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TETRAHEDRON

α-Oligodeoxynucleotides Containing 5-Propynyl Analogs of α-Deoxyuridine and α-Deoxycytidine: Synthesis and Base Pairing Properties

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Abstract: From propyne and 5-iodo-α,2-deoxyuridine, obtained by glycosylation, 5-propynyl-α,2'-deoxyuridine was synthesized following the procedure of Hobbs. One part was then transformed through displacement of its C4-triazolo derivative with ammonia into 5-propynyl-α,2'-deoxycytidine derivative. Finally, the corresponding α 5-propynyl nucleoside phosphoramidites were prepared, and 5-propynyl-α oligonucleotides (12-mer) with either phosphodiester and phosphorothioate were synthesized. The melting temperatures showed that duplexes with complementary DNA are stabilized between 0.65 and 1.2°C/mod, and duplexes with RNA are stabilized between 1.2 and 1.4°C/mod.

INTRODUCTION

Oligonucleotides are of considerable interest as therapeutic agents [1-3]. A considerable effort has been made to design a modified oligo that fulfills all of the requirements for antisense activity specially high nuclease resistance, high affinity with RNA target, activation of RNase H and increased in permeability with respect to cell membranes. Our efforts led us to synthesize α-oligos [4,5] which exhibit antisense activity by RNase H-independent mechanism by targeting the translation initiation codon and the 5' untranslated region of RNA [6]. This mechanism requires that antisense oligo tightly binds to the RNA target. We have, therefore, sought

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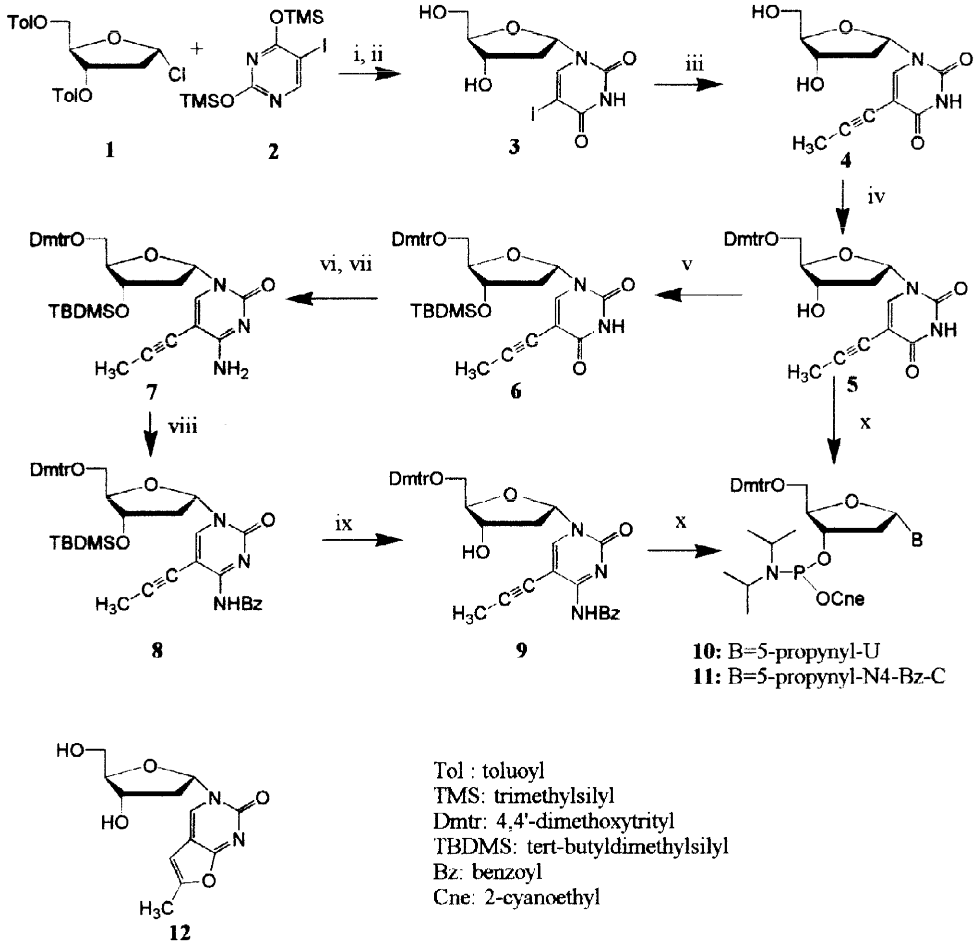
factors that can increase binding affinity while still retaining target selectivity. It has been shown, for β-oligos, that the introduction of C5 propynyl pyrimidine nucleosides strongly stabilizes the corresponding duplexes with RNA between 1.5 and 1.7 °C per substitution [7]. Since the C5 propyne β-oligos are not nuclease resistant, they must be used as phosphorothioate analogs to be active. One of the drawbacks of phosphorothioate analogs is their facility to interact non-specifically with proteins producing non-sequence-specific activities. Furthermore, it was demonstrated that the presence of sulfur increases the toxicity of the oligos and the general trend at present is to reduce the amount of sulfur into oligonucleotide using the gapmer approach or by replacement of phosphorothioate backbones by neutral backbones [8]. Since α-oligos are resistant to nuclease [9] it is not necessary to use them as their phosphorothioate analogs and thus it is expected that C5 propyne α analogs would present high affinity with RNA target and low nonspecific binding with proteins. Herewith we report the synthesis of 5-propynyl-α-deoxynucleoside pyrimidines (i.e. 5-propynyl-α-deoxyuracil and 5-propynyl-α-deoxycytidine) and of the corresponding phosphoramidite units. Such, α-dodecamers, complementary to the splice acceptor site of HIV-1 *tat*, containing eight 5-propynyl nucleobases with either phosphodiester or phosphorothioate backbone were synthesized. Finally, the Tm of these α-oligos with complementary DNA and RNA sequences were determined by UV melting curve and compared to duplexes with the corresponding wild type DNA oligonucleotide.

