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Synthesis of 2′-*O*-guanidinopropyl-modified nucleoside phosphoramidites and their incorporation into siRNAs targeting hepatitis B virus

Jolanta Brzezinska [a], Jennifer D'Onofrio [a], Maximilian C.R. Buff [a], Justin Hean [b], Abdullah Ely [b], Musa Marimani [b], Patrick Arbuthnot [b], Joachim W. Engels [a,*]

[a] Goethe-University, Institute of Organic Chemistry & Chemical Biology, Max-von-Laue-Str. 7, 60438 Frankfurt am Main, Germany
[b] Antiviral Gene Therapy Research Unit, School of Pathology, Health Sciences Faculty, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, South Africa

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ABSTRACT

Synthetic RNAi activators have shown considerable potential for therapeutic application to silencing of pathology-causing genes. Typically these exogenous RNAi activators comprise duplex RNA of approximately 21 bp with 2 nt overhangs at the 3′ ends. To improve efficacy of siRNAs, chemical modification at the 2′-OH group of ribose has been employed. Enhanced stability, gene silencing and attenuated immunostimulation have been demonstrated using this approach. Although promising, efficient and controlled delivery of highly negatively charged nucleic acid gene silencers remains problematic. To assess the potential utility of introducing positively charged groups at the 2′ position, our investigations aimed at assessing efficacy of novel siRNAs containing 2′-*O*-guanidinopropyl (GP) moieties. We describe the formation of all four GP-modified nucleosides using the synthesis sequence of Michael addition with acrylonitrile followed by Raney-Ni reduction and guanidinylation. These precursors were used successfully to generate antihepatitis B virus (HBV) siRNAs. Testing in a cell culture model of viral replication demonstrated that the GP modifications improved silencing. Moreover, thermodynamic stability was not affected by the GP moieties and their introduction into each position of the seed region of the siRNA guide strand did not alter the silencing efficacy of the intended HBV target. These results demonstrate that modification of siRNAs with GP groups confers properties that may be useful for advancing therapeutic application of synthetic RNAi activators.

1. Introduction

Use of synthetic small interfering RNAs (siRNAs) to trigger RNA interference- (RNAi-) mediated gene silencing has shown considerable potential for therapeutic application.[1–3] Typically, siRNAs are synthetic mimics of natural Dicer products and comprise 21–25 nt duplexes with 2 nt 3′ overhangs. Progress with use of synthetic siRNAs has profited from vast experience gained from developing antisense RNA molecules. Consequently advances have been rapid and improving siRNA efficacy has benefited from valuable biological and synthetic chemistry insights. Advantages of synthetic siRNAs over expressed RNAi activators are that they are amenable to chemical modification to improve stability, safety and specificity.[4,5] Also, controlled large scale preparation necessary for clinical use is feasible with chemical synthetic procedures. Nevertheless, despite significant advances, the delivery of these polyanionic nucleic acids across lipid-rich cell membranes remains problematic. Vectors used to transport synthetic RNAi activators to target cells have included cationic lipid-containing lipoplexes,[6] conjugations to peptides[7] or oligocationic compounds such as spermidine.[8] However, success using these methods has been variable. To overcome difficulties of the excessive negative charge of nucleic acids, while at the same time improving thermal and serum stability, we previously investigated an approach that entailed 2′-modification of ribose with cationic groups.[9,10] Initially we generated always 2′-*O*-aminoethyl-adenosine and 2′-*O*-aminoethyl uridine. Synthesis entailed initial alkylation by methyl bromoacetate, which was followed by a series of transformation reactions. Using a luciferase reporter assay to measure knockdown, it was demonstrated that the 2′-*O*-aminoethyl modifications were at least as efficient as 2′-OMe siRNA modifications. An important property of the 2′-*O*-aminoethyl derivatives was their ability to rescue less active siRNAs when the chemical modifications were placed at the 3′ end of the siRNA passenger strand.[11] Subsequently this approach was advanced by developing methods that enabled successful alkylation of all four ribonucleosides.[12] This was achieved using phalimidoethyltriflate as an alkylating agent and with this methodology all four phosphoramidites bearing 2′-*O*-aminoethyl side chains were formed. Although encouraging, a problem of using these siRNA reagents is that the yields of the multistep chemical synthesis are typically low. Moreover scaling up the synthesis reaction is difficult. To address these

* Corresponding author. Tel.: +49 69 798 29150; fax: +49 69 798 29148.
E-mail address: Joachim.Engels@chemie.uni-frankfurt.de (J.W. Engels).

