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Synthesis, binding and cellular uptake properties of oligodeoxynucleotides containing cationic bicyclo-thymidine residues

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ABSTRACT

The synthesis and incorporation into oligodeoxynucleotides of two novel derivatives of bicyclothymidine carrying a cationic diaminopropyl or lysine unit in the C(6′)-β position is described. Compared to unmodified DNA these oligonucleotides show T_m-neutral behavior when paired against complementary DNA and are destabilizing when paired against RNA. Unaided uptake experiments of a decamer containing five lys-bcT units into HeLa and HEK293T cells showed substantial internalization with mostly cytosolic distribution which was not observed in the case of an unmodified control oligonucleotide.

1. Introduction

Oligonucleotide based gene silencing, discovered in the late seventies,[1,2] has evolved over the years into oligonucleotide based therapeutic strategies, the two most important ones being antisense and RNA interference.[3,4] In the therapeutic context it is believed that chemically modified oligonucleotides have superior properties compared to natural DNA or RNA as they allow to address and improve on critical parameters as RNA target affinity, nuclease resistance and cellular uptake and distribution. As a consequence, a large variety of chemically modified oligonucleotide analogues have been synthesized in the past and their biological properties evaluated. One successful concept applied to the design of oligonucleotide analogues is that of conformational restriction, resulting in structures such as locked-nucleic acids (LNA),[5,6] hexitol nucleic acids (HNA),[7] and tricyclo-DNA (tc-DNA, Fig. 1).[8,9] These analogues typically exhibit increased affinity to RNA and feature higher nuclease resistance.

While in this way the challenges linked to target affinity and nuclease resistance have largely been met, other requirements such as improving cellular uptake and distribution are yet elusive. Promising strategies to ameliorate cellular delivery include bioconjugation of therapeutic oligonucleotides to specific or unspecific cell targeting molecular entities,[10,11] or packaging into cationic and/or lipophilic complexes or nanoparticles.[12] While in the former case, covalent conjugation is often limited to the ends of an oligonucleotide, the latter technology frequently suffers from low loading of oligonucleotide per average molecular weight of the particles.

For cellular transfection experiments, oligonucleotides are typically delivered in complex with cationic lipids such as lipofectamine. Due to the toxic properties of these lipids this is, however, no method for therapeutic delivery. An alternative therefore may be to covalently add cationic lipid-like groups throughout

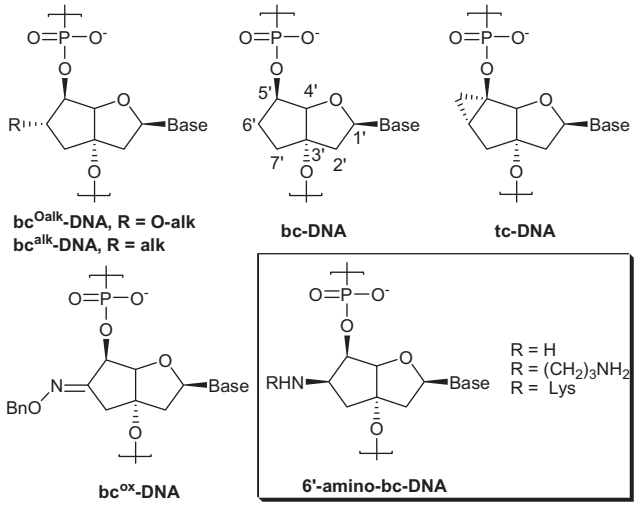

Figure 1. Selected derivatives of the bi- and tricyclo-DNA family.

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the sequence of an oligonucleotide to emulate such complexes. Given the structure of the nucleic acids, two points of attachment seem to be exquisitely suited for such manipulations, namely the internucleosidic phosphate and the 2′-OH group (in RNA).

Substitution of non-bridging phosphate oxygens by alkylamino or alkylguanidinium groups leading to uncharged or positively charged phosphoramidates,[13–15] or complete replacement of the phosphate by guanidine groups[16,17] have been explored in the past in this context and have proven successful in increasing RNA affinity due to reduced electrostatic repulsion and in some cases also in promoting cellular delivery. Also the 2′-OH function in oligoribonucleotides has been modified with cationic side chains in the past, and increased RNA binding and improved antisense effects were reported for selected cases.[18–21]

In the context of oligonucleotide therapeutics our laboratory has developed over the years the bicyclo-DNA scaffold (Fig. 1). The five-membered carbocyclic ring in bicyclo-DNA offers unique possibilities for further chemical modification, specifically at C(6′) and C(7′), to introduce additional functional groups for various purposes. In recent work we reported on the DNA and RNA affinity of 6-alkyl or -oxyalkyl modified bc-DNA,[22,23] and particularly on C(6′)-oxime modified bc-DNA (bc^{ox}-DNA), where the uncharged lipophilic benzyl group was shown to improve cellular uptake relative to unmodified oligonucleotides when delivered with lipofectamine as carrier.[24] In extension of these investigations we became interested in positioning positively charged residues at C(6′) in β-configuration. Model building suggests such residues to be located at the outer rim of the grooves pointing towards the solvent with a slight bias towards the major groove (Fig. 2). Here we report on the synthesis and incorporation into oligodeoxynucleotides of two novel C(6′)-amino-modified bicyclonucleosides carrying either a doubly cationic diaminopropyl or lysin group. We found that complementary base-pairing to DNA is associated with essentially no loss or gain in affinity against DNA while RNA affinity is compromised with respect to unmodified oligonucleotides. More importantly, a decamer with five lys-bcT modifications was shown to undergo unaided cellular uptake into HeLa and HEK293T cells, a property that was not observed in the case of the unmodified control oligonucleotide.

2. Results and discussion

2.1. Synthesis of nucleosides and building blocks

The synthesis of the two building blocks **5** and **7**, containing either a diaminopropyl or a lysine unit at C(6′) of the bicyclo-DNA skeleton, started from the already known nucleoside **1** (2:1 mixture of β- and α-anomers, Scheme 1).[25] Removal of the N-protecting group by ammonolysis lead to aminobicyclothymidines **2** that could be separated into the pure anomeric forms by column chromatography. The β-anomer of **2** was subsequently converted to **3** by reductive amination with Fmoc protected β-amino propanal. The basic amino function in **3** was again protected as a trifluoroacetamide (→ **4**) and the synthesis of building block **5** completed by standard phosphitylation. Alternatively, **2** was converted into the lysine-derived nucleoside **6** by carbodiimide mediated condensation of *N*,*N*-bis-Fmoc protected lysine in excellent yield. Again, standard transformation to the phosphoramidite **7** concluded the synthesis of this building block.

