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Synthesis of 2′-*O*-modified adenosine building blocks and application for RNA interference

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Abstract—RNA-Interference has been recognized as a powerful tool to control gene function and has been used for gene silencing by knocking down mRNA. Chemically modified RNAs, especially 2′-*O*-modification, successfully improved their physicochemical and pharmaceutical properties such as stability, nuclease resistance and delivery. Here, we report the synthesis of adenosine building blocks with different 2′-tethered modifications like aminoethyl and guanidinoethyl and show that they are compatible with RNAi function. They enhance the half life of the siRNA in serum suggesting that these modifications can enhance the pharmacokinetic properties and knock down activity of siRNAs in vivo.
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1. Introduction

Since the recognition of RNA interference (RNAi) in 1998, the process by which specific mRNAs are targeted and degraded by complementary short-interfering RNAs so called siRNA became a powerful tool to control gene function and has great hopes as therapeutic approach for gene silencing.[1,2] For developing the most effective siRNA construct it is necessary to understand the mechanism of RNAi. The generally accepted mechanism of RNAi can be divided into two main steps. In the first double stranded RNA (dsRNA) is cleaved into short 21–24 nt siRNAs. This process is catalysed by Dicer, an endonuclease of the RNase III family.[3] The resultant siRNAs duplexes have 3′-overhangs of 2 nt with 3′-hydroxyl termini and a 5′-phosphate at both ends. In the second step, siRNAs are incorporated into the RNA-induced silencing complex (RISC). A helicase in RISC unwinds the duplex siRNA, which than pairs to messenger RNAs (mRNAs) that bear a high degree of sequence complementarity to the siRNA.[4] In humans the degradation of the target mRNA is mediated by the Agonaute 2 protein associated with RISC. The target mRNA is cleaved in the complementary region at the phosphodiester bond that lies across from nucleotides 10 and 11 of the 5′-end of the siRNA.[5] For RNAi-mediated mRNA cleavage and degradation to be successful, a 5′-phosphate must be present on the antisense strand, and the double helical antisense-target mRNA duplex must be in the A-form.[6]

The main challenge for therapeutic applications are the rapid degradation of RNA by extra- and intra-cellular enzymes and the delivery inside to the relevant cells in whole organism.

Chemically modified nucleosides have shown to be of great importance for antisense strategies and are now being applied for RNA interference mediated gene silencing. The rationale for the synthesis of 2′-*O*-cationic modified nucleosides is on one hand a higher expected nuclease resistance and on the other hand a potentially better cellular uptake due to an overall reduced negative charge based on internal charge compensation.

A large number of oligonucleotide derivatives bearing 2′-*O*-tethered modifications are reported.[7–10] As a result

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of the higher nuclease resistance and affinity for complementary oligonucleotides of 2′-*O*-aminopropyl[11] and 2′-*O*-guanidinoethyl,[12] we decided to synthesise 2′-*O*-modified adenosine with cationic modifications and to analyse their stabilising effect on the duplex. Here, we report the preparation of adenosine building blocks carrying as 2′-modifications either cationic groups like [aminoethyl(**D1**), guanidinoethyl(**D2**)] or neutral ones like [cyanoethyl(**D3**), allyl(**D4**)] (Scheme 1).

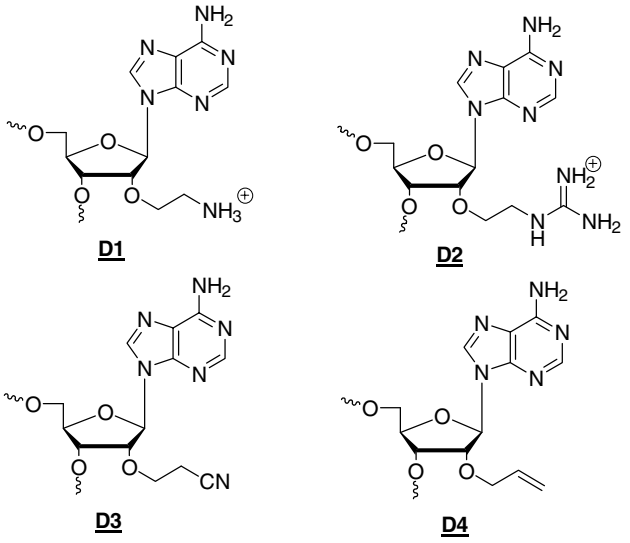
These modified building blocks were synthesised and incorporated into RNA in order to test their effects on RNA stability and siRNA silencing. 12mer RNA oligonucleotides were synthesised for measuring thermal stability as well as their CD spectra. 21mer RNA oligonucleotides were prepared for siRNA silencing and measuring nuclease resistance.

2. Synthesis

Preparation of the adenosine nucleoside phosphoramidites with an aminoethyl modification on the 2′-OH group is shown in Scheme 2. Different strategies for an efficient method to introduce the aminoethyl group on the 2′-*O*-position were reported earlier.[13]

Adenosine **1** was alkylated at the 2′-position with NaH and bromoacetate[14] to provide **2** in 47% yield. The nucleoside **2** could be separated easily from the isomers by silica gel chromatography.

Nucleoside **2** was protected at the 5′-position with dimethoxytriphenylmethyl (DMTr) to produce **3** in 75%. Nucleoside **3** was reduced with $LiBH_4$ to provide the 2′-(hydroxyethyl) nucleoside **4** in 80% yield. Reduction using $LiAlH_4$ instead of $LiBH_4$ was not so efficient because of a side reaction producing 2′-vinyl adenosine in 20%. The nitrogen was introduced by phthalimide in a Mitsunobu reaction to yield the 2′-(phthalimidoethyl) nucleoside **5** in 89%. For protection of the nucleobase N^6 we choose the dimethylformamidine group to provide **6** in 86% yield. The final step of Scheme 1 is the phosphitylation of nucleoside **6** using 2-cyanoethyldiisopropylchloro-phosphoramidite to yield the adenosine tethered phthalimidoethyl nucleoside phosphoramidite **7**.



Scheme 1. Synthesised 2′-*O*-modified building blocks.

The synthesis of compound **14** is shown in Scheme 3. Adenosine **1** was alkylated at the 2′-*O*-position under the same conditions reported for compound **2.**Nucleoside **2** was protected at the 5′- and 3′-position with *tert*-butyl-dimethylsilyl (TBDMS) to provide **8** in 92% yield. Nucleoside **8** was reduced with $LiAlH_4$ to produce the 2′-*O*-hydroxyethyl nucleoside **9** in 86% yield. For the incorporation of the guanidino group we used tribocguanidin, which we had synthesised before,[15] in a Mitsunobu type reaction first under conventional conditions heating for 8 h to produce nucleoside **10** in 78% yield. The microwave assisted method was even more efficient. We chose a power of 150 W at 80 °C for 1.5 h to provide nucleoside **10** in 92% yield.

Removal of the 3′- and 5′-silyl groups with TBAF, gave **11** in 90% yield and was followed by 5′-DMTr protection to produce **12** in 75% yield and the protection of the N^6 position of the base with DMF to provide **13** in 86% yield. The final step was the phosphitylation yielding **14** in 65%.

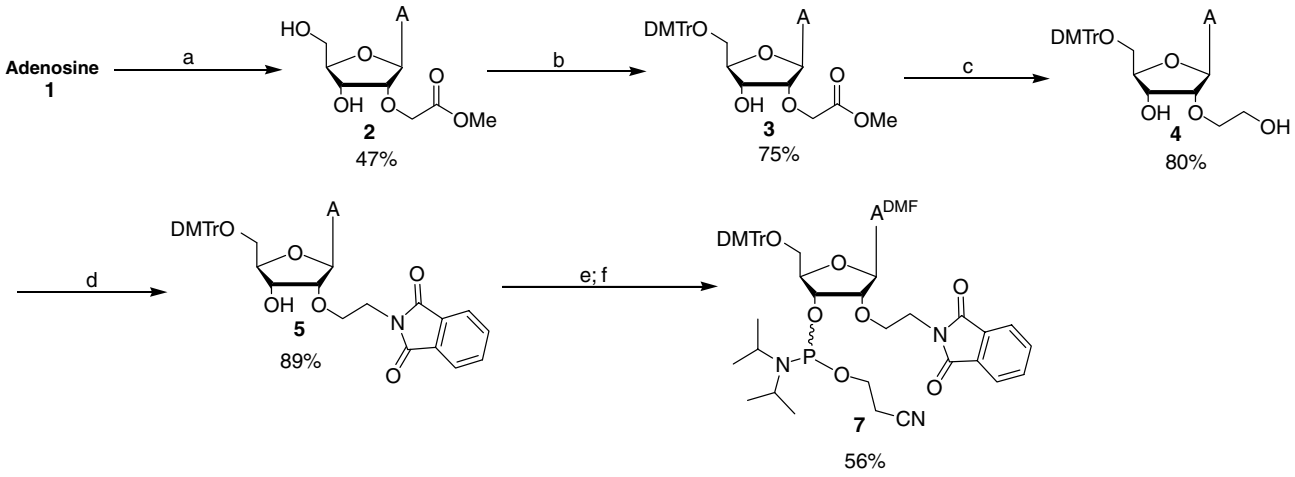
The synthesis of the neutral building blocks **20** and **23** is shown in Scheme 4.

