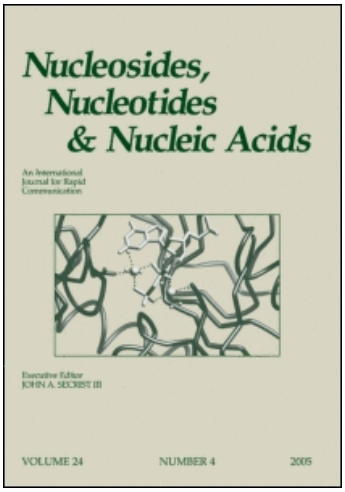

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Ermolinsky, Boris S. , Fomitcheva, Marina V. , Efimtseva, Ekaterina V. , Meshkov, Sergey V. , Mikhailov, Sergey N. , Esipov, Dmitriy S. , Boldyreva, Elena F. and Korobko, Vyacheslav G.(1996) 'Oligodeoxyribonucleosides Containing 1-β-D-Glucopyranosylthymine Synthesis and Substrate Properties', Nucleosides, Nucleotides and Nucleic Acids, 15: 10, 1619 — 1634
To link to this Article: DOI: 10.1080/07328319608002462
URL: http://dx.doi.org/10.1080/07328319608002462

NUCLEOSIDES & NUCLEOTIDES, 15(10), 1619-1634 (1996)

OLIGODEOXYRIBONUCLEOSIDES CONTAINING 1-β-D-GLUCOPYRANOSYLTHYMINE: SYNTHESIS AND SUBSTRATE PROPERTIES

Boris S.Ermolinsky[1], Marina V.Fomitcheva[1], Ekaterina V.Efimtseva[1], Sergey V.Meshkov[1], Sergey N.Mikhailov[1]*, Dmitriy S.Esipov[2], Elena F.Boldyreva[2], and Vyacheslav G.Korobko[2]

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Abstract : Regioselective method for 1-β-D-glucopyranosylthymine incorporation into oligonucleotides has been developed and substrate properties of the latters in DNA synthesis and hydrolysis reactions were investigated.

Recently several sets of oligodeoxyribonucleosides (ODNs) containing dideoxyhexopyranosyl[1-3] and hexopyranosyl[2] nucleosides have been prepared. It has been shown that fully substituted ODNs with hexopyranosylnucleoside residues do not form a complex with complementary DNA[1-3], but ODNs that contain only one dideoxyhexopyranosyl residue in the middle of the chain may form a stable duplex[1,3]. Here we present our results concerning preparation of 1-β-D-glucopyranosylthymine containing ODNs and their substrate properties in DNA synthesis and hydrolysis reactions. Our first attempt to prepare such ODNs by enzymatic polymerization (copolymerization with natural NTP) of 1-(β-D-glucopyranosyl) thymine 6'-triphosphate using E.coli (Klenow fragment) DNA polymerase I, DNA polymerase α, and avian myeloblastosis virus reverse transcriptase was unsuccessful[4].

An efficient preparative six-step synthesis of 1-(2,3-di-O-acyl-β-D-glucopyranosyl)thymine has been achieved starting from D-glucose. Fully acetylated D-glucopyranose was condensed with bis-trimethylsilylthymine under Vorbruggen's

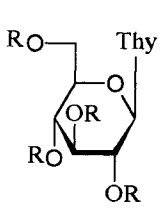
conditions[5,6] to give **1**, deacetylation of which produced 1-(β-D-glucopyranosyl) thymine (**2**) in a high overall yield. Selective 4,6-O-protection was achieved using Markiewicz blocking group[7]. Benzoylation of **3** with an excess of benzoyl cyanide[8] in the presence of triethylamine yielded only 2'-O-monosubstituted **4**, with benzoyl chloride a mixture of **4** and **5** was formed. The reaction of **3** with acetic anhydride proceeded rather slow (1-2 days at room temperature) to yield **6** in a high overall yield. With more bulky iso-butyric anhydride the reaction at 20°C was stopped on the stage of a monosubstituted derivative **7**, further addition of acetic anhydride resulted in formation of **8** in a high yield. Selective desilylation resulted in generation of building blocks **9** and **10** suitable for ODN synthesis. Conventional tritylations of **9** yielded **11** and **12**, the latter was further converted to amidite **13**. Several steps in this scheme were characterized by high yields which made us possible to perform several reactions in succession (**2→3→6→9,** overall yield 67% and **3→7→8,** overall yield 87 %) without product isolation.

The structure of compounds prepared was proven by NMR spectroscopy. Chemical shifts and coupling constants are presented in tables 1 and 2. Several conclusions may be drawn from their analysis:

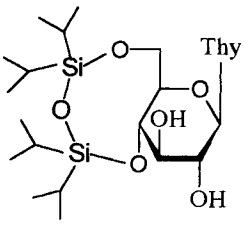
1. The coupling constants of glucopyranosyl residue are in the range of 9-10 Hz, which is typical of equatorial location of all substituents.
2. Introduction of acyl groups shifts the neighbouring protons to the low field, the opposite effect is observed for trityl groups. Their influence on the coupling constants is negligible.
3. Introduction of 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl group causes only small changes in chemical shifts (lowfield shift) and coupling constants, the most pronounced is a decrease of coupling constants $J_{5',6'a}$ and $J_{5',6'b}$. In the case of natural ribonucleosides the conformational changes are more visible[7,9].

Starting with **11** and peracetylated 2'-deoxyadenosine 5'-phosphate, a model dinucleoside phosphate 1-(β-D-glucopyranosyl)thymine-4'-O-phosphorylyl-(4'-5')-2'-deoxyadenosine (**14**) was prepared. The structure and position of internucleotide phosphodiester bond was proven by NMR spectroscopy. The same compound was also prepared by solid phase synthesis and was shown by HPLC to be identical with that prepared in solution.

Several sets of 1-(β-D-glucopyranosyl)thymine containing ONs have been prepared using solid phase synthesis. Their structure was proven using phosphodiesterase digestion followed by HPLC analysis. The correct ratio of natural nucleosides and the modified one was observed. The structures of selected ONs and their complexes with short DNAs are shown on scheme 2. These



1 R = Ac
2 R = H



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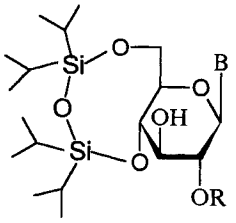
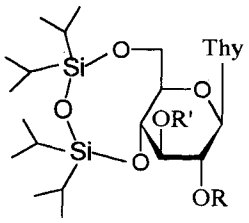
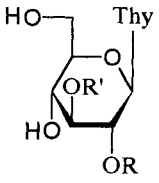


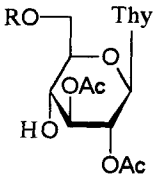
4 R = Bz, B = Thy
5 R = Bz, B = N-Bz-Thy
7 R = *i*Bu



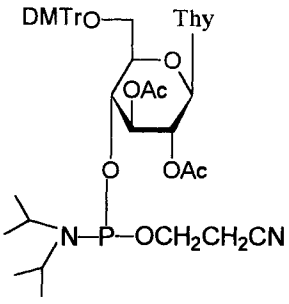
6 R = R' = Ac
8 R = *i*Bu, R' = Ac



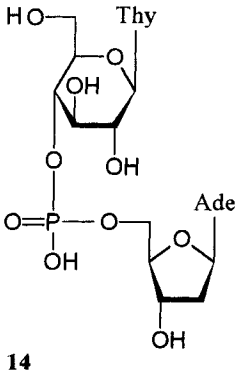
9
10



11 R = MMTr
12 R = DMTr



13



14

SCHEME I

complexes were designed to study elongation by DNA polymerase and they also contain cleavage sites for different endonucleases. Different length of ONs makes possible investigation of cleavage of both strands in one experiment.

The effects of 2',3'-dideoxyglucopyranosylthymine substitution for natural thymidine on duplex stability were evaluated[1,3]. Replacement by one or two

TABLE 1. Chemical shifts in 400.13 MHz PMR spectra at 295° K.

	solvent	H-1'	H-2'	H-3'	H-4'	H-5'	H-6'a	H-6'b	H-6	Me-5
1	$CDCl_3$	5.85d	5.19t	5.38t	5.14dd	3.92ddd	4.28dd	4.12dd	7.14q	1.61d
2	D_2O	5.38d	3.43t	3.49t	3.30t	3.41ddd	3.68dd	3.54dd	7.40q	1.68d
3	$CDCl_3$	5.64d	3.76t	3.86t	3.56t	3.42ddd	4.00dd	3.92dd	7.10q	1.82d
4	$CDCl_3$	5.92d	5.20dd	4.00t	3.94dd	3.46ddd	4.10dd	3.96dd	7.17q	1.88d
5	$CDCl_3$	5.92d	5.22dd	3.99t	3.97dd	3.46ddd	4.11dd	3.99dd	7.28q	1.93d
6	$CDCl_3$	5.80d	4.99t	5.33t	4.05t	3.47ddd	4.08dd	3.94dd	7.07q	1.88d
7	$CDCl_3$	5.82d	5.02t	4.04t	3.92t	3.47ddd	4.16dd	3.90dd	7.15q	1.89d
8	$CDCl_3$	5.80d	4.98t	5.36t	4.06t	3.47ddd	4.09dd	3.94dd	7.07q	1.89d
9	$CDCl_3$-CD_3OD	5.68d	4.96t	5.13t	3.64dd	3.47ddd	3.76dd	3.70dd	7.19q	1.79d
10	$CDCl_3$-CD_3OD	5.70d	4.97t	5.18t	3.66dd	3.48ddd	3.78dd	3.73dd	7.20q	1.81d
11	$CDCl_3$	5.79d	5.05t	5.22t	3.84dd	3.68dt	3.41dd	3.39dd	7.15q	1.88d
14	D_2O	5.56d	3.71t	3.75t	3.98dt	3.45ddd	3.73dd	3.68dd	7.61q	1.89d

Other signals in **1:** 8.37brs (1H, NH), 2.10s (3H, Ac), 2.06s (3H, Ac), 2.02s (3H, Ac); **3**: 9.89 brs (1H, NH), 1.13-1.05m (28H, iPr); **4**: 8.17brs (1H, NH), 7.95d (2H, J = 7.4 Hz), 7.53t (1H, J = 7.4 Hz) and 7.40t (2H, Bz), 2.58brs (1H, OH), 1.15-1.06m (28H, iPr); **5**: 7.96d (2H, J = 7.4 Hz) and 7.58-7.35m (8H, Bz), 2.70brs (1H, OH), 1.13-1.01m (28H, iPr); **6**: 8.36brs (1H, NH), 2.03s (3H, Ac), 1.96s (3H, Ac), 1.12-0.95m (28H, iPr); **7**: 8.92brs (1H, NH), 2.42brs (1H, OH), 2.52sep (1H, J = 7.0 Hz, CH in iBu), 1.01d (3H, Me in iBu), 1.00d (3H, Me in iBu), 1.11-0.98m (28H, iPr); **8**: 8.37brs (1H, NH), 2.45sep (1H, J = 7.0 Hz, CH in iBu), 2.00s (3H, Ac), 1.02d (3H, Me in iBu), 1.00d (3H, Me in iBu), 1.11-0.95m (28H, iPr); **9**: 1.97s (3H, Ac), 1.87s (3H, Ac); **10**: 2.37sep (1H, J = 7.0 Hz, CH in iBu), 1.97s (3H, Ac), 0.97d (3H, Me in iBu), 0.96d (3H, Me in iBu); **11**: 8.91brs (1H, NH), 7.40-7.20m (12H, MMTr), 6.80d (2H, J = 9.0 Hz, MMTr), 3.06brs (1H, OH), 2.06s (3H, Ac), 1.97s (3H, Ac); **14**: for other signals see experimental part.

TABLE 2. Coupling constants in 400.13 MHz PMR spectra at 295° K.

	$J_{1',2'}$	$J_{2',3'}$	$J_{3',4'}$	$J_{4',5'}$	$J_{5',6'a}$	$J_{5',6'b}$	$J_{6'a,6'b}$	$J_{5,6}$
1	9.6	9.6	9.6	10.4	5.3	2.3	-12.7	1.2
2	9.0	9.0	9.0	9.0	2.2	5.3	-12.7	1.2
3	9.2	9.2	9.2	9.2	2.1	1.3	-12.7	1.2
4	9.4	9.2	9.2	8.9	2.0	1.5	-12.9	1.2
5	9.4	9.2	9.2	8.9	2.0	1.4	-12.8	1.2
6	9.4	9.4	9.4	9.4	2.0	1.3	-12.9	1.2
7	9.4	9.4	9.4	9.4	2.0	1.6	-12.7	1.2
8	9.4	9.4	9.4	9.4	2.0	1.3	-12.9	1.2
9	9.4	9.4	9.4	9.9	2.6	4.0	-12.4	1.2
10	9.4	9.4	9.4	9.9	2.7	4.0	-12.4	1.2
11	9.4	9.4	9.4	9.7	4.2	4.2	-12.4	1.2
14	9.0	9.1	9.1	9.6	2.4	4.7	-12.8	1.2

14: $J_{4',p}$ = 9.0 Hz

hexoses at either end of T_{13} resulted in melting temperature similar to that of unsubstituted ones. Substitution in the middle gave a more pronounced destabilization effect, resulting in a drop of Tm of about 15°C[1,3]. In the case of complexes **II - VI** this effect was in the range of 3-5°C (scheme 2).

The **I - V** complexes formed may be recognised and elongated by E.coli DNA polymerase (Klenow fragment)[10] (scheme 2, fig. 1). In the presence of dCTP, dCTP+dATP or dCTP+dATP+TTP the short strand (primer) was elongated by one, three, and four nucleotide residues, respectively (Fig. 1, lanes 5-7 for duplex **V**). Only in the last two cases (**VI** and **VII**) where the modification is located close to 3'-end of the primer (5th position) no polymerization was observed (Fig. 1, lanes 1-3 for duplex **VI**). This observation can be explained by local destabilization of 3'-terminal region of the primer.

It was of interest to study the substrate properties of synthetic oligonucleotide duplexes (**II-VII**), containing β-D-glucopyranosylthymine at different positions, in reactions catalyzed by restriction enzymes. In this connection, these duplexes were subjected to hydrolysis with restriction endonuclease *Eco*RI[11]. The reactions were carried out under conditions of complete cleavage of nonmodified duplex (**I**) (Fig.

```
                              ←a                                  b→
CACAGAATTCTAGATATCACACACT   CACAGAATTCTAGATATCACA-3'       CACAG       AATTCTAGATATCACA
GTGTCTTAAGATCTATAGTGTGTGA   GTGTCTTAAGATCTATAGTGTGTGA-5'   GTGTCTTAA       GATCTATAGTGTGTGA
                                      I    Tm = 56°C

CACAGAAXTCTAGATATCACACACT   CACAGAAXTCTAGATATCACA-3'       CACAG       AAXTCTAGATATCACA
GTGTCTTAAGATCTATAGTGTGTGA   GTGTCTTAAGATCTATAGTGTGTGA-5'         GTGTCTTAAGATCTATAGTGTGTGA
                                      II   Tm = 49°C

CACAGAATXCTAGATATCACACACT   CACAGAATXCTAGATATCACA-3'       CACAGAATXCTAGATATCACA
GTGTCTTAAGATCTATAGTGTGTGA   GTGTCTTAAGATCTATAGTGTGTGA-5'   GTGTCTTAAGATCTATAGTGTGTGA
                                      III  Tm = 52°C

CACAGAATTCXAGATATCACACACT   CACAGAATTCXAGATATCACA-3'       CACAGAATTCXAGATATCACA
GTGTCTTAAGATCTATAGTGTGTGA   GTGTCTTAAGATCTATAGTGTGTGA-5'   GTGTCTTAA       GATCTATAGTGTGTGA
                                      IV   Tm = 52°C

CACAGAATTCTAGAXATCACACACT   CACAGAATTCTAGAXATCACA-3'       CACAG       AATTCTAGAXATCACA
GTGTCTTAAGATCTATAGTGTGTGA   GTGTCTTAAGATCTATAGTGTGTGA-5'   GTGTCTTAA       GATCTATAGTGTGTGA
                                      V    Tm = 53°C

CACAGAATTCTAGATAXCACA       CACAGAATTCTAGATAXCACA-3'       CACAG       AATTCTAGATAXCACA
GTGTCTTAAGATCTATAGTGTGTGA   GTGTCTTAAGATCTATAGTGTGTGA-5'   GTGTCTTAA       GATCTATAGTGTGTGA
                                      VI   Tm = 53°C

CACAGAAXTCTAGATAXCACA       CACAGAAXTCTAGATAXCACA-3'       CACAG       AAXTCTAGATAXCACA
GTGTCTTAAGATCTATAGTGTGTGA   GTGTCTTAAGATCTATAGTGTGTGA-5'         GTGTCTTAAGATCTATAGTGTGTGA
                                      VII  Tm = 45°C
```

X - 1-(β-D-glucopyranosyl)thymine residue

a. E.coli DNA polymerase , b. *Eco*R1 endonuclease

Scheme 2

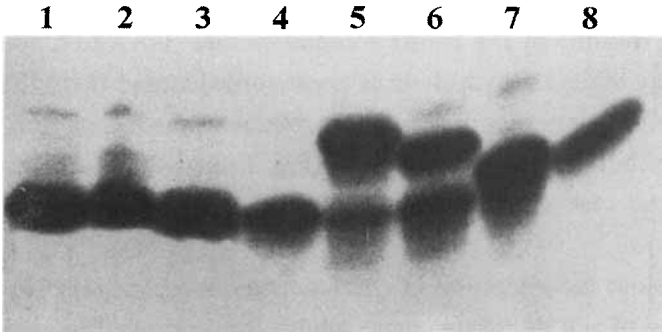


FIG. 1. Autoradiogram of duplex elongation reactions carried out by E. coli DNA-polymerase I (Klenow fragment) : duplex V (lanes 5 - 7) and duplex VI (lanes 1 -3); lanes 4 and 8 for duplexes VI and V, respectively; lanes 3 and 7 - elongation in the presence of dCTP only; lanes 2 and 6 - elongation in the presence of dCTP+dATP; lanes 1 and 5 - elongation in the presence of dCTP+TTP+dATP.

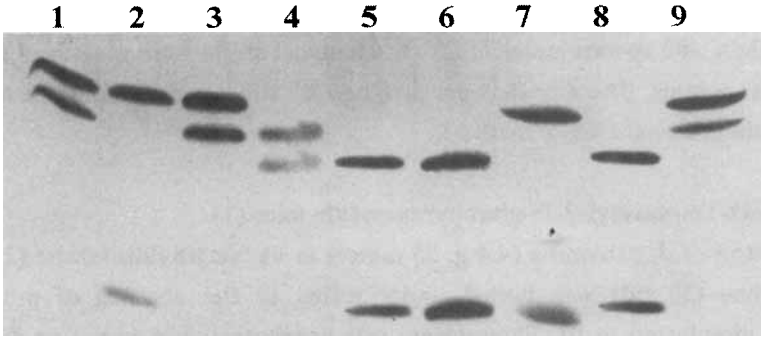


FIG. 2. Separation of *Eco*RI digests of duplexes I - VII in 20% polyacrylamide-urea gel: lanes 2-7 - digests of duplexes II-VII, respectively; lane 8 - *Eco*RI-digest of duplex I; lanes 1 and 9 - duplexes II and I, respectively.

1, lane 8). The hydrolysis of both chains proceeded in quantitative yield when a modified unit was placed apart from the recognition site (duplexes **V** and **VI**) (Fig. 1, lanes 5 and 6, respectively). The modified nucleoside unit insertion close to the endonuclease recognition site (duplex **IV**) inhibited cleavage of the modified strand (Fig. 1, lane 4). On the contrary, replacement of the first thymidine by glucopyranosylthymine residue in the *Eco*RI recognition site GAAXTC (duplexes **II** and **VII**) completely inhibited hydrolysis of nonmodified strand (Fig. 1, lanes 2 and 7). The modification of the second thymidine residue in the recognition site prevented the cleavage of both strands in duplex **III** (Fig. 1, lane 3). Similar results have been obtained in the case of endonucleases *Eco*32I and *Xba*I and will be presented elsewhere.

In conclusion, incorporation of β-D-glucopyranosyl nucleoside residue into a specific place of ODN chain may inhibit the primer elongation by DNA polymerase and prevent the cleavage of modified, unmodified or both strands by restriction endonucleases.

EXPERIMENTAL

Melting points (uncorrected) were determined with an Electrothermal melting point apparatus. UV spectra were recorded on Specord UV/VIS spectrophotometer. Column chromatography was performed on silica gel (0.06-0.20 mm), TLC was carried out on Kieselgel 260 F (Merck) with detection by UV light using A, $CHCl_3$; B, 95:5 $CHCl_3$-EtOH. NMR spectra were recorded on Bruker AMX 400 spectrometer at 22°C. Chemical shifts were measured relative to the solvent signals. The signals were assigned by the double resonance techniques and two dimensional COSY method.

1-(2,3,4,6-O-Tetraacetyl-β-D-glucopyranosyl)thymine (1).

A suspension of dry thymine (4.4 g, 35 mmol) in hexamethyldisilazane (20 ml) and dry pyridine (30 ml) was boiled under reflux in the absence of moisture till complete dissolution (6 h). The mixture was concentrated in vacuo to dryness ans dry toluene (2 x 20 ml) was evaporated from the residue. A solution of 1,2,3,4,6-penta-O-acetyl-β-D-glucopyranose (11.7 g, 30 mmol) in dry 1,2-dichloroethane (150 ml) and $SnCl_4$ (4.2 ml, 35 mmol) were added to the residue, and the mixture was stored for 16 h at 20°C. Chloroform (50 ml) and saturated aqueous sodium hydrogencarbonate (50 ml) were added, the mixture was stirred for 20 min at 20°C, and then filtered through Hyflo Super Cel. The organic layer was separated, washed with aqueous sodium hydrogencarbonate (30 ml) and water, dried, and

evaporated. The residue was crystallized from 90% aqueous ethanol to afford **1** (11.1 g, 81%). M.p. 155-156°C. Lit.[12]: 156-158°C.

1-(β-D-Glucopyranosyl)thymine (2).

The solution of **1** (9.1 g, 20 mmol) in methanol (60 ml) semi-saturated with ammonia at 0°C was kept for 20 h at 20°C and then concentrated in vacuo to dryness. The residue was crystallized from ethanol to yield **2** (5.0 g, 87%). M.p. > 220°C. Lit.[12]: 270-272°C. UV (pH 1-7): λ max 267 nm (ε 9800); (pH 13): λ max 267 nm (ε 7400).

1-[4,6-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-β-D-glucopyranosyl]-thymine (3).

To the solution of dry **2** (0.864 g, 3 mmol) in dry pyridine (15 ml) 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (0.97 ml, 3.1 mmol) was added and the mixture was kept at 20°C for 1.5 hrs until the reaction was complete according to TLC. The solvent was evaporated to dryness, the residue was dissolved in chloroform (30 ml), the organic layer was washed successfully with water, 10% aqueous solution of sodium bicarbonate, and again with water. The organic layer was dried with Na_2SO_4, filtered, the filtrates were evaporated to dryness and evaporated with toluene. The products were purified by column chromatography on silica gel using solvent B to give **3** as a foam (1.45 g, 91%). R_F 0.18 (solvent B).

1-[2-O-Benzoyl-4,6-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-glucopyranosyl]-thymine (4).

Triethylamine (0.21 ml, 1.5 mmol) and benzoylcyanide (197 mg, 1.5 mmol) were added to the solution of **3** (265 mg, 0.5 mmol) in dry dioxane (5 ml). The solution was allowed to stand for 1 h at 20°C, then methanol (0.1 ml) was added, and after 10 min the mixture was evaporated in vacuo to dryness. The residue was chromatographed on a column with silica gel (20 g) in solvent A to yield **4** as a foam (0.35 g, 95%). R_F 0.61 (solvent B). ^{13}C NMR (100.61 MHz) ($CDCl_3$) (100.61 MHz) : 165.81 (C=O), 164.88 (C-4), 150.30 (C-2), 134.94 (C-6), 133.75, 129.98 and 128.56 (Bz), 111.95 (C-5), 80.62 (C-1'), 79.52 (C-5'), 75.55 (C-3'), 72.27 (C-2'), 69.34 (C-4'), 60.31 (C-6'), 17.36, 17.26, 17.18, 13.56, 13.20, 12.69 and 12.63 (iPr), 12.52 (Me-5).

N^3-Benzoyl-1-[2-O-benzoyl-4,6-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-glucopyranosyl]-thymine (5).

Benzoyl chloride (0.26 ml, 2.25 mmol) was added to the solution of **3** (400 mg, 0.75 mmol) in dry pyridine (7 ml) and the mixture was kept for 16 h at 20°C. After

usual work up and column chromatography on silica gel in solvent A compound **5** was obtained as a foam (250 mg, 45%). R_F 0.78 (solvent B). ^{13}C NMR ($CDCl_3$): 168.25 (C=O), 166.01 (C=O), 162.37 (C-4), 149.49 (C-2), 134.84 (C-6), 134.67, 133.78, 133.56, 131.09, 130.12, 130.07,128.87, 128.67, 128.57 and 128.43 (Bz), 112.01 (C-5), 80.69 (C-1'), 79.63 (C-5'), 75.31 (C-3'), 72.72 (C-2'), 69.28 (C-4'), 60.34 (C-6'), 17.37, 17.32, 17.28, 17.18, 17.14, 17.12, 17.04, 13.56, 13.20, 12.80 and 12.64 (iPr), 12.52 (Me-5).

Further elution with solvent A gave **4** as a foam (250 mg, 53%).

1-[2,3-Di-O-acetyl-4,6-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-glucopyranosyl]-thymine (6).

A. A mixture of **3** (1.45 g, 2.74 mmol) and acetic anhydride (5 ml) in dry pyridine (15 ml) was kept for 48 h at 20°C. After usual work up and column chromatography on silica gel in solvent A compound **6** was obtained as a foam (1.50 g, 89%). R_F 0.69 (solvent B). ^{13}C NMR ($CDCl_3$): 174.61 and 169.70 (C=O), 163.00 (C-4), 150.24 (C-2), 134.75 (C-6), 111.98 (C-5), 80.48 (C-1'), 79.59 (C-5'), 74.81 (C-3'), 70.42 (C-2'), 67.12 (C-4'), 60.23 (C-6'), 20.93 and 20.43 (Me, Ac), 17.23, 17.05, 16.95, 13.57, 13.18 and 12.64 (iPr), 12.59 (Me-5).

B. To the solution of dry **2** (0.864 g, 3 mmol) in dry pyridine (15 ml) 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (0.97 ml, 3.1 mmol) was added and the mixture was kept at 20°C for 1.5 h until the reaction was complete according to TLC (system B). Then acetic anhydride (5 ml) was added and the solution was kept for 48 h at 20°C. After usual work up and column chromatography on silica gel in chloroform compound **6** was obtained as a foam (1.80 g, 98%).

1-[2-O-iso-Butyryl-4,6-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-glucopyranosyl]-thymine (7).

A mixture of **3** (1.06 g, 2.0 mmol) with iso-butyric anhydride (2 ml) in dry pyridine (5 ml) was kept for 24 h at 20°C. After usual work up and column chromatography on silica gel in solvent A compound **7** was obtained as a foam (1.10 g, 92%). R_F 0.63 (solvent B).

1-[3-O-Acetyl-2-O-iso-butyryl-4,6-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-glucopyranosyl]-thymine (8).

A. A mixture of **7** (1.20 g, 2.0 mmol) and acetic anhydride (2 ml) in dry pyridine (5 ml) was kept for 48 h at 20°C. After usual work up and column chromatography on silica gel in solvent A compound **8** was obtained as a foam (1.50 g, 90%). R_F

0.73 (solvent B). ^{13}C NMR ($CDCl_3$): 175.95 and 169.58 (C=O), 162.98 (C-4), 150.20 (C-2), 134.90 (C-6), 111.81 (C-5), 80.52 (C-1'), 79.67 (C-5'), 74.51 (C-3'), 70.08 (C-2'), 67.18 (C-4'), 60.26 (C-6'), 33.88 (CH, iBu), 20.93 (Me, Ac), 18.66 and 18.55 (Me, iBu), 17.23, 17.04, 16.95, 13.57, 13.18 and 12.65 (iPr), 12.60 (Me-5).

B. A mixture of **3** (0.90 g, 1.7 mmol) and iso-butyric anhydride (0.5 ml) in dry pyridine (3 ml) was kept for 36 h at 20°C until the reaction was complete according to TLC. Then acetic anhydride (2 ml) was added and the solution was kept for 48 h at 20°C. After usual work up and column chromatography on silica gel in system A compound **8** was obtained as a foam (0.95 g, 87%).

1-(2,3-Di-O-acetyl-β-D-glucopyranosyl)thymine (9).

A. Nucleoside **6** (900 mg, 1.47 mmol) was dissolved in 0.5 M tetrabutylammonium fluoride trihydrate in tetrahydrofuran (8 ml). The solution was kept for 30 min at 20°C, evaporated to dryness, coevaporated with chloroform (10 ml) and applied onto a column with silica gel (20 g). The column was washed with system A (200 ml) and then eluted with system B to give **9** after crystallization from chloroform. Yield 0.40 g (73%). R_F 0.04 (solvent B). M.p. > 200°C. LSIMS: (M + H) 373. ^{13}C NMR ($CDCl_3$-CD_3OD): 170.75 and 169.96 (C=O), 164.07 (C-4), 150.74 (C-2), 135.55 (C-6), 111.58 (C-5), 80.36 (C-1'), 78.99 (C-5'), 75.13 (C-3'), 69.85 (C-2'), 67.65 (C-4'), 60.68 (C-6'), 20.53 and 20.21 (Me, Ac), 12.08 (Me-5).

B. To a solution of dry **2** (1.73 g, 6 mmol) in dry pyridine (30 ml) 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (2.0 ml, 6.37 mmol) was added and the solution was kept at 20°C for 2 h. Then acetic anhydride (5 ml) was added and the solution was kept for 48 h at 20°C. After extraction with chloroform an organic layer was evaporated in vacuo to dryness and evaporated with toluene. The residue was dissolved in 0.5 M tetrabutylammonium fluoride trihydrate in tetrahydrofuran (30 ml). The solution was kept for 30 min at 20°C, evaporated to dryness, coevaporated with chloroform (10 ml) and applied onto a column with silica gel (20 g). The column was washed with system A (200 ml) and then eluted with system B to give **9** after crystallization from chloroform. Yield 1.50 g (67%).

1-(3-O-Acetyl-2-O-iso-butyryl-β-D-glucopyranosyl)thymine (10).

Analogous desilylation of **8** yielded **10** as white powder (65%). R_F 0.06 (solvent B). M.p. > 200°C. LSIMS: (M + H) 401. ^{13}C NMR ($CDCl_3$-CD_3OD): 175.94 and 170.59 (C=O), 163.91 (C-4), 150.60 (C-2), 135.54 (C-6), 111.44 (C-5), 80.23 (C-1'), 79.00 (C-5'), 74.86 (C-3'), 69.38 (C-2'), 67.72 (C-4'), 60.73 (C-6'), 33.73 (CH, iBu), 20.48 (Me, Ac), 18.73 and 18.38 (Me, iBu), 12.06 (Me-5).

1-(2,3-Di-O-acetyl-6-O-monomethoxytrityl-β-D-glucopyranosyl)thymine (11).
A solution of **9** (550 mg, 1.48 mmol) and monomethoxytrityl chloride (602 mg, 1.95 mmol) in dry pyridine (3 ml) was kept for 16 h at 20°C. The reaction mixture was diluted with chloroform, washed with the saturated sodium bicarbonate solution and water. The organic layer was dried, evaporated to dryness and purified by column chromatography in system B to give to give **11** as a foam (0.75 g, 79%). ^{13}C NMR ($CDCl_3$): 170.75 and 169.88 (C=O), 163.27 (C-4), 150.32 (C-2), 134.74 (C-6), 158.71, 143.76, 130.27, 128.19, 127.97, 127.17 and 113.24 (MMTr), 111.87 (C-5), 87.01 (Ph_3C), 80.20 (C-1'), 77.32 (C-5'), 75.10 (C-3'), 70.30 (C-2'), 69.62 (C-4'), 63.30 (C-6'), 55.20 (OMe), 20.77 and 20.42 (Me, Ac), 12.57 (Me-5).

1-(3,4-Di-O-acetyl-6-O-dimethoxytrityl-β-D-glucopyranosyl)thymine (12).
A solution of **9** (400 mg, 1.08 mmol) and dimethoxytrityl chloride (422 mg, 1.25 mmol) in dry pyridine (5ml) was kept for 16 h at 20°C. The reaction mixture was diluted with methylene chloride (20 ml) and washed twice with the saturated sodium bicarbonate solution (2 x 10 ml). The organic layer was dried, evaporated to dryness and purified by column chromatography using system B (containing 1% of Et_3N) to give 680mg (94%) of the title compound. ^1H NMR ($CDCl_3$): 8.85 brs (1H, NH), 7.45-7.17 m (10H, H-6, DMTr), 6.76 d (2H, J = 9.0 Hz, DMTr), 6.74 d (2H, J = 9.0 Hz, DMTr), 5.80 d (1H, $J_{1',2'}$ = 9.4 Hz, H-1'), 5.20 t (1H, $J_{3',2'} = J_{3',4'}$ = 9.4 Hz, H-3'), 5.03 t (1H, H-2'), 3.84 dd (1H, $J_{4',5'}$ = 9.7 Hz, H-4'), 3.77 s (6H, OMe), 3.67 dt (1H, $J_{5',6a'} = J_{5',6'b}$ = 4.2 Hz, H-5'), 3.40 dd (1H, $J_{6'a,6'b}$ = -12.4 Hz, H-6'a), 3.39 dd (1H, H-6'b), 3.00 brs (1H, HO-4'), 2.07 s (3H, Ac), 1.98 s (3H, Ac), 1.90 d (3H, $J_{5,6}$ = 1.2 Hz, Me-5).

[1-(2,3-di-O-acetyl-6-O-dimethoxytrityl-β-D-glucopyranosyl)thymine]-4'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (13).
To a solution of **12** (480 mg, 0.71 mmol) in dry MeCN (5 ml) was added diisopropylamine hydrotetrazolide (59.5 mg, 0.35 mmol) and 2-cyanoethyl-bis-(N,N-diisopropyl)-phosphoramidite (0.254 ml, 0.8 mmol), the mixture was stirred for 2 h at 20°C and diluted with CH_2Cl_2 (20 ml). The organic layer was washed with a saturated sodium bicarbonate solution (3 x10 ml) and water (10 ml), dried, evaporated and purified by flash column chromatography (CHCl3-EtOAc-Et3N 45:45:10) affording **13** (380 mg, 61%) as a white foam. ^1H NMR ($CDCl_3$): 8.35 brs (1H, NH), 7.45-7.19 m (10H, H-6, DMTr), 6.78 d (2H, J = 9.0 Hz, DMTr), 6.77 d (2H, J = 9.0 Hz, DMTr), 5.86 d (1H, $J_{1',2'}$ = 9.4 Hz, H-1'), 5.37 t (1H, $J_{3',2'} = J_{3',4'}$ = 9.4 Hz, H-3'), 5.03 t (1H, H-2'), 3.92 m (1H, H-4'), 3.81 m (1H, H-5'), 3.77 s (6H,

OMe), 3.68 m (2H, $POCH_2$), 3.51 m (2H, H-6'a,6'b), 3.24 m (2H, CH in iPr), 2.31 t (1H, J = 6.2 Hz, CH_2CN), 2.30 t (1H, J = 6.7 Hz, CH_2CN), 2.02 s (3H, Ac), 1.96 s (3H, Ac), 1.62 d (3H, $J_{5,6}$ = 1.2 Hz, Me-5), 1.04 d (6H, J = 6.8 Hz, Me in iPr), 0.96 d (6H, J = 6.8 Hz, Me in iPr).

1-(β-D-glucopyranosyl)thymine-4'-O-phosphorylyl-(4'-5')-2'-deoxyadenosine (14).
To a mixture of predried pyridinium salt of N^2,3'-O-diacetyl-2'-deoxyadenosine 5'-phosphate (0.8 mmol) and **11** (400 mg, 0.62 mmol) in dry pyridine (5 ml) N,N'-dicyclohexylcarbodiimide (0.82 g, 4 mmol) was added, and the mixture was stirred for 7 days at 20°C. The mixture was treated with water (5 ml), stirred for 5 h at 20°C and filtered. The precipitate was washed with 20% aqueous pyridine and the combined filtrates were evaporated in vacuo to dryness. The residue was dissolved in methanol (30 ml) semi-saturated with ammonia at 0°C, the resulted solution was kept for 48 h at 20°C and then concentrated in vacuo to dryness. The residue was solved in 80% acetic acid (10 ml) and the solution was kept for 16 h at 20°C. After evaporation and coevaporation with iso-propanol (5 x 10 ml) the deprotected product was dissolved in a mixture of water (20 ml) and ether (20 ml). The aqueous layer was separated, washed again with ether (20 ml) and applied onto a column of DEAE cellulose (50 ml, HCO_3^- form). The column was washed with water and eluted with a linear gradient of NH_4HCO_3 (from 0.01 to 0.1 M/L). The pooled fractions (eluted at 0.05 M/L) were evaporated to dryness, coevaporated with water (5 x 10 ml) and freeze-dried. Yield 35%. R_F 0.50 (TLC, silica gel, 2-propanol-NH_4OH-H_2O 7:1:2). ^1H NMR (400.13 MHz) (D_2O): 8.41 s (1H, H-8 A), 8.22 s (1H, H-2 A), 7.61 q (1H, $J_{6,5}$ = 1.2 Hz, H-6 T), 6.50 t (1H, $J_{1',2'a} = J_{1',2'b}$ = 6.7 Hz, H-1' A), 5.56 d (1H, $J_{1',2'}$ = 9.0 Hz, H-1' T), 4.73 ddd (1H, $J_{3',2'a}$ = 6.7 Hz, $J_{3',2'b}$ = 3.8 Hz, $J_{3',4'}$ = 2.9 Hz, H-3' A), 4.28 dddd (1H, $J_{4',5'a}$ = 3.5 Hz, $J_{4',5'b}$ = 4.7 Hz, $J_{4',P}$ = 1.3 Hz, H-4' A), 4.13 ddd (1H, $J_{5'a,5'b}$ = -11.5 Hz, $J_{5'a,P}$ = 5.3 Hz, H-5'a A), 4.10 ddd (1H, $J_{5'b,P}$ = 5.7 Hz, H-5'b A), 3.98 dt (1H, $J_{4',3'}$ = 9.1 Hz, $J_{4',5'}$ = 9.6 Hz, $J_{4',P}$ = 9.0 Hz, H-4' T), 3.75 t (1H, $J_{3',2'}$ = 9.1 Hz, H-3'), 3.73 dd (1H, $J_{6'a,5'}$ = 2.4 Hz, $J_{6'a,6'b}$ = -12.8 Hz, H-6'a T), 3.71 t (1H, H-2' T), 3.68 dd (1H, $J_{6'b,5'}$ = 4.7 Hz, H-6'b T), 3.45 ddd (1H, H-5' T), 2.89 ddd (1H, $J_{5'a,5'b}$ = -14.1 Hz, H-2'a A), 2.62 ddd (1H, H-2'b A), 1.89 d (3H, Me-5). ^{31}P NMR (161.98 MHz) (D_2O) chemical shift in ppm from 80% phosphoric acid: 0.35.

DNA synthesis.
Oligonucleotide synthesis was performed on the Biosan DNA synthesizer model ASM-102U in 0.2 μmol-scale (5 μmole amidite per cycle) using commercial 2-

cyanoethylphosphoroamidites and **13**. The obtained sequences were deprotected and cleaved from the solid support by treatment with concentrated ammonia for 16h at 60°C. The obtained tritylated oligonucleotides were purified on Zorbax ODS column using concentration gradient of MeCN (15%-40% in 35 min) in 0.1 M triethylammonium acetate. Further detritylation of oligonucleotides was achieved by 80% acetic acid (25 min at room temperature). The final purification was performed on the above mentioned column (5%-25% of MeCN in 30 min) with the subsequent gel filtration of products on the Toyopearl HW-40 column in water.

Enzymatic degradation of oligonucleotides.

An oligonucleotide (0.2 OD) solution in 400 μl of the following buffer (50 mM Tris-HCl pH 8.6; 50 mM sodium chloride; 7mM $MgCl_2$) was digested by 1.0 U of snake venom phosphodiesterase (Pharmacia) at 37°C for 16h. This digestion was followed by the treatment with 1.0 U of alkaline phosphatase (Boehringer Manheim) for 4 h at 37°C. Aliquots of this solution (50 μl) were analyzed by HPLC. All oligonucleotides showed a correct ratio of unmodified nucleosides over the modified one.

HPLC analysis.

The reverse phase HPLC on an analytical Zorbax ODS column (4 x 250 mm) was perfomed using Beckman Gold System. The separation was carried out in 0.1 M triethylammonium acetate - MeCN (from 0% till 25% in 25 min, flow rate 1 ml/min). The retention time was 4.3 min for **2** and 5.1, 8.5, 9.1, 11.0 min for cytosine, guanine, thymidine and adenine respectively.

Melting experiments.

Oligomers were dissolved in 0.2 M NaCl, containing 0.01 M potassium phosphate, pH 7.0. ON concentrations were determined spectrophotometrically at 260 nm. Melting curves were measured on Gilford spectrophotometer. Cuvettes were thermostated with water circulating through the cuvette holder and the temperature was measured directly in the cuvette. The samples were heated at a typical rate of 1°C/min.

^{32}P-5′-Phosphorylation of oligonucleotides.

^{32}P-5′-Phosphorylation of oligonucleotides (250 pmoles) was carried out in 20 μl of buffer, containing 20 mM tris-HCl (pH 9.5), 10 mM $MgCl_2$ and 2 mM dithiothreitol (DTT) in the presence of 10 μCi γ-^{32}P[rATP] and 5 U of

polynucleotide kinase for 30 min at 37°C. Thereafter, 5 μl of 0.1 mM rATP was added and the mixture was kept for 20 min at 37°C. The reaction was stopped by addition of 5 M sodium acetate (2.5 μl, pH 5.0), and labelled oligonucleotides were precipitated with ethanol.

Elongation reactions with Klenow fragment of *E.coli* DNA Polymerase I.

The elongation reactions for duplexes (**I-VII**) (2.5 pmoles, ^{32}P-labelled short strand) were performed in 20 μl of buffer, containing 50 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 2 mM DTT, and 0.1 mM of dNTP. Three elongations were performed for each duplex in the presense of dCTP, dCTP+dATP and dCTP+dATP+TTP. The reactions were carried out for 40 min at 12°C. The products were analyzed by electrophoresis in 20% polyacrylamide gel (PAG) containing 7 M urea.

Hydrolysis by restriction endonucleases *Eco*RI, *Xba*I и *Eco*32I.

^{32}P-5'-Labelled (both strands) oligonucleotide duplexes (**I-VII**) (2.5 pmoles of each) were heated for 5 min at 72°C in 20 μl of buffer, containing 50 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 100 mM NaCl and slowly cooled to 15°C. Mercaptoethanol was added to this solution till final concentration 5 mM, then 5-10 units of corresponding restriction endonuclease were introduced into reaction mixture. The reaction was kept for 1.5 h at 37°C and the products were analyzed by electrophoresis in 20% PAG containing 7 M urea.

Acknowledgements

We are grateful to Prof. P.Herdewijn for helpful discussions. Financial support from INTAS (project 93-1500) and RFBR (projects 93-04-7607 and 95-03-08347A) is gratefully acknowledged.

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Received February 26, 1996
Accepted July 15, 1996