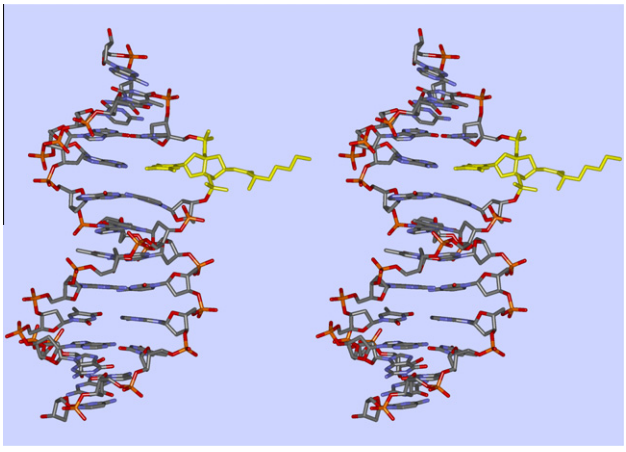


Figure 2. Stereoview of an energy minimized DNA duplex containing a lys-bcT residue. The lysyl group points from the rim of the grooves into the solvent.

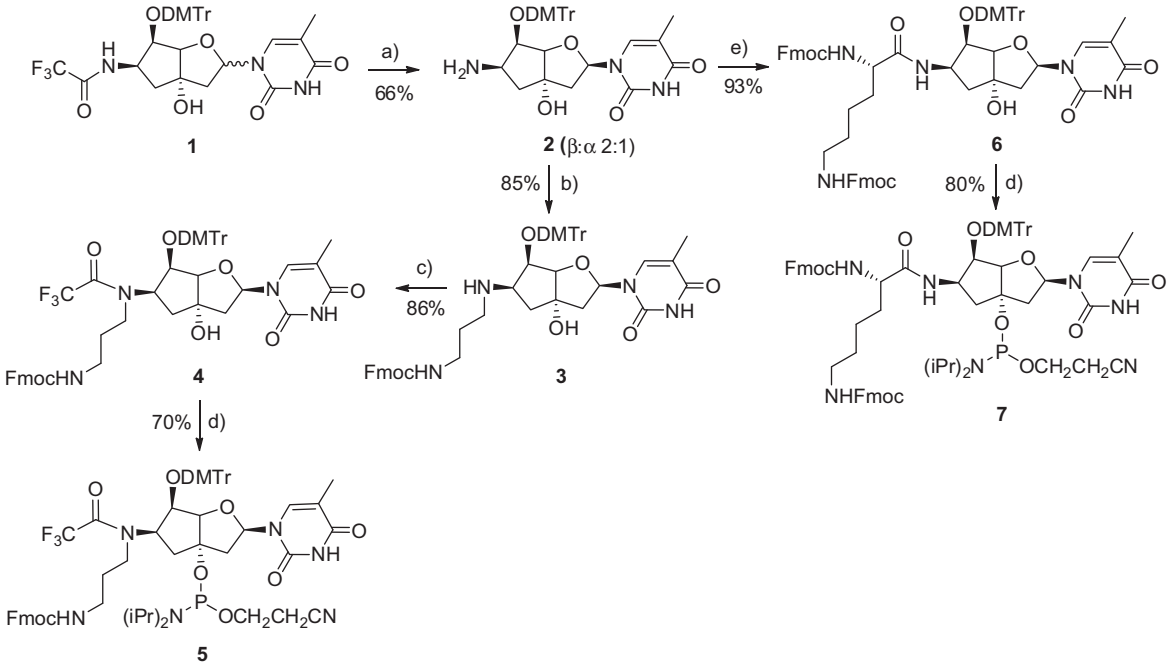
2.2. Oligonucleotide synthesis and deprotection

Oligodeoxyribonucleotides **ON1–9** (Table 1) containing single to triple substitutions in different nearest neighbor contexts were synthesized on a 1.3 μmol scale by standard automated phosphoramidite chemistry. For incorporation of the modified building blocks **5** and **7** the coupling time was extended to 9 min. No further changes to the synthesis cycle were necessary. Coupling yields of **5** were between 90% and 95%. For the lysine containing building block **7** coupling yields were in the same range (⩾90%) with the exception for **ON6**, containing two consecutive modifications, where the yield dropped to 68% for the second coupling. This is most likely due to increased steric crowding around O(5′) by the double Fmoc protected lysine unit. The 3′fluorescein labeled **ON10** containing five lys-bcT residues was synthesized analogously for the purpose of following cellular uptake by fluorescence microscopy. Deprotection after chain assembly was carried out by standard treatment with concd NH_3 (55 °C, 16 h). The crude oligonucleotides were purified by ion-exchange HPLC and obtained in >95% purity. Table 1 gives an overview over the sequences **ON1–10** obtained in this way as well as a confirmation of their respective structures by ESI$^-$ mass spectrometry.

2.2.1. T_m measurements

UV-melting curves were measured at 260 nm in standard saline buffer (10 mM Na-phosphate, 150 mM NaCl, pH 7.0). The T_m data (Table 1) of singly and doubly substituted oligonucleotides (**ON1–8**) against complementary DNA indicated almost negligible thermal affinity changes compared to an unmodified DNA unit in the respective positions. ΔT_m values per modification range between −1.2 and +1.5 °C. This clearly demonstrates that neither an aminopropyl nor a lysyl residue at the C(6′)-β-amino function sterically interferes with duplex formation, indicating smooth accommodation within the major groove. On the other hand, increasing the number of positive charges from one (**ON1**) to two (**ON2/3**), does not lead to coherent additional stability, indicating that the electrostatic shielding of phosphate groups is limited in these cases. This could be a consequence of the relative positioning and the conformational flexibility of the cationic alkyl groups. We further note that consecutive modifications as in **ON4–6** have a stronger destabilizing effect (ΔT_m/mod ca. −1.0 °C) relative to single modifications. It is not excluded that this is the consequence of local electrostatic repulsion of the positive charges or, also less likely, to steric interference of the alkyl chains.