We protected the exocyclic amino group of the base with dimethylformamidine to avoid undesired derivatization on N^6 in the alkylation step and obtained compound **15** in 99% yield. The protection of the 5′- and 3′-position with TIPDS produced nucleoside **16** in 95% yield. For the 2′-*O*-allyl modified building block we chose the palladium-catalyzed allylation[16] to provide **17** in 80% yield. A Michael addition with acrylonitrile and Cs_2CO_3 in *tert*-butanol[7] produced nucleoside **21** in 70% yield. The TIPDS 3′,5-protecting group was removed with NEt_3*3HF, producing **18** and **22** with 90% yield. Selective reprotection of the 5′-hydroxyl group with DMTr led to nucleosides **19** and **23** followed by phosphitylation providing **20** and **24** in a yield of 52%.

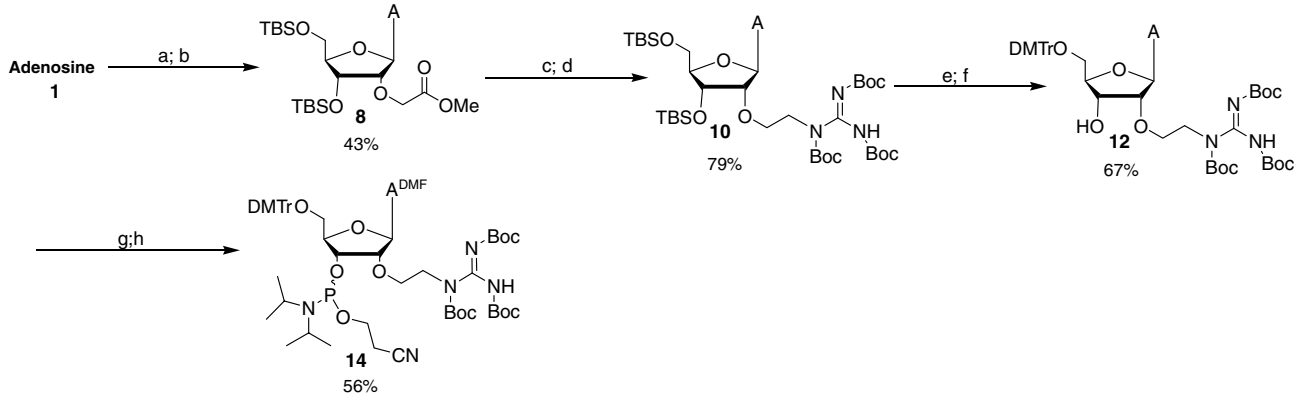
3. Results and discussion

The route of our synthesis from **1** to **7** takes 6 steps with 6 chromatographic purifications in a 14% overall yield. For the nucleoside **14** it takes 8 steps with 7 chromatographic purifications in a 13% overall yield. The synthesis from **1** to **20** and **24** takes 6 steps with 5 chromatographic purifications in a 35% overall yield for **20** and 31% for **24**.

All the synthesized nucleoside phosphoramidites **7**, **14**, **20** and **24** were incorporated into 12mer RNA oligonucleotides and their thermal stability as well as their CD spectra measured. The sequence of the RNA 12mer, the Tm values and the resulting ΔTm are shown in Scheme 5.



Scheme 2. Synthesis of the aminoethyl modified building block **7**. Reagents and conditions: (a) NaH, $BrCH_2COOMe$, DMF, rt 8 h; (b) DMTr-Cl, NEt_3, pyridine, rt, 16 h; (c) $LiBH_4$, THF/MeOH, 0 °C, 3 h; (d) phthalimide, PPh_3, DEAD, THF, rt, 1.5 h; (e) DMF-dimethyl-acetale, DMF, 60 °C, 1 h; (f) CEP-Cl, sym collidine, 1-methylimidazole, acetonitrile, 0 °C, 0.5 h, rt, 0.25 h.



Scheme 3. Synthesis of the guanidinoethyl modified building block **14**. Reagents and conditions: (a) NaH, $BrCH_2COOMe$, DMF, rt 8 h; (b) TBDMS-Cl, imidazole, DMF, rt, 16 h; (c) $LiAlH_4$, ether, 0 °C, 0.5 h ; (d) *N*,*N*′,*N*″-triboc-guanidin, PPh_3, DEAD, THF, rt, 1.5 h, p:1 bar, P: 150 W; (e) TBAF/THF(1 mol), THF, rt, 16 h; (f) DMTr-Cl, NEt_3, pyridine, rt, 16 h; (g) DMF-dimethyl-acetale, DMF, 60 °C, 1 h; (h) CEP-Cl, sym collidine, 1-methylimidazole, acetonitrile, 0 °C, 0.75 h.

The results of the Tm and CD measurement show that the aminoethyl and guanidinoethyl modified building blocks **D1** and **D2** stabilize the 12mer duplex. Guanidinoethyl modified building block **D2** gives the highest Tm value and therefore the highest stabilization (ΔTm:+3.1 °C). The aminoethyl modified building block **D1** has a stabilization of (ΔTm:+1 °C). The zneutral modified building blocks were synthesized to improve the effect of the positive charge in combination with increasing the lipophilicity.

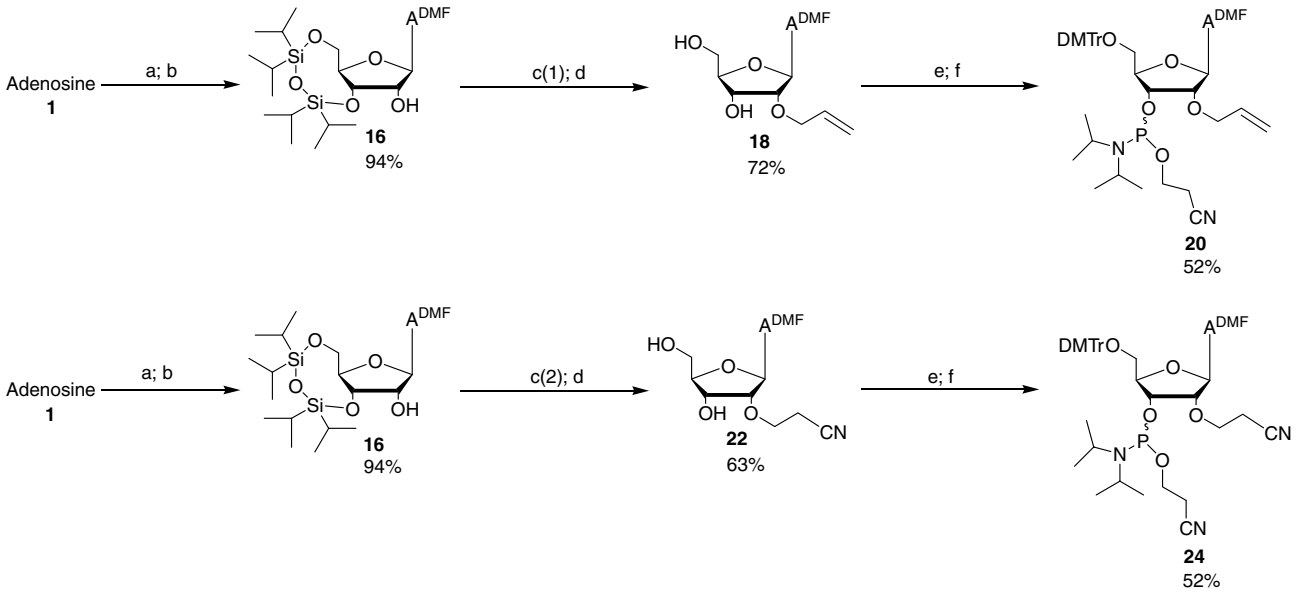
The results indicate that the allyl modification **D4** has the lowest stabilization, whereas the cyanoethyl modified building block **D3** shows a stabilisation of (ΔTm:+0.9 °C). The CD spectra depicted in Scheme 6 support the A-helix geometry of the duplex and confirm the results of the Tm measurements.

The oligonucleotides with the above 2′-*O*-modifications were used for biological tests including stability in serum and inhibitory activity in RNA interference. For this purpose we synthesized four 21mer siRNAs, directed towards a previously established functional target in the eGFP mRNA[17] with modified building blocks at different positions in the sense strand and two antisense strands, see Scheme 7.

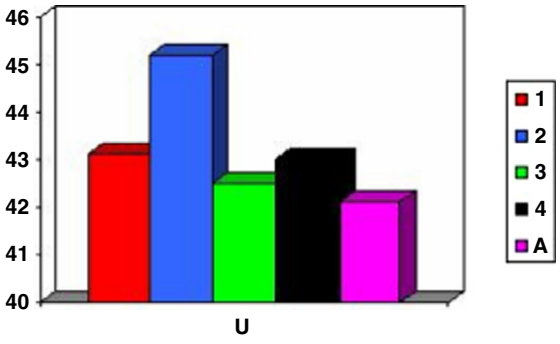
The 3′-terminal additional U was included for synthetic reasons and was shown to have no effect on siRNA knock down.[18] The synthesis of the modified building blocks C and G will be published (manuscript in preparation). These sense RNAs DO002-4 were annealed to the antisense RNA modified oligos JE1001 and JE1002, to obtain siRNAs.

4. Biological test

Incorporation of unnatural nucleotides in the siRNA will presumably inhibit the activity of RNases and extent the halflife of the duplex in vivo. To test this hypothesis siRNA duplexes were incubated at 37 °C in 80% fetal calf serum to mimic in vivo conditions and followed by taking time aliquots. The integrity of the siRNAs was subsequently analyzed by staining the RNA after size fractionation on a 15% native polyacrylamide



Scheme 4. Synthesis of the neutral modified building blocks **20** and **23**. Reagents and conditions: **a** DMF-dimethyl-acetale, DMF, 60 °C, 1 h **b** TIPDS-Cl, pyridine, 20 h, rt **c(1)** allyl ethyl carbonate, $Pd_2(dpa)_2$, dppb, THF, 1 h, 70 °C **c(2)** Acrylonitrile, Cs_2CO_3, *tert*-butanol, rt, 16 h **d** NEt_3*3HF, THF, rt, 0.5 h **e** DMTr-Cl, NEt_3, pyridine, rt, 16 h **f** CEP-Cl, sym collidine, 1-methylimidazole, acetonitrile, 0 °C, 0.5 h.



