex,[22] appeared as the reagent of choice, removing both *t*BDMS groups within several hours without any noticeable side reactions. The synthetic pathway to the 5′-*O*-dimethoxytrityl-4-cyanoethylthio-5-fluoro-2′-*O*-methyluridine **8b** and the phosporamidate **14** is shown in Scheme 1.

Phosphitylation of the protected nucleoside was achieved with a known procedure[23] and gave good yield of product in the form of an approximately 1:1 mixture of diastereoisomers. The phosphoramidite synthon was applied for the automated synthesis of oligonucleotides intended for photocrosslinking studies (for the sequences of the oligonucleotides see Table 1).

The reactivity of the 5-fluoro-4-thiopyrimidine system, had to be taken int account during de-blocking of the synthesized oligomer and 'soft' amino-protecting groups were applied as a precaution. The thiol-protecting 2-cyanoethyl group was removed first by treating the oligomer with DBU base (0.4 M in anhydr acetonitrile) for 15 min while still attached to the solid support. Removing the oligomer from the solid support and de-blocking of base-labile groups followed a routine protocol for 'soft' blocking groups. The optimal procedure consisted of thoroughly washing the support from DBU with anhydrous acetonitrile and drying before deprotection with methanolic ammonia as the next step. With phenoxyacetyl (for adenosine) and 4-isopropylphenoxyacetyl (for guanosine) protection, a 3 h treatment with methanolic ammonia at room temperature was optimal (sufficient for deprotection and removal from the solid support without undue sulfur loss). After evaporation of the ammonia solution, the residue was dissolved in 0.1 M ammonium acetate and freed from low molecular weight contaminants by gel filtration. Final purification was achieved on HPLC (see Fig. 5). As a convenient test for identification of the appropriate HPLC peak and approximate verification of the content of the

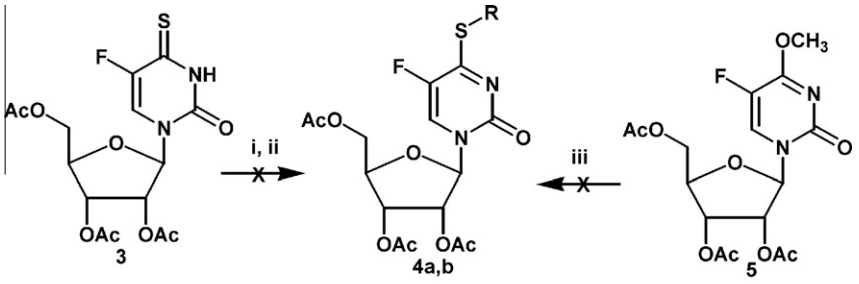


Figure 2. Reagents and conditions: (i) Pivalyloxy methyl chloride, K_2CO_3; (ii) 2-cyanobromoethane, K_2CO_3; (iii) 2-cyanoethanethiol, K_2CO_3 or DIPEA, or TEA, solvents: DMF, THF, neat thiol, w. or w/o MW.

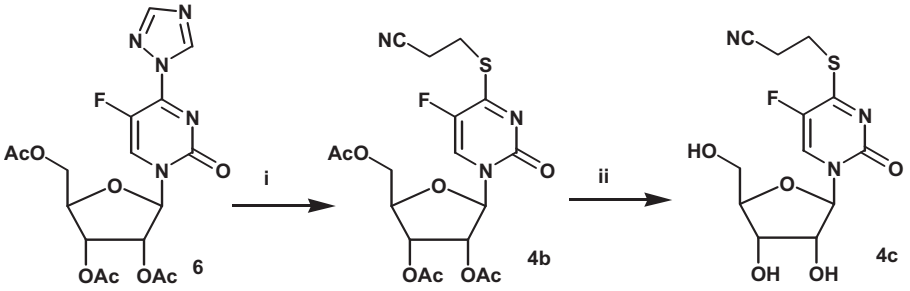


Figure 3. Reagents and conditions: (i) 2-cyanoethanethiol, DIPEA, CH_3CN; (ii) 1.5% aq ammonia.

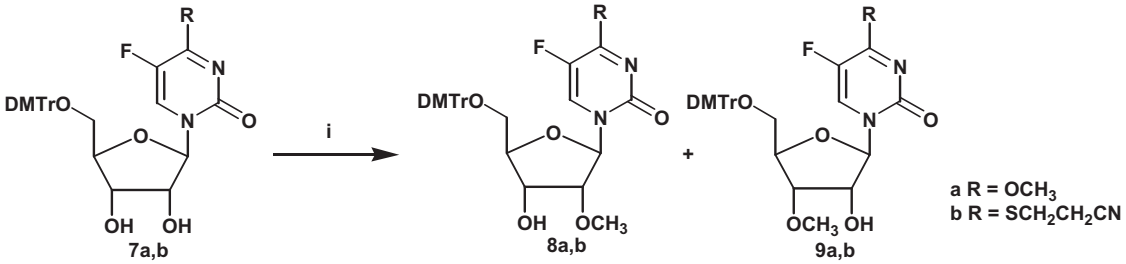
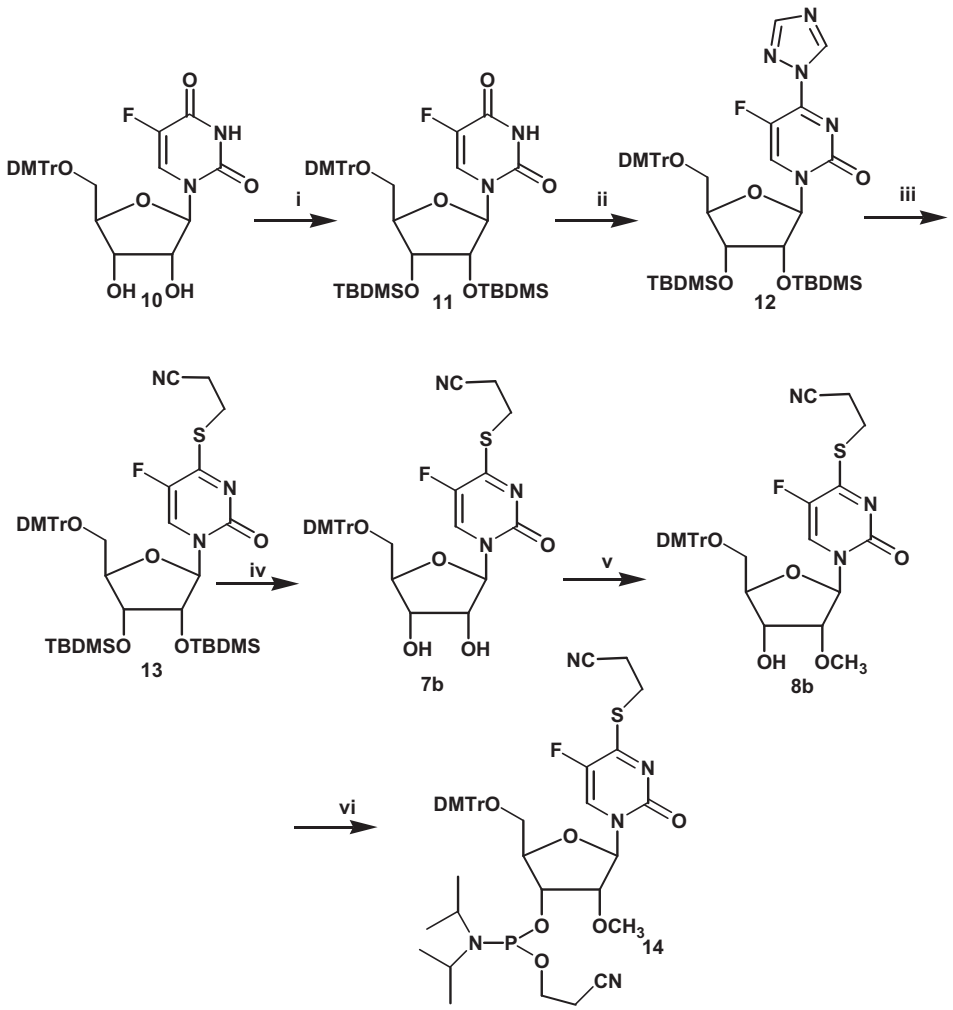


Figure 4. Reagents and conditions: (i) diazomethane (Et_2O solution), $SnCl_2$ hydrate, CH_2Cl_2.



Scheme 1. Reagents and conditions: (i) TBDMSCl, imidazole, DMF; (ii) $POCl_3$, TEA, 1,2,4-triazole; (iii) CH_3CN, $NC(CH_2)_2SH$, DIPEA; (iv) TEA–3HF; (v) CH_2N_2, $SnCl_2{\cdot}2H_2O$, CH_2Cl_2; separation of isomers; (vi) DIPEA, $NC(CH_2)_2OP(Cl)\,N(iPr)_2$, THF.

Table 1

Oligonucleotide	Sequence (X = 5-fluoro-4-thio-2′-*O*-methyl-U), MALDI peaks	OD after gel filtration	OD after purification and removal of TEAA
BS-3	5′-ATA GCA XAG C-3′; 3085 ($M+H^+$, calcd for $C_{98}H_{123}FN_{40}O_{56}P_9S^+$ 3085.5339), 3107 ($M+Na^+$), 3123 ($M+K^+$)	89	62
BS-4	5′-CGA TAC GAX A-3′; 3085 ($M+H^+$, calcd for $C_{98}H_{123}FN_{40}O_{56}P_9S^+$ 3085.5339), 3107 ($M+Na^+$), 3123 ($M+K^+$)	84	53
BS-5	5′-AXA TGC ATA T-3′; 3075 ($M+H^+$, calcd for $C_{99}H_{125}FN_{36}O_{58}P_9S^+$ 3075.5271), 3097 ($M+Na^+$), 3113 ($M+K^+$)	119	41
BS-6	5′-AXA GCA TAG C-3′; 3085 ($M+H^+$, calcd. for $C_{98}H_{123}FN_{40}O_{56}P_9S^+$ 3085.5339), 3107 ($M+Na^+$), 3123 ($M+K^+$)	111	56

4-S modification, absorbance ratio A_{260}/A_{340} was applied. A mean value of 5.11:1 was observed (Fig. 6).

3. Photocrosslinking

To examine the feasibility of photocrosslinking, the photoreaction of BS-4 with its complementary strand ODN 1 was performed. According to the earlier results,[5] the label X can form interstrand crosslink either with thymidine at 5′ side:

BS-4	5' C G A T A C G A **X** A 3'
ODN 1	3' T G C T A T G C **T** A T T 5'
or at 3' side	
BS-4	5' C G A T A C G A **X A 3'**
ODN 1	3' T G C T A T G C T A **T** T 5'

or both crosslinks can be formed, each of them in two possible diastereomeric forms.

Duplex BS-4—ODN 1 ($A_{260} \approx 2.3$ in 0.1 M phosphate buffer (pH 7)) was irradiated using argon–ion laser emitting the light at 351 nm. To be sure that all molecules are in duplex form small excess of complementary strand ODN 1 was added (1.2 equiv) and reaction was carried out at 15 °C (the estimated melting temperature of duplex was above 30 °C). The course of the reaction was followed with UV spectrophotometry. During the reaction, along with the formation of photocrosslink, the pronounced rise of fluorescence was observed (Fig. 7). HPLC analysis (Fig. 8) showed that BS-4 and ODN 1 were consumed after 60 s of irradiation. The reaction gave two fluorescent photoproducts (ODN 2 and ODN 3), with one of them (ODN 3) dominating (it was formed with 90% yield). MALDI-TOF MS indicated that both isolated photoproducts have the same molecular mass which corresponds to calculated mass of single intermolecular crosslink [calcd 6693 for $(M+H)^+$, found 6694.75] and is equal to the sum of mass of BS-4 and ODN 1 minus HF (eliminated during crosslink formation). In the absorption and

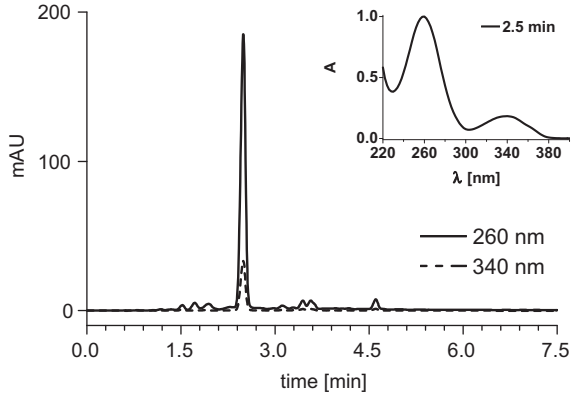


Figure 5. Example HPLC trace for the BS3 oligomer. Separation conditions: Sample injected: 0.5 μl of oligodeoxynucleotide in 1 mL H_2O/Acetonitrile, 95/5, v/v; Waters XBridge OST C_{18} Column, 2.5 μm, 4.6 × 50 mm, mobile phases: A = 0.1 M TEAA, B = 0.1 M TEAA/Acetonitrile, 50/50, v/v, Flow rate: 1 mL/min, column temp = 40 °C, gradient: 15–25%B in 5 min, then to 50%B in 5 min.

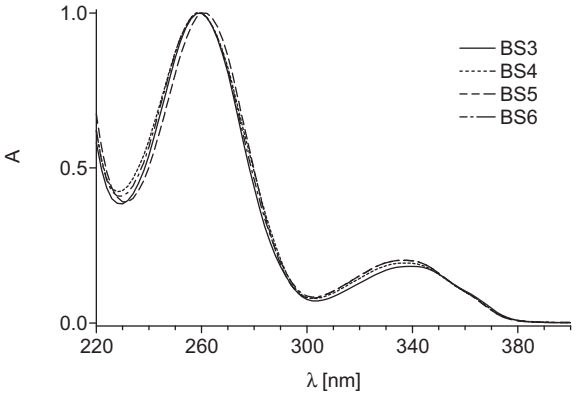


Figure 6. Normalized UV spectra of oligodeoxynucleotides BS3–BS6. Mean A_{260}/A_{340} = 5.11:1. (Theoretical A_{260}/A_{340}, calculated from the sum of molar coefficients of individual nucleosides, without adjusting for hipochromicity was 5.32:1.)

fluorescence spectra (Fig. 9) of photoproducts the shifts of maximum bands of fluorescence and absorption are noticeable (ODN 2: $A\ \lambda_{max}$ = 374 nm, $F\ \lambda_{max}$ = 462 nm; ODN 3: $A\ \lambda_{max}$ = 370 nm, $F\ \lambda_{max}$ = 457 nm). The same properties had been observed on nucleotide level.[5] It prompted us to the suggestion that photoproducts ODN 2 and ODN 3 are diastereoisomers.

Enzymatic digestion of the isolated crosslink ODN 3 revealed the formation of appropriate amounts dC, dG, T, dA and dI as a result of deamination of dA (Fig. 10). Fluorescent remaining fragment (dAMP-crosslink) was assumed to be a crosslink core bound to adenosine monophosphate (Fig. 11) on the basis of MALDI-TOF MS [calcd 828.71 for $(M+H)^+$, found 828.96]. The same mixture was obtained in case of enzymatic digestion of ODN 2 with formation of dAMP-crosslink fragment in second diastereomeric form, respectively.

4. Conclusions

Different approaches for the synthesis of potential useful photoaffinity probe, phosphoramidite of 5-fluoro-4-thio-2′-*O*-methyluridine showed that the system possesses unique reactivity of the pyrimidine ring, easily leading to decomposition or poor synthetic yield. Adjustment of synthetic strategy required proper choice of protecting groups and the optimal arrangement was found to be 5′-*O*-DMTr, 2′,3′-di-*O*-TBDMS and 4-*S*-(2-cyanoethyl). When applying this set of protections, phosphoramidite synthon was synthesized with good yield and purity and was applied in the oligonucleotide synthesis. Optimizing the deprotection and purification procedure of the product oligonucleotide showed that when using 'soft' exoamine function protection and deprotection with methanolic ammonia at room temperature, nucleophilic substitution of sulphur atom does not occur and the 5-fluoro-4-thiouracil system is preserved.

When FSU-containing oligonucleotides were irradiated as a duplex with their complementary strand, efficient formation of crosslink with the thymidine next to the opposite adenosine occurred. Properties of the photoproduct followed the properties of analogous product obtained on the nucleoside level and prelimin-

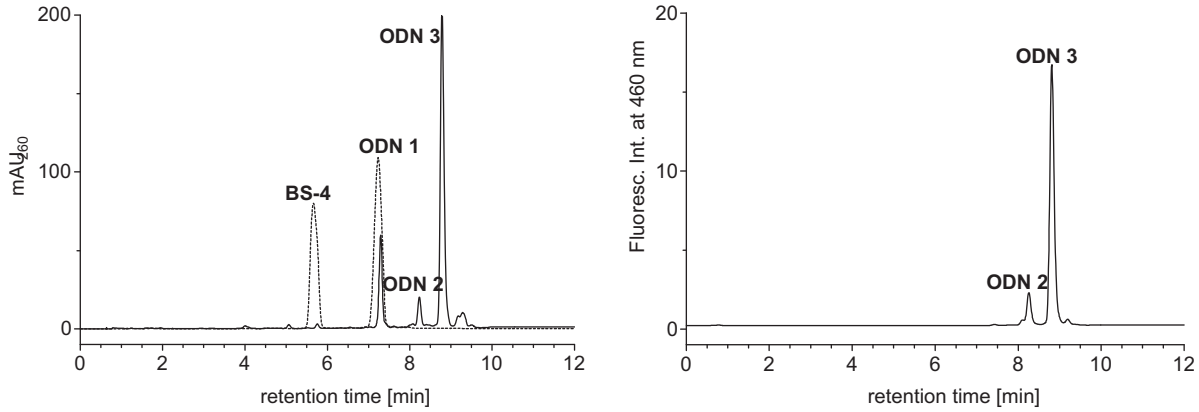


Figure 7. Upper part—HPLC absorption elution profile for irradiation of duplex BS4-ODN 1. Dotted line refers to mixture before irradiation and solid line to mixture after 60 s of irradiation to ca. 98% conversion of BS-4. Lower part—HPLC fluorescence elution profile after irradiation of duplex recorded at 460 nm (λ_{Ex} = 370 nm). Separation conditions: Waters XBridge OST C_{18} Column, 2.5 μm, 4.6 × 50 mm, mobile phases: A = 0.1 M TEAA, B = 0.1 M TEAA/Acetonitrile, 50/50, v/v, flow rate: 1 ml/min, column temp = 45 °C, gradient: 13–20%B in 10 min.

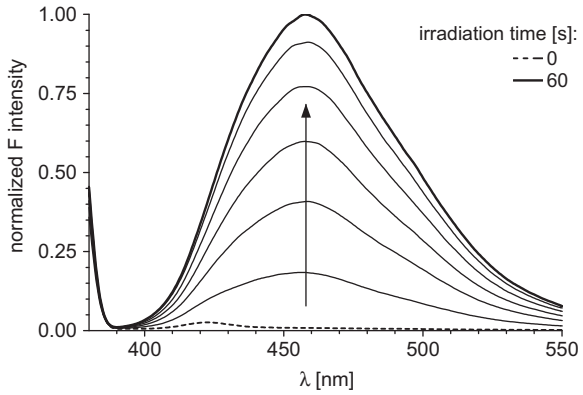


Figure 8. Changes in fluorescence spectra of duplex BS-4-ODN 1 during irradiation.