RESULTS AND DISCUSSION

The synthetic pathway is presented on scheme 1. 5-Iodo-α-dU **3** was obtained through direct condensation of α-D-1-chloro-3,5-di-O-(p-toluoyl)-2-deoxyribofuranose **1** [10,11] with bis-trimethylsilylated 5-iodouracil **2** in presence of p-nitrophenol as a catalyst and pyridine [12]. This reaction promoted formation of α nucleoside which was isolated by fractional crystallization. Further deprotection under basic conditions afforded crystalline **3** which was coupled with propyne by treatment with tetrakis(triphenylphosphine)palladium(0), copper(I) iodide and triethylamine in dimethylformamide [13]. 5-(Propyn-1-yl)-α-deoxyuridine **4** was generated as a major compound (65%), but not as a single nucleoside product although it proceeded under mild conditions and a 2:1 ratio of copper(I) and palladium(0) was maintained. Unlike the coupling reaction described by Hobbs [13] two side products were formed. The first one, **12**, was fluorescent and resulted from a intramolecular cyclization of **4** between 5-(propyn-1-yl) and O-4. Indeed Robins and Barr [14,15] reported that such cyclized furano[2,3-d] pyrimidin-2-one derivatives appeared during coupling reactions of terminal alkynes with 5-iodo-1-methyluracil or protected 5-iodouracil nucleosides in the presence of triethylamine and catalyzed by bis(triphenylphosphine)palladium(II) chloride and copper(I) iodide. The second byproduct isolated as a yellow crystalline material was probably a complex of **4** with two molecules of triphenylphosphine and copper(I): $C_{12}H_{13}N_2O_5[(C_6H_5)_3P]_2Cu(I)$. Nevertheless compound **4** was isolated by silica gel chromatography and purified by crystallization in 65% yield.



10: B=5-propynyl-U
11: B=5-propynyl-N4-Bz-C

Tol : toluoyl
TMS: trimethylsilyl
Dmtr: 4,4'-dimethoxytrityl
TBDMS: tert-butyldimethylsilyl
Bz: benzoyl
Cne: 2-cyanoethyl

Scheme 1: (i) p-nitrophenol, pyridine, CH_2Cl_2 (ii) MeONa / MeOH (iii) $[(C_6H_5)_3P]_4Pd(0)$ /CuI / Et_3N/, propyne, DMF (iv) DMTrCl, pyridine (v) TBDMSCl, imidazole, pyridine (vi) phosphorus oxychloride, 1,2,4-triazole, pyridine (vii) NH_4OH, dioxane (viii) benzoic anhydride, pyridine (ix) tetrabutylammonium fluoride, THF (x) diisopropyl ammonium tetrazolide, 2-cyanoethyl tetraisopropylaminophosphorodiamidite CH_2Cl_2.

5'-O-dimethoxytritylated 5-(propyn-1-yl)-α-dU **5** prepared to obtain phosphoramidite building block **10** was used also as a starting material in a synthesis of 5-(propyn-1-yl)-α-2'-deoxycytidine (scheme 1). Protection of 3'-hydroxyl function of **5** afforded 3'-O-*tert*-butyldimethylsilyl derivative **6**. The latter was transformed upon treatment with phosphorus oxychloride and 1,2,4-triazole in pyridine into C-4 triazolo derivative which subsequently was converted to cytosine derivative **7** by ammonia under mild conditions [16-18]. Benzoylation of amino group of **7** with benzoic anhydride in pyridine afforded fluorescent, fully protected 5-(propyn-1-yl)-α-dC **8**. Its 3'-hydroxyl function was then deprotected with tetrabutylammonium fluoride and **9** was isolated. Finally, **5** and **9** were converted by reaction with 2-cyanoethyl tetraisopropylaminophosphorodiamidite activated with diisopropyl ammonium tetrazolide into phosphoramidite building blocks 10 and 11 with respectively a 72% and 89% yield.

DNA and RNA targets: *tat* HIV-1 sequence (position 5357 to 5368 of genomic RNA)

	5'-d(AGAATTGGGTGT)-3'	DNA
	5'-r(AGAAUUGGGUGU)-3'	RNA
Complementary oligos		
name	*sequence*	*linkages*
β-PO	5'-d(ACACCCAATTCT)-3'	phosphodiester
α-PO:	5'-d(TCTTAACCCACA)-3'	phosphodiester.
α-PS:	5'-d(TCTTAACCCACA)-3'	phosphorothioate.
α-5pro-PO :	5'-d(^{5p}U^{5p}C^{5p}U^{5p}UAA^{5p}C^{5p}C^{5p}CA^{5p}CA)-3'	phosphodiester.
α-5pro-PS:	5'-d(^{5p}U^{5p}C^{5p}U^{5p}UAA^{5p}C^{5p}C^{5p}CA^{5p}CA)-3'	phosphorothioate

Table 1: Sequences of DNA and RNA target and complementary β-, α- and α-5-propynyl-oligos.