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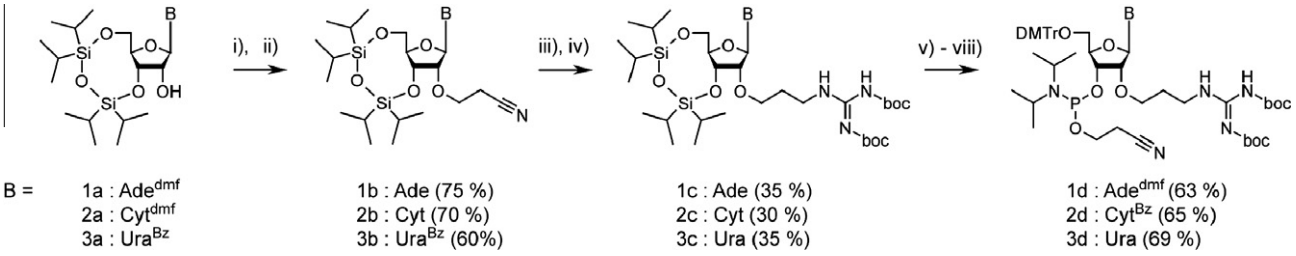
concerns, we have investigated utility of an alternative 2′-*O*-guanidinopropyl (GP) nucleoside modification method. Using the novel approach reported here, we describe the formation of all four GP-modified nucleosides using the synthesis sequence of Michael addition with acrylonitrile[13–15] followed by Raney-Ni reduction[16] and guanidinylation. Efficiency of the GP siRNAs was assessed in a cell culture model of hepatitis B virus (HBV) replication using target sequences that have previously been shown to be suitable for RNAi-based inhibition of viral replication.[17–20] Results demonstrate more effective silencing of markers of viral replication than unmodified counterparts. Moreover, the GP-modified siRNAs were more stable to serum conditions than the unmodified controls.

2. Results

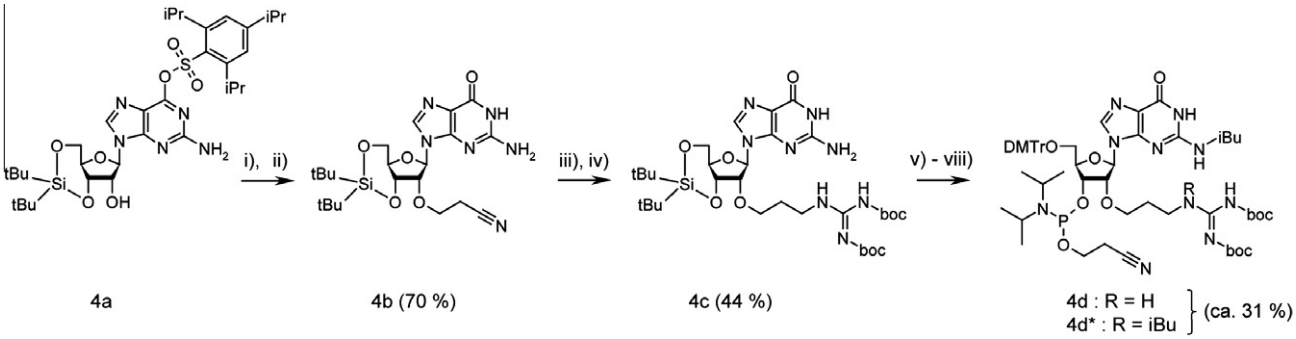
2.1. Synthesis of the four 2′-*O*-guanidinopropyl-nucleoside-phosphoramidites