	Base	Tm	Tm
1	U	43.1	+1.0
2	U	45.2	+3.1
3	U	42.5	+0.4
4	U	43.0	+0.9
A	U	42.1	-------

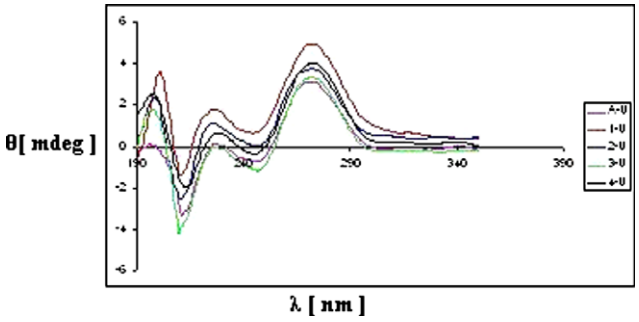
5'-AAG-AAX-GAA-AAG-3'

3'-UUC-UUU-CUU-UUC-5'

* Tm +/- 0.2

1:X=Aminoethyl 2: X= Guanidinoethyl 3: X= Ally 4: X= Cyanoethyl

Scheme 5. RNA 12mer sequence and the resulting Tm values.



Scheme 6. CD spectra of the modified and unmodified 12mer RNA oligonucleotides.

gel at the indicated time points. All modified siRNA duplexes exhibited prolonged half life compared to unmodified siRNA, with the 5′-terminal modified siRNA (containing JE1002) being most stable. Interestingly, we also found that 2-*O*-aminoethyladenosine modified siRNA (DO0003) and 2′-*O*-guanidinoethyladenosine (DO004) were more resistant than siRNA containing 2′-*O*-allyladenosine (DO0002) modification (Scheme 8; compare panels a–f and g) with JE1001 as antisense strand.

The biological activity of the chemically modified siRNA were tested together with unmodified and mismatched control siRNAs by transfecting them into a H1299 lung carcinoma cell line that stably expresses destabilized EGFP, and the level of EGFP protein expression was monitored on the basis of flow cytometry (Scheme 9). Treating the cells with a single dose of 10 nM of the various siRNAs gave a comparable 80% knock down in the average EGFP expression for all constructs containing the lightly-modified JE1001 antisense strand 48 h after transfection and the decline in protein level correlated closely with the level of eGFP mRNA expression (Scheme 9, lanes 1–3). This result

siRNA:

5`- GAC GUA AAC GGC CAC AAG UUC-3`
3`-CG CUG CAU UUG CCG GUG UUC A -5`

Sense A Allyl:

5`-GAC GUA AAC GGC CAC AAG UUC-3` DO0002

Sense A Aminoethyl:

5`-GAC GUA AAC GGC CAC AAG UUC-3` DO0003

Sense A Guanidinoethyl:

5`-GAC GUA AAC GGC CAC AAG UUC-3` DO0004

Antisense C,G Aminoethyl:

3`-UCG CUG CAU UUG CCG GUG UUC A-5` JE1001

Antisense C,G, A Aminoethyl:

3`-UCG CUG CAU UUG CCG GUG UUC A-5` JE1002

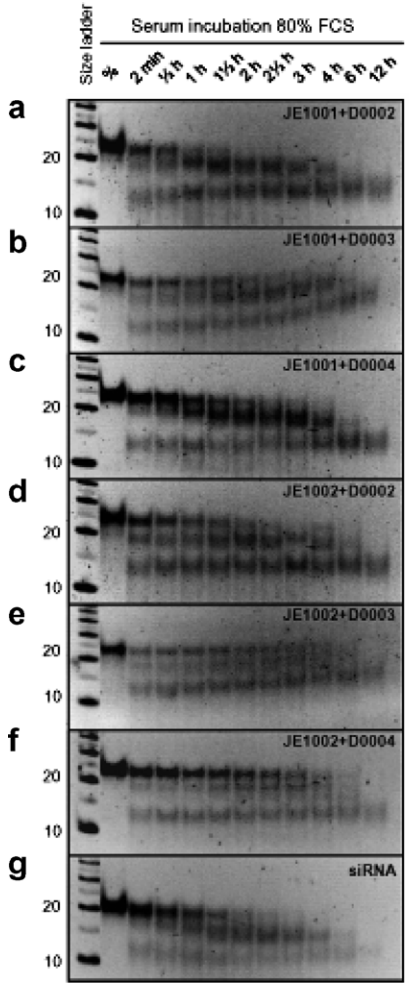
Scheme 7. siRNA sequences and tested 21mers.

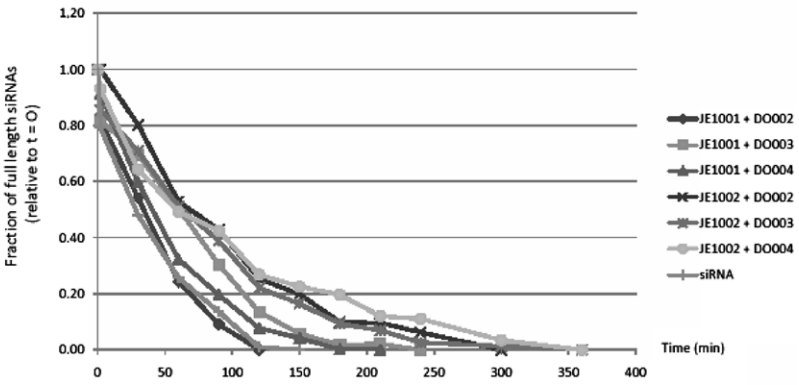
was indistinguishable from the knockdown obtained with unmodified RNA suggesting that the substitutions were fully compatible with siRNA function (Scheme 9, lane7). In contrast, all siRNAs containing the 5-terminal antisense JE1002 gave only small (10-20%), but reproducible, knock down in protein and mRNA expression (Scheme 9, lanes 4–6), implying that a 5′-terminal modification in the antisense strand interferes with RNAi activity.

The knock down efficiencies between different siRNA (denoted below) were compared by targeting eGFP mRNA. The eGFP protein expression was measured by flow cytometry (mean fluorescence of approximately 50.000 cells) and the eGFP mRNA levels were measured by Northern blotting. The siRNA mismatch represents a siRNA that contains four mismatches to the eGFP target and green cells denote untreated cells.

5. Conclusion

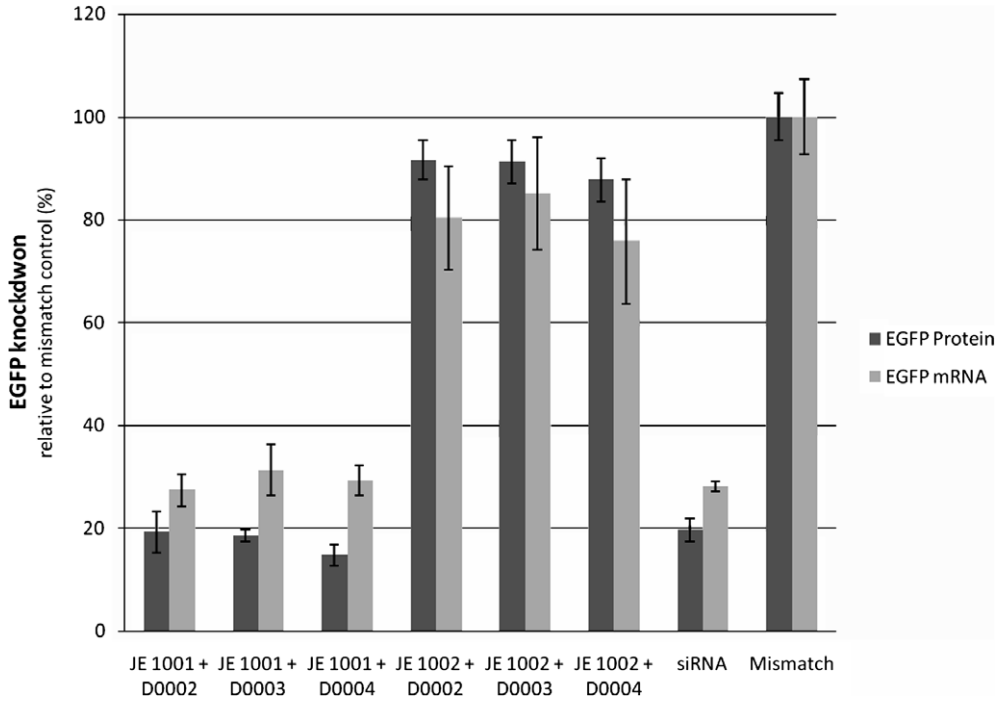
We have described a set of experimental procedures for synthesizing 2′-*O*-cationic and neutral modified adeno-





siRNA duplex	t½ (min)
JE1001 + DO002	33
JE1001 + DO003	49
JE1001 + DO004	39
JE1002 + DO002	63
JE1002 + DO003	58
JE1002 + DO004	61
siRNA	30

Scheme 8. Serum stability of siRNAs and density scan.