Complexes of **ON1–9** with complementary RNA were in all cases destabilizing with ΔT_m/mod of −1.5 to −5.6 °C. Again, consecutive substitutions are less well tolerated than single or multiple, discontinuous substitutions. Also, there is no significant difference between the aminopropyl or the lysyl unit relative to the primary amine (R = H) indicating that changing the steric demand near to C(6′) does not influence the affinity. There seems to be a trend that destabilization diminishes with increasing the



Scheme 1. Synthesis of building blocks **5** and **7**. Reagents and conditions: (a) NH_3 concd in MeOH, 55 °C; (b) 3-(Fmoc-NH$(CH_2)_2$CHO, $NaBH_3CN$, MeOH, rt, 5 h; (c) TFAA, pyridine, 0 °C, 1 h; (d) $(iPr)_2NP(Cl)CH_2CH_2CN$, iPr_2NEt, THF, rt, 45 min; (e) N^{α},N^{ω}-Fmoc-Lys-OH, EDC, DMAP, CH_2Cl_2, rt, 1 h;

Table 1
T_m data from UV-melting curves (260 nm) of modified dodecamer duplexes with complementary DNA and RNA

	Sequence	*t* = RHN (R)	ESI⁻-MS *m/z* calc	ESI⁻-MS *m/z* found	T_m (°C) vs DNA[a,b] (ΔT_m/mod)	T_m (°C) vs RNA[a,c] (ΔT_m/mod)
ON1	d(GGATGTTCtCGA)	R = H	3718.5	3718.0	48.3 (+0.8)	46.5 (−3.0)
ON2		R = 3-aminopropyl	3774.6	3774.4	47.3 (−0.2)	45.7 (−3.8)
ON3		R = Lys	3845.7	3846.0	49.0 (+1.5)	46.0 (−3.5)
ON4	d(GGATGttGTCGA)	R = H	3760.6	3759.0	45.5 (−1.0)	38.8 (−5.4)
ON5		R = 3-aminopropyl	3872.8	3873.0	45.1 (−1.2)	38.3 (−5.6)
ON6		R = Lys	4014.9	4015.0	45.5 (−1.0)	38.5 (−5.5)
ON7	d(GGAtGTTGtCGA)	R = 3-aminopropyl	3872.8	3872.4	46.1 (−0.7)	43.3 (−3.1)
ON8		R = Lys	4014.9	4015.0	47.3 (−0.1)	46.0 (−1.7)
ON9	d(GGAtGtTGtCGA)	R = Lys	4184.2	4182.0	46.5 (−0.3)	40.1 (−3.1)
ON10	d(tTtTtTtTtT)-FAM	R = Lys	4424.7	4423.0	—	—

[a] Duplex concn 2 μM in 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.0. estimated error in T_m = ±0.5 °C.
[b] T_m of unmodified duplex: 47.5 °C.
[c] T_m of unmodified duplex: 49.5 °C.

number of discontinuous modifications (**ON7–9**). This suggests that in these cases the global reduction of negative charge of the oligonucleotide becomes beneficial. This is important as only 50% modification of an oligonucleotide is needed to render it mono cationic.

There are clear structural differences between a pure DNA and a DNA/RNA double helix, the former being of B-conformation and the latter being an intermediate between A and B conformation. An unsubstituted bcT unit in the same sequence context has been shown previously to be nearly T_m neutral.[23] Thus the relative differences in affinity between complementary RNA and DNA observed here are not a consequence of the bicyclic core structure but that of the substituents. Previous C(6′) substitutions on the bicyclo-skeleton in α-configuration with aminoalkoxyl chains were found to be slightly less destabilizing when paired against RNA.[23] In both the C(6′)α- and β-cases the substituents point towards the solvent with α-substituents showing a slight bias towards the minor groove and β-substituents towards the major groove. Both configurations seem therefore to be well suited to present aminoalkyl groups towards a potential nucleic acid binding interface, but obviously the linker lengths still need to be optimized to take full advantage of nucleic acid charge screening.

2.3. Cellular uptake

One of the main aims of this work was to investigate whether multiple positive charges change the cellular uptake properties. We therefore prepared the fluorescein labeled 10-mer model oligonucleotide **ON10**, containing five alternately positioned lys-bcT units. The sequence was specifically designed to be short in order to exclude antisense effects potentially obstructing cellular metabolism and/or cellular distribution of the oligonucleotide. Two

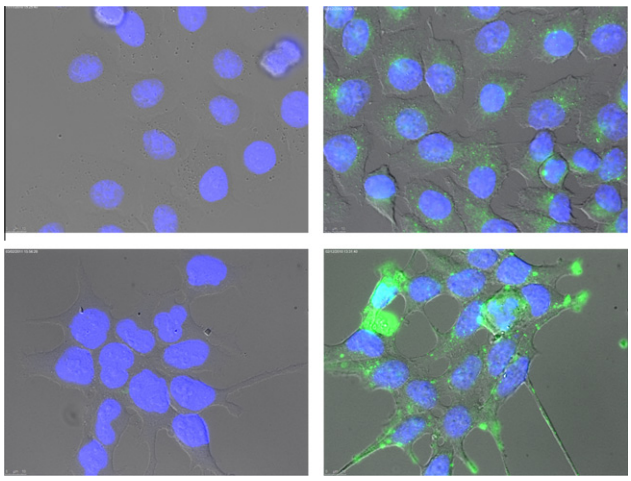


Figure 3. Fluorescence microscopic images (overlay of contour, green and blue fluorescence) of HeLa cells (top row) and HEK293T cells (bottom row) treated with d(T_{10})-FAM (left column) or **ODN10** (right column) 48 h after transfection with oligonucleotides (10 μM) without any transfection agent. Cells were incubated live and only later fixed with formaldehyde for imaging. Nuclei (blue) were DAPI stained.

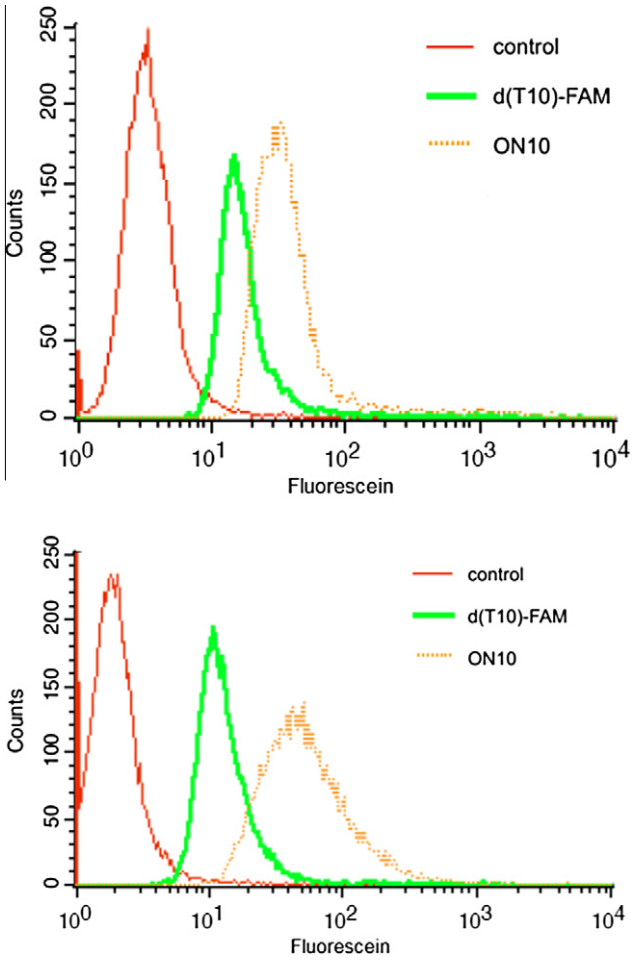


Figure 4. Histograms representing HeLa cells (top) and HEK293T cells (bottom) treated with **ON10** (orange) or d(T_{10})-FAM (green). Control: untreated cells (red). 20,000 cells were counted in each case.

different cell lines, HeLa and HEK293T, were transfected with **ON10** (10 μM) and with the unmodified d(T_{10})-FAM as a control, in the absence of any transfection agent. After 48 h cells were washed fixed and analyzed by fluorescence microscopy (Fig. 3).

As can be seen from Figure 3 (left column), the natural control oligonucleotide showed no visible internalization. In contrast **ON10** showed considerable uptake after the same exposure time into both cell lines (Fig. 3, right column). The cellular distribution was predominantly cytosolic and less nuclear. We quantified the fraction of cells associated with fluorescein fluorescence by FACS (Fig. 4) and found that in the case of **ON10**, 95% of the cells shifted towards higher intensity in both cell lines. In the case of the control d(T_{10})-FAM only 50–75% of the cells were shifted towards higher fluorescence. The absence of cytosolic and nuclear fluorescence spots in these cases suggests that the residual fluorescence is associated with surface bound, labeled material. The fact that no internalization is observed with d(T_{10})-FAM proves that cellular uptake of **ON10** is not an artifact of the fluorescent label.

It has been shown previously that fixation of cells influences the uptake and distribution of cationic peptide-PNA conjugates, leading to artifactual results.[26] In a more recent study with oligonucleotide phosphorohioates, however, such effects were absent.[27] We therefore assume that fixation does not alter uptake and distribution in the cases reported here, but we cannot rule it out completely.

3. Conclusions

We successfully prepared the two phosphoramidite building blocks **5** and **7** containing a β-6′-diaminopropyl or -lysyl substituent on the bcT skeleton and incorporated them into oligodeoxynucleotides. Both modifications were essentially T_m neutral when paired against DNA but were destabilizing when paired against RNA. Cellular uptake studies in two cell lines with a decamer carrying five lys-bcT units showed consistent, unaided cellular internalization which was absent in the case of the corresponding unmodified decamer. From these results we conclude that aminoalkyl side chains are indeed a measure to increase cellular uptake of oligonucleotides. We further conclude that position C(6′), which localizes the substituent in a unique and for natural nucleosides inaccessible position relative to the double helix, is well suited for this. The decrease of T_m against complementary RNA (which is largely absent if DNA is the complement) is most likely a consequence of inefficient screening of the negative phosphate charges in the specific duplex conformation of an RNA/DNA hybrid. To remedy this it will be necessary to further vary chain length and position of the positive charges within the substituent.

Our experiments clearly demonstrate that the lysine unit at C(6′) of bcT improves unaided cellular uptake. These results are in line with earlier findings on the internalization of fully modified cationic phosphoramidate α-oligonucleotides,[14] and with improved antisense or siRNA activity of oligoribonucleotides carrying cationic residues (including lysine units) attached to the 2′-OH group.[19,20]

4. Experimental

4.1. General

All reactions were performed under Ar in dried glassware. Anhydrous solvents for reactions were obtained by filtration through activated aluminum oxide, or by storage over 3 Å molecular sieves. Column chromatography (CC) was performed on silica gel (Fluka) with an average particle size of 40 μm. All solvents for CC were of technical grade and distilled prior to use. Thin layer chromatography (TLC) was performed on silica gel plates (Macherey–Nagel, 0.25 mm, UV254). Visualization was performed either

by UV or by staining in dip solution (10.5 g Cer(IV)-sulfate, 21 g phosphormolybdenic acid, 60 ml concd sulfuric acid, 900 ml H_2O) followed by heating with a heat gun. NMR spectra were recorded on a Bruker DRX 400 or a Bruker AC 300 spectrometer at 400 MHz or 300 MHz (^{1}H NMR) or 100 MHz (^{13}C NMR) in either $CDCl_3$, CD_3OD or DMSO-d_6. δ in ppm relative to residual undeuterated solvent [$CHCl_3$: 7.26 ppm (^{1}H) and 77.0 ppm (^{13}C); CHD_2OD: 3.35 ppm (^{1}H) and 49.3 ppm (^{13}C); $(CHD_2)_2SO$: 2.54 ppm (^{1}H) and 40.45 ppm (^{13}C)], J in Hz. Signal assignments are based on DEPT and on ^{1}H–^{1}H and ^{1}H–^{13}C correlation experiments (COSY/HSQC). High resolution electrospray ionization (ESI^-) mass spectra (MS, m/z) were recorded on an Applied Biosystems Sciex QSTAR Pulsar instrument.

4.1.1. 1-[(3′*S*,5′*R*,6′*R*)-5′-*O*-[(4,4′-dimethoxytriphenyl)methyl]-6′-amino-2′-deoxy-3′,5′-ethano-β-D-ribofuranosyl]thymine 2

To a solution of 6′-aminobicyclothymidine **1** (3.37 g, 4.9 mmol, 2:1 mixture of β/α anomers) in MeOH (18 ml) was added concd NH_3 (150 ml) at rt. The mixture slowly turned purple and was stirred for 1 h at rt, then at 55 °C overnight. After evaporation of the solvents the white solid was suspended in EtOH (150 ml), adsorbed on silica gel (30 g) and purified by CC (CH_2Cl_2/EtOH, 2%→10%) to give **2β** (1.28 g, 44%) and **2α**-anomer (632 mg, 22%) both as a white foams. Analytical data of **2β**: TLC (CH_2Cl_2/EtOH 9:1) R_f = 0.25. ^{1}H NMR (300 MHz, CD_3OD) δ: 7.96 (d, J = 0.8 Hz, 1H, H–C(6)), 7.56–7.54 (m, 2H, H-arom), 7.46–7.42 (m, 4H, H-arom), 7.29–7.17 (m, 3H, H-arom), 6.85 (dd, J = 8.9, 1.3 Hz, 4H, H-arom), 5.93 (dd, J = 9.0, 6.5 Hz, 1H, H–C(1′)), 4.08 (t, J = 5.6 Hz, 1H, H-C(5′)), 3.75 (s, 6H, *Me*O-DMTr), 3.69 (d, J = 5.3 Hz, 1H, H–C(4′)), 2.84 (dd, J = 13.5, 9.2 Hz, 1H, H–C(2′)), 2.57 (t, J = 5.9 Hz, 1H, H–C(6′)), 2.33 (dd, J = 13.5, 6.5 Hz, 1H, H–C(2′)), 2.08 (d, J = 14.8 Hz, 1H, H–C(7′)), 1.88 (s, 3H, *Me*-C(5)), 1.65 (dd, J = 14.9, 6.4 Hz, 1H, H–C(7′)). ^{13}C NMR (100 MHz, CD_3OD) δ: 167.58 (C(4)), 160.61 (C-arom), 153.53 (C(2)), 146.91 (C-arom), 141.45 (C(6)), 137.76, 137.53, 131.44, 131.41, 129.24, 129.14, 128.24, 114.54, 114.52 (C-arom), 111.91 (C(5)), 90.02, 89.93 (C(4′), C(1′)), 89.18, 85.08 (C(3′), *C*(Ph)$_3$), 75.62 (C(5′)), 55.92 (MeO), 55.56 (C(6′)), 47.63 (C(2′)), 43.95 (C(7′)), 12.64 (*Me*-C(5)). ESI^+-HRMS: calcd for $C_{33}H_{37}O_7N_3$ ([M+H]$^+$) 586.2548, found 586.2535.

4.1.2. 1-[(3′*S*,5′*R*,6′*R*)-5′-*O*-[(4,4′-dimethoxytriphenyl)methyl]-6′-[3″-(*N*-Fmoc-aminopropyl)]amino-2′-deoxy-3′,5′-ethano-β-D-ribofuranosyl]thymine 3

To a solution of nucleoside **2** (168 mg, 0.286 mmol) in dry methanol (8 ml) containing molecular sieves (3 Å) was added $FmocNH(CH_2)_2CHO$ (169 mg, 0.572 mmol, 2 equiv) at rt. After stirring for 90 min, $NaCNBH_3$ (54 mg, 0.859 mmol, 3 equiv) was added in three equal portions over 2 h. After 3 h the mixture was diluted with 25 ml EtOAc and washed with satd aq $NaHCO_3$ (2 × 25 ml), and sat aq Na_2CO_3 solution (2 × 25 ml). The aqueous phases were extracted with EtOAc (2 × 100 ml) and the combined organic phases dried over $MgSO_4$, filtered, evaporated and the faintly yellow residue purified by CC (hexane/EtOAc, 75%→90%) to give the title compound **3** (210 mg, 85%) as a white foam. TLC (CH_2Cl_2/EtOH 9:1) R = 0.48. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 9.12 (s, 1H, NH), 8.15 (s, 1H, H–C(6)), 7.74 (d, J = 7.5 Hz, 2H-arom(Fmoc)), 7.54 (t, J = 8.5 Hz, 4H-arom(Fmoc)), 7.44–7.40, 7.40–7.34 (2 m, 6H-arom), 7.30–7.25, 7.25–7.18 (2 m, 3H-arom), 6.82 (dd, J = 9.2, 3.5 Hz, 4H$_{arom}$(DMTr)), 6.55 (dd, J = 9.8, 5.4 Hz, *H*–C(1′)), 4.83–4.80 (m, 1H, H–N), 4.39 (d, J = 6.9 Hz, 2H, CH_2-Fmoc), 4.21–4.16 (m, 2H, H–C(4′), CH-Fmoc), 4.03 (t, J = 5.9 Hz, 1H, H–C(5′)), 3.76 (s, 6H, OCH_3), 3.13 (m, 2H, H–C(3″)), 2.65 (dd, J = 14.1, 10.4 Hz, 1H, H–C(2′)), 2.38 (dd, J = 13.2, 5.2 Hz, 1H, H–C(2′)), 2.32–2.24 (m, 1H, 1H–C(1″)), 1.98 (s, 4H, H_3C–C(5), 1H–C(7′)), 1.86–1.80, 1.78–1.75, (2 m, 2H, H–C(6′), 1H–C(1″)), 1.56–1.47 (m, 2H, H–C(2″)), 1.25–1.19 (m, 1H, H–C(7′)). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 163.99 (C(4)), 159.00 (CH_3O–*C*-arom), 151.04 (C(2)), 145.50, 144.14, 144.11, 141.53, 136.83, 136.54 (C-arom), 137.62 (C(6)), 129.87, 127.97, 127.89, 125.17, 120.17 (C-arom(Fmoc)), 128.32, 127.33, 127.23, 113.66, 113.61 (C-arom(DMTr)), 111.05 (C(5)), 89.45 (C(4′)), 87.53, 86.23 (C(3′), C(Ph)$_3$), 85.51 (C(1′)), 74.22 (C(5′)), 66.77 (CH_2-Fmoc), 61.63 (C(6′)), 55.48 (CH_3O-DMTr), 48.54 (C(2′)), 47.46 (CH-Fmoc), 45.86 (C(8′)), 39.38 (C(10′)), 38.25 (C(7′)), 31.25 (C(9′)), 12.86 (CH_3–C(5)). ESI^+-HRMS: calcd for $C_{51}H_{53}O_9N_4$ ([M+H]$^+$) 865.3807, found 865.3806.

4.1.3. 1-[(3′*S*,5′*R*,6′*R*)-5′-*O*-[(4,4′-dimethoxytriphenyl)methyl]-6′-[3″-(*N*-Fmoc-aminopropyl)]trifluoroacetylamino-2′-deoxy-3′,5′-ethano-β-D-ribofuranosyl]thymine 4

Trifluoroacetic anhydride (0.161 ml, 1.15 mmol, 5 equiv) was carefully added to a mixture of nucleoside **3** (200 mg, 0,231 mmol) in dry pyridine (3.6 ml) at 0 °C. The solution immediately turned yellow and the reaction was complete within 1 h. After dilution with EtOAc (30 ml) the mixture was washed with sat aq $NaHCO_3$ (2 × 25 ml) and the aqueous phases were extracted with EtOAc (2 × 50 ml). The combined organic phases were dried over $MgSO_4$, filtered and evaporated. Purification by CC (EtOAc hexane/EtOAc, 60%→100%) afforded the title compound **4** (190 mg; 86%) as a yellow foam. TLC (CH_2Cl_2/EtOH 9:1) R = 0.67. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 7.88–7.86 (*m*, 2H, H-arom(Fmoc)), 7.67 (d, J = 7.3 Hz, 2H, H-arom(Fmoc)), 7.39–7.11 (m, 14H, H-arom(Fmoc), H-arom(DMTr), H–C(6)), 6.80–6.74 (m, 4H, H-arom(DMTr)), 5.74–5.65 (m, 1H, H–C(1′)), 4.39–4.18 (m, 4H, H–C(5′), CH_2-Fmoc, C*H*-Fmoc), 3.71, 3.70, 3.68 (3s, 6H, *Me*O-DMTr), 3.76 (m, 1H, H–C(1″)), 3.57 (m, 1H, H–C(1″)), 3.04–2.96 (m, 2.3H, H–C(3″), H–C(4′)), 2.89 (d, J = 4.2 Hz, 0.7H, H–C(4′)), 2.60–2.53 (m, 1H, H–C(7′)), 2.27 (dd, J = 13.4, 7.9 Hz, 1H, H–C(2′)), 2.09 (dd, J = 13.5, 7.2 Hz, 1H, H–C(2′)), 2.02 (dd, J = 12.7, 5.5 Hz, 1H, H–C(7′)), 1.79, 1.70 (2s, 3H, Me-C(5)), 1.75–1.59 (m, 2H, H–C(2″)). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 163.59 (C(4)), 158.19 (C-arom), 156.17, 156.13 (*C*OCF$_3$), 150.12 (C(2)), 143.77, 140.66 (C-arom), 136.47, 136.08 (C(6)), 135.68, 135.38 (C-arom), 130.61, 130.41, 128.26, 128.17, 127.54, 127.44, 126.98, 125.01, 120.05 (*C*H-arom), 112.70 (C-arom(DMTr)), 109.32 (C(5)), 87.85 (*C*(Ph)$_3$), 87.31, 86.56 & 86.02 (C(4′)), 84.58 (C(1′)), 81.12 (C(3′)), 74.11 (C(5′)), 65.37 (*C*H2-Fmoc), 58.09 (C(6′)), 54.92 (*Me*O-DMTr), 46.68 (*C*H-Fmoc), 44.84 (C(1″)), 43.05 (C(2′)), 41.22 (C(7′)), 38.29 (C(3″)), 27.86 (C(2″)), 11.98, 11.88 (*Me*-C(5)).^{19}F NMR (376 MHz, DMSO-d_6) δ −67.49, −67.51. ESI^+-HRMS: calcd for $C_{53}H_{51}O_{10}N_4F_3Na$ ([M+Na]$^+$) 983.3449, found 983.3461.

4.1.4. 1-[(3′*S*,5′*R*,6′*R*)-5′-*O*-[(4,4′-dimethoxytriphenyl)methyl]-6′-[3″-(*N*-Fmoc-aminopropyl)]amino- 3′-*O*-(2-cyanoethoxy) diisopropylaminophosphanyl-2′-deoxy-3′,5′-ethano-β-D-ribofuranosyl]thymine 5

To a mixture of nucleoside **4** (125 mg, 0,13 mmol) and $EtNiPr_2$ (0.089 ml, 0.52 mmol, 4 equiv) in THF (2 ml) was added 2-cyanoethoxy diisopropylamino chlorophosphine (0.058 ml, 0.26 mmol, 2 equiv) at rt. After 45 min the mixture was diluted with EtOAc (25 ml) and washed with a satd aq $NaHCO_3$ (2 × 25 ml). The aqueous phases were extracted with EtOAc (2 × 50 ml) and the combined organic phases dried over $MgSO_4$, filtered, evaporated and the crude material purified by CC (hexane/EtOAc 50%→75%) to give phosphoramidite **5** (106 mg, 70%) as a white foam. TLC (EtOAc/Hexane 4:1) R = 0.74. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.90–7.87 (m, 2H, H-arom(Fmoc)), 7.69–7.67 (m, 2H, H-arom(Fmoc)), 7.44–7.38 (m, 3H, H-arom, H–C(6)), 7.32–7.29 (m, 4H, H-arom), 7.25–7.14 (m, 7H, H-arom), 6.79–6.76 (m, 4H, H-arom(DMTr)), 5.71 (m, 1H, H–C(1′)), 4.38–4.28 (m, 4H, CH_2-Fmoc, H–C(6′), H–C(5′)), 4.23–4.20 (m, 1H, CH-Fmoc), 3.81 (br, 1H, H–C(3″)), 3.71–3.69 (3s, 6H, MeO-DMTr), 3.64–3.45 (m, 5H, O*CH*$_2$CH$_2$CN, (Me$_2$*CH*)$_2$N, H–C(3″)), 3.10–3.02 (m, 3H, H–C(1″), H–C(4′)), 2.78–2.75 (m, 1H,

H–C(2′)), 2.71–2.70 (m, 2H, OCH_2CH_2CN), 2.47–2.33 (m, 2.3H, H–C(2′), H–C(7′)), 1.86 (s_{br} 1H, H–C(2″)), 1.80, 1.73 (2s, 3H, Me-C(5)), 1.65–1.57 (m, 1H, H–C(2″)), 1.10–1.01 (m, 12H, $(Me_2CH)_2N$). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 163.62 (C(4)), 158.35, 158.26, 156.15 (C-arom), 150.16, 150.10 (C(2)), 144.82, 143.88, 143.81, 140.67 (C-arom), 136.91, 136.53 (C(6)), 135.55, 135.25 (C-arom), 130.49, 130.28, 128.15, 127.55, 127.01, 125.13, 120.07 (CH-arom), 118.86, 118.75 (CN), 112.77 (C-arom(DMTr), 109.58, 109.46 (C(5)), 88.08 (C(3′)), 85.44 (C(1′)), 85.19, 85.00 (C(4′), $C(Ph)_3$), 73.70, 73.53 (C(5′)), 65.40 (CH_2-Fmoc), 57.86, 57.70 (OCH_2CH_2CN), 54.95, 54.92 (CH_3O-DMTr), 46.70 (CH-Fmoc), 45.05 (C(10′)), 42.77, 42.76 ($(Me_2CH)_2N$), 40.76 (C(7′)), 39.85 (C(2′)), 38.29 (C(8′)), 27.82 (C(9′)), 24.09, 23.93, 23.85 ($(Me_2CH)_2N$), 19.67, 19.60 (OCH_2CH_2CN), 11.93 (Me-C(5)). ^{19}F NMR (376 MHz, DMSO-d_6) δ −67.46, −67.53. ^{31}P NMR (122 MHz, DMSO-d_6) δ 142.01, 141.32, 141.28, 140.94. ESI$^+$-HRMS: calcd for $C_{62}H_{68}O_{11}N_6F_3NaP$ ($[M+H]^+$) 1183.4528, found 1183.4561.