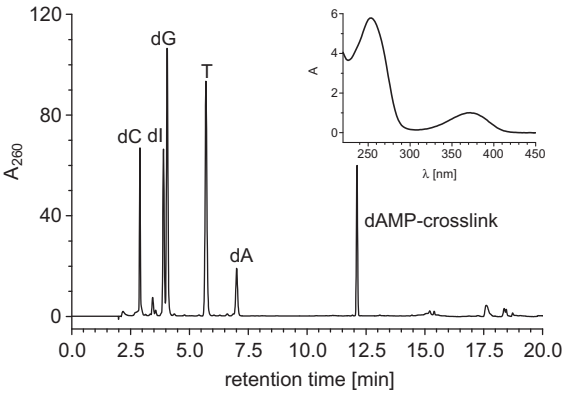


Figure 10. HPLC absorption elution profile for ODN 3 after enzymatic digestion. Insert: UV spectrum of dAMP-crosslink. Separation conditions: Agilent Poroshell 120 EC-C_{18} Column, 2.7 μm, 4.6 × 150 mm, mobile phases: A = 0.1 M CH_3COONH_4/Acetonitrile, 95/5, B = 0.1 M CH_3COONH_4/Acetonitrile, 50/50, v/v, flow rate: 0.7 ml/min, column temp = 40 °C, gradient: 0–3%B in 5 min, then to 15%B in 5 min and to 100%B in 5 min.

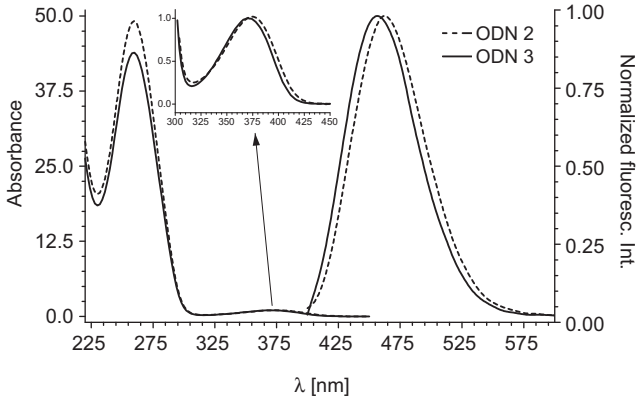


Figure 9. Normalized absorption and fluorescence emission spectra of photoproducts ODN 2 and ODN 3.

ary analytical data confirm formation of two diastereoisomes, with one of them dominating.

5. Experimental

5.1. Instruments

^1H, ^{13}C, ^{19}F and ^{31}P NMR spectra were recorded on Varian 300 MHz Mercury system in deuterated solvents as indicated. All chemical shifts are reported in parts per million relative to TMS (^1H and ^{13}C spectra), CF_3COOH (^{19}F) or 85% H_3PO_4 (^{31}P).

High-performance liquid chromatography (HPLC) was performed with an Agilent 1200 system with a binary gradient-forming module and diode-array UV–Vis detector. The preparative separation was carried out using the same conditions (specified at Fig. 5).

The MS analyses were performed using the MALDI-TOF MS instrument model Autoflex II equipped with a reflectron (resolution about 5000 at *m/z* 1000), on a MALDI metal target plate (Bruker, Bremen, Germany). The instrument was equipped with a SmartBeam laser and operated under FlexControl. Spectra were calibrated in FlexAnalysis using the Protein Calibration Standard I from Bruker.

Steady-state irradiation and UV–vis and fluorescence spectra: UV–vis spectra were measured at room temperature using a Cary 300 Bio Varian spectrophotometer. Fluorescence emission spectra were measured at room temperature using a Luminescence Spectrometer LS 50B Perkin Elmer.

Steady-state photochemical irradiation experiments were carried out in 1 cm × 1 cm rectangular fluorescence cell with stirring bar on a standard optical bench system equipped with an argon ion laser (Coherent INNOVA 400, equipped with a special UV grade tube and UV resonator optics). A double Pellin–Broca prismline separator was used to select narrow band irradiation wavelengths

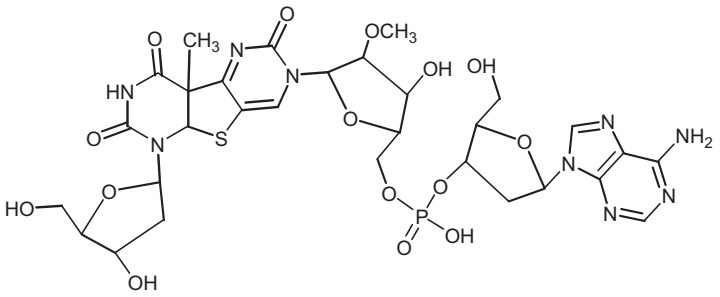


Figure 11. Tentative structure of fragment (dAMP-crosslink) containing photocrosslink produced on enzymatic digestion of ODN 2 and ODN 3.

(351.1 and 351.4 nm) with approximately 100 mW optical power, as measured by a Newport 1918-C laser-power meter with an 818P-010-12 thermopile head. The beam profile was expanded (5×) in a home-built beam expander. The approximately circular cross-sectional beam with 1 cm diameter was directed to the temperature-controlled cell holder (Quantum Northwest, model TC 125), placed on a micrometer one-axis translation stage.

A small portion (<1%) of the laser beam was reflected by a beam splitter to the monitoring CCD spectrometer (Edmund Optics, model BRC111 A-USB) to monitor the wavelength.

5.2. Materials

Kieselgel 60H (MERCK) was used for flash chromatography. Acetonitrile for HPLC (J. T. Baker) was used as received. Pyridine was dried by successive distillation from KOH and CaH_2, methylene chloride was distilled from K_2CO_3. THF was distilled from CaH_2 directly before use. Triethylamine and DIPEA were distilled from CaH_2 and stored over 4 Å molecular sieves. Water used in the preparation of aqueous solutions for HPLC solutions was distilled and purified using a Millipore Simpak1 system.

Eluting solvents used for TLC were: chloroform–ethyl acetate 7:3 (A), methylene chloride–methanol 19:1 (B), methylene chloride–methanol 9:1 (C), hexane–ethyl acetate–triethylamine 50:40:3 (D). TLC was performed on aluminum-backed SiO_2 plates (HF_{254} Merck 5554) cut to a size 3 × 10 cm.

5.2.1. 2′,3′,5′-Tri-*O*-acetyl-5-fluoro-4-(1,2,4-triazol-1-yl) uridine 6

1,2,4-Triazole (3.7 g, 53.62 mmol) was placed in 50 mL flask, dried in vacuo for 3 h, dissolved in anhyd acetonitrile (17 mL) and triethylamine (7.2 mL, 5.22 g, 51.6 mmol) was added. The flask was closed with a rubber septum, solution was cooled to 0 °C and phosphorus oxychloride (1.2 mL, 1.97 g, 12.87 mmol) was added with syringe. After 10 min stirring at 0 °C, solution of 2,3,5-tri-*O*-acetyl-5-fluorouridine (1.7 g, 4.38 mmol) in pyridine–acetonitrile (2:1, 35 mL) was added during 5 min. The mixture was allowed to reach room temperature and was left stirring for 20 h. Water (0.34 mL) was added to the dark solution and after 30 min the solvents were evaporated, residue dissolved in dichloromethane (40 mL), washed with satd $NaHCO_3$, water, dried with $MgSO_4$ and evaporated. The crude product (1.9 g) showed one spot on TLC (SiO_2, solvent A, R_f = 0.12, blue fluorescence) and was essentially pure by NMR spectrum. It was used for the next step without purification.

^{1}H NMR ($CDCl_3$): 9.27 (s, 1H); 8.43 (d, J = 6.5 Hz, 1H), 8.23 (s, 1H), 6.14 (d, J = 2.4 Hz, 1H), 5.47 (dd J = 2.4 Hz, J = 5.5 Hz, 1H), 5.31 (d, J = 5.5 Hz, 1H), 4.52 (m, 1H), 4.45 (m, 2H), 2.20, 2.16, 2.11 (3 × s, 3 × 3H).

^{19}F NMR ($CDCl_3$): −157.19 (d, $J_{H\text{-}F}$ = 6.5 Hz).

5.2.2. 2′,3′,5′-Tri-*O*-acetyl-4-(2-cyanoetylthio-)-5-fluorouridine 4b

Compound **6** (1.9 g crude), was dissolved in anhyd. acetonitrile (10 mL), 2-cyanoethanothiol (2 mL, 2 g, 23 mmol) and *N*,*N*-diisopropylethylamine (DIPEA) (4 mL, 2.97 g, 22.96 mmol) were added. After 3 h TLC (solvent B) showed no starting material. Solvents were evaporated, residue dissolved in methylene chloride, successively washed with 0.1 M KH_2PO_4, water and evaporated. Chromatography (gradient of methanol in dichloromethane from 0% to 2%) gave compound **4b** (2.3 g) as yellow foam. NMR showed that it contained free mercaptane, and after repeated chromatography 1.7 g (3.71 mmol) of pure **4b** was obtained. TLC: R_f = 0.43 (B).

^{1}H NMR ($CDCl_3$): 7.78 (d, J = 4.5 Hz, 1H); 6.05 (dd, J = 1.3 Hz, J = 3.6 Hz, 1H); 5.39 (dd, J = 3.6 Hz, J = 5.2 Hz, 1H); 5.29 (t, J = 5.6 Hz, 1H); 4.46–4.39 (m, 3H); 3.47 (t, J = 6.6 Hz, 2H); 2.80 (t, J = 6.6 Hz, 2H); 2.17; 2.14; 2.10 (3 × s, 3 × 3H).

^{19}F NMR ($CDCl_3$): −155.91 (d, $J_{H\text{-}F}$ = 4.5 Hz). ^{13}C NMR ($CDCl_3$) see Table 2.

Elemental Anal. Calcd for $C_{18}H_{20}FN_3O_8S$: C, 47.26; H, 4.41; N, 9.19; S, 7.01. Found: C, 47.11; H, 4.63; N, 9.23; S, 7.20.

5.2.3. (2-Cyanoetylthio-)-5-fluorouridine 4c

Compound **4b** (1.5 g, 3.28 mmol) was dissolved in dimethoxyethane–water 2:1 (20 mL), containing 1.5% ammonia. After 1.5 h at room temp the solvents were evaporated, the residue was dissolved in 1 mL of methanol, 7 mL of dichloromethane was added and the resulting emulsion was applied onto a chromatography column. Elution with gradient of methanol in dichloromethane from 0% to 6% gave 400 mg of compound **4c**. TLC R_f = 0.27 (solvent C).

^{1}H NMR (DMSO-d_6): 8.70 (d, J = 5 Hz, 1H); 5.65 (m, 2H); 5.39 (m, 1H); 5.05 (m, 1H); 3.95 (m, 3H); 3.79 (dt, J = 2.3 Hz, J = 10 Hz 1H); 3.64 (dt, J = 2.2 Hz, J = 10 Hz, 1H); 3.44 (t, J = 6.6 Hz); 2.98 (t, J = 6.6 Hz, 2H).

^{19}F NMR (DMSO-d_6): −158.24 (d, $J_{H\text{-}F}$ = 5.3 Hz). ^{13}C NMR ($CDCl_3$) see Table 2.

Elemental Anal. Calcd for $C_{12}H_{14}FN_3O_5S$: C, 43.50; H, 4.26; N, 12.68; S, 9.62. Found: C, 43.21; H, 4.53; N, 12.87; S, 9.50.

5.2.4. 5′-*O*-Dimethoxytrityl-4-(2-cyanoetylthio-)-5-fluorouridine 7b

*From **4c***. Compound **4c** (400 mg, 1.03 mmol) was coevaporated with pyridine three times and dissolved in pyridine (12 mL). Dimethoxytrityl chloride (375 mg, 1.13 mmol) was added and the mixture left for 5 h. Pyridine was evaporated to thick oil, which was dissolved in dichloromethane (25 mL), washed with KH_2PO_4, dried over $MgSO_4$ and solvent was evaporated, leaving yellow oil (770 mg). Separation on silicagel (gradient of methanol in dichloromethane 0–2%) gave product **7b** as a foam (720 mg,). TLC R_f = 0.67 (solvent B).