Scheme 9. RNAi test.

sine building blocks. Furthermore we have shown that our cationic modifications have a stabilizing effect on a RNA 12mer duplex. The modifications led generally to higher stability in serum, an effect that was strongest for the 2′-*O*-aminoethyladenosine modification. As long as the modifications were restricted to the 3′-end of the antisense strand the siRNA duplexes were all fully active in RNA interference when tested in cell lines. This suggests that the new modifications are compatible with RNAi function and may prove advantageous for in vivo applications.

6. Experimental

6.1. Materials and methods

Oligonucleotides synthesis. The RNA oligomers were synthesized on Expedite synthesizer by phosphoamidite chemistry with coupling time for modified monomers of 12 min.[19] The RNA oligomers were cleaved from the controlled-pore-glass (CPG) support with ethanol:ammonia solution (1:3) at 40 °C for 24 h. The 2′-TBDMS groups were deprotected with triethylamine, *N*-methylpyrrolidinone and $Et_3N \cdot 3HF$ mixture for 1.5 h at 65 °C.[20] The RNA oligomers were precipitated with BuOH at −80 °C over 30 min and purified by anion exchange HPLC (NucleoPac-PA-100) and desalted (Sephadex-G25). All ribonucleotides were characterized by MALDI-TOF-MS.

UV Melting curves. UV Melting profiles of the RNA duplexes were recorded in a phosphate buffer containing NaCl (140 mM, pH 7.0) at oligonucleotide concentrations 2 μM for each strand at wavelength 260 nm.[21] Each melting curve was determined twice. The temperature range was 0–60 °C with a heating rate 0.5 °C. The thermodynamic data were extracted from the melting curves by means of a two state model for the transition from duplex to single strands.

CD spectra. CD spectra of RNA duplexes were recorded at 350–190 nm with oligonucleotide concentration of 2 μM for each strand in a phosphate buffer containing NaCl (140 mM, pH 7.0). The measurements were performed at 10 °C to ensure that only duplex RNA was present.

Transfection. The human lung cancer cell line H1299 produced to stably express EGFP (EGFP half-life 2 h; a gift from Dr Anne Chauchereau, CNRS, Villejuif, France) were plated in 6-well plates and grown in RPMI-1640 containing 10% FBS, 1% penicillin/streptomycin to 60–80% confluence. Immediately before transfection, the cells were replenished in 1.25 ml of complete growth media per well. Sense and antisense strands where mixed in annealing buffer (10 mM Tris–HCl, pH 7.3, 50 mM NaCl) at 20 mM equimolar concentration and incubated at 95 °C for 1 min and at 1 h at 37 °C. Per well in a 6-well plate, the following solution was prepared: 6 μl of TransIT-TKO in 250 μl serum free RPMI media. siRNA was added, mixed carefully, incubated for 20 min at rt, and applied to the cells at a final concentration of 10 nM. After 24 h incubation at 37 °C, the media was changed and the cells were incubated for another 24 h at 37 °C. The cells were removed by trypsination and the expression of EGFP protein was quantified by flow cytometric analysis counting approximately 50,000 cells and averaged (mean fluorescence).

siRNA stability assay. siRNA sense and antisense strands where mixed in annealing buffer (10 mM Tris–HCl, pH 7.3, 50 mM NaCl) at 20 μM equimolar concentration and incubated at 95 °C for 1 min and at 1 h at 37 °C. siRNA duplexes were incubated at 37 °C in 80% fetal calf serum in DMEM (Gibco). Aliquots of 5 μl (each containing 20 pmol of siRNA) were diluted in 25 μl 1.2× TBE loading buffer (1.2× TBE, 10% glycerol, bromophenol blue) and snap-frozen on dry ice immediately upon sample taking. Samples were run on a 15% native polyacrylamide gel and stained using SYBR Gold® (Invitrogen).

6.2. General procedures

All ^{1}H and ^{13}C NMR spectra were measured on Bruker AM 250 (250 MHz) or AMX 400 (400 MHz) spectrometers. Chemical shifts (δ) are reported in parts per million (ppm). For peak multiplicity are used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broadened. *J* values are given in Hz. MALDI mass spectra were recorded on a Fisons VG Tofspec spectrometer and ESI mass spectra on a Fisons VG Plattform II spectrometer. The microwave-supported synthesis was accomplished in equipment of the company CEM, model Discover. All solvents and reagents were obtained from commercial sources used without further purifications.

6.3. Chemistry

6.3.1. 2′-*O*-(Methoxycarbonylmethyl)-adenosine (2). Sodium hydride 0.25 g (10.5 mmol) was added to a suspension of adenosine (2 g, 7.5 mmol) in 40 ml DMF at 0 °C. The suspension was stirred at 0 °C for 1 h and methyl bromoacetate (1.1 ml, 11.25 mmol) was added. This mixture was warmed to room temperature and stirred for 8 h. After the reaction was complete, the suspension was quenched with 1 ml acetic acid and 3 ml methanol. The solvents were concentrated under vacuum, and the residue was purified by a dry-load silica gel chromatography with 10% MeOH in CH_2Cl_2 as the eluant to provide 1.2 g (47%) **2** as a white foam. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 8.35 (s, 1H, H2), 8.12 (s, 1H, H8), 7.32 (s, 2H, NH_2), 6.06 (d, 1H, J = 6.3 Hz, 1′H), 5.39 (m,1H, 5′OH), 5.28 (d, 1H, J = 4.77 Hz, 3′OH), 4.67 (m, 1H, 2′H), 4.39 (m, 1H, 3′H): 4.29 (m, 2H, 2″H), 4.08 (m, 1H, 4′H), 3.68 (m, 2H, 5′H), 3.52 (s, 3H, OCH_3).^{13}C NMR (100.62 MHz, DMSO-d_6) δ [ppm]: 170.79 (C═O), 156.63 (C6), 152.91 (C8), 149.58 (C5),140.33 (C2), 119.72 (C4), 86.73 (C1′), 86.22 (C4′), 81.56 (C2′), 69.37 (C3′), 67.24 (C2″), 61.93 (C5′), 51.81 (O–CH_3). ESI(+)-MS: Calcd $C_{13}H_{17}N_5O_6$: 339.03. Found: 340.03 $[M+H]^+$.

6.3.2. 5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-2′-*O*-(methoxycarbonylmethyl)-adenosine (3). A portion of **2** (0.98 g, 2.88 mmol) was dissolved in 15 ml pyridine. Triethylamine (0.64 ml, 4.32 mmol) and 4,4′-dimethoxytrityl chloride (1.16 g, 3.45 mmol) were added at room temperature. The mixture was stirred for 16 h at room temperature, and quenched with 5 ml methanol and 10 ml saturated aqueous $NaHCO_3$. The mixture was extracted with CH_2Cl_2. The combined organic extracts were dried over $MgSO_4$ and concentrated under vacuum. The resulting orange oil was purified by chromatography with 10% MeOH in CH_2Cl_2 as the eluent to produce 1.34 g (73%) **3** as a white foam. ^{1}H NMR (250 MHz, DMSO-d_6) δ [ppm] 8.29 (s, 1H, H2), 8.11 (s, 1H, H8), 7.37 (s, 2H, NH_2), 7.35 (m, 8H, H_{DMTr}), 6.85 (m, 5H, H_{DMTr}), 6.11 (s, 1H, 1′H), 5.38 (m, 1H, 3′OH), 4.78 (m, 1H, 2′H), 4.49 (m, 1H, 3′H), 4.24 (m, 3H, 4′H, 5′H), 4.08 (m, 2H, 2″H), 3.73 (s, 6H, OCH_3), 3.53 (s, 3H, OCH_3). ^{13}C NMR (62.9 MHz, DMSO-d_6) δ[ppm] 170.42 (C═O), 156.67 (C6), 152.41 (C8), 149.57 (C5) 144.81 (C_{DMTr}) 139.72 (C2), 135.5–123.9(C_{DMTr}), 119.7 (C4), 113.6 (C_{DMTr}), 85.84 (C1′), 85.49 (C4′), 80.65 (C2′), 69.04 (C3′), 67.02 (C2″), 63.51 (C5′), 54.94 (OCH_{3DMTr}), 51.82 (O–CH_3). MALDI-MS: Calcd $C_{34}H_{35}N_5O_8$: 641.34. Found: 642.34 $[M+H]^+$.

6.3.3. 5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-2′-*O*-(hydroxyethyl)-adenosine (4). Lithium borhydride (0.39 g, 17.52 mmol) was added to a solution of **3** (1.41 g, 2.19 mmol) in 60 ml THF/MeOH (4:1) cooled to 0 °C, the reaction mixture was stirred for 3 h at 0 °C and then saturated aqueous NH_4Cl was added dropwise. The solution was warmed to room temperature and extracted with CH_2Cl_2. The combined organic extracts were dried over $MgSO_4$ and concentrated under vacuum. The resulting oil was purified by chromatography with 10% MeOH in CH_2Cl_2 as the eluent to produce 0.89 g (66%) **4** as a white foam. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 8.31 (s, 1H, H2), 8.15 (s, 1H, H8), 7.42 (s, 2H, NH_2), 7.41 (m, 8H, H_{DMTr}), 6.89 (m, 5H, H_{DMTr}), 6.10 (d, 1H, J = 5.8 Hz, 1′H), 5.21 (d, 1H, J = 6.02 Hz, 3′OH), 4.77 (t, 1H, J = 5.33 Hz, 3″OH), 4.70 (m, 1H, 2′H), 4.48 (m, 1H, 3′H), 4.12 (m, 1H, 4′H), 3.77 (s, 6H, OCH_3), 3.66 (m, 2H, 2″H), 3.62 (m, 2H, 3″H), 3.27 (m, 2H, 5′H). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ [ppm] 156.57 (C6), 153.14 (C8), 149.70 (C5), 145.29 (C_{DMTr}), 140.06 (C2), 136.13–127.12 (C_{DMTr}), 119.65 (C4), 113.69 (C_{DMTr}) 86.46 (C1′), 83.72 (C4′), 81.13 (C2′), 72.33 (C2″), 69.82 (C3′), 64.01 (C5′), 60.68 (C3″), 55.48(O–CH_3). MALDI-MS: Calcd $C_{33}H_{35}N_5O_7$: 613.33. Found: 614.33 $[M+H]^+$.

