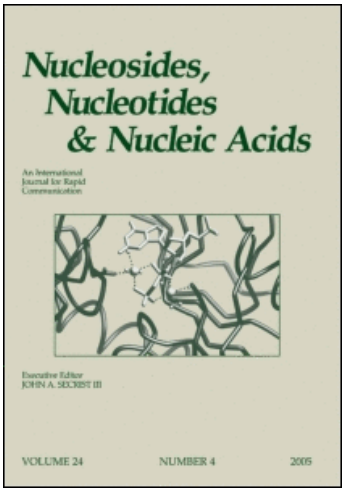

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Synthesis and DNA Duplex Stabilities of Oligonucleotides Containing C-5-(3-Methoxypropynyl)-2'-deoxyuridine Residues

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SYNTHESIS AND DNA DUPLEX STABILITIES OF OLIGONUCLEOTIDES CONTAINING C-5-(3-METHOXYPROPYNYL)-2'-DEOXYURIDINE RESIDUES.

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ABSTRACT: 5'-Dimethoxytrityl-5-(3-methoxypropynyl)-2'-deoxyuridine phosphoroamidite was synthesized with the use of commercial 3-methoxypropyne. Oligonucleotides (ODNs) containing 5-(3-methoxypropynyl)-2'-deoxyuridine in different positions were prepared. The stabilities of the duplexes formed by these ODNs with the complementary templates are increased in comparison with the unmodified counterparts. On average modified residue incorporated, the Tm is raised by 1°C.

INTRODUCTION

Synthetic oligonucleotides with increased affinity to their complementary DNA targets have considerable potentials as improved antisense agents[1-4], hybridization probes[5-7], and primers for PCR amplification[8] and nucleic acid sequencing[9].

Earlier we[6-9] and others[5,10-15] made use of the substitution of 5-methylcytosine for cytosine and 2-aminoadenine for adenine as a tool for

enhancement of the strength of the ODN:DNA duplexes. It was shown[10-15] that each such a substitution increases Tm of the duplex by approximately 1°C as compared to the natural counterpart. The 5-methylcytosine and 2-aminoadenine containing oligonucleotides were successfully used for different purposes and appeared to be better hybridization probes[5-7] and DNA-polymerase primers[6-9] than their nonmodified analogs. However, it is quite desirable in many cases to have also modified thymidine incorporated into an oligonucleotide to improve the strength of A:T base pairs. Such a modification would be especially useful for hybridization or priming in A rich DNA regions. Several modifications changing 5-methyl group with different chemical groups were evaluated in search of such a substitution[14,16-21]. It was demonstrated that most of them weaken the A:T base pair strength, however the 5-bromo tends to slightly increase the Tm of the correspondingly modified ODNs. Most successful was the substitution of C-5 propyne groups for the 5-methyl. In this case the Tm was raised 1.7 °C per modified dT residue[22,23]. C-5 Propynyl ODNs containing substituted uracyl residues were successfully used as antisense inhibitors of gene expression *in vitro*[1-4]. However, the routine synthesis of the propyne derivatives is rather complicated. In continuation of our search for efficient hybridization probes for DNA fingerprinting, possessing improved binding ability and retaining the specificity of duplex formation[7], we were looking for a reliable and safe synthetic route of C-5 propyne like analogs of 2'-deoxyuridine.

In this paper we report on the synthesis and DNA duplex formation properties of ODNs containing 5-(3-methoxypropynyl)-2'-deoxyuridine.

RESULTS AND DISCUSSION

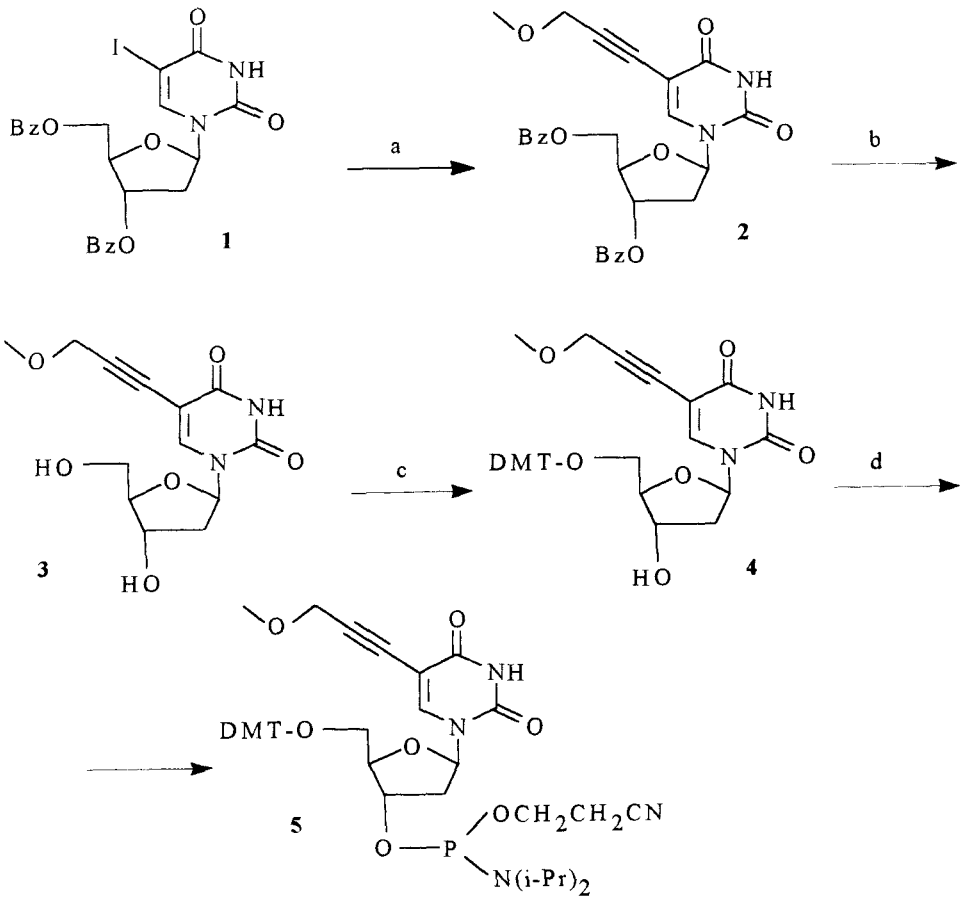
The remarkable enhancement of the stability of DNA duplexes containing C-5 propynyl uridine residues makes such a modification attractive for preparation of oligonucleotides aimed at utilization either as antisense agents or

as DNA-polymerase primers. However, the synthesis of such derivatives is not very convenient. Propyne is an explosive gas and is not commercially available. After applying molecular modeling (Hyperchem 4.5) we found 3-methoxypropyne can be introduced in C-5 position of pyrimidines instead of propyne in order to provide a similar enhancement of double helix formation. The advantage would be that 3-methoxypropyne is liquid and is commercially available.

Synthesis of 5-(3-methoxypropynyl)-2'-deoxyuridine (**3**) (see Scheme 1) was started from benzoyl protected 5-iodo-2'-deoxyuridine (**1**). It was coupled with 3-methoxypropyne in the presence of bis(triphenylphosphine)palladium(II) chloride and copper(I) iodide as described by De Clercq, et al.[24]. It was found that the low solubility of the parent compound **1** in triethylamine, used as solvent for this reaction, complicated the coupling procedure and lowered the yield for the desired product. Utilization of a mixture of dimethylformamide and triethylamine (1:1, v./v.) allowed us to overcome this solubility problem. The resulting product **2** was isolated in 81% yield after column chromatography and was deprotected with methanolic ammonia. Deprotection with methanolic sodium methoxide[24] was found to lead to by-products elevation. After column chromatography 5-(3-methoxypropynyl)-2'-deoxyuridine was isolated in 85% yield. Product **3** was reacted with 4,4'-dimethoxytrityl chloride affording the protected nucleoside **4** in 89% yield after column chromatography purification. After reaction of **4** with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphoro diamidite in the presence of diisopropylammonium tetrazolide, the phosphoroamidite building block **5** was isolated in 97% yield after column chromatography and precipitation from pentane.

Oligonucleotides were prepared, with 5-(3-methoxypropynyl)-2'-deoxyuridine incorporated in different positions (see Table 1), deprotected and purified using standard protocols[25].

The strengths of binding of these oligonucleotides to target DNA were assessed by thermal denaturation analysis (see Figure 1). The melting temperatures (Tm) of the duplexes were calculated on the base of variation of absorbance with the



a. $CH_3OCH_2C{\equiv}CH$, $(Ph_3P)_2PdCl_2$, CuI, Et_3N/DMF,
b. NH_4OH, MeOH, c. DMT-Cl, pyridine,
d. $P(OCH_2CH_2CN)[N(i\text{-}Pr)_2]_2$, diisopropylammonium tetrazolide, CH_2Cl_2

Scheme 1

temperature (see Table 1). The Tm results demonstrate that insertion of 5-(3-methoxypropynyl)-2'-deoxyuridine increases the Tm by 1°C/substitution as compared to the non-modified oligomers (see Table 1). It is noteworthy that shorter ODN **10** binds to target DNA with the strength analogous for ODN **6** probably due to increased relative U content in the sequence.

TABLE 1: Tm of the duplexes formed by the modified oligonucleotides, containing 5-(3-methoxypropynyl)-2'-deoxyuridine with the complementary 20-mer 5' GAAATGAAGAAAAGGTCTGG 3'.

No	ODN sequence	Tm (°C)	△Tm (oC/subst)
6	5' TTTCTTCATTTC 3' (control)	39.5	-
7	5' UUTCTTCAUUUC 3'	43	+1
8	5' TUUCUUCAUTTC 3'	44.5	+1
9	5' UUUCUUCAUUUC 3'	46.5	+1
10	5' UUCAUUUC 3'	38	+1.7*

U indicates the position of 5-(3-methoxypropynyl)-2'-deoxyuridine.

* - calculated[26] Tm for TTCATTTC is 30.5°C

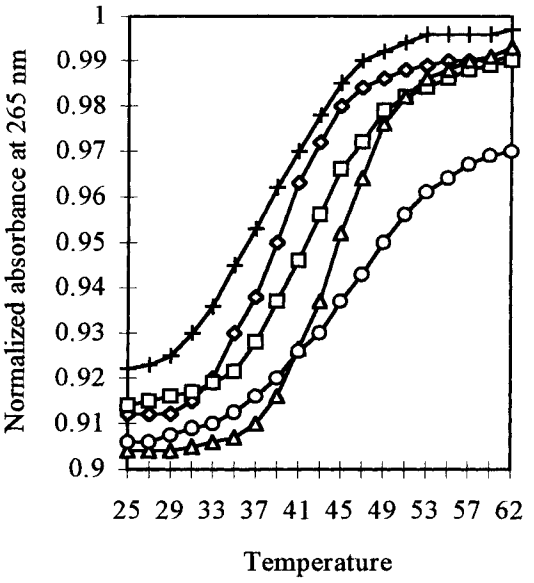


FIGURE 1: UV Melting curves of DNA duplexes

—◇— 6 —□— 7 —△— 8 —○— 9 —+— 10

The data obtained demonstrate that oligonucleotides containing 5-(3-methoxypropynyl)uracyl residues have enhanced binding affinity for target DNA. Their properties as hybridization probes, as antisense agents and DNA-polymerase primers will be the subject for further evaluation.

EXPERIMENTAL

Reagent grade pyridine, triethylamine and diisopropylethylamine were refluxed over and then distilled from calcium hydride. Reagent grade dimethylformamide was vacuum distilled from BaO and next from ninhydrine. TLC analyses were performed on Kieslgel 60 F_{254} plates (Merck). The spots were visualized by UV light and/or spraying with 1M cysteine in water/sulfuric acid, 50:50 with the following warm up. The presence of 4,4'-dimethoxytrityl group was visualized with vapors of trifluoroacetic acid. Column chromatography was performed using silica gel 60 (0.040-0.063 mm, Merck) with the UV monitoring (UVICORD 83000, LKB). Analytical HPLC was performed on a Separon SGX C-18 reverse-phase column (Tessek Ltd.) by using mobile phase: TEAA (0.1M pH=7.5)/ CH_3CN gradient. The NMR spectra were recorded on a Varian VXR-400 instrument using $CDCl_3$ or CD_3CN as solvent with TMS as internal standard. The purity of the products **2-5** was confirmed by analytical reversed phase HPLC (not shown).

3',5'-Di-*O*-benzoyl-5-(3-methoxypropynyl)-2'-deoxyuridine (2). To 6 ml of deoxygenated by dry argon mixture of Et_3N and DMF (1:1, v/v) were added 650 mg (1.15 mmol) of 3',5'-di-*O*-benzoyl-5-(iodo)-2'-deoxyuridine, 26 mg of $(Ph_3P)_2PdCl_2$, and 26 mg of CuI at 20°C and stirring. Dry argon was bubbled through the mixture within 30 min and the temperature was elevated up to 50°C. 0.4 ml of 3-methoxypropyne was added to the resulted stirred solution. The

solution was kept at 50°C for 4 hours under argon and monitored by TLC. It was evaporated to dryness at 40°C. The residue was dissolved in CH_2Cl_2, and washed with 5% EDTA/H_2O, and water, dried over Na_2SO_4, and concentrated to small volume. This solution was applied for column chromatography with methanol gradient in CH_2Cl_2. Product was eluted with 2% MeOH in CH_2Cl_2. Appropriate fraction was collected. The solvent was removed *in vacuo* to give 470 mg (81%) of **2** as a colorless foam. ^1H NMR ($CDCl_3$) δ = 2.45 and 2.82 (m, 2H, H2',2''), 3.32 (s, 3H, OCH_3), 4.14 (s, 2H, $C{\equiv}CCH_2OC$), 4.62 (m, 1H, H4'), 4.78 (m, 2H, H5',5''), 5.64 (m, 1H, H3'), 6.40 (t, 1H, H1'), 7.42-7.70 (m, 12H, Ph), 7.97 (s, 1H, H6), 7.90-8.10 (m, 6H, Ph).

5-(3-methoxypropynyl)-2'-deoxyuridine (3). To 630 mg (1.25 mmol) of **2** were added 3 ml of methanol and 7 ml of concentrated aqueous ammonia. This mixture was sealed and shaken within 1 hour at 20°C until the solution become clear and left overnight at 20°C. It was evaporated to dryness at 20°C and residue was dissolved in MeOH. The solution was carefully neutralized by addition of Dowex 50-X8 (H^+) resin until moistened pH paper indicated pH~6. The solution was filtered out and concentrated to dryness. Residue was dissolved in small volume of CH_2Cl_2 and was applied for column chromatography with methanol gradient in CH_2Cl_2. Product was eluted with 3% MeOH in CH_2Cl_2. Appropriate fraction was collected. The solvent was removed *in vacuo* to give 313 mg (85%) of **3** as a colorless foam. ^1H NMR (CD_3CN) δ = 2.10, (m, 2H, H2',2''), 3.33 (s, 3H, OCH_3), 3.70 (m, 2H, H5',5''), 3.86 (m, 1H, H4'), 4.23 (s, 2H, $C{\equiv}CCH_2OC$), 4.36 (m, 1H, H3'), 6.12 (t, 1H, H1'), 8.17 (s, 1H, H6).

5'-(4,4'-dimethoxytrityl)-5-(3-methoxypropynyl)-2'-deoxyuridine (4). 208 mg (0.71 mmol) of **3** was dissolved in 5 ml of pyridine. To this solution 287 mg of 4,4'-dimethoxytrityl chloride was added. Reaction was monitored by TLC. After 2 hours, 5 ml of water was added and the reaction mixture was extracted with ether. Organic layer was dried under Na_2SO_4, and concentrated to small volume.

It was applied for column chromatography with methanol gradient in mixture of CH_2Cl_2 and Et_3N (0.1%, v/v). Product was eluted with 2% MeOH. Appropriate fraction was collected and upon concentration gave 374 mg (89%) of **4** as a colorless foam. ^1H NMR ($CDCl_3$) δ = 2.29 and 2.50 (m, 2H, H2',2''), 3.13 (s, 3H, OCH_3), 3.39 (m, 2H, H5',5''), 3.80 (s, 6H, 2xOCH_3), 4.02 (s, 2H, C≡CCH_2OC), 4.09 (m, 1H, H4'), 4.54 (m, 1H, H3'), 6.30 (t, 1H, H1'), 6.85 (m, 4H, Ar), 7.20-7.42 (m, 9H, Ar), 8.06 (s, 1H, H6).

2-cyanoethyl (5'-(4,4'-dimethoxytrityl)-5-(3-methoxypropynyl)-2'-deoxyuridin-3'-yl) (*N,N'*-diisopropyl) phosphoroamidite (5). To the solution of 370 mg (0.62 mmol) of **4** in 6 ml of CH_2Cl_2 was added 53 mg of diisopropylammonium tetrazolide. 0.24 ml of 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphoro diamidite was added at stirring. Reaction mixture was stirred for 2 hours. Monitored by TLC. It was diluted by 100 ml of CH_2Cl_2, washed with saturated aqueous $NaHCO_3$, saturated aqueous NaCl, dried under Na_2SO_4 and evaporated. Residue was dissolved in 1 ml of CH_2Cl_2 and precipitated by 200 ml of pentane at -20°C. Precipitate was collected, dissolved in CH_2Cl_2 and solution was evaporated. Residue was dried out in high vacuum. It resulted in 540 mg (97%) of **5** as a cream foam. TLC showed a single spot of **5.** HPLC (mobile phase: CH_3CN/TEAA (0.1M pH=7.5) 90:10, isocratic) showed two peaks of the corresponding diastereomers with the minor admixtures. ^{31}P NMR (CD_3CN) δ = 151.682, 151.682. This product without further purification was used in an automated synthesis.

Oligonucleotide Synthesis. Oligonucleotides **6-10** were synthesized on the controlled pores glass support with a ASM-102 U DNA synthesizer (Biosan Ltd.). After the cleavage from support and deprotection by aqueous ammonia at 20°C for 20 hours, the oligonucleotides were purified by reversed phase HPLC on Separon C-18 column followed by the removal of 4,4'-dimethoxytrityl group and desalting. The purity of oligonucleotides was confirmed by analytical reversed phase HPLC (not shown).

Melting Points of Duplexes. Melting point (Tm) values were determined for duplexes formed by modified oligonucleotides and a complementary unmodified 20-mer d(GAAATGAAGAAAAGGTCTGG) by measuring melting curves in TM buffer (140 mM KCl, 5mM Na_2HPO_4, pH 7.0 / 5 mM $MgCl_2$) on Perkin-Elmer 552 UV/VIS spectrometer at 265 nm[9].

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