As model for our binding study, we have chosen a target corresponding to the splice acceptor site of HIV-1 *tat* protein [19,9]. This sequence is a 12-mer containing eight pyrimidines which were replaced by 5-propynyl-pyrimidines (Table 1). Since α-oligos hybridize to complementary β-strands with a parallel orientation [20], the α-oligonucleotides were constructed parallel to their β-target (Table 1). The 5-propynyl α-oligonucleotide were synthesized using a freshly prepared 0.05M solution of α-phosphoramidites in anhydrous acetonitrile and a solid support functionalized with protected α-deoxyadenosine [5], using standard elongation cycle. Thus syntheses were performed with only a 10 molar excess of phosphoramidite and was monitored by dosage of dimethoxytrityl cation released. The average coupling yield was better than 97.7 %. To obtain phosphorothioate oligos the standard oxidation step was substituted with a treatment with an acetonitrile solution of 0.05 M Beaucage reagent [21] for 30 sec.. After synthesis oligos were cleaved from solid support by treatment with concentrated ammonia (1.5 h) at room temperature and then 5 h at 55°C to cause the deprotection of the nucleobases. Crude syntheses were analyzed and purified by HPLC using a C18 reverse phase column and a gradient of acetonitrile in triethylammonium acetate (TEA AC 0.05 M pH 7). Purity of each oligo was checked by analytical C18 HPLC, gel capillary electrophoresis and electro-spray mass spectrometry.

The binding properties of 5-propynyl α-oligos (α-5pro-PO and α-5pro-PS) were determined with either DNA or RNA target, by UV melting curve experiments (Figure 1), carried out in 100 mM sodium chloride, 10 mM sodium cacodylate at pH 7.0 with a 3μM concentration of each strand. Likewise binding properties of α-oligos (α-PO and α-PS) and of β-DNA oligo (β-PO) were determined in the same conditions for comparison (Table 2).

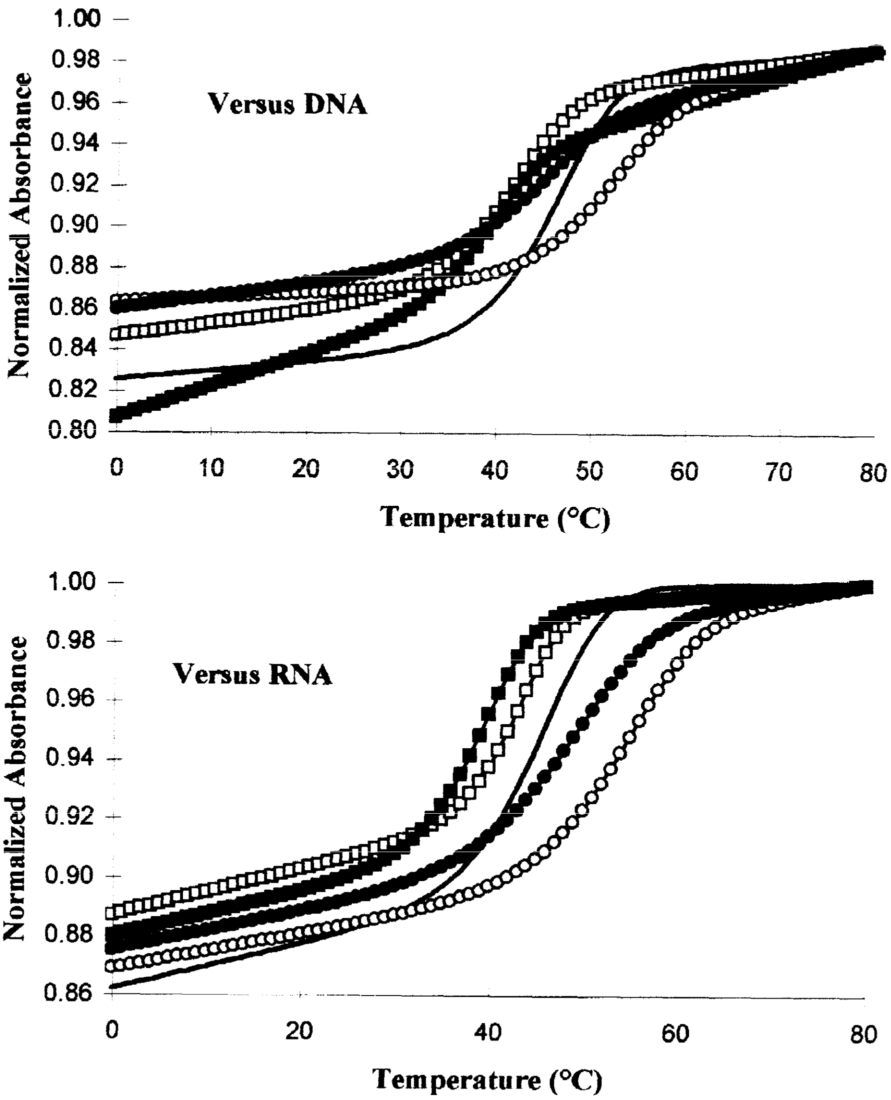


Figure 1: Melting curves of β-PO —, α-PO -□-, α-PS -■-, α-5pro-PO -o- and α-5pro-PS -●- with complementary DNA (top) and RNA (bottom) strand in 100 mM NaCl, 10 mM sodium cacodylate, pH 7.0.

Inversion of anomeric carbon from β to α configuration yielded a slight destabilization of duplexes (ΔTm -0.38 and -0.23°C/modification for duplexes with DNA and RNA respectively, Table 3). As already reported [9], introduction of phosphorothioate internucleosidic linkages in α-series destabilized the resulting duplexes (ΔTm -0.20 to -0.33°C/mod, Table 3) but this destabilization was lower than in β-series where it was about -0.9°C/mod [9]. When 5-propynyl pyrimidines were present in α-series the destabilizing effect of phosphorothioate was stronger (ΔTm -0.63 and -0.49°C/mod) and comparable to that observed with β-5-propynyl phosphorothioate oligos (ΔTm -0.56 °C/mod) when hybridized with RNA [22].

oligo	versus DNA				versus RNA			
	ΔH (kcal/mol)	ΔS (cal/K.mol)	ΔG_{298K} (kcal/mol)	Tm (°C)	ΔH (kcal/mol)	ΔS (cal/K.mol)	ΔG_{298K} (kcal/mol)	Tm (°C)
β-PO	-84.3	-237	-13.6	46.4	-82.0	-230	-13.3	45.9
α-PO	-82.5	-235	-12.3	41.8	-112.6	-330	-14.5	43.2
α-PS	-91.1	-265	-12.2	39.6	-97.1	-284	-12.5	39.6
α-5pro-PO	-77.2	-211	-14.3	51.7	-72.5	-195	-14.5	54.7
α-5pro-PS	-65.9	-181	-12.1	44.8	-63.5	-170	-12.7	49.3

Table 2 : Thermodynamic parameters and Tm of duplexes between a DNA or RNA target and the complementary β- or α-phosphodiester or α-phosphorothioate oligos or 5-propynyl-α-phosphodiester or -phosphorothioate oligos in 100 mM NaCl, 10 mM sodium cacodylate pH 7.

	Versus DNA ΔTm (°C)	Versus DNA ΔTm/mod (°C)	Versus RNA ΔTm (°C)	Versus RNA ΔTm/mod (°C)
Anomeric inversion:				
β-PO → α-PO	-4.6	-0.38	-2.7	-0.23
Phosphorothioate effect in α-series:				
α-PO → α-PS	-2.2	-0.20	-3.6	-0.33
α-5pro-PO → α-5pro-PS	-6.9	-0.63	-5.4	-0.49
5-propynyl effect in α-series:				
α-PO→α-5pro-PO	9.9	1.2	11.5	1.4
α-PS →α-5pro-PS	5.2	0.65	9.7	1.2
multiple effects:				
β-PO→α-5pro-PO	5.3	2-fold modified	8.8	2-fold modified
β-PO→α-5pro-PS	-1.6	3-fold modified	3.4	3-fold modified

Table 3: Effect of inversion of anomeric carbon and of introduction of phosphorothioate and/or 5-propynyl in α-series on the Tm values of duplexes with a DNA or a RNA complementary strand.

Finally, the introduction of 5-propynyl pyrimidines led to a neat stabilization with a ΔTm of +1.2 and +1.4 °C/mod from α-PO to α-5pro-PO and a ΔTm of +0.65 and +1.21 °C/mod from α-PS to α-5pro-PS according to the DNA or RNA complementary strand (Table 3). The twofold modified α-5-pro-PO bound to DNA and RNA more strongly (ΔTm +5.3 and +8.8 °C respectively) than β-PO. In contrast, the threefold modified α-5-pro-PS bound to DNA (ΔTm -1.6 °C) less tightly than β-PO and to RNA (ΔTm +3.4°C) more strongly than β-PO. The stability of duplexes with an RNA strand were always higher

In each case enthalpic factors were unfavorable. Nevertheless, the duplexes containing 5-propynyl pyrimidines were always more stable. This increase of stability is mainly due to a favorable entropic factor which is further stronger in RNA duplexes than in DNA ones (Table 2). This entropic gain could be explained by the hydrophobic nature of the propynyl group that displaces water molecules from the duplex major groove.

Likewise in β-series, introduction of 5-propynyl pyrimidine nucleosides in α-series led to more stable duplexes. Furthermore, since α-oligos are resistant to nucleases, it is not necessary to use them as their phosphorothioate analogs. This data allow us to envisage their use as antisense agents with a non- RNase H-dependent mechanism.

EXPERIMENTAL SECTION

NMR spectra were recorded at 250 MHz for ^1H and at 100 MHz for ^{31}P on a Brucker AC 250 spectrometer in Me_2SO-d_6 as a solvent. δ values are in ppm relative to tetramethylsilane as an internal standard (^1H-NMR) and relative to 85% H_3PO_4 as an external standard (^{31}P-NMR).

FAB mass spectra were recorded on a JEOL JMS 300 DX spectrometer and high resolution FAB mass spectra were recorded on a JEOL SX 102 spectrometer using thioglycerol (GT) or cesium iodide (ICs) as matrix. ESI mass spectra were recorded on a Finnigan Mat SSQ 7000 spectrometer. Absorption spectra were recorded on a Kontron 931 spectrophotometer.

Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected.

Column chromatography was performed on silica gel 60H (0.015-0.040, 0.040-0.063 mm) purchased from E.Merck company. Thin layer chromatography (TLC) were performed on E.Merck silica gel F_{254} plates (0.5 mm) and silica gel F_{254} sheets, respectively.

β-Oligonucleotides (DNA and RNA) were synthesized from commercial 2-cyanoethyl phosphoramidites (from Glen Research) using standard protocols. 2-Cyanoethyl phosphoramidite of protected α-deoxyadenosine and solid support were already synthesized as described in reference [5].

5-iodo-α,2'-deoxyuridine (3). A mixture of 5-iodouracil (9.80g, 41.18 mmol), hexamethyldisilazane (27.87g, 172.6 mmol) and trimethylchlorosilane (0.169g, 1.56 mmol) was refluxed under argon atmosphere for 2h. The excess of HMDS was removed *in vacuo* (10 Torr, 70°C). The crude product **2** was dissolved in anhydrous dichloromethane (103 ml), *p*-nitrophenol (1.84g, 13.27 mmol) and dry pyridine (0.96 ml, 11.90 mmol) were added, followed by 2-deoxy-3,5-di-O-(*p*-toluoyl)-α-D-*erythro*-pentofuranosyl chloride [10] (14.57g, 38.48 mmol). The reaction mixture was stirred under argon atmosphere for 20 h, protected from light. It was then evaporated to an oily residue. 3,5-di-*O*-(*p*-toluoyl)-5-iodo-α,2-deoxyuridine was isolated by a fractional crystallization from ethanol. It was deprotected with 0.1 M sodium methoxide (1.5 eq) in methanol at 22°C. After 45 min. the solution was neutralized with Dowex 50Wx2 (H^+), the resin was filtered off. Compound **3** crystallized from the filtrate upon concentration. Fractional crystallization from methanol afforded 6.35g (17.9

mmol.) of 3 (43.5% if calcd. from 5-iodouracil): m.p. dec 165-170°C (lit.[23] dec.170°C). ^{1}H NMR δ: 11.70 (s, 1H, NH), 8.33 (s, 1H, H_6), 6.12 (dd: $J_{1'2''}$=7.7, $J_{1'2'}$=2.0 Hz, 1H, $H_{1'}$), 5.43 (d, 1H, $OH_{3'}$), 4.89 (s, 1H, $OH_{5'}$), 4.30-4.25 (m, 2H, $H_{3'}$, $H_{4'}$), 3.42 (m, H_2O, $H_{5',5''}$), 2.66-2.50 (m, Me_2SO-d_5, $H_{2''}$), 1.93 (d: $J_{2'2''}$=14.4 Hz, 1H, $H_{2'}$). HR-MS FAB$^+$ (GT) $C_9H_{12}N_2O_5I$ cal.: 354.9791, found 354.9779.

5-(propyn-1-yl)-α,2'-deoxyuridine (4). A stirred solution of 3 (0.438g, 1.24 mmol) in anhydrous DMF (6 ml), in a two neck flask was deoxygenated with argon for 1.5 h. Catalysts: $(Ph_3P)_4Pd(0)$ (0.143g, 0.12 mmol) and CuI (0.047g, 0.24 mmol) were added. Argon was removed by vacuum, anhydrous triethylamine (0.34 ml, 2.48 mmol) was injected and the flask was filled up with propyne gas (ca. 200 cm^3, used in excess). The reaction mixture was protected from light and stirred for 22 h. Then triphenylphosphine on polystyrene (0.610g) was added to deactivate catalysts. The mixture was stirred in argon atmosphere for 3 h. The resin was filtered off, washed with DMF (30 ml). The filtrate was concentrated to an oily residue below 43°C. Compound **4** was isolated by silica gel chromatography with a step-gradient of methanol (0-10%) in dichloromethane. and was crystallized from hot methanol. 0.213g (65% yield) 2 crops: m.p. soft. 201°C, dec. 214-215°C. UV (H_2O): λ_{max} 233 and 292 nm (ε 11300 and 11400), λ_{min} 267 nm.. ^{1}H NMR δ: 11,50 (s, 1H, NH), 8.08 (s, 1H, H_6), 6.11 (dd: $J_{1'2''}$=7.6, $J_{1'2'}$=1.9 Hz, 1H, $H_{1'}$), 4.86 (d, 1H, $OH_{3'}$), 4.37 (s, 1H, $OH_{5'}$), 4.27-4.13(m, 2H, $H_{3'}$, $H_{4'}$), 3.42-3.30 (m. H_2O, $H_{5',5''}$), 2.63-2.52 (m, Me_2SO-d_5,$H_{2''}$), 1.97 (s, 3H, CH_3), 1.89 (d: $J_{2'2''}$=14.5 Hz,1H, $H_{2'}$). HR-MS FAB$^+$ (GT) $C_{12}H_{15}N_2O_5$ cal.: 267.0981 found: 267.0986.