Each of the four 2′-*O*-guanidinopropyl-nucleoside phosphoramidites was synthesised using essentially analogous methodology. The synthesis of the adenosine (A), cytidine (C) and uridine (U) derivatives is depicted in Scheme 1. Since a different protecting group strategy was employed to synthesise the guanosine (G) derivative, it is shown in a separate scheme (Scheme 2). Each synthesis was initiated by simultaneous protection of 5′- and the 3′-OH-groups with 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl (TIPS) (for A,C and U) or di-*tert*-butylsilanediyl (DTBS) for guanosine. DTBS was selected for protection of G as this group has been reported to improve selectivity for the subsequent 2,4,6-triisopropylbenzenesulfonyl (TPS) protection of O^6-position of guanosine.[21] The exocyclic amino functions of A and C were protected with dimethylaminomethylene groups employing standard conditions and a benzoyl group was attached to N^3-position of U using the two phase system reported by Sekine.[22] The resulting nucleotide precursors (**1a**–**4a**) were then subjected to the first crucial step of the 2′-*O*-guanidinopropyl derivatisation. Employing the procedure reported by Sekine et al.,[23] a Michael addition under mild conditions (Cs_2CO_3, *tert*-butanol, room temperature) was performed using acrylonitrile to obtain the 2′-*O*-cyanoethyl derivatives. In a subsequent step the dimethylaminomethylene group of the A and C derivatives was removed with hydrazine to form the 2′-*O*-cyanoethyl derivatives **1b** and **2b**. This additional deprotection step was necessary to avoid formation of a mixture of dimethylaminomethylene protected and unprotected derivatives that result from proceeding directly to the next reduction step. For the uridine derivative **3b** no intermediate deprotection of the N^3-benzoyl group was necessary. This is because the benzoyl group was completely removed under the ammonia conditions of the following step. The O^6-TPS group of the guanosine derivative was removed without further purification of the Michael reaction product. This was achieved after filtration and evaporation of solvents using formic acid in a mixture of dioxane and water to yield the 2′-*O*-cyanoethyl-guanosine derivative **4b**.

In the next step, the 2′-*O*-cyanoethyl group was transformed into a 2′-*O*-aminopropyl group. Reduction with hydrogen (30 bar) with Raney-nickel as catalyst in ammonia and methanol was used to achieve this according to a procedure we previously described.[24] The hydrogenation step was sensitive to reaction conditions such as the ratio of amount of starting material to catalyst, the size of the autoclave employed and reaction time. Under optimised conditions, yields from reduction of each nucleotide derivative were moderate (about 50%). A loss of the desired product was also



Scheme 1. Synthesis of the 2′-*O*-guanidinopropyl adenosine-, cytidine- and uridine- phosphoramidites for oligoribonucleotide synthesis. (i) acrylonitrile, Cs_2CO_3, *tert*-butyl alcohol, rt; (ii) $H_2N–NH_2·H_2O$, methanol, rt (adenosine and cytidine derivative); no deprotection of the uridine derivative; (iii) H_2 (30 bar), NH_3, methanol, 30–60 min, rt; (iv) *N*,*N*′-di-Boc-*N*″-triflylguanidine, Et_3N, CH_2Cl_2, 0 °C (30 min) to rt (30 min); (v) DMF-dimethyl diacetale, methanol, rt (adenosine derivative); benzoyl chloride, pyridine, 0 °C (30 min) to rt (30 min) (cytidine derivative); no protection group was applied to the uridine derivative; (vi) $Et_3N·3HF$, THF, rt; (vii) 4,4′-dimethoxytrityl chloride, pyridine, rt; (viii) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropyl phosphane, 4,5-dicyanoimidazole, CH_2Cl_2, rt.



Scheme 2. Synthesis of the 2′-*O*-guanidinopropyl guanosine phosphoramidite for oligoribonucleotide synthesis. (i) Acrylonitrile, Cs_2CO_3, *tert*-butyl alcohol, rt; (ii) formic acid (70%), dioxane/water; (iii) H_2 (30 bar), NH_3, methanol, 30–60 min, rt; (iv) *N*,*N*′-di-Boc-*N*″-triflylguanidine, Et_3N, CH_2Cl_2, 0 °C (30 min) to rt (30 min); (v) isobutyryl chloride, pyridine, 0 °C (1 h) to rt (1 h); (vi) $Et_3N·3HF$, THF, rt; (vii) 4,4′-dimethoxytrityl chloride, pyridine, rt; (viii) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropyl phosphane, 4,5-dicyanoimidazole, CