SYNTHESIS, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF DNA AND RNA OLIGONUCLEOTIDES CONTAINING SHORT ALKYLAMINO INTERNUCLEOTIDE BOND

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Received April 28, 2011
Accepted July 14, 2011
Published online December 7, 2011

This paper is dedicated to Professor Antonín Holý, on the occasion of his 75th birthday.

The condensation of the 5′-*O*-DMT-3′-deoxy-3′-aminothymidine with 3′-*O*-TBDMS-thymidine-5′-aldehyde, followed by reduction of the resultant imine derivative and removal of *tert*-butyldimethylsilyl (TBDMS) protecting group, provided a dimer (denoted as $T_{NH}T$), which is a congener of dithymidine phosphate with the phosphate linkage 3′-O-P(O)(OH)-O-5′ replaced with an amino group (–NH–). After phosphitylation of the 3′-OH group, the dimer $T_{NH}T$ was introduced (by the standard phosphoramidite approach) into a central part of the nonadecathymidylate. This oligomer exhibited lower affinity to the complementary single and double stranded DNA complements as compared to unmodified T_{19} oligonucleotide. The cleavage of modified oligomer with the snake venom and calf spleen phosphodiesterases was completely suppressed at the site of modification. RNA oligomers containing the $T_{NH}T$ dimer were used for preparation of siRNA molecules directed towards mRNA of BACE1 (beta-site amyloid precursor protein cleaving enzyme). The presence of the $T_{NH}T$ units at the 3′-ends of the RNA strands of the siRNA molecule (the siRNA itself is an effective gene expression inhibitor for BACE1) preserved the gene silencing activity and improved the stability of the modified siRNA in 10% fetal bovine serum.

Keywords: Schiff base; Oligonucleotide; Bioorganic chemistry; Modified oligonucleotide; Modified backbone; Thymidine dimer.

Oligonucleotides are useful tools for molecular biology, biotechnology and medicinal chemistry. In the latter application, oligonucleotides are used as sequence-specific inhibitors of gene expression at the transcription level

doi:10.1135/cccc2011090

(antigene, decoy and CpG oligonucleotides) as well as at the post-transcriptional level (antisense oligonucleotides, ribozymes and short interfering RNAs). One of the most discouraging features of the therapeutic oligonucleotides is their fast degradation by cellular nucleases. This disadvantage is less severe when more nucleases resistant oligonucleotides containing chemically modified internucleotide linkages are used. Oligonucleotide phosphorothioates were the first chemically modified constructs with enhanced resistance in body fluids. Because of the polyanionic character of oligonucleotides, some carriers (e.g. lipids) are used to facilitate their cellular up-take. Well known replacement of the phosphate internucleotide bond with methylphosphonate function gives rise to the nuclease resistant, non-charged oligomers with improved ability to cross the cell membrane. However, both phosphorothioate and methylphosphonate analogs are P-chiral, and the corresponding oligomers, if prepared on non-stereocontrolled way, exist as mixtures of hundreds or thousands P-diastereomeric species[1]. Thus, nucleases resistant oligonucleotides with achiral and non-ionic internucleotide linkages can be considered as a next generation of antisense oligonucleotides[2]. Oligodeoxyribonucleotides having the phosphate group replaced with carbonate[3], carbomethoxyl[4], carbamate or oxyamide function[5], were first constructs of this type, followed by those with alkylamino-[6] or amido-type[7] internucleotide linkages. Recently, it was demonstrated that amide residues are excellent mimics of the phosphate linkages in the RNA backbone[8]. Heterocyclic internucleotide linkages, based on imidazole[6e] or triazole rings, constitute another type of phosphate mimics[9]. The latter were successfully synthesized by the click chemistry ([3+2] azide-alkyne cycloaddition). In this paper we describe the chemical synthesis and physico-chemical and biological properties of DNA and RNA oligonucleotides containing a novel unit, in which the internucleotide phosphate bond 3′-O-P(O)(OH)-O-5′ is replaced with an amino function (–NH–).

RESULTS AND DISCUSSION

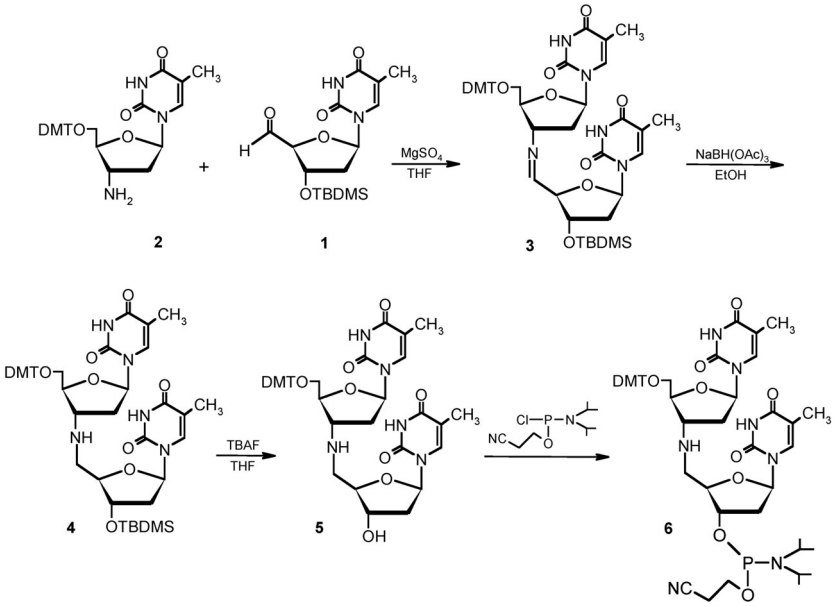
Chemical Synthesis of a Dimer $T_{NH}T$

Condensation of 5′-*O*-DMT-3′-amino-3′-deoxythymidine[10] **1** (Scheme 1) with 3′-*tert*-butyldimethylsilylthymidine-5′-aldehyde[11] **2** (the latter obtained from 3′-*tert*-butyldimethylsilylthymidine by Swern's oxidation[12]) resulted in the formation of a Schiff base **3**. Reduction of the imine group

in **3** with $NaBH(OAc)_3$ and chromatographic purification provided the dimer **4**. It was characterized by FAB MS (in a negative ions mode) and the expected signal *m/z* 880 (m.w. 881) was found. Removal of the 3′-protecting group with TBAF in THF [13] provided the dimer **5**, which after routine purification (a silica gel column) was phosphitylated with *O*-(2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite[14]. The crude **6** was purified by chromatography on a silica gel column and lyophilized from benzene. It was further used for the synthesis of the model oligonucleotides via the phosphoramidite method (Table I).

*Chemical Synthesis of Oligonucleotides **7–9***

Synthesis of oligonucleotides was performed according to the manufacturer's recommendations, except for the coupling time of the modified unit, which was extended to 600 s. An oligodeoxyribonucleotide $T_9T_{NH}TT_8$ was synthesized on the LCA-CPG solid support, then routinely deprotected and purified by the two step procedure[15]. The 5′-*O*-DMT-protected (DMT-on) and fully deprotected (DMT-off) oligomer **7** was subjected to



SCHEME 1
Chemical synthesis of 3′-*O*-phosphoramidite derivative of 5′-*O*-DMT-protected dimer $T_{NH}T$

RP-HPLC on an ODS Hypersil C18 column. The structure of this oligomer was confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry at both steps (Table I).

TABLE I
The sequences of prepared oligonucleotides containing the $T_{NH}T$ dimer and their MALDI-TOF mass spectral analysis data

ODN No.	Sequence	M.w.	MALDI-TOF MS, *m/z*
7 (DNA)	5′-$T_9T_{NH}TT_8$-3′	5937 (DMT-on) 5634 (DMT-off)	5944 (DMT-on) 5639 (DMT-off)
8 (siRNA sense strand)	5′-AAUCAGACAAGUUCUUCAUT_{NH}T-3′	6519	6515
9 (siRNA antisense strand)	3′-T_{NH}TUUAGUCUGUUCAAGAAGUA-5′	6576	6574

The RNA oligonucleotides **8** and **9**, corresponding to the sense and antisense strands of siRNA directed towards beta-site amyloid precursor protein cleaving enzyme (BACE1) mRNA [16], were synthesized on a universal solid support[17] (Glen Research) and cleaved from the support according to the manufacturer's protocol (NH_3 in MeOH, 30 min, room temperature). After removal of the 2′-protecting group with $Et_3N{\cdot}3HF$, the resulting product was purified by RP-HPLC (a PRP-1 column, Hamilton). The structures of **8** and **9** were confirmed by MALDI-TOF mass spectrometry (Table I), and their purity was assessed by electrophoresis in 20% polyacrylamide/7 M urea gel (data not shown).

Thermal Stability of the Complexes of $T_9T_{NH}TT_8$ with dA_{19} and $d(A_{21}C_4T_{21})$ Templates

Thermal stability of the complexes of fully deprotected oligonucleotide **7** with the complementary single stranded dA_{19} and double stranded $d(A_{21}C_4T_{21})$ oligodeoxyribonucleotides was determined to assess the affinity of the modified oligonucleotide towards target molecules. This parameter is important when antisense/antigene properties are considered. The dA_{21} tract of the $d(A_{21}C_4T_{21})$ hairpin oligonucleotide is able to bind **7** (as the third strand) by the Hoogsteen hydrogen bonds. The melting temperature measurements were performed in Tris-HCl buffer, pH 7.4. Since a typical pK_a of dialkyl amines is above 10 (for diethyl amine pK_a = 10.87 [18]) one

may assume that the –NH– linkage in the $T_{NH}T$ dimer exists in a protonated form. We found that the $T_{NH}T$ modification present in the central part of the duplex **7**/dA_{19} decreases its melting temperature by 4.8 °C, compared to the T_m of the reference T_{19}/dA_{19} duplex (T_m = 48.1 vs 52.9 °C), while the triplex **7**/d($A_{21}C_4T_{21}$) melts at the temperature only slightly lower than its non-modified counterpart (T_m = 31.0 vs 32.6 °C). These data indicate that the inserted $T_{NH}T$ dimer (in which there is only a two bonds linkage between the C3′ atom of the upstream T unit and the C5′ atom of the downstream T unit) decreases the hybridization affinities of the modified strand. It is rather expected feature, although there are exceptions, like 5′-*nor*-oligonucleotides with a shortened sugar-phosphate backbone (due to the lack of the 5′-methylene group), which form only slightly less stable complexes with DNA templates (ΔT_m = 9 °C between d(5′-*nor*-A)$_{24}$/T_{24} and dA_{24}/T_{24}, less than 0.4 °C/modification)[19]. In a case of the triplex **7**/d($A_{21}C_4T_{21}$), the destabilizing effect of the shortening (ΔT_m = 1.6 °C) is not very big. Perhaps, to a certain extent it is compensated by electrostatic attraction between the protonated NH function and the negatively charged phosphate groups in the template.

Circular Dichroism Spectra of Complexes (Duplex and Triplex) Containing $T_9T_{NH}TT_8$ strand

The overall structures of the complexes of oligonucleotide **7** with dA_{19} and d($A_{21}C_4T_{21}$) were analyzed by circular dichroism (CD) spectroscopy and compared to the structures of the parent non-modified complexes. The spectra for complexes with the strands stoichiometry 1:1, recorded in a range of 220–320 nm at 25 °C, are shown in Fig. 1. The CD spectra for duplexes **7**/dA_{19} and T_{19}/dA_{19} are similar and have a profile typical for the B-type DNA helix (Fig. 1a). The CD spectra for triplexes **7**/d($A_{21}C_4T_{21}$) and T_{19}/d($A_{21}C_4T_{21}$) differ slightly in a range of 220–230 nm (an increase of intensity) as well as in 245–263 nm (a decrease of intensity for the modified triplex; Fig. 1b), indicating that the modified unit disturbs the helical structure of the B-type DNA [20].

Nucleolytic Stability of Oligonucleotides Containing $T_{NH}T$ Moieties

*A) Stability of **7** in the presence of snake venom and calf spleen phosphodiesterases*. Oligonucleotide **7** was treated with snake venom phosphodiesterase (svPDE, PDE I, 3′-exonuclease) and with calf spleen phosphodiesterase (csPDE, PDE II, 5′-exonuclease). The reaction was stopped at

given time points and the products of digestion were analyzed by MALDI-TOF mass spectrometry. Figures 2a and 2b show the series of four consecutive MS spectra for digestion products, recorded after 10, 30, 45 and 120 min of incubation of **7** with the csPDE and svPDE nucleases, respec-

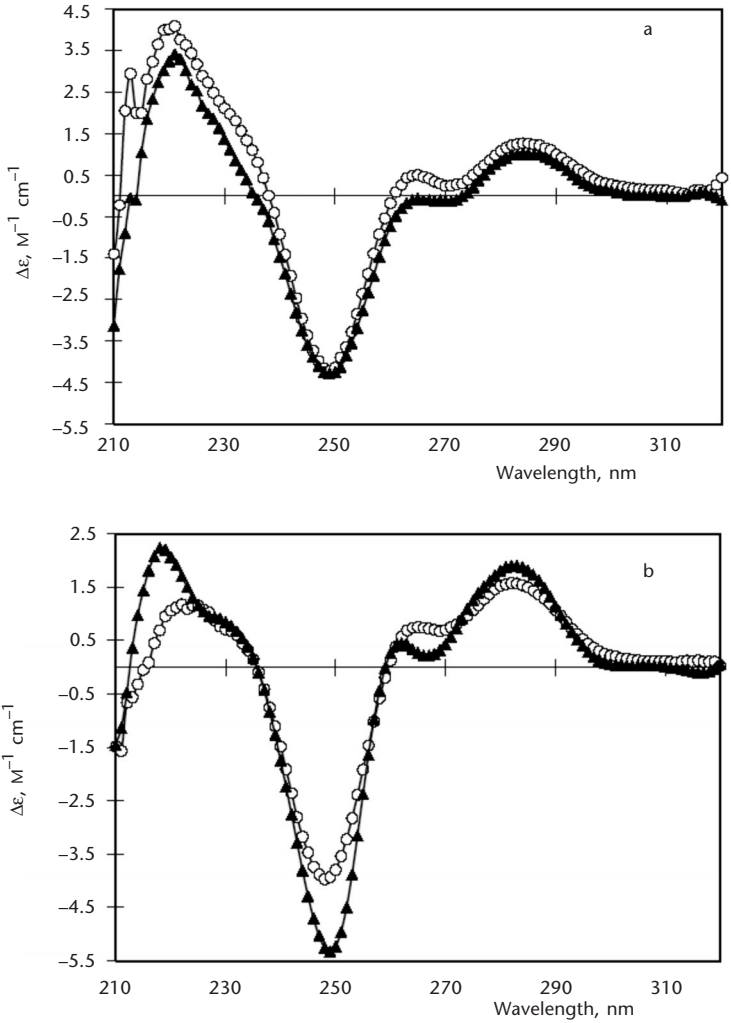


FIG. 1

CD spectra of a duplexes T_{19}/dA_{19} (○) and **7**/dA_{19} (▲) and b triplexes $T_{19}/d(A_{21}C_4T_{21})$ (○) and **7**/$d(A_{21}C_4T_{21})$ (m). Equimolar amounts of modified oligonucleotides and complementary templates (c = 1 μM of each strand) were dissolved in 10 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$ and 100 mM NaCl; the spectra were recorded in a range of 220–320 nm at 25 °C

tively. The substrate **7** (m.w. 5634, *m/z* 5634) is cleaved to yield shorter oligonucleotides, which differ by 304 atomic mass units (u), until the enzyme reaches the modification site. Upon digestion with csPDE and svPDE, the nucleolytically stable products $T_{NH}TT_8$ (*m/z* 2895) (Fig. 2a) and $T_9T_{NH}T$ (*m/z* 3199) (Fig. 2b), respectively, were found. This means that, as it was expected, the modified internucleotide bond (structurally far different from

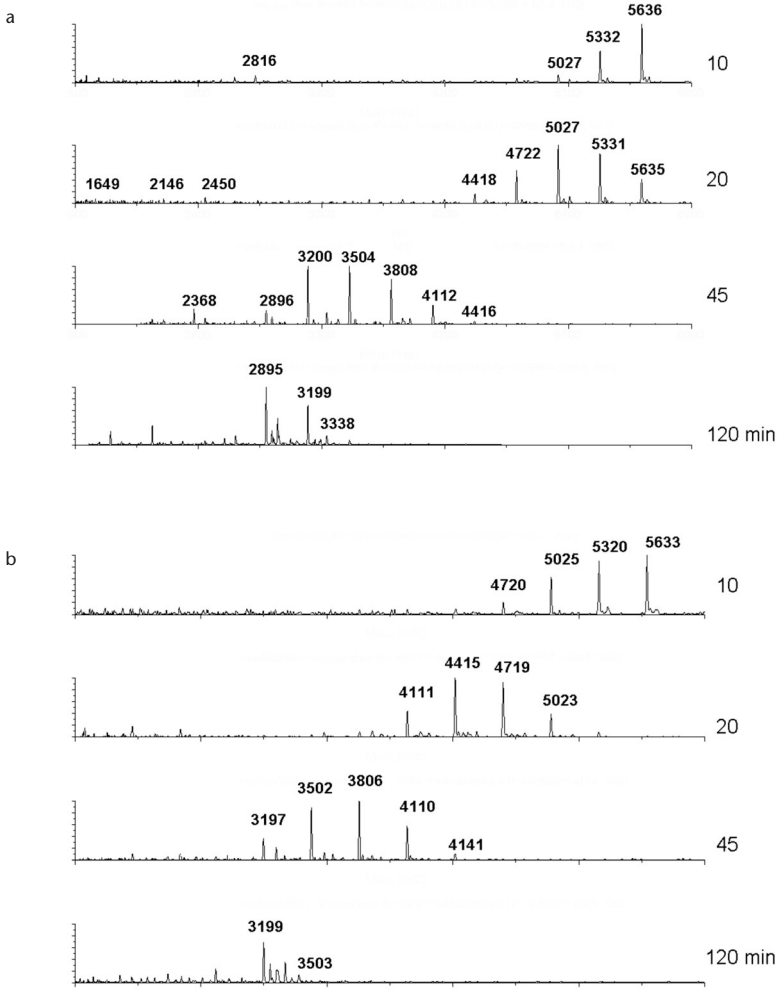


FIG. 2
MALDI-TOF mass spectra of products of the digestion of **7** with calf spleen phosphodiesterase (a) and snake venom phosphodiesterase (b). The samples were digested for 10, 30, 45 and 120 min at 25 °C

the phosphate moiety) remained undigested and prevented the partially hydrolyzed products from further nucleolytic cleavage.

B) Stability of siRNA containing $T_{NH}T$ dimer at the 3′-ends in 10% fetal bovine serum. Short interfering RNAs (siRNAs) are executory molecules of the RNA interference, which was discovered recently as a gene expression regulatory mechanism. siRNAs are commonly used as exogenous gene silencers. They consist of complementary RNA strands, which contain two non-paired units at their 3′-ends. It was already shown that siRNAs with deoxyribonucleotide T_PT overhangs are as active as their full-RNA congeners. To examine the influence of the $T_{NH}T$ modification on the silencing activity and nucleolytic stability of siRNA, we tested duplexes modified at the 3′-ends in one or both strands. Three siRNA duplexes designed for silencing of BACE1 protein[16,21] were prepared by annealing the sense (**8** or **10**) and antisense (**9** or **11**) strands (Table II), and their stability in 10% fetal bovine serum (FBS) was quantified.

TABLE II
The sequences of siRNA duplexes used in these studies. Non-modified parent single stranded RNAs are designed as sense (**10**) and antisense (**11**) strands

siRNA	Sequence
S1 (**10**/**11**)	5′-AAUCAGACAAGUUCUUCAU**TT**-3′ 3′-**TT**UUAGUCUGUUCAAGAAGUA-5′
S2 (**8**/**9**)	5′-AAUCAGACAAGUUCUUCAUT$_{NH}$**T**-3′ 3′-**T**$_{NH}$**T**UUAGUCUGUUCAAGAAGUA-5′
S3 (**10**/**9**)	5′-AAUCAGACAAGUUCUUCAU**TT**-3′ 3′-**T**$_{NH}$**T**UUAGUCUGUUCAAGAAGUA-5′

SiRNAs **S1**, **S2** and **S3** as well as single stranded oligonucleotides **8**, **9**, **10** and **11** were treated with 10% FBS in RPMI medium at 37 °C for up to 24 h. At consecutive time points, aliquots were removed and examined for the intact single stranded RNA or the duplex by native polyacrylamide gel electrophoresis (PAGE; Fig. 3).

Despite of the presence of the $T_{NH}T$ modification in the single stranded RNAs **8** and **9**, these oligomers were degraded as efficiently as non-modified RNAs **10** and **11**. Double stranded RNAs were more resistant to nucleolytic degradation. There was an initial loss of the duplexes – in each case the difference in amount of the intact duplex was observed between a sample without FBS and a sample "0" with cold FBS, incubated in ice. Remaining

amounts of duplexes were degraded slowly, but no significant difference in stability was noticed between the wild type and modified siRNAs. The half life times for all duplexes were around 5 h. These results indicate that the $T_{NH}T$ modification present at the 3′-ends of siRNAs increases their stability toward FBS to a little extent only.

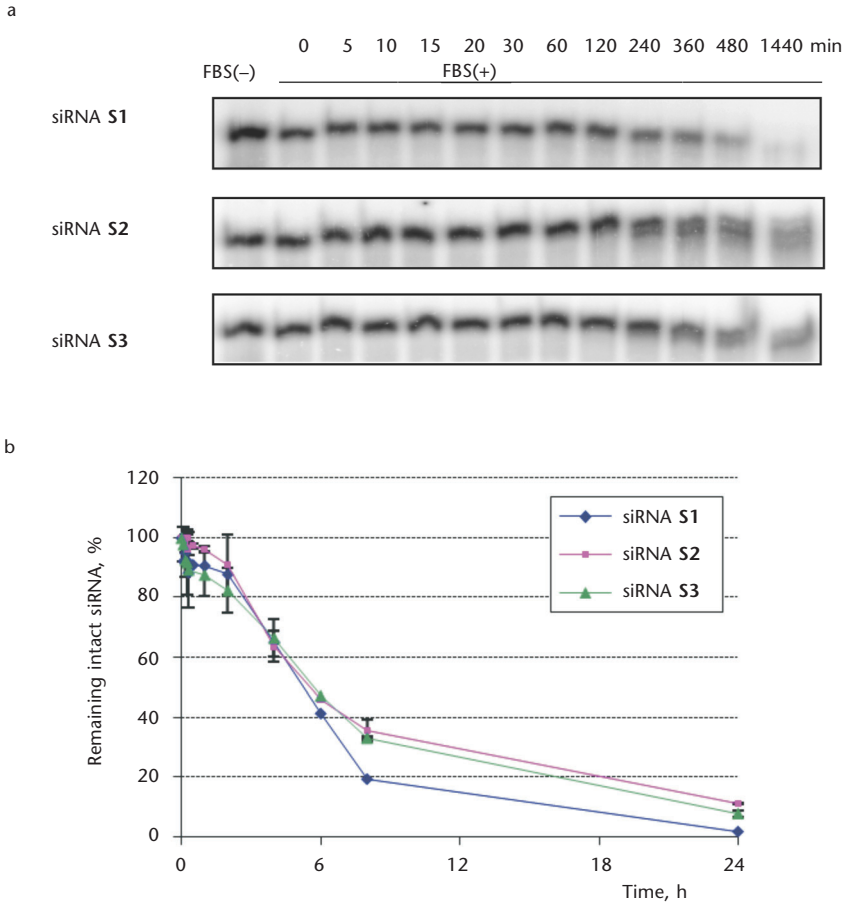


FIG. 3

Stability of siRNA duplexes in 10% FBS. a Double stranded RNA (siRNAs **S1**, **S2** and **S3**) were treated with 10% FBS at 37 °C for indicated times 0, 5, 10, 15, 20, 30, 60, 120, 240, 360, 480 min and 24 h, then were examined by non-denaturating gel electrophoresis to determine the amount of intact duplexes. b The bands were quantified by ImageQuant v. 5.0 software (Molecular Dynamics) and the percentage of the intact siRNA (compared to the initial amount) was calculated. Data represent mean values from two independent experiments

*Gene Silencing Activity of siRNA **S2** Containing $T_{NH}T$ Units at 3′-End of Each Strand*

Gene silencing activity of **S2** was determined in a dual fluorescence assay, conducted in HeLa cells[16,21], and was compared to the activity of the parent, non-modified **S1** and siRNA "**B3**". The latter duplex was already described in the literature as an active BACE1 silencer[22]. Cells treated with lipofectamine only were used as a control (C). The duplexes were tested at 1 nM and 100 pM concentrations. The results (presented in Fig. 4) are mean values from three independent transfection experiments.

These results indicate that at 1 nM concentration the duplex containing the $T_{NH}T$ dimer at the 3′-ends of both strands induces gene silencing almost as efficiently as the non-modified counterpart (~5 vs 2% of BACE-GFP expression). The duplex is still active, but to a smaller extent (40 vs 15% of BACE-GFP expression) at ten-fold lower concentration (100 pM).

In conclusion, in this paper we have investigated a new type of the internucleotide bond, in which the phosphate moiety 3′-O-P(O)(OH)-O-5′ is replaced with an amino group –NH–. The thymidine dimer ($T_{NH}T$) containing such a short bond, which most likely exists in the protonated form, was introduced into DNA and RNA oligomers by standard phosphoramidite approach. DNA oligomer $T_9T_{NH}TT_8$ exhibited lower affinity to the comple-

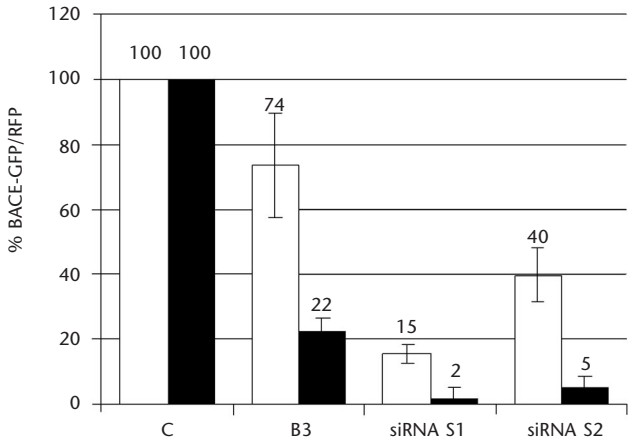


FIG. 4
Gene silencing activity of siRNA **S1**, siRNA **S2** and siRNA "**B3**" used in 1.0 (■) and 0.1 nM (□) concentration determined by a dual fluorescence assay in HeLa cells; control cells treated with lipofectamine only (C)

mentary single and double stranded DNA complements as compared to T_{19} parent oligonucleotide. Its hydrolysis with snake venom and calf spleen phosphodiesterases was completely suppressed at the site of modification. RNA oligomers containing $T_{NH}T$ dimer at the 3′-ends were used for preparation of siRNA molecules, directed towards BACE1 mRNA. Such $T_{NH}T$-modified siRNAs exhibited similar silencing activity as non-modified siRNA duplex, while their stability in 10% fetal bovine serum was slightly enhanced. Therefore, oligonucleotides with this nuclease stable linkage can be considered as candidates for biochemical probing or therapeutic use.

EXPERIMENTAL

The nuclear magnetic resonance spectra were recorded with a Bruker AC-200 instrument at 200.13 MHz for ^1H and 81.33 MHz for ^{31}P. Chemical shifts (δ-scale) are given in ppm, coupling constants (*J*) in Hz. Unless otherwise indicated, the samples were dissolved in $CDCl_3$. The solvent signal was used as the internal standard for ^1H (δ $CDCl_3$ = 7.26 ppm) and 85% H_3PO_4 as the external standard for ^{31}P. Fast atom bombardment (FAB) mass spectra were recorded on a Finnigan MAT 95 spectrometer and MALDI-TOF mass spectra were recorded in positive and negative ion modes on a Voyager–Elite instrument (Perseptive Biosystem, Inc., Framingham (MA), USA). Unmodified oligomer T_{19} was prepared by the phosphoramidite method on an ABI 394 synthesizer. Preparative purifications and separations were performed on a ThermoQuest Hypersil ODS 5 μ column (250 × 4.6 mm) using a binary analytical HPLC system and a UV/VIS-151 detector (Gilson). Aqueous triethylamine bicarbonate (0.1 M, pH 7.5)/acetonitrile buffer system was used as an eluent at a flow rate 1 ml/min. Evaporations were carried out at 40 °C. TLC was carried out on silica gel $60F_{254}$ plates (Merck, Germany) in chloroform/methanol (95:5 (A) or 90:10 (B)) solvent system. Nucleosides were purchased from Pharma Waldhof (Germany). Chloroform used for chromatography always contained triethylamine (1%, v/v).

Synthesis of 3′-*O*-*tert*-Butyldimethylsilylthymidine-5′-aldehyde (**1**)

Thymidine derivative **1** was obtained according to the already published procedure[12]. Briefly, 0.2 ml of oxalyl chloride (1.65 mmol, 3 eq.) was dissolved in freshly distilled CH_2Cl_2 (10 ml), dried over CaH_2, and after 15 min the solution was cooled down to –78 °C. Then, 0.25 ml of dimethylsulfoxide (DMSO; 3.3 mmol, 6 eq.) was added, the reaction mixture was stirred for 10 min, and 200 mg of 3′-*O*-*tert*-butyldimethylsilylthymidine[13] (0.55 mmol, 1 eq., dried overnight at vacuum over P_2O_5) dissolved in 2 ml of anhydrous CH_2Cl_2 was added dropwise over ca. 5 min. The mixture was stirred for 30 min and 0.4 ml of triethylamine (2.75 mmol, 5 eq.) were added. After next 30 min the reaction mixture was warmed up to the room temperature, diluted with CH_2Cl_2 (20 ml) and washed with 1% aqueous HCl (10 ml) and 5% aqueous $NaHCO_3$ (10 ml). The organic layer was dried with $MgSO_4$ and the solvent was evaporated to yield the aldehyde **1** (174 mg, 87%). R_F 0.53 (A). ^1H NMR ($CDCl_3$, 200 MHz): 9.49 (1 H, s, H-C(O)), 7.40 (1 H, d, $J_{CH3\text{-}H6}$ = 1.2, H-6), 6.22–6.19 (1 H, m, H-1′), 4.51–4.47 (1 H, m, H-3′), 3.97–3.71 (2 H, m, H-4′, H-5′), 2.47–2.18 (2 H, m, H-2′, H-2″), 1.90 (3 H, d, $J_{CH3\text{-}H6}$ = 1.2, CH_3 Thy), 0.89 (9 H, s, *t*-Bu), 0.08 (6 H, s, Si-CH_3).

Condensation of Thymidine-5′-aldehyde **1** with 5′-*O*-(4,4′-Dimethoxytrityl)-3′-amino-3′-deoxythymidine **2**

To the solution of 106 mg of thymidine derivative **1** (0.38 mmol, dried in vacuum for 2 h) in 8 ml of tetrahydrofurane (THF), anhydrous $MgSO_4$ (ca. 100 mg) and 5′-*O*-DMT-3′-amino-3′-deoxythymidine **2** (207 mg, 0.38 mmol) were added. The reaction mixture was stirred at room temperature overnight, filtered and concentrated to dryness at reduced pressure. The residue was applied on a silica gel column and chromatographed with a gradient of methanol in chloroform (0 to 5% MeOH). The dimer **3** was obtained in 68% yield (246 mg). R_F 0.52 (A). ^{1}H NMR ($CDCl_3$, 200 MHz): 7.73 (1 H, m, H-5′, 3′-T), 7.69 (1 H, d, $J_{CH3\text{-}H6}$ = 1.0, H-6), 6.93 (1 H, d, $J_{CH3\text{-}H6}$ = 1.0, H-6), 7.36–6.80 (13 H, m, aromatic DMT), 6.82 (1 H, m, H-1′, 3′-T), 6.31 (1 H, m, H-1′, 5′-T), 5.58 (1 H, m, H-3′, 3′-T), 5.30 (1 H, m, H-4′, 3′-T), 4.17 (1 H, m, H-3′, 5′-T), 4.17 (1 H, m, H-4′, 5′-T), 3.78 (6 H, s, 2 × CH_3 of DMT), 3.63 and 3.10 (2 H, 2 × m, H-5′ and H-5″, 5′-T), 3.35 and 2.76 (2 H, 2 × m, H-2′ and H-2″, 3′-T), 2.62 and 2.42 (1 H, m, H-2′ and H-2″, 5′-T), 1.91 (3 H, d, $J_{CH3\text{-}H6}$ = 1.0, CH_3 Thy), 1.53 (3 H, d, $J_{CH3\text{-}H6}$ = 1.0, CH_3 Thy), 0.87 (9 H, s, *t*-Bu), 0.07 (6 H, s, 2 × CH_3(Si)). FAB MS: 878.7 $[M - H]^-$, m.w. for $C_{47}H_{57}N_5O_{10}Si$ 879.

Reduction of the Thymidine Dimer **3**

Thymidine dimer **3** (240 mg, 0.3 mmol) was dissolved in anhydrous ethanol (10 ml) and treated with $NaBH(OAc)_3$ (130 mg, 0.6 mmol, 2 eq.). After 2 h of stirring at room temperature, the mixture was concentrated under reduced pressure and the crude reaction product was chromatographed on a silica gel column with a gradient of methanol in chloroform (0 to 5% MeOH). The dimer **4** was obtained in 88% yield (211 mg). R_F 0.50 (A). FAB MS: 880.4 $[M - H]^-$, m.w. for $C_{47}H_{59}N_5O_{10}Si$ 881.

Removal of the *tert*-Butyldimethysilyl Group from **4**

Thymidine dimer **4** (88 mg, 0.1 mmol) was dried in vacuum overnight, dissolved in anhydrous THF (1 ml) and treated with 1 M solution of tetra-(*n*-butylammonium) fluoride (TBAF) in THF (0.45 mmol, 0.45 ml). After 2 h of stirring at room temperature, the mixture was diluted with chloroform (20 ml) and washed with water (10 ml). The organic layer was dried with $MgSO_4$, the solvent was evaporated and the crude product was chromatographed on a silica gel column with a gradient of methanol in chloroform (0 to 7% MeOH). The product **5** was obtained in 94% yield (173 mg). R_F 0.31 (A). ^{1}H NMR ($CDCl_3$, 200 MHz): 7.61 (1 H, d, $J_{CH3\text{-}H6}$ = 1.0, H-6), 7.24 (1 H, d, $J_{CH3\text{-}H6}$ = 1.0, H-6), 7.36–6.80 (13 H, m, aromatic DMT), 6.29 (1 H, m, H-1′, 5′-T), 6.11 (1 H, m, H-1′, 3′-T), 4.43 (1 H, m, H-3′, 3′-T), 3.80 (1 H, m, H-4′, 3′-T), 3.80 (1 H, m, H-3′, 5′-T), 3.78 (6 H, s, 2 × CH_3 of DMT), 3.55 (1 H, m, H-5′, 5′-T), 3.45 (1 H, m, H-5″, 5′-T), 3.32 (1 H, m, H-4′, 5′-T), 2.89 (2 H, m, H-5′ and H-5″, 3′-T), 2.35–2.21 (2 H, m, H-2′ and H-2″, 3′-T), 2.35–2.21 (2 H, m, H-2′ and H-2″, 5′-T), 1.83 (3 H, d, $J_{CH3\text{-}H6}$ = 1.0, CH_3 Thy), 1.47 (3 H, d, $J_{CH3\text{-}H6}$ = 1.0, CH_3 Thy). FAB MS: 768.3 $[M + H]^+$ and 766.3 $[M - H]^-$, m.w. for $C_{41}H_{45}N_5O_{10}$ 767.

Synthesis of the Monomer **6**

To the solution of thymidine dimer **5** (76 mg, 0.01 mmol) and *N*,*N*-diisopropylethylamine (60 µl, 0.03 mmol, 3 eq.) in anhydrous acetonitrile (2 ml) *O*-(2-cyanoethyl)-*N*,*N*-diiso-

propylchlorophosphoramidite (25 μl, 0.015 mmol, 1.5 eq.) was added dropwise using a gas-tight syringe. After 30 min of stirring, the TLC analysis showed the presence of the substrate **5**, therefore, the next portion of the phosphitylating reagent (10 μl) was added dropwise and the reaction mixture was stirred for additional 30 min. Subsequently, the reaction mixture was applied onto a silica gel column and the product was chromatographed with a gradient addition of methanol in ethyl acetate/hexane (1:1, v/v) (0 to 7% MeOH). The product **6** was isolated in 67% yield (34 mg). R_F 0.36 (B). ^{31}P NMR ($CDCl_3$, 200 MHz): 148.98 and 148.78.

Synthesis and Purification of Oligonucleotide $T_9T_{NH}TT_8$

The synthesis of oligomer $T_9T_{NH}TT_8$ (**7**) was performed at 1-μmol scale on an ABI 394 synthesizer (Applied Biosystems Inc., Foster City (CA), USA) using commercially available 5′-*O*-DMT-thymidine-3′-*O*-(*O*-2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (Glen Research) and the monomer **6**. A succinyl-linked LCA-CPG was used as a solid support. The only change in the manufacturer recommended protocol of the synthesis was a prolonged coupling time (600 s) for the modified monomer **6**. The coupling efficiency of **6** determined by DMT-ion assay was 92%. The resultant oligomer carrying the 5′-terminal DMT group[15] was cleaved off from the solid support by treatment with 30% ammonium hydroxide (1 ml) at 55 °C for 16 h and purified by a standard RP-HPLC. The 5′-DMT group was removed by treatment with 50% acetic acid at room temperature for 30 min, followed by RP HPLC purification on a PRP-1 Hamilton column (305 × 7 mm). Fully deprotected oligonucleotide was obtained in 76% yield. The structure and purity of the oligomer were confirmed by MALDI-TOF mass spectrometry, 20% polyacrylamide/7 M urea gel electrophoresis (PAGE), and RP HPLC analysis. Spectral and chromatographic characteristics of the oligomer $T_9T_{NH}TT_8$ (**7**) are given in Table I.

Synthesis and Purification of RNA Oligonucleotides **8** and **9**

RNA oligonucleotides were synthesized on a 1-μmol scale, using an Applied Biosystems 394 instrument under conditions recommended by the manufacturer. The synthesis was performed on the Universal solid support II (Glen Research)[17]. The first coupling performed with the thymidine dimer **6** was followed by commercial RNA phosphoramidite monomers (Glen Research). The oligomers (the DMT protecting group was removed at the end of synthesis) were deprotected within the nucleobases and cleaved from the solid support by treatment with NH_3 in MeOH at room temperature for 30 min. The solutions were decanted and the solvent was evaporated under reduced pressure (Speed Vac). The resulting products were treated with 250 μl of hydrogen fluoride–triethylamine complex (Aldrich) at room temperature for 24 h and then with 1 ml of *n*-butanol. The reaction mixtures were kept in a freezer at –20 °C for 30 min. The pellets were spun down and washed once with diethyl ether. The crude products were dissolved in deionized water and isolated by means of RP-HPLC (a PRP-1 Hamilton column, 305 × 7 mm). The structure of oligomers was confirmed by MALDI-TOF mass spectrometry and their purity was assessed by electrophoresis in 20% polyacrylamide/7 M urea gel. Spectral and chromatographic characteristics of oligomers **8** and **9** are given in Table I.

Melting Temperature Measurements

UV thermal stability studies of oligonucleotides (Table I) were performed on a Cintra 40 UV-Vis spectrophotometer (GBC, Australia). The duplexes consisting of the modified oligonucleotide and the complementary template (Table I) were dissolved in 10 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl and 5 mM $MgCl_2$, whereas the heteroduplexes of the modified oligonucleotide and a single DNA target (Table II) were dissolved in an appropriate buffer. For proper annealing, the samples were heated to 70 °C and cooled to 5 °C at a rate of 0.5 °C/min. The samples were kept for 5 min at 5 °C and heated to 86 °C at a rate of 0.2 °C/min. Melting profiles for the heteroduplexes were recorded for equimolar amounts of modified oligonucleotides and complementary template (1 μM of each strand) at 260 nm. The melting temperatures obtained from the melting curves using the first order derivative method allowed for calculation of thermodynamic parameters (data not shown).

CD Spectra Recording

CD spectra were recorded on a CD6 dichrograph (Jobin–Yvon, Longjumeau, France) using a cell with 0.5-cm path length. The bandwidth of 2 nm and 1–2 s integration time were applied. Each spectrum was smoothed with a 25-point algorithm (included in the manufacturer software, version 2.2.1). The spectra for duplexes (1.0 μM each strand) of modified oligonucleotide with complementary target strand (Table II) in appropriate restriction buffer were recorded in a range of 200–320 nm at 25 °C.

Assay for digestion of oligonucleotide $T_9T_{NH}TT_8$ with snake venom phosphodiesterase (svPDE) or calf spleen phosphodiesterase (csPDE) and MALDI-TOF analysis[23].

Samples of oligonucleotide $T_9T_{NH}TT_8$ (0.1 A_{260}) were mixed with svPDE (0.1 mU, 5 μl buffer solution 2.5 mM Tris HCl, pH 8.5, 0.5 mM $MgCl_2$) or with csPDE (0.4 mU, 5 μl acetate buffer (50 mM, pH 5.0)) and incubated at 37 °C. After 10, 30, 45 and 240 min, 1 μl aliquots were withdrawn, mixed with 1 μl of the matrix (2,4,6-trihydroxyacetophenone (10 mg/ml in water/acetonitrile, 1:1) – ammonium citrate dibasic (50 mg/ml in water), 8:1, v/v) and applied directly to the sample plate. After 10 min of drying, the samples were analyzed by MALDI-TOF mass spectrometry using a Voyager Elite instrument (PerSeptive Biosystems, USA).

siRNA Stability in 10% FBS

Unmodified ssRNA (3′ T_pT) and modified ssRNA (3′ $T_{NH}T$) strands were labeled at the 5′-OH group with [γ-^{32}P]ATP by T4 polynucleotide kinase (Amersham) (1 h, 37 °C). Duplexes were formed by annealing of equimolar amounts of unmodified or modified sense and antisense strands to form three types of siRNA: **S1** (**10**/**11**), **S2** (**8**/**9**), **S3** (**10**/**9**) (Table II). Duplex formation was confirmed by 20% PAGE under native conditions. Then duplexes (3 pmol/reaction) were diluted with the RPMI medium (Gibco) supplemented with 10% FBS (Gibco) and incubated at 37 °C. Aliquots of 10 μl were collected after 0, 5, 10, 15, 20, 30, 60, 120, 240, 360, 480 min and 24 h, diluted in 1× loading buffer (Fermentas), frozen in liquid nitrogen and kept at –20 °C until analyzed. Control sample "0" was prepared in ice in RPMI medium and collected immediately after mixing with cold fetal bovine serum (FBS). The samples were separated in 20% PAGE under non-denaturing conditions. Gels were analyzed with PhosphoImager (Molecular Dynamics) and quantified by ImageQuant v. 5.0 software.

siRNA Activity Determined in a Dual Fluorescence Assay

HeLa (human cervical carcinoma) cells were cultured in RPMI 1640 medium (Gibco, BRL, Paisley), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, BRL, Paisley) and 100 U/ml penicillin and 100 μg/ml streptomycin (Polfa) at 37 °C and 5% CO_2. One day before transfection, the cells were trypsinized, diluted with fresh media and plated in black-wall 96-well plates (with a transparent bottom, Perkin–Elmer) at a density of 1.5×10^4 cells per well. Before transfection, the culture medium was replaced with fresh, antibiotics-free medium. Cells were co-transfected with plasmids coding reporter and target genes pDsRed2-N1 (15 ng per well; Clontech Laboratories, Inc.) and pBACE-GFP [24] (70 ng per well, provided by Dr. Weihong Song, The University of British Columbia, Vancouver, Canada) and siRNAs (final concentration 0.1 and 1.0 nM) using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer protocol. After 48 h of incubation, the cells were washed with PBS and lysed in NP-40 buffer. The cell lysates were used for fluorescence measurements. Fluorescence values of enhanced green fluorescent protein (EGFP) and red fluorescent protein (RFP) were measured using a Synergy HT reader (BIO-TEK); data quantification was performed using KC4 software. Excitation and emission wavelengths were as follows: GFP λ_{Ex} = 485/20 nm and λ_{Em} = 528/20 nm; RFP λ_{Ex} = 530/25 nm and λ_{Em} = 590/30 nm. The siRNA activity was calculated as the ratio of GFP to RFP fluorescence values, averaged over eight parallel repetitions. The relative level of fluorescence (GFP/RFP) in control cells (transfected with pBACE-GFP, pDsRed2-N1 and control, non-silencing siRNA) was taken as the reference (100%).

The work was supported by PBZ-MNiSW-07/I/2007 for years 2008–2010 to B. N. and by statutory funds from Technical University of Lodz and Centre of Molecular and Macromolecular Studies of PAS. The authors are grateful to Mr. M. Nowak for recording MALDI-TOF mass spectra, and Dr. P. Guga and Prof. W. J. Stec for critical reading of the manuscript.

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