Side product **12** was purified on a preparative silica gel plate with ethyl acetate sat. with water. Appropriate band was eluted with 20% MeOH in CH_2Cl_2. UV (MeOH): λ_{max} 327, 243, 224 nm (ε 3460, 6400, 6800), λ_{min} 266, 236 nm. MS FAB$^+$ GT matrix m/z: 267 $(M+H)^+$, 151 $(Base+H)^+$. ^{1}H NMR δ: 8.49 (s, 1H, H_4), 6.46 (d: J=1.2 Hz, 1H, H_5), 6.07 (dd: $J_{1',2''}$= 5.8, $J_{1',2'}$= 1.1 Hz, 1H, $H_{1'}$), 5.10 (s, 1H, $OH_{3'}$), 4.95 (t, 1H, $OH_{5'}$), 4.40 (t, J= 4.7 Hz, 1H, $H_{4'}$), 4.24 (d, J= 4.9 Hz, 1H, $H_{3'}$), 3.45 (m, 2H, $H_{5',5''}$), 2.60 (ddd, $J_{2'',2'}$= 14.3 Hz,. Me_2SO-d_5, 1H, $H_{2''}$), 2.33 (s, 3H, Me_6), 2.02 (d: $J_{2',2''}$= 14.3 Hz, 1H, $H_{2'}$).

Side product **$C_{12}H_{13}N_2O_5[(C_6H_5)_3P]_2Cu(I)$** was separated from **4** and **12** by a column chromatography and crystallized from $MeOH/CH_2Cl_2$ mixture (yellow crystals dec. above 130°C). MS FAB$^+$ GT matrix m/z: 852 [$(M+H)^+$, M: $C_{48}H_{43}N_2O_5P_2\,^{63}Cu$], 737 $(Base+H)^+$. ^{1}H NMR δ: 8.01 (s, 1H, H_6), 7.50 (m, 30H, 6x phenyl), 6.13 (dd:$J_{1',2''}$=7.8, $J_{1',2'}$=1.8 Hz, 1H, $H_{1'}$), 5.78 (d, 1H, $OH_{3'}$), 5.05 (t, 1H, $OH_{5'}$), 4.40 (m, 1H, $H_{3'}$), 4.28 (m, 1H, $H_{4'}$), 3.55 (m, 2H, $H_{5',5''}$), 2.72 (m, 1H, $H_{2''}$), 1.71 (m, 4H, $H_{2'}$, Me). ^{31}P NMR (Me_2SO-d_6) : 24.74 ppm.

5'-*O*-(4,4'-dimethoxytrityl)-5-(propyn-1-yl)-α,2'-deoxyuridine (5). To a solution of **4** (0.573g, 2.1 mmol) in dry pyridine (8 ml) dimethoxytrityl chloride (0.644g, 1.9 mmol) was added. After 24 h the reaction mixture was cooled and neutralized with a $NaHCO_3$ sat. aqueous solution (12 ml), extracted with dichloromethane and dried over Na_2SO_4. The organic solution was evaporated and the residue was purified by chromatography with a step-gradient of methanol (0-1.5%) in dichloromethane (with 0.1% Et_3N). It afforded 0.938g (83% yield) of **5** as a foam. ^{1}H NMR δ: 11.55 (s, 1H, NH), 8.05 (s, 1H, H_6), 7.45-7.11 (m, 9H, DMTr), 6.90 (m, 4H, DMTr), 6.19 (dd: $J_{1',2''}$=6.1 Hz, 1H, $H_{1'}$), 5.43 (d, 1H, $OH_{3'}$), 4.33 (m, 1H, $H_{4'}$), 4.18 (m, 1H, $H_{3'}$), 3.73 (s, 6H, 2x OCH_3), 3.00 (m, 2H, $H_{5',5''}$), 2.59 (m,. Me_2SO-d_5, $H_{2''}$), 1.93 (m, 4H, $H_{2'}$, CH_3). HR-MS FAB$^-$ (CsI) $C_{33}H_{31}N_2O_7$ cal.: 567.2131 ; found: 567.2212.

5'-*O*-(4,4'-dimethoxytrityl)-3'-*O-tert*-butyldimethylsilyl-5-(propyn-1-yl)-α,2'-deoxyuridine (6). To a solution of **5** (3.80g,6.69 mmol) and imidazole (1.262g, 18.53 mmol) in dry pyridine (37 ml), cooled to 0°C, *tert*-butyldimethylsilyl chloride (1.446g, 9.57 mmol) was added portionwise. The mixture was allowed to react at room temp. After 24 h TBDMSCl (0.135g, 1.3 eq) and imidazole (0.127g, 2.8 eq) were added and the reaction was continued for another 24 h. It was quenched by addition of a $NaHCO_3$ sat. aqueous solution (60 ml), and product **6** was extracted with dichloromethane and dried over Na_2SO_4. The organic solution was concentrated and the residue was purified by chromatography with a step-gradient of methanol (0-2%) in dichloromethane (with 0.1% Et_3N). It afforded 4.0g (88% yield) of **6** as a foam. ^{1}H NMR δ: 11.55 (s, 1H, NH), 7.96 (s, 1H, H_6), 7.45-7.11 (m, 9H, DMTr), 6.90 (m, 4H, DMTr), 6.17 (d, $J_{1',2''}$=6.5 Hz, 1H, $H_{1'}$), 4.24 (m, 2H, $H_{4'}$, $H_{3'}$), 3.73 (s, 6H, 2x OCH_3), 3.06 (m, 2H, $H_{5',5''}$), 2.65-2.45 (m,. Me_2SO-d_5, $H_{2''}$), 1.95 (m, 4H, $H_{2'}$, CH_3), 0.81 (s, 9H, t-Bu), -0.01 (s, 6H, 2x Si-CH_3). HR-MS FAB$^+$ (CsI) $C_{39}H_{47}N_2O_7Si$ cal.: 683.3153 found: 683.3236.