6.3.4. 5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-2′-*O*-(phthalimidoethyl)-adenosine (5). To a solution of **4** (0.15 g, 0.24 mmol) in 10 ml THF, triphenylphosphine (0.093 g, 0.36 mmol) and phthalimide (0.036 g, 0.24 mmol) were added at room temperature, stirred for 5 min and then diethylazodicarboxylate (0.063 g, 0.36 mmol) was added dropwise to the mixture. The mixture was stirred for 1 h at room temperature, and quenched with 3 ml saturated aqueous $NaHCO_3$. The mixture was extracted with CH_2Cl_2. The combined organic extracts were dried over $MgSO_4$ and concentrated under vacuum. The resulting orange oil was purified by chromatography with 10% MeOH in CH_2Cl_2 as the eluent to produce 0.16 g (90%) **5** as a white foam.^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 8.16 (s, 1H, H2), 7.96 (s, 1H, H8), 7.78 (m, 4H, $H_{phthalimide}$), 7.34 (s, 2H, NH_2), 7.25 (m, 8H, H_{DMTr}), 6.83 (m, 5H, H_{DMTr}), 5.91 (d, 1H, J = 5.5 Hz, 1′H), 5.19 (d, 1H, J = 4.92 Hz, 3′OH),

4.66 (m, 1H, 2′H), 4.40 (m, 1H, 3′H), 3.98 (m, 1H, 4′H), 3.84 (m, 4H, 2″H, 3″H), 3.72 (s, 6H, OCH_3), 3.16 (m, 2H, 5′H). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ [ppm] 168.18 (C=O), 158.48 ($C_{phthalimide}$), 156.57 (C6), 153.14 (C8), 149.70 (C5), 145.29 (C_{DMTr}), 140.06 (C2), 136.13–127.18 (C_{DMTr}), 119.65 (C4), 113.69 (C_{DMTr}) 86.46 (C1′), 83.71 (C4′), 81.13 (C2′), 72.33 (C2″), 69.82 (C3′), 64.01 (C5′), 60.68 (C3″), 55.48(O–CH_3). MALDI-MS: Calcd $C_{41}H_{38}N_6O_8$: 742.41. Found: 743.52 $[M+H]^+$.

6.3.5. 5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-2′-*O*-(phthalimidoethyl)-N^6-dimethylformamidine adenosine (6). Dimethylformamidine acetal (0.15 ml, 1.07 mmol) was added to a solution of **5** (0.12 g, 0.16 mmol) in 10 ml DMF. The mixture was stirred for 1 h at 60 °C and the solvents were concentrated under vacuum. The resulting oil was purified by chromatography with 10% MeOH in CH_2Cl_2 as the eluent to produce 0.11 g (86%) **6** as a white foam.

^1H NMR (400 MHz, DMSO-d_6) δ [ppm] 8.87 (s, 1H, $H_{amidine}$), 8.27 (s, 1H, H2), 8.23 (s, 1H, H8), 7.76 (m, 4H, $H_{phthalimide}$), 7.35 (m, 8H, H_{DMTr}), 6.79 (m, 5H, H_{DMTr}), 5.98 (d, 1H, J = 5.7 Hz, 1′H), 5.22 (d, 1H, J = 5.12 Hz, 3′OH), 4.70 (t, 1H, J = 4.7 Hz, 2′H), 4.42 (m, 1H, 3′H), 3.91 (m, 3H, 4′H, 5′H), 3.72 (s, 6H, OCH_3), 3.31 (m, 10H, 2″H, 3″H, $N(CH_3)_2$). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ [ppm] 168.18 (C=O), 157,89 ($C_{amidine}$), 156.57 (C6), 153.14 (C8), 149.70 (C5), 145.29 (C_{DMTr}), 140.06 (C2), 136.13 (C_{DMTr}), 130.17 ($C_{phthalimide}$), 128.21 (C_{DMTr}), 119.65 (C4), 113.69 (C_{DMTr}), 86.46 (C1′), 83.72 (C4′), 81.13 (C2′), 72.33 (C2″), 69.82 (C3′), 64.01 (C5′), 60.68 (C3″), 55.48(O–CH_3), 34.53 ($N(CH_3)_2$). MALDI-MS: Calcd $C_{44}H_{43}N_7O_8$: 797.44. Found: 798.48 $[M+H]^+$.

6.3.6. 3′-*O*-(2-Cyanoethoxydiisoproylphosphine)-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-2′-*O*-(phthalimidoethyl)-N^6-dimethylformamidine adenosine (7). Sym.-collidine (0.16 ml, 1.2 mmol) and 1-methylimidazole (5 μl, 0.062 mmol) were added dropwise to a solution of **6** (0.1 g, 0.12 mmol) in 5 ml acetonitrile. After the mixture was cooled to 0 °C, 2-cyanoethyldiisopropylchloro-phosphoramidite (0.04 ml, 0.18 mmol) was added dropwise. The mixture was stirred for 30 min at 0 °C, 15 min at room temperature and quenched with 1.5 ml saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic extracts were washed with 0.01 M citric acid, dried over $MgSO_4$ and concentrated under vacuum. The resulting oil was purified by chromatography with 2% MeOH in CH_2Cl_2 as the eluent to produce 78 mg (65%) **7** as a white foam. ^{31}P NMR (400 MHz, $CDCl_3$) δ [ppm] 141.55, 140.89 (ratio 1:1.5). MALDI-MS: Calcd $C_{53}H_{60}N_9O_9P$: 997.53. Found: 998.56 $[M+H]^+$.

6.3.7. 5′,3′-*O*-(*tert*-Butyldimethylsilyl)-2′-*O*-(methoxycarbonylmethyl)-adenosine (8). *tert*-Butyldimethylsilyl chloride (0.71 g, 4.7 mmol) and imidazole (0.31 g, 2.2 mmol) were added to a stirred solution of nucleoside **2** (0.5 g, 1.47 mmol) in 10 ml DMF. After 16 h, the solvents were removed under vacuum, and the resulting paste was partitioned between water and CH_2Cl_2. The combined organic extracts were washed with saturated aqueous NaCl, dried over $MgSO_4$ and concentrated under vacuum. The resulting oil was purified by chromatography with 5% MeOH in CH_2Cl_2 as the eluent to produce 0.77 g (92%) **8** as a white solid. ^1H NMR (250 MHz, DMSO-d_6) δ [ppm] 8.16 (s, 1H, H2), 7.98 (s, 1H, H8), 7.17 (s, 2H, NH_2), 5.94 (d, 1H, J = 6.2 Hz, 1′H), 4.65 (m, 1H, 2′H), 4.51(m, 1H, 3′H), 4.07 (m, 2H, 2″H), 3.72 (m, 3H, 4′H, 5′H), 3.41 (s, 3H, OCH_3), 0.94 (m, 18H, $H_{tert\text{-}butyl}$), 0.09 (m, 12H, $H_{dimethylsilyl}$). ^{13}C NMR (62,90 MHz, DMSO-d_6) [δ ppm]: 169.85 (C=O), 156.03 (C6), 152.54 (C8), 149.24 (C5),139.52 (C2), 119.04 (C4), 85.17 (C1′), 84.75 (C4′), 80.29 (C2′), 70.24 (C3′), 66.91 (C2″), 62.02 (C5′), 51.36 (O–CH_3), 25.76 (C_{TBS}), 17.76 (C_{TBS}), -5.12 (C_{TBS}). ESI(+)-MS: Calcd $C_{25}H_{46}N_5O_6Si_2$: 567.29. Found: 568.30 $[M+H]^+$.

6.3.8. 5′,3′-*O*-(*tert*-Butyldimethylsilyl)-2′-*O*-(hydroxyethyl)-adenosine (9). A portion of **8** (0.77 g, 1.35 mmol) was dissolved in 10 ml ether and cooled to 0 °C. After adding Lithium aluminium hydride (0.14 g, 3.38 mmol) the solution was stirred for 0.5 h at 0 °C and quenched by addition of saturated aqueous NH_4Cl. After the solution was warmed to room temperature, it was diluted with ether. The combined organic extracts were washed with saturated aqueous NaCl, dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by chromatography in 5% MeOH in CH_2Cl_2 as the eluent to produce 0.61 g (86%) **9** as fine needeld crystalls. ^1H NMR (400 MHz, DMSO-d_6) δ [ppm] 8.20 (s, 1H, H2), 8.01 (s, 1H, H8), 7.18 (s, 2H, NH_2), 5.89 (d, 1H, J = 4.8 Hz, 1′H), 4.52 (m, 1H, 2′H), 4.45(m, 2H, 3′H, OH), 3.82 (m, 2H, 2″H), 3.38 (m, 3H, 4′H, 5′H), 0.96 (m, 18H, $H_{tert\text{-}butyl}$), 0.08 (m, 12H, $H_{dimethylsilyl}$). ^{13}C NMR (62,90 MHz, DMSO-d_6) δ [ppm]: 156.07 (C6), 152.61 (C8), 149.18 (C5), 139.38 (C2), 119.08 (C4), 85.59 (C1′), 84.52 (C4′), 80.29 (C2′), 71.79 (C3′), 70.11 (C3″), 61.97 (C2″), 60.02 (C5′), 25.76 (C_{TBS}), 17.76 (C_{TBS}), -5.12 (C_{TBS}). MALDI-MS: Calcd $C_{24}H_{46}N_5O_5Si_2$: 539.30. Found: 540.08 $[M+H]^+$.

6.3.9. 5′,3′-*O*-(*tert*-Butyldimethylsilyl)-2′-*O*-(N,N′,N″-triboc-guanidinoethyl)-adenosine (10). A portion of **9** (0.7 g, 1.3 mmol) was dissolved in 45 ml of THF. Triphenylphosphine (0.6 g, 1.95 mmol) and N,N′,N″triboc-guanidine (1.4 g, 3.9 mmol) were added and cooled to −5 °C. After diethylazodicarboxylate (0.35 g, 1.95 mmol) was added dropwise to the mixture, it was refluxed for 1.5 h at 80 °C with a power P: 150W under microwave conditions. The reaction was quenched by adding saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic extracts washed with saturated aqueous NaCl, dried over $MgSO_4$ and concentrated under vacuum. The resulting oil was purified by chromatography with 5% MeOH in CH_2Cl_2 as the eluent to produce 1.05 g (92%) **10** as a white foam. ^1H NMR (250 MHz, DMSO-d_6) δ [ppm] 10.03 (br s, 1H, NH), 8.15 (s, 1H, H2), 7.99 (s, 1H, H8), 7.18 (s, 2H,

NH_2), 5.88 (s, 1H, 1′H), 4.51 (m, 2H, 2′H, 3′H), 3.78 (m, 2H, 2″H), 3.54 (m, 5H, 4′H, 5′H, 3″H), 1.22 (m, 27H, H_{Boc}), 0.89 (m, 18H, $H_{tert\text{-}butyl}$), 0.06 (m, 12H, $H_{dimethylsilyl}$). ^{13}C NMR (62,90 MHz, DMSO-d_6) δ [ppm]: 159.67 (C═O), 156.07 (C6), 152.51 (C8), 151.89 (C_{Gua}), 148.99 (C5), 139.32 (C2), 119.18 (C4), 85.94 (C1′), 84.08 (C4′), 82.24 (C2′), 70.80 (C3′), 69.14 (C3″), 61.93 (C2″), 60.09 (C5′), 30.66 (C_{Boc}), 27.70 (C_{Boc}), 25.76 (C_{TBS}), 17.76 (C_{TBS}), −5.12 (C_{TBS}) MALDI-MS: Calcd $C_{40}H_{72}N_8O_{10}Si_2$: 880.49. Found: 881.58 $[M+H]^+$.

6.3.10. 2′-*O*-(N,N′,N″-tri-boc-guanidinoethyl)-adenosine (11). TBAF (1 M) in THF (3.26 ml, 11.3 mmol) was added dropwise to a solution of **10** (1 g, 1.13 mmol) in 25 ml THF cooled to 0 °C. The solution was warmed to room temperature and stirred for 20 h. The solvents were removed under vacuum and the resulting oil was purified by chromatography with 10% MeOH in CH_2Cl_2 as the eluent to produce 0.66 g (90%) **11** as a white foam. ^1H NMR (250 MHz, DMSO-d_6) δ [ppm] 10.17 (br s, 1H, NH), 8.32 (s, 1H, H2), 8.11 (s, 1H, H8), 7.32 (br s, 2H, NH_2), 5.99 (d, 1H, J = 5.05 Hz, 1′H), 5.34 (m, 1H, 5′OH), 5.07 (d, 1H, J = 5.37 Hz, 3′OH), 4.44 (t, 1H, J = 5.05 Hz, 2′H), 4.33 (m, 1H, 3′H), 3.94 (m, 1H, 4′H), 3.60 (m, 6H, 5′H, 2″H, 3″H), 1.35 (m, 27H, H_{Boc}). ^{13}C NMR (62,90 MHz, DMSO-d_6) δ [ppm] 159.67 (C═O), 156.16 (C6), 152.44 (C8), 152.03 (C_{Gua}), 148.78 (C5), 139.56 (C2), 119.35 (C4), 86.52 (C1′), 85.42 (C4′), 81.86 (C2′), 69.31 (C3′), 68.14 (C3″), 61.93 (C2″), 61.12 (C5′), 30.65 (C_{Boc}), 27.70 (C_{Boc}). MALDI-MS: Calcd $C_{28}H_{44}N_8O_{10}$: 652.70. Found: 653.82 $[M+H]^+$.

6.3.11. 5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-2′-*O*-(N,N′,N″-tri-boc-guanidinoethyl) adenosine (12). 4,4′Dimethoxytrityl-chloride (0.41 g, 1.17 mmol) and NEt_3 (0.24 ml, 1.47 mmol) were added to a solution of **11** (0.64 g, 0.98mmol) in 10 ml pyridine cooled to 0 °C. The solution was warmed to room temperature and stirred for 24 h. The reaction was quenched by adding saturated aqueous $NaHCO_3$ and MeOH. The resulting solution was extracted with CH_2Cl_2 and the combined organic extracts were washed with saturated aqueous NaCl, dried over $MgSO_4$ and concentrated under vacuum. The residual yellow oil was coevaporated two times with toluene and purified by chromatography with 5% MeOH in CH_2Cl_2 as the eluent to produce 0.7 g (75%) **12** as a white foam. ^1H NMR (250 MHz, DMSO-d_6) δ [ppm] 10.20 (br s, 1H, NH), 8.21 (s, 1H, H2), 8.10 (s, 1H, H8), 7.32 (m, 2H, NH_2), 7.20 (m, 8H, H_{DMTr}), 6.82 (m, 5H, H_{DMTr}), 6.07 (m, 1H, 1′H), 5.10 (d, 1H, J = 6.32 Hz, 3′OH), 4.55 (m, 2H, 2′H, 3′H), 4.06 (m, 2H, 2″H), 3.68 (m, 9H, 4′H, 5′H, OCH_3), 3.22 (m, 2H, 3″H), 1.33 (m, 27H, H_{Boc})^{13}C NMR (62,90 MHz, DMSO-d_6) δ [ppm] 158.55 (C═O), 156.10 (C6), 152.58 (C8), 152.08 (C_{Gua}), 148.78 (C5), 144.82 (C_{DMTr}), 139.54 (C2), 135.54–126.60 (C_{DMTr}), 119.28 (C4), 113.05 (C_{DMTr}), 85.37 (C1′), 81.83 (C4′), 80.88 (C2′), 69.34 (C3′), 68.14 (C3″), 61.93 (C2″), 61.12 (C5′), 30.66 (C_{Boc}), 27.75 (C_{Boc}). MALDI-MS: Calcd $C_{49}H_{62}N_8O_{12}$: 954.45. Found: 953.91 $[M-H]^+$.

6.3.12. 5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-2′-O-(N,N′,N″-tri-boc-guanidinoethyl)-N^6-dimethylformamidine-adenosine (13). Dimethylformamidine acetal (0.19 ml, 1.41 mmol) was added to a solution of **12** (0.2 g, 0.21 mmol) in 15 ml DMF. The mixture was stirred for 1 h at 60 °C and the solvents were removed under vacuum. The resulting oil was purified by chromatography with 5% MeOH in CH_2Cl_2 as the eluent to produce 0.18 g (86%) **13** as a white foam. ^1H NMR (250 MHz, Acetone-d_6) δ [ppm] 8.81 (s, 1H, $H_{amidine}$), 8.29 (s, 1H, H2), 8.09 (s, 1H, H8), 7.25 (m, 8H, H_{DMTr}), 6.71 (m, 5H, H_{DMTr}), 6.09 (s, 1H, 1′H), 4.55 (m, 2H, 2′H, 3′H), 3.98 (m, 5H, 4′H, 5′H, 2″H,), 3.68 (s, 6H, OCH_3), 3.31 (m, 2H, 3″H), 3.13 (s, 3H, NCH_3), 3.08 (s, 3H, NCH_3), 1.32 (m, 27H, H_{Boc}). ^{13}C NMR (62,90 MHz, Acetone-d_6) δ [ppm] 159.17 ($C_{amidine}$), 158.95 (C═O), 156.15 (C6), 153.59 (C8), 153.06 (C_{Gua}), 146.15 (C5), 141.56 (C_{DMTr}), 139.58 (C2), 135.54–126.60 (C_{DMTr}), 118.38 (C4), 113.86 (C_{DMTr}), 87.95 (C1′), 86.95 (C4′), 83.33 (C2′), 69.34 (C3′), 68.14 (C3″), 64.51 (C2″), 62.51 (C5′), 55.49 (OCH_3), 34.86 ($N(CH_3)_2$), 30.76 (C_{Boc}), 27.75 (C_{Boc}). MALDI-MS: Calcd $C_{52}H_{67}N_9O_{12}$: 1010.14. Found: 1010.59.

6.3.13. 3′-*O*-(2-Cyanoethoxydiisoproylphosphine)-5′-*O*-(4,4′dimethoxytriphenylmethyl)-2′-*O*-(N,N′, N″-tri-boc-guanidinoethyl)-N^6-dimethylformamidine-adenosine (14). Sym. Collidine (0.13 ml, 1 mmol) and 1-methylimidazole (4.2 μl, 0.052 mmol) were added dropwise to a solution of **13** (0.1 g, 0.1 mmol) in 5 ml acetonitrile. After the mixture was cooled to 0 °C, 2-cyanoethyldiisopropyl-chloro-phosphoramidite (0.03 ml, 0.15 mmol) was added slowly and dropwise. The mixture was stirred for 30 min at 0 °C, 15 min at room temperature quenched with 1.5 ml saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic extracts were washed with 0.01 M citric acid, dried over $MgSO_4$ and concentrated under vacuum. The resulting oil was purified by chromatography with 2% MeOH in CH_2Cl_2 as the eluent to produce 78 mg (65%) **14** as a white foam. ^{31}P NMR(400 MHz, $CDCl_3$) δ [ppm] 141.55, 140.89 (ratio 1:1,5). MALDI-MS: Calcd $C_{61}H_{84}N_{11}O_{13}P$: 1210.85. Found: 1210.98.

6.3.14. N^6-dimethylformamidine-adenosine (15). Dimethylformamidine acetal (10 ml, 75 mmol) was added to a solution of **1** (3 g, 11.25 mmol) in 50 ml DMF. The mixture was stirred for 1h at 60 °C and the solvents were removed under vacuum to produce 3.58 g (99%) **15** as a white solid. ^1H NMR (250 MHz, DMSO-d_6) δ [ppm] 8.97 (s, 1H, $H_{amidine}$), 8.53 (s, 1H, H2), 8.47 (s, 1H, H8), 5.97 (d, 1H, J = 6.0 Hz, 1′H), 5.52 (d, 1H, J = 6.3 Hz, 2′OH), 5.35 (t, 1H, J = 4.7 Hz, 5′OH), 5.25(d, 1H, J = 4.7 Hz, 3′OH), 4.67 (m, 1H, 2′H), 4.22 (m, 1H, 3′H), 4.02 (m, 1H, 4′H), 3.65 (m, 2H, 5′H), 3.26-3.19 (m, 6H, CH_3). ^{13}C NMR (62.9 MHz, DMSO-d_6) δ [ppm] 159.29 ($C_{amidine}$), 158.14 (C6), 151.73 (C8), 151.18 (C5), 141.54 (C2), 125.92 (C4), 87.69 (C1′), 85.73 (C4′), 73.42 (C2′), 70.52 (C3′), 61.53 (C5′), 34.55 ((CH_3)$_2$N) MALDI-MS: Calcd $C_{14}H_{19}N_5O_4$: 321.33. Found: 322.35 $[M+H]^+$.

6.3.15. 5′,3′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-N^6-dimethylformamidine-adenosine (16). TIPDS-Cl_2 (3.9 ml, 11.95 mmol) were added dropwise to a solution of **15** (3.5 g, 10.86 mmol) in 25 ml pyridine at 0 °C. The solution was warmed to room temperature and stirred for 24 h, quenched with water and extracted with CH_2Cl_2. The combined organic extracts were washed with saturated aqueous NaCl, dried over $MgSO_4$ and concentrated under vacuum. The residual oil was coevaporated two times with toluene and purified by chromatography with 5% MeOH in CH_2Cl_2 as the eluent to produce 5.8 g (95%) **16** as a white foam. ^{1}H NMR (250 MHz, DMSO-d_6) δ [ppm] 8.91 (s, 1H, $H_{amidine}$), 8.43 (s, 1H, H2), 8.39 (s, 1H, H8), 5.92 (s, 1H, 1′H), 5.62 (d, 1H, J = 6.1 Hz, 2′OH), 4.80 (m, 1H, 2′H), 4.55 (m, 1H, 3′H), 3.96 (m, 3H, 4′H, 5′H), 3.20 (s, 3H, CH_3), 3.10 (s, 3H, CH_3), 1.08 (m, 27H, SiCH(CH_3)$_2$). ^{13}C NMR (62.9 MHz, DMSO-d_6) δ [ppm] 159.24 ($C_{amidine}$), 157.92 (C6), 151.74 (C8), 150.62 (C5), 141.13 (C2), 125.94 (C4), 86.69 (C1′), 80.78 (C4′), 73.51 (C2′), 69.85 (C3′), 61.50 (C5′), 34.57 ((CH$_3$)$_2$N), 16.89 (SiCH<u>C</u>H$_3$), 12.24 (Si<u>C</u>HCH$_3$) MALDI-MS: Calcd $C_{26}H_{45}N_5O_5Si_2$: 563.84. Found: 565.61 [M+2H]$^+$.

6.3.16. 5′,3′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-(allyl)-N^6-dimethylformamidine-adenosine (17). A portion of **16** (1.3 g, 2.3 mmol) was dissolved in a solution of allyl-ethyl-carbonate (0.58 ml, 4.6 mmol) in 10 ml THF. The resulting solution was added dropwise to a suspension composed of tris(dibenzylidinacetone)-dipalladium(0) (24.6 mg, 0.023 mmol) and 1,4- bis-diphenylphosphino-butane (39.17 mg, 0.092 mmol) in 5 ml THF and stirred for 1 h at 70 °C. The solution was removed under vacuum and the residual green oil was purified by chromatography with 5% MeOH in CH_2Cl_2 to produce 1.1 g (79%) **17** as a white foam. ^{1}H NMR (250 MHz, DMSO-d_6) δ [ppm] 8.89 (s, 1H, $H_{amidine}$), 8.32 (s, 1H, H2), 8.29 (s, 1H, H8), 6.02 (s, 1H, 1′H), 5.91 (m, 1H, 3″H), 5.21 (m, 2H, 4″H), 4.51 (m, 1H, 3′H), 4.32 (m, 2H, 2″H), 3.89 (m, 3H, 4′H, 5′H), 3.21 (s, 3H, CH_3), 3.12 (s, 3H, CH_3), 1.12 (m, 27H, SiCH(CH_3)$_2$). ^{13}C NMR (62.9 MHz, DMSO-d_6) δ [ppm] 159.24 ($C_{amidine}$), 157.90 (C6), 151.75 (C8), 150.48 (C5), 141.14 (C2), 134.15 (C3″), 125.93 (C4), 116.41 (C4″), 87.71 (C1′), 80.67 (C4′), 80.14 (C2′), 70.97 (C2″), 69.98 (C3′), 59.93 (C5′), 34.55 ((CH$_3$)$_2$N), 17.06 (SiCH<u>C</u>H$_3$), 12.19 (Si<u>C</u>HCH$_3$). MALDI-MS: Calcd $C_{29}H_{49}N_5O_5Si_2$: 603.90. Found: 606.08.

6.3.17. 2′-*O*-(allyl)-N^6-dimethylformamidine-adenosine (18). NEt_3* 3HF (1.1 ml, 6.37 mmol) were added dropwise to a solution of **17** (1 g, 1.65 mmol) and NEt_3 (0.37 ml, 2.73 mmol) in 20 ml THF and stirred for 1 h at room temperature. The solvent was concentrated under vacuum and the resulting oil was purified by chromatography with 10% MeOH in CH_2Cl_2 as the eluent to produce 0.53 g (90%) **18** as a white foam. ^{1}H NMR (250 MHz, DMSO-d_6) δ [ppm] 8.89 (s, 1H, $H_{amidine}$), 8.51 (s, 1H, H2), 8.42 (s, 1H, H8), 6.09 (d, 1H, J = 6.3 Hz, 1′H), 5.75 (m, 1H, 3″H), 5.41 (m, 2H, 3′OH, 5′OH), 5.12 (m, 2H, 4″H), 4.51 (m, 1H, 2′H), 4.32 (m, 1H, 3′H), 4.13 (m, 3H, 4′H, 2″H), 3.70 (m, 2H, 5′H), 3.22 (s, 3H, CH_3), 3.12 (s, 3H, CH_3). ^{13}C NMR (62.9MHz, DMSO-d_6) δ [ppm] 159.30 ($C_{amidine}$), 158.03 (C6), 151.81 (C8), 151.03 (C5), 141.30 (C2), 134.63 (C3″), 125.75 (C4), 116.64 (C4″), 86.21 (C1′), 85.94 (C4′), 80.33 (C2′), 70.19 (C2″), 68.94 (C3′), 61.30 (C5′), 34.55 ((CH$_3$)$_2$N). MALDI-MS: Calcd $C_{16}H_{20}N_6O_4$: 360.16. Found: 361.27 [M+H]$^+$.

6.3.18. 5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-2′-*O*-(allyl)-N^6-dimethylformamidine-adenosine (19). 4,4′Dimethoxytrityl-chloride (0.6 g, 1.73 mmol) and NEt_3 (0.35 ml, 2.16 mmol) were added to a solution of **18** (0.52 g, 1.44 mmol) in 10 ml pyridine cooled to 0 °C. The solution was warmed to room temperature and stirred for 24 h. The reaction was quenched by adding saturated aqueous $NaHCO_3$ and MeOH. The resulting solution was extracted with CH_2Cl_2 and the combined organic extracts were washed with saturated aqueous NaCl, dried over $MgSO_4$ and concentrated under vacuum. The residual yellow oil was coevaporated two times with toluene and purified via chromatography with 5% MeOH in CH_2Cl_2 as the eluent to produce 0.73 g (75%) **19** as a white foam. ^{1}H NMR (250 MHz, DMSO-d_6) δ [ppm] 8.90 (s, 1H, $H_{amidine}$), 8.35 (m, 2H, H2, H8), 7.29 (m, 9H, H_{DMTr}), 6.28 (m, 4H, H_{DMTr}), 6.12 (d, 1H, J = 6.2Hz, 1′H), 5.82 (m, 1H, 3″H), 5.31 (d, 1H, J = 5.8 Hz, 3′OH), 5.12 (m, 2H, 4″H), 4.65 (m, 1H, 2′H), 4.45 (m, 1H, 3′H), 4.12 (m, 3H, 4′H, 2″H), 3.7 (m, 6H, OCH_3), 3.33 (m, 2H, 5′H), 3.21 (s, 3H, CH_3), 3.14 (s, 3H, CH_3). ^{13}C NMR (62.9 MHz, DMSO-d_6) δ [ppm] 159.22 ($C_{amidine}$), 158.03 (C6), 151.94 (C8), 151.15 (C5), 144.71 (C_{DMTr}), 141.29 (C2), 134.46 (C3″), 129.62–126.60 (C_{DMTr}), 125.71 (C4), 116.76 (C4″), 113.08 (C_{DMTr}), 86.03 (C1′), 85.47 (C4′), 83.48 (C2′), 70.41 (C2″), 69.18 (C3′), 63.46 (C5′), 52.95 (OCH_3) 34.55 ((CH$_3$)$_2$N). MALDI-MS: Calcd $C_{37}H_{39}N_6O_6$: 663.37. Found: 664.21 [M+H]$^+$.