*From **12***. Compound **12** (420 mg, 0.49 mmol) was dissolved in neat triethylamine–3HF complex (0.6 mL). After 8 h methylene

Table 2
^{13}C NMR shifts (ppm from TMS) and ^{13}C–^{19}F coupling constants (Hz) for new compounds

Carbon atom	**4b***	**4c**	**7b**	**8b**	**9b**	**11**	**13**
C-2	152.32	152.88	153.38	151.55	152.32	148,52	151.6742
C-4	167.60 (d, J_{C-F} = 18 Hz)	167.48 (d, J_{C-F} = 18 Hz)	167.64 (d, J_{C-F} = 18 Hz)	167.68 (d, J_{C-F} = 18 Hz)	167.50 (d, J_{C-F} = 18 Hz)	156.57 (d, J_{C-F} = 27 Hz)	167.27 (d, J_{C-F} = 18 Hz)
C-5	143.38 (d, J_{C-F} = 241 Hz)	143.51 (d, J_{C-F} = 242 Hz)	143.60 (d, J_{C-F} = 243 Hz)	143.30 (d, J_{C-F} = 243 Hz)	143.34 (d, J_{C-F} = 243 Hz)	140.40 (d, J_{C-F} = 238 Hz)	143.16 (d, J_{C-F} = 248 Hz)
C-6	135.02 (d, J_{C-F} = 25 Hz)	135.12 (d, J_{C-F} = 26 Hz)	134.94 (d, J_{C-F}=27 Hz)	135.22 (d, J_{C-F} = 26 Hz)	135.10 (d, J_{C-F} = 25 Hz)	124.22 (d, J_{C-F} = 31 Hz)	125.89 (d, J_{C-F} = 33 Hz)
Arom.DMTr (*i*-OCH_3)	—	—	158.64	158.60	158.68	158.74	158.66
Arom. DMTr	—	—	143.97	144.21	144.07	143.97	143.78
Arom. DMTr	—	—	129.80	129.98	129.90	134.98	134.90
Arom. DMTr	—	—	127.80	128.02	129.10	130.08	130.12
Arom. DMTr	—	—	127.13	127.93	128.05	129.11	128.18
Arom. DMTr	—	—	126.38	127.04	127.82	128.02	127.80
Arom. DMTr	—	—	125.93	126.06	127.74	127.18	127.06
Arom. DMTr (*o*- OCH_3)	—	—	113.59	113.32	113.32	113,30	113.31
Ar_3C-$OCH_2$5′	—	—	93.85	88.42	92.05	88.96	87.02
SCH_2CH_2CN	117.70	117.70	117.69	117.73	117.77	—	117.72
SCH_2CH_2CN	24.61	24.60	24.63	24.62	24.57	—	24.43
SCH_2CH_2CN	18.09	18.08	18.09	18.07	18.10	—	17.98
C1′	88.19	90.00	87.20	84.41	87.14	87.39	91.76
C2′	73.63	74.40	77.28	67.76	79.08	75.94	75.77
C3′	69.62	59.36	72.50	83.36	74.97	71.75	69.60
C4′	79.84	84.31	86.68	83.57	82.28	84.17	81.95
C5′	62.63	59.36	62.98	60.64	62.09	62.40	60.95
2′(3′)-OCH_3	—	—	—	58.93	58.54	—	—
DMTr OCH_3	—	—	55.22	55.24	55.26	55.24	55.12
Si-C-$(CH_3)_3$	—	—	—	—	—	25.72; 25.79	25.63; 25.77
Si-*C*-$(CH_3)_3$	—	—	—	—	—	17.91; 17.95	17.75; 17.93
Si-$(CH_3)_2$	—	—	—	—	—	−4.25; −4.62; −4.77; −4.89	−3.94; −4.18; −5.19; −5.32

* Acetyl C=O: 170.02; 169.53; 169.45; acetyl CH_3: 20.72; 20.48; 20.49.

chloride was added (10 mL) and the solution was washed with satd $NaHCO_3$, dried with $MgSO_4$ and evaporated, giving oily residue (500 mg). Separation on silicagel (gradient of methanol in dichloromethane 0–2%) gave product **7b** as a foam (270 mg,). TLC R_f = 0.67 (solvent B).

^1H NMR ($CDCl_3$): 7.88 (d, J = 4.6 Hz), 7.25 (m, 9H); 6.80 (m, 4H); 5.72 (d, J = 4.6 Hz, 1H); 5.35 (s, 1H); 4.57 (t, J = 4.6 Hz,1H); 4.43–4.38 (m, 2 H); 3.80 (s, 3H); 3.50 (m, 2H); 3.31 (m, 3H); 2.92 (t, J = 6.6 Hz, 2H).

^{19}F NMR ($CDCl_3$): 155.41 (d, J_{H-F} = 4.6 Hz). ^{13}C NMR ($CDCl_3$) see Table 2.

Elemental Anal. Calcd for $C_{33}H_{32}FN_3O_7S$: C, 62.55; H, 5.09; N, 6.63; S, 5.06. Found: C, 62.36; H, 5.29; N, 6.87; S, 5.01.

5.2.5. 5′-*O*-Dimethoxytrityl-2′-*O*-methyl-4-(2-cyanoetylthio-)-5-fluorouridine 8b and 5′-*O*-dimethoxytrityl-3′-*O*-methyl-4-(2-cyanoetylthio-)-5-fluorouridine 9b

Compound **7b** (592 mg, 0.93 mmol) was dissolved in anhydrous methylene chloride (100 mL). The solution was cooled in ice and $SnCl_2$ dihydrate (68 mg, 0.3 mmol) was added. To the stirred solution diazomethane in diethyl ether (approx 0.5 M) was added in aliquots of 2 mL in 15 min intervals. The progress of the reaction was monitored by TLC. When no starting material remained (6 additions) the solution was successively washed with satd $NaHCO_3$, water, dried over $MgSO_4$ and evaporated, giving 610 mg of yellow oil. Separation on a SiO_2 column (gradient of ethanol in dichloromethane 0–2%) gave pure product **8b** (490 mg) and **9b** (48 mg). Analytical sample was dissolved in benzene, frozen and freeze-dried to give solvent free fluffy solid.

Compound **8b** ^1H NMR ($CDCl_3$): 8.26 (d, J = 4.2 Hz, 1H); 7.42–7.24 (m, 9H); 6.80 (m, 4H); 5.84 (s, 1H); 4.46 (m, 1H); 4.03 (m, 1H); 3.86 (d, J = 5 Hz, 1H); 3.80 (s, 3H); 3.75 (s, 3H); 3.56 (d, J = 2.2 Hz, 2H); 3.44 (t, J = 6.5 Hz, 2H); 2.91 (t, J = 6.6 Hz, 2H); 2.53 (d, J = 9.7 Hz, exchangeable with D_2O).

^{19}F NMR ($CDCl_3$): −155.63 (d, J = 4.2 Hz). ^{13}C NMR ($CDCl_3$) see Table 2.

Elemental Anal. Calcd for $C_{34}H_{34}FN_3O_7S$: C, 63.05; H, 5.29; N, 6.49; S, 4.95. Found: C, 63.44; H, 5.35; N, 6.31; S, 4.82.

Compound **9b**: ^1H NMR ($CDCl_3$): 8.06 (d, J = 4.2 Hz, 1H); 7.37–7.24 (m, 9H); 6.83 (m, 4H); 5.85 (d, J = 2.5 Hz, 1H); 4.45 (m, 1H); 4.30 (m, 1H); 4.01 (t, J = 5.2 Hz, 1H); 3.80 (s, 3H);); 3.55 (d, J = 3.5 Hz, 1H); 3.51–3.40 (m, 5H, overlapped with s, 3H at 3.47); 2.90 (t, m, 2H).

^{19}F NMR ($CDCl_3$): −155.63 (d, J_{H-F} = 4.2 Hz). ^{13}C NMR ($CDCl_3$) see Table 2.

Elemental Anal. Calcd for $C_{34}H_{34}FN_3O_7S$: C, 63.05; H, 5.29; N, 6.49; S, 4.95. Found: C, 63.43; H, 5.47; N, 6.30; S, 4.86.

5.2.6. 5′-*O*-Dimethoxytrityl-2′,3′-di-*O*-*t*-butyldimethylsilyl-5-fluorouridine 11

5′-*O*-Dimethoxytrityl-5-fluorouridine (700 mg, 1.24 mmol) and imidazole (700 mg, 10.28 mmol) were placed in flask and dried in vacuo (30 °C, 1 torr) for 2 h. Anhydrous DMF (2 mL) and *t*-butyldimethylsilyl chloride (500 mg, 3.32 mmol) were added and the reaction mixture was left overnight at rt. TLC showed complete disappearance of starting nucleoside. Satd $NaHCO_3$ (30 mL) was added and the mixture was extracted with ethyl acetate (2 × 10 mL) and combined organic layers were washed with H_2O, dried with $MgSO_4$ and evaporated. Chromatography on SiO_2 (gradient of methanol in dichloromethane (0–3%) gave pure disililated product (580 mg, 0.73 mmol, 58%) followed by mixture of 2′,3′-O-monosilylated isomers (300 mg, 0.44 mmol, 36%).

^1H NMR ($CDCl_3$): 8.05 (d, J = 6.2 Hz, 1H) 7.40–7.24 (m, 9H); 6.80 (m, 4H); 5.90 (d, J = 4.6 Hz, 1H); 4.24 (t, J = 4.1 Hz, 1H); 4.09 (m,

Table 3
Enzymatic digestion of oligonucleotides

Oligonucleotide	Nucleoside	Area (HPLC)	$\varepsilon \times 10^{-3}$	Area/$\varepsilon \times 10^{-3}$	Experimental nucleoside count**	Theoretical nucleoside count
BS3	dC	237.7	9.1	0.026	1.62	2
	dI	306.9	7.4	0.041	2.57	4
	dA	373.4	14.9	0.025	1.55	
	dG	334.5	13.6	0.025	1.52	2
	T	156.6	9.7	0.016	1.00	1
	X	378.7	20.92*	0.018	1.12	1
BS4	dC	277.3	9.1	0.030	1.93	2
	dI	201.8	7.4	0.027	1.73	4
	dA	554.5	14.9	0.037	2.36	
	dG	361.2	13.6	0.027	1.69	2
	T	152.8	9.7	0.016	1.00	1
	X	375.0	20.92*	0.018	1.14	1
BS5	dC	116.1	9.1	0.013	0.71	1
	dI	336.8	7.4	0.046	2.54	4
	dA	354.6	14.9	0.024	1.32	
	dG	166.2	13.6	0.012	0.68	1
	T	521.4	9.7	0.054	3.00	3
	X	452.2	20.92*	0.022	1.20	1
BS6	dC	354.3	9.1	0.039	1.86	2
	dI	252.0	7.4	0.034	1.62	4
	dA	764.2	14.9	0.051	2.44	
	dG	483.0	13.6	0.036	1.69	2
	T	203.5	9.7	0.021	1.00	1
	X	467.3	20.92*	0.022	1.06	1

* ε of 5-fluoro-4-thiouridine was assumed for 5-fluoro-4-thio-2′-*O*-methyluridine (X).
** was determined relative to signal of thymidine.

2H); 3.79 (s, 6H); 3.53 (d, J = 8.8 Hz, 1H); 3.33 (d, J = 8.8 Hz, 1H); 0.89 (s, 9H); 0.81 (s, 9H); 0.09 (s, 3H); 0.08 (s, 3H); 0.04 (s, 3H); −0.04 (s, 3H).

^{19}F NMR ($CDCl_3$): −155.56 (d, $J_{H\text{-}F}$ = 4.7 Hz). ^{13}C NMR ($CDCl_3$) see Table 2.

5.2.7. 5′-*O*-Dimethoxytrityl-2′,3′-di-*O*-*t*-butyldimethylsilyl-5-fluoro-4-(1,2,4-triazol-1-yl) uridine 12

1,2,4-Triazole (1.3 g, 18.81 mmol) was dried in vacuo (30 °C, 1 torr) for 3 h. Dry acetonitrile (6 mL) was added, followed by triethylamine (2.7 mL, 1.96 g, 19.36 mmol), the flask was closed with septum, cooled in ice and phosphoryl chloride (0.5 mL, 822 mg, 5.36 mmol) was added. White suspension was stirred at 0 °C for 10 min and solution of compound **11** (570 mg, 0.718 mmol) in 1:2 mixture of acetonitrile and pyridine (10 mL) was added. The mixture was stirred at rt overnight. TLC (hexane–ethyl acetate 30:1) showed complete reaction. Water (0.5 mL) was added, after 30 min solvents were evaporated, residue treated with satd $NaHCO_3$ (20 mL) and extracted with ethyl acetate (2 × 10 mL). Organic phases were combined, dried with $MgSO_4$, and evaporated, leaving essentially pure **12** (TLC) as white foam (698 mg), which was used without purification.

^{1}H NMR ($CDCl_3$): 9.22 (s, 1H); 8.86 (d, J = 6.3 Hz, 1H), 8.20 (s, 1H), 7.40–7.24 (m, 9H); 6.80 (m, 4H); 5.79 (s, 1H), 4.33 (dd J = 1.1 Hz, J = 3.8 Hz, 1H), 4.30 (t, J = 2.3 Hz, 1H); 4.14 (dd J = 12.0 Hz, J = 3.8 Hz, 1H); 3.80 (s, 6H); 3.77 (dd, J = 11.1 Hz, J = 2.6 Hz, 1H); 3.40 (dd, J = 11.1 Hz, J = 2.6 Hz 1H), 0.92 (s, 9H); 0.76 (s, 9H); 0.27 (s, 3H); 0.16 (s, 3H); 0.02 (s, 3H); −0.05 (s, 3H). ^{19}F NMR ($CDCl_3$): −157.21 (d, $J_{H\text{-}F}$ = 5.0 Hz).

5.2.8. 5′-*O*-Dimethoxytrityl-2′,3′-di-*O*-*t*-butyldimethylsilyl-4-(2-cyanoetylthio-)-5-fluorouridine 13

Compound **12** (690 mg, obtained from 0.710 mmol of **11**) was dissolved in dry acetonitrile (2 mL), 2-cyanoethanethiol (0.5 mL, 500 mg, 5.73 mmol) and DIPEA (0.33 mL, 245 mg, 1.89 mmol) were added and the mixture was left at rt. After 1.5 h TLC showed complete disappearance of the substrate **12**. Solvent was evaporated (hood, stench), residue was dissolved in methylene chloride, washed with 0.5 M KH_2PO_4 solution and evaporated. Chromatography (SiO_2, methylene chloride as eluent) gave pure **13** as cream-white foam (425 mg).

^{1}H NMR ($CDCl_3$): 8.43 (d, J = 4.7 Hz, 1H); 7.40–7.24 (m, 9H); 6.80 (m, 4H); 5.66 (s, 1H); 4.28–4.24 (m, 2H); 4.12 (dd, J = 3.8 Hz, J = 8.8 Hz, 1H); 3.80 (s overlapped with m, 6H+1H); 3.50–3.30 (m, 3H); 2.96–2.83 (m, 2H); 0.91 (s, 9H); 0.73 (s, 9H); 0.28 (s, 3H); 0.15 (s, 3H); 0.01 (s, 3H); −0.07 (s, 3H).

^{19}F NMR ($CDCl_3$): −155.88 (d, $J_{H\text{-}F}$ = 4.7 Hz). ^{13}C NMR ($CDCl_3$) see Table 2.

Elemental Anal. Calcd for $C_{45}H_{60}FN_3O_7SSi_2$: C, 62.69; H, 7.01; N, 4.87; S, 3.72. Found: C, 62.56; H, 7.29; N, 4.87; S, 3.60.

5.2.9. 5′-*O*-Dimethoxytrityl-2′-*O*-methyl-3′-*O*-[(2-cyanoethoxy), (*N*,*N*-diisopropylamino)phosphino]-4-(2-cyanoetylthio-)-5-fluorouridine 14

Nucleoside **8b** (480 mg, 0.74 mmol) was placed in round-bottomed flask (50 mL) and dried in vacuo (0.5 mmHg) for 3 h at room temp. Argon was let to the flask, which was closed with septum. Anhydrous THF (5 mL, freshly distilled from CaH_2) was added, followed by DIPEA (210 μL, 155 mg, 1.20 mmol) and chlorophosphine (230 μL, 244 mg, 1.04 mmol) and the solution was left at room temperature. After 3 h no starting nucleoside was visible by TLC (solvent D). Reaction was quenched with methanol (0.5 mL), then additional triethylamine (1 mL) and ethyl acetate (30 mL) were added. Solution was washed with satd $NaHCO_3$, brine, dried over $MgSO_4$ and solvent was evaporated, leaving 685 mg of yellow foam. Chromatography (SiO_2, cyclohexane containing 2% of triethylamine with gradient of dichloromethane (20–40%) gave product **14** (white solid after freeze drying of benzene solution). Yield 250 mg.

^{31}P NMR ($CDCl_3$) 150.61, 150.02; ^{19}F NMR ($CDCl_3$) −156.03 ($J_{H\text{-}F}$ = 4.2 Hz).

Elemental Anal. Calcd for $C_{43}H_{51}FN_5O_9PS$: C, 59.78; H, 5.95; N, 8.19; S, 3.71. Found: C, 60.13; H, 6.06; N, 8.15; S, 3.62.

5.3. Oligonucleotide synthesis

Syntheses were performed on ABI 391PCR Mate instrument applying built-in 1 μmol protocol and commercial CPG supports. Iodine solution of 0.05 M concentration was used in the oxidation step.

5.4. Deprotection and purification of oligonucleotides

Column containing solid support with the synthesized oligonucleotide was attached to a syringe containing 2 mL of 0.4 M solution of DBU in anhydr CH_3CN. Approx. 0.7 mL of the solution was pushed into the column, replaced by a new portion after 5 min and another one after next 5 min. After total time of 15 min the support was washed with dry CH_3CN (10 mL) and dried in vacuo. The dried support was placed into a vial, 1 mL of approx. 22% methanolic ammonia was added and the vial closed tightly. After 3 h at rt the solid was filtered off, washed with methanol and water and the filtrates were evaporated. The residue was dissolved in 0.1 m ammonium acetate (1 mL) and passed through a NAP 25 column. The column was washed with the same solution and 6 fractions of 1.4 mL were collected and checked by UV. The oligomer was present mainly in the fractions 3 and 4, with some residue in 5th fraction. Combined fractions were evaporated, dissolved in H_2O/acetonitrile, 95/5, v/v (1 mL) and purified by HPLC (two 500 μl injections, Waters XBridge OST C_{18} Column, 2.5 μm, 10 × 50 mm, phase A = 0.1 M TEAA, B = 0.1 M TEAA/Acetonitrile, 50/50, v/v, flow rate 1 ml/min, $T = 40\ ^\circ C$, gradient 17–20%B in 10 min, then to 50%B in 5 min. Combined fractions were concentrated to approx. 0.5 mL and desalted by passage through HPLC column. Yields of the oligomers BS3–BS6 see Table 1. The oligomers were verified by enzymatic digestion, results see Table 3. Enzymatic digestion conditions: 0.2 OD of oligonucleotide in 150 μL of buffer (10 mM KH_2PO_4, 10 mM $MgCl_2$, pH 7) was digested with alkaline phosphatase bovine intestinal mucosa (27 DEA units, Sigma–Aldrich, BioUltra) and phosphodiesterase I from *Crotalus adamanteus* venom (0.0055 units, Sigma–Aldrich, Purified) for 18 h at 37 °C. The enzyme caused partial deamination of dA to dI (confirmed in model experiment).

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