5'-*O*-(4,4'-dimethoxytrityl)-3'-*O-tert*-butyldimethylsilyl-5-(propyn-1-yl)-α,2'-deoxycytidine (7). To a solution of **6** (0.410g, 0.60 mmol) and 1,2,4-triazole (0.292g, 4.23 mmol) in dry pyridine (3 ml), cooled at 0°C, phosphoryl chloride (0.216g, 1.41 mmol) was added dropwise in 10 min. After 15 min. the ice-water bath was removed and the mixture was allowed to react at room temperature overnight. The reaction mixture was diluted with dichloromethane (35 ml) and washed with a $NaHCO_3$ sat. aqueous solution. The organic solution was dried over Na_2SO_4 and concentrated to dryness. The residue (0.457g) was dissolved in dioxane (5 ml) and treated with 20% aqueous ammonia (1.1 ml). After 1 h under stirring the mixture was diluted with dichloromethane (12 ml) and washed with water. The organic solution was dried over Na_2SO_4 and concentrated to dryness. The residue was chromatographied with a step-gradient of methanol (0-5%) in dichloromethane (with 0.1% Et_3N) to give 0.246g (60% yield) of the pure 7 as a foam. ^{1}H NMR δ: 7.85 (s, 1H, H_6), 7.55-7.00 (m, 9H, DMTr), 7.61 (broad s, 1H, NH), 6.93 (m, 4H, DMTr), 6.82 (broad s, 1H, NH), 6.14 (d: $J_{1',2''}$=6.3 Hz, 1H, $H_{1'}$), 4.30 (m, 1H, $H_{4'}$), 4.22 (m, 1H, $H_{3'}$), 3.76 (s, 6H, 2x OCH_3), 3.09 (m, 2H, $H_{5',5''}$), 2.63-2.47 (m,. Me_2SO-d_5, $H_{2''}$), 2.01 (s, 3H, CH_3), 1.89 (d: $J_{2',2''}$=14.3 Hz, 1H, $H_{2'}$), 0.81 (s, 9H, t-Bu), 0.03 and 0.01 (two s, 6H, Si-CH_3). HR-MS FAB$^+$ (CsI) $C_{39}H_{48}N_3O_6Si$ cal.: 682.3312 found: 682.3296.

5'-*O*-(4,4'-dimethoxytrityl)-3'-*O-tert*-butyldimethylsilyl-N-4-benzoyl-5-(propyn-1-yl)-α,2'-deoxycytidine (8). Compound 7 (0.656g, 0.96 mmol) dissolved in dry pyridine (5 ml) was treated with benzoic anhydride (0.283g, 1.25 mmol). After 19 h 0.2 eq of benzoic anhydride was added (0.19 mmol, 0.0435g). After 43 h, the reaction was almost completed and was quenched by adding 3 drops of methanol, followed by dichloromethane (6 ml). The mixture was washed with a $NaHCO_3$ sat. aqueous solution. The organic layer was dried over Na_2SO_4 and evaporated. The oily residue was chromatographied with a step-gradient of methanol (0-2%) in dichloromethane (with 0.1% Et_3N) to yielded the fluorescent compound **8**: 0.644g (85% yield). ^1H NMR δ: 8.03 (m, 4H, Bz, H_6, NH), 7.68-7.10 (m, 12H: Bz, DMTr), 6.90 (m, 4H, DMTr), 6.17 (d: $J_{1',2''}$=6.0 Hz, 1H, $H_{1'}$), 4.36 (m,1H, $H_{4'}$), 4.23 (m, 1H, $H_{3'}$) 3.75 (s, 6H, 2x OCH_3), 3.10 (m, 2H, $H_{5',5''}$), 2.68-2.48 (m,. Me_2SO-d_5, $H_{2''}$), 2.02 (d: $J_{2',2''}$=14.5 Hz, 1H, $H_{2'}$), 1.90 (s, 3H, CH_3), 0.80 (s, 9H, t-Bu), 0.01 and -0.01 (2s, 6H, Si-CH_3). HR-MS FAB^+ (CsI) $C_{46}H_{52}N_3O_7Si$ cal.: 786.3575 found: 786.3480.