6.3.19. 3′-*O*-(2-Cyanoethoxydiisoproylphosphine)-5′-*O*-(4, 4′dimethoxytriphenylmethyl)-2′-*O*-(allyl)-N^6-dimethylformamidine-adenosine (20). Sym. Collidine (0.4 ml, 3 mmol) and 1-methylimidazole (12.6 μl, 0.16 mmol) were added dropwise to a solution of **19** (0.2 g, 0.3 mmol) in 8 ml acetonitrile. After the mixture was cooled to 0 °C, 2-cyanoethyldiisopropylchloro-phosphoramidite (0.1 ml, 0.45 mmol) was added dropwise. The mixture was stirred for 30 min at 0 °C, quenched with 1.5 ml saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic extracts were washed with 0.01 M citric acid, dried over $MgSO_4$ and concentrated under vacuum. The resulting oil was purified by chromatography with 2% MeOH in CH_2Cl_2 as the eluent to produce 0.18 g (69%) **20** as a white foam. ^{31}P NMR(400 MHz, $CDCl_3$) δ [ppm] 150.75, 149.98 (ratio 1:1,5). MALDI-MS: Calcd $C_{46}H_{56}N_8O_7P$: 863.43. Found: 864.43 [M+H]$^+$.

6.3.20. 5′,3′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-(cyanoethyl)-N^6-dimethylformamidine-adenosine (21). A portion of **16** (1 g, 1.77 mmol) was dissolved in 10 ml *tert*-butanol. Acrylnitrile (1.9 ml, 35.4 mmol) and Cs_2CO_3 (0.58 g, 1.77 mmol) were added to the solution and stirred for 24 h at room temperature. After the reaction was done the solution was filtrated over celite and

washed with CH_2Cl_2 and concentrated under vacuum. The resulting oil was purified by chromatography with 5% MeOH in CH_2Cl_2 as the eluent to produce 0.76 g (70%) **17** as a white foam. ^1H NMR (250 MHz, DMSO-d_6) δ [ppm] 8.89 (s, 1H, $H_{amidine}$), 8.56 (s, 1H, H2), 8.44 (s, 1H, H8), 6.06 (d, 1H, $J = 5.6$ Hz, 1′H), 5.02 (m, 1H, 2′H), 4.61 (m, 1H, 3′H), 3.91 (m, 5H, 4′H, 5′H, 2″H), 3.23 (s, 3H, CH_3), 3.12 (s, 3H, CH_3), 2.82 (m, 2H, 3″H), 1.04 (m, 28H, $SiCH(CH_3)_2$). ^{13}C NMR (62.9 MHz, DMSO-d_6) δ [ppm] 159.21 ($C_{amidine}$), 157.89 (C6), 151.72 (C8), 150.51 (C5), 141.23 (C2), 125.96 (C4), 118.87 (CN), 87.61(C1′), 80.52 (C4′), 70.11 (C2′), 65.68 (C3′), 65.04 (C2″), 60.08 (C5′), 34.55 ($(CH_3)_2N$), 18.11 (C3″), 17.25 (17.06 ($SiCH\underline{CH}_3$), 12.33 ($Si\underline{C}HCH_3$). MALDI-MS: Calcd $C_{28}H_{47}N_7O_5Si_2$: 617.44. Found: 618.95 $[M+H]^+$.

6.3.21. 2′-*O*-(Cyanoethyl)-N^6-dimethylformamidine-adenosine (22). NEt_3* 3HF (1.07 ml, 6.23 mmol) were added dropwise to a solution of **21** (1.1 g, 1.78 mmol) and NEt_3 (0.36 ml, 2.67 mmol) in 20 ml THF and stirred for 1 h at room temperature. The solvent was removed under vacuum and the resulting oil was purified by chromatography with 10% MeOH in CH_2Cl_2 as the eluent to produce 0.6 g (90%) **22** as a white foam. ^1H NMR (250 MHz, DMSO-d_6) δ [ppm] 8.91 (s,1H,$H_{amidine}$), 8.57 (s, 1H, H2), 8.42 (s, 1H, H8), 6.06 (d, 1H, $J = 5.5$ Hz, 1′H), 5.35 (m, 2H, 3′OH, 5′OH), 4.61 (m, 1H, 2′H), 4.35 (m, 1H, 3′H), 3.98 (m, 1H, 4′H), 3.72 (m, 4H, 5′H, 2″H), 3.21 (s, 3H, CH_3), 3.10 (s, 3H, CH_3), 2.82 (m, 2H, 3″H). ^{13}C NMR (62.9 MHz, DMSO-d_6) δ [ppm] 159.90 ($C_{amidine}$), 158.04 (C6), 151.87 (C8), 151.10 (C5), 141.25 (C2), 125.77 (C4), 118.83 (CN), 85.93 (C1′), 85.75 (C4′), 80.99 (C2′), 68.74 (C3′), 64.70 (C2″), 61.15 (C5′), 34.55 ($(CH_3)_2N$), 18.11 (C3″). MALDI-MS: Calcd $C_{16}H_{21}N_7O_4$: 375.16. Found: 376.59 $[M+H]^+$.

6.3.22. 5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-2′-*O*-(cyanoethyl)-N^6-dimethylformamidine-adenosine (23). 4,4′Dimethoxytrityl-chloride (0.23 g, 0.67 mmol) and NEt_3 (0.13 ml, 0.84 mmol) were added to a solution of **22** (0.21 g, 0.56 mmol) in 10 ml pyridine cooled to 0 °C. The solution was warmed to room temperature and stirred for 24 h. The reaction was quenched by adding saturated aqueous $NaHCO_3$ and MeOH. The resulting solution was extracted with CH_2Cl_2 and the combined organic extracts were washed with saturated aqueous NaCl, dried over $MgSO_4$ and concentrated under vacuum. The residual yellow oil was coevaporated two times with toluene and purified by chromatography with 5% MeOH in CH_2Cl_2 as the eluent to produce 0.28 g (75%) **23** as a white foam. ^1H NMR (250 MHz, DMSO-d_6) δ [ppm] 8.82 (s, 1H, $H_{amidine}$), 8.24 (s, 1H, H2), 8.13 (s, 1H, H8), 7.36 (m, 9H, H_{DMTr}), 6.71 (m, 4H, H_{DMTr}), 6.09 (d, 1H, $J = 5.4$ Hz, 1′H), 5.35 (d, 1H, $J = 4.7$ Hz, 3′OH), 4.75 (m, 1H, 2′H), 4.61 (m, 1H, 3′H), 4.21 (m, 3H, 4′H, 5′H), 3.85 (m, 2H, 2″H), 3.65 (s, 6H, OCH_3), 3.15 (s, 3H, CH_3), 3.05 (s, 3H, CH_3), 2.72 (m, 2H, 3″H). ^{13}C NMR (62.9 MHz, DMSO-d_6) δ [ppm] 159.80 ($C_{amidine}$), 158.93 (C6), 153.03 (C8), 152.48 (C5), 146.10 (C_{DMTr}), 141.77 (C2), 136.92–128.55 (C_{DMTr}), 127.51 (C4), 118.83 (CN), 113.85 (C_{DMTr}), 87.88 (C1′), 86.97 (C4′), 82.33 (C2′), 70.80 (C3′), 66.61 (C2″), 64.39 (C5′), 55.48 (OCH_3), 34.88 ($(CH_3)_2N$), 19.10 (C3″). MALDI-MS: Calcd $C_{37}H_{39}N_7O_6$: 677.37. Found: 678.52 $[M+H]^+$.

6.3.23. 3′-*O*-(2-cyanoethoxydiisoproylphosphine)-5′-*O*-(4,4′dimethoxytriphenylmethyl)-2′-*O*-(cyanoethyl)-N^6-dimethylformamidine-adenosine (24). Sym. Collidine (0.31 ml, 2.36 mmol) and 1-methylimidazole (9.9 μl, 0.123 mmol) were added dropwise to a solution of **23** (0.16 g, 0.24 mmol) in 8 ml acetonitrile. After the mixture was cooled to 0 °C, 2-cyanoethyldiisopropylchloro-phosphoramidite (0.08 ml, 0.35 mmol) was added dropwise. The mixture was stirred for 30 min at 0 °C, quenched with 1.5 ml saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic extracts were washed with 0.01 M citric acid, dried over $MgSO_4$ and concentrated under vacuum. The resulting oil was purified by chromatography with 5% MeOH in CH_2Cl_2 as the eluent to produce 0.14 g (69%) **24** as a white foam. ^{31}P NMR(400 MHz, $CDCl_3$) δ [ppm] 150.10, 149.89 (ratio 1:1,5) MALDI-MS: Calcd $C_{46}H_{56}N_9O_7P$: 877.43. Found: 878.43 $[M+H]^+$.

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