4.1.5. 1-[(3′*S*,5′*R*,6′*R*)-5′-*O*-[(4,4′-dimethoxytriphenyl)methyl]-6′-[(N_α,N_ε-di-Fmoc)-L-lysine]amino-2′-deoxy-3′,5′-ethano-β-D-ribofuranosyl]thymine 6

To a solution of nucleoside **2** (204 mg, 0.35 mmol) in dry THF (3.2 ml), was added N^α,N^ω-Fmoc-protected lysine (246 mg, 0.42 mmol, 1.2 equiv) and 4-dimethylaminopyridine (21 mg, 0.17 mmol, 0.5 equiv) at rt. The mixture was cooled to 0 °C and a solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 100 mg, 0.52 mmol, 1.5 equiv) in dry CH_2Cl_2 (2 ml) was slowly added. After 10 min at 0 °C the cooling bath was removed and after 1 h the mixture was diluted with EtOAc (100 ml) and washed with sat aq $NaHCO_3$ (2 × 100 ml) The aq phases were extracted with EtOAc (2 × 200 ml), the combined organic phases dried over $MgSO_4$, filtered, evaporated and the crude material purified by CC (EtOAc + 1% Et_3N→EtOAc/EtOH 98:2) to give the title compound **6** (373 mg, 93%) as a white foam. ^1H NMR (300 MHz, CD_3OD) δ: 7.74 (d, J = 7.4 Hz, 4H, H-arom), 7.61–7.48 (m, 7H, H-arom), 7.41–7.18 (m, 15H, H-arom), 6.80–6.75 (m, 4H, H-arom(DMTr), 5.66 (dd, J = 8.8, 5.2 Hz, 1H, H–C(1′)), 4.33–4.29 (m, 2H, CH_2-Fmoc), 4.24–4.17 (m, 4H, CH_2-Fmoc, CH-Fmoc, H–C(α)), 4.11–4.04 (m, 1H, H–C(5′)), 3.99 (t, J = 6.4 Hz, 1H, CH-Fmoc), 3.88–3.85 (m, 1H, CH_2-Fmoc), 3.67, 3.66 (2s, 6H, MeO-DMTr), 3.53–3.51 (m, 1H, H–C(4′)), 3.44–3.40 (m, 1H, H–C(6′)), 3.14–3.06 (m, 2H, H–C(ε)), 2.52–2.45 (m, 1H, H–C(2′)), 2.30 (dd, J = 13.2, 5.2 Hz, 1H, H–C(2′)), 2.07 (d, J = 14.6 Hz, 1H, H–C(7′)), 1.91, 1.88 (2s_{br}, 3H, Me-C(5)), 1.75–1.33 (m, 7H, CH_2-lysine, H–C(7′)). ^{13}C NMR (101 MHz, CD_3OD) δ 180.10, 177.40, 166.59, 166.47 (C(4)), 160.43 (MeO-*C*-arom), 149.23 (C(2)), 147.03, 146.98, 145.53, 145.13, 142.76, 141.57, 139.47 (C-arom), 137.57 (C(6)), 131.53, 130.06, 129.32, 129.02, 128.87, 128.27, 126.35 (CH-arom), 122.19, 121.03 (CH-arom(Fmoc)), 114.42 (CH-arom(DMTr), 89.80 (C(1′)), 89.19 (C(4′)), 84.80, 74.47 (C(5′)), 67.82 (CH_2-Fmoc), 55.88 (C(α)), 55.81 (CH_3O-DMTr), 54.20 (C(6′)), 48.10, 48.02 (CH-Fmoc), 47.38 (C(2′)), 43.10 (C(7′)), 41.44 (C(ε)), 31.70 (CH_2-lysine), 30.20 (CH_2-lysine), 24.21 (CH_2-lysine), 12.63 (Me-C(5)). ESI$^+$-HRMS: calcd for $C_{69}H_{67}O_5N_{12}Na$ ($[M+Na]^+$) 1180.4678, found 1180.4660.

4.1.6. 1-[(3′*S*,5′*R*,6′*R*)-5′-*O*-[(4,4′-dimethoxytriphenyl)methyl]-6′-[(N_α,N_ε-di-Fmoc)-L-lysine]amino-3′-*O*-(2-cyanoethoxy) diisopropylaminophosphanyl-2′-deoxy-3′,5′-ethano-β-D-ribofuranosyl]thymine7

To a solution of DMT-protected nucleoside **6** (505 mg, 0.43 mmol) in dry THF (8.5 ml) was added, iPr$_2$NEt (0.3 ml, 1.73 mmol, 4 equiv) and 2-cyanoethoxy diisopropylamino chlorophosphine (0.2 ml, 0.87 mmol, 2 equiv) at rt. The solution was stirred for 45 min, diluted with EtOAc (100 ml) and washed with satd aq $NaHCO_3$ (2 × 100 ml). The aqueous phases were extracted with EtOAc (2 × 200 ml) and the combined organic phases dried over $MgSO_4$, filtered and evaporated. The crude yellow oil was purified by CC (EtOAc/hexane 3:1→5:1) to yield phosphoramidite **7** (470 mg, 80%) as a white foam. ^1H NMR (300 MHz, DMSO-d_6) δ: 11.49, 11.48 (br, 1H, H–N), 7.89–7.86 (m, 4H, H-arom(Fmoc)), 7.68–7.61 (m, 6H, H–C(6), H-arom), 7.44–7.23 (m, 18H, H-arom(DMTr), H-arom(Fmoc)), 7.16 (t, 2H,), 6.99 (m, 1H,), 6.83–6.77 (m, 4H, H-arom(DMTr)), 5.82–5.76 (m, 1H, H–C(1′)), 4.28–4.26 (m, 2.5H, CH_2-Fmoc), 4.21–4.19 (m, 2H, CH_2-Fmoc, CH-Fmoc), 4.09, 4.04 (2t, 1H, CH-Fmoc), 3.97–3.90 (m, 2H, H–C(α), H–C(5′)), 3.73–3.67 (m, 0.5H, CH_2-Fmoc), 3.65, 3.64 (2s, 6H, MeO-DMTr), 3.61–3.42 (m, 5H, OCH_2CH_2CN, $(Me_2CH)_2N$, H–C(4′)), 3.32–3.24 (m, 1H, H–C(6′)), 2.95 (m, 2H, H–C(ε), 2.69–2.62 (m, 3H, H–C(2′), OCH_2CH_2CN), 2.33–2.21 (m, 1.5H, H–C(2′)), 2.16–2.11 (m, 1H, H–C(7′)), 1.91–1.85 (m 1H, H–C(7′)), 1.82 (br, 3H, Me-C(5)), 1.60 (br, 2H, H–C(β)), 1.37 (br, 2H, H–C(δ)), 1.24 (m, 2H, H–C(γ)), 1.08–0.99 (m, 12H, $(Me_2CH)_2N$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.27 (C(8′)), 163.66 (C(4)), 158.21, 158.16 (MeO-C-arom), 156.35, 156.02 (C-arom, HN-COO-Fmoc), 150.27, 150.23 (C(2)), 145.33, 143.90, 143.67, 140.69, 140.62 (C-arom), 135.69, 135.62 (C(6)) 129.79, 129.60, 127.68, 127.52, 126.97, 125.09 (CH-arom), 120.04, 119.95 (CH-arom(Fmoc)), 118.88, 118.85 (CN), 113.19, 113.13 (CH-arom(DMTr)), 110.00 (C(5)), 87.21, 87.15 (C(4′)), 85.70 (C(1′)), 72.35, 72.29 (C(5′)), 65.71, 65.15 (CH_2-Fmoc), 57.84 (OCH_2CH_2CN), 54.92 (MeO-DMTr), 54.30 (C(α)), 52.44 (C(6′)), 44.96, 44.89 (C(2′)), 46.73, 46.49 (CH-Fmoc), 42.78, 42.65 ($(Me_2CH)_2N$), 40.88 (C(7′)), 40.14–38.88 (C(ε)), 30.23, 30.10 (C(β)), 29.21 (C(δ)), 24.23–23.77 ($(Me_2CH)_2N$), 22.72 (C(γ)), 19.64, 19.57 (OCH_2CH_2CN), 11.93 (*Me*-C(5)). ^{31}P NMR (122 MHz, DMSO-d_6) δ 141.73, 140.95.

4.1.7. Oligonucleotides synthesis and purification

Oligonucleotide syntheses were performed on the 1.3 μmol scale on a LKB Gene Assembler Plus (Pharmacia) DNA synthesizer using standard solid-phase phosphoramidite chemistry. Natural phosphoramidites were from Vivotide or Applied Biosystems. Solid support was from Glen Research. Reagents were prepared according to the manufacturer's protocols. As coupling agent, 5-(ethylthio)-1*H*-tetrazole (Azco PharmChem, 0.25 M in CH_3CN) was used. Fluorescein (FAM)-labeled oligonucleotides were synthesized on FAM-solid support (Roche diagnostics). For the modified phosphoramidites **5** and **7**, an extended coupling time of 9 min was applied and average coupling efficiencies, as monitored by trityl assay, were ⩾93% for **7** (except for consecutive couplings, where the yield dropped to 68%) and 90–95% for **5**. Deprotection of the oligonucleotides and detachment from the solid support was carried out in concd aq NH_3 for 16 h at 55 °C. Crude oligomers were purified by ion-exchange HPLC on an Äkta basic 10/100 system (GE healthcare) with a DNAPAC PA200, 4 × 250 mm analytical column (Dionex). Buffer A) 25 mM Trizma (2-amino-2-hydroxymethyl-1,3-propanediol) in H_2O, pH 8.0; buffer B) 25 mM Trizma, 1.25 M NaCl in H_2O, pH 8.0. Linear gradients of B in A were used, with a 1 ml/min flow rate and detection at 260 nm. Purified oligonucleotides were desalted over Sep-Pak C18 Cartridges (Waters), quantified on a Nanodrop spectrophotometer (Thermo Scientific), and analyzed by ESI- mass spectrometry.

4.1.8. UV-melting curves

UV-melting curves were recorded on a Varian Cary 100 Bio UV–vis spectrophotometer. Absorbances were monitored at 260 nm and the heating rate was set to 0.5 °C/min. A cooling–heating–cooling cycle in the temperature range 15–80 °C was applied. T_m values were obtained from the derivative curves using the Varian WinUV software. To avoid evaporation, the sample solutions were covered with a layer of dimethylpolysiloxane. All measurements were carried out in 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.0 with duplex concentrations of 2 μM

4.1.9. Cell cultures and transfection

Hela and HEK293T cells were grown at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% (v/v) Fetal Calf Serum (Amimed), 100 units/ml penicillin (Invitrogen) and 100 μg/ml streptomycin (Invitrogen). For transfection experiments, 1×10^5 HeLa and 2×10^5 HEK293T cells were seeded in duplicate in six-well plates, half of them containing cover slips, 24 h before transfection. Then, the medium was replaced by a solution of fluorescein-labeled oligonucleotide **ON10** or d(T_{10})FAM (10 μM final concentration) in DMEM +/+ (FCS, P/S). The transfection medium was removed after 48 h at 37 °C and cells washed with 2×1 ml PBS and resuspended in 1 ml fresh DMEM +/+.

4.1.10. Fluorescence measurements and FACS analysis

Fixation of the cells on the cover slips was carried out using a solution of paraformaldehyde (1 ml, 3.7% in PBS) for 10 min followed by washing with PBS (2×1 ml), permeabilization of the cell membrane with Triton x-100 (0.2%, Promega) for 10 min and washing with PBS (2×1 ml).The cover slips were treated with a few drops of polyvinylalcohol (Mowiol) and nuclear stain 4′,6′-diamidino-2-phenylindole (DAPI). Cells were analyzed by fluorescence microscopy (Leica DMI6000 B, Leica Microsystems; software: Leica Application Suite) 48 h post transfection. Cellular uptake of fluorescein-labeled oligonucleotides was determined by fluorescence-activated cell sorting (FACS, BD FACSCalibur, BD Biosciences). HeLa and HEK293T cells grown without cover slips were washed in PBS and trypsinized. After dilution in 1 ml DMEM +/+, the suspension was centrifuged, the supernatant removed and the pellets resuspended in 1 ml cold PBS. They were then stored on ice. Fluorescence intensity was measured using FACSscan and Cell Quest Pro software. Viable cells were gated for forward, side scatter and fluorescence activity. 20,000 events were captured per sample.

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