5'-*O*-(4,4'-dimethoxytrityl)-N-4-benzoyl-5-(propyn-1-yl)-α,2'-deoxycytidine (9). Compound **8** (0.644g, 0.82 mmol) was treated for 5h, under magnetic stirring, with an excess of 1 M tetrabutylammonium fluoride in solution in THF (2.46 ml, 3 eq). Then the mixture was diluted with dichloromethane and washed with a $NaHCO_3$ sat. aqueous solution. The organic layer was separated, dried over Na_2SO_4,evaporated and the residue was chromatographied with methanol (0 to 2%) in dichloromethane to yielded pure **9** after precipitation from petroleum ether (< 50°) as a colorless powder 0.431g (78% yield). ^1H NMR ($CDCl_3$) δ: 8.20 (m, 2H, H_6, NH), 7.63-7.21 (m, 14H: Bz, DMTr), 6.88 (m, 4H, DMTr), 6.24(d: $J_{1',2''}$=6.2 Hz, 1H, $H_{1'}$), 4.61 (m, 1H, $H_{4'}$), 4.48 (d: $J_{3',2''}$=5.0 Hz, 1H, $H_{3'}$), 3.82 (s, 6H, 2x OCH_3), 3.23 (two dd: $J_{5'',5'}$=10.3, $J_{5'',4'}$=4.6, $J_{5',4'}$=4.3 Hz, 2H, $H_{5',5''}$), 2.82 (ddd: $J_{2'',1'}$=6.2, $J_{2'',2'}$=15.0, $J_{2'',3'}$=5.0 Hz, 1H, $H_{2''}$), 2.38 (d: $J_{2',2''}$=15.0 Hz, 1H, $H_{2'}$), 2.05 (s, 3H, CH_3). ^1H NMR (Me_2SO-d_6) δ: 5.40 (d, 1H, $OH_{3'}$). HR-FAB^+(GT) $C_{40}H_{38}N_3O_7$ cal.: 672.2710 found:672.2641.

Synthesis of 5-propynyl-α-deoxyuracil and 5-propynyl-α-deoxycytidine phosphoramidite building blocks (10 and 11). To a solution of 5-propynyl α-protected nucleoside **5** or **9** (1 mmol) in dry dichloromethane (5 ml), diisopropylammonium tetrazolide (0.5 mmol.) and cyanoethyl bis-diisopropylamidite (1.2 mmol.) were added under argon. After 5 to 6 h of magnetic stirring, the mixture was diluted with ethyl acetate (30 ml) and washed with sodium hydrogencarbonate (30 ml) and brine (3 x 30 ml). Organic layer was dried over sodium sulfate and solution was evaporated under reduced pressure. Resulting phosphoramidite derivative was precipitated in cold heptane and dried under vacuum under P_2O_5. **10** (yield 72%), ^{31}P NMR 150.4 and 150.2 ppm, HR-MS FAB^+ (CsI) $C_{42}H_{50}N_4O_8P$ cal.: 769.3366 found: 769.3455. **11** (yield 89%), ^{31}P NMR 150.2 and 150.1 ppm. HR-MS FAB^+ (CsI) $C_{49}H_{55}N_5O_8P$ cal.: 872.3788 found: 872.3862.

Oligonucleotides synthesis. Syntheses were performed on a ABI 381A DNA synthesizer on 1 μmol scale using fresh solutions of α-5-propynyl-phosphoramidites (0.05 M, 10 eq) in anhydrous acetonitrile and following standard protocol [5]. Phosphorothioate oligo was synthesized using Beaucage's reagent as sulfurizing agent [21]. The average coupling yield calculated from release of dimethoxytrityl cation, was 97.8% and 97.7% respectively for phosphodiester and phosphorothioate oligonucleotide. Oligos were cleaved from solid support by treatment with 32% ammonia for 2 h at room temperature and then heated at 55°C for 5 h to allow base deprotection. After evaporation of ammonia, oligos were purified by HPLC on C18 reverse phase column (Waters, Delta Pak, 15μ, 7.8 x 300 mm, C18 100Å) using a linear gradient of acetonitrile (10% to 15% in 10 min for phosphodiester oligo α-5pro-PO and 12 % to 17 % in 10 min for phosphorothioate oligo α-5pro-PS) in 50 mM triethylammonium acetate pH 7.0. Purity of each oligo was controlled by analytical HPLC (Macherey Nagel, Nucleosil, 5µ, 4.6 x 150 mm, C18), by gel capillary electrophoresis (capillary: 75 µm I.D. x 375 µm O.D., 400V/cm, 10%T 0%C polyacrylamide in 100 mM tris-borate, 7 M urea) and by MS-ESI negative mode for α-5pro-PO calculated: 3811.7 found: 3812 and for α-5pro-PS calculated: 3988.4 found: 3988.0.

UV melting experiments. Concentration of each separated oligonucleotide was determined spectrophotometrically at 260 nm and at the temperature of 85 °C assuming the molar extinction coefficient (ε) of each oligomer is the sum of the molar extinction coefficient of the constitutive nucleotides ε_{260nm}: α-^{5p}dU 3500 l/mol.cm; α-^{5p}dC 5500 l/mol.cm and α-dA 15400 l/mol.cm. Optical measurements were performed on a UVIKON 931 spectrophotometer (KONTRON). The temperature control was done with a HUBER PD 415 temperature programmer connected to a refrigerated ethylene glycol-water bath (HUBER Ministat). Cuvettes were 1 cm pathlength quartz cells and nitrogen was continuously circulated through the cuvette compartment. Prior to the experiments, the oligonucleotides to be studied were mixed together each at a final concentration of 3 µM, in 10 mM sodium cacodylate, 100 mM sodium chloride pH 7.0 and allowed to incubate at 90°C for 30 min. During the melting and hybridizing experiments the heating rate was 0.5°C min.$^{-1}$. Digitized absorbance and temperature values were stored in a computer for subsequent plotting and analysis.

Thermodynamic analysis. Melting and hybridization curves showed one reversible transition. A non linear least-square method was used to fit the curves, applying a two-state model, [24,25]. The root-mean-square difference between the data and calculated curve is less than 0.4 %. The Tm values given are the temperature for α (fraction of single strand in duplex) equals 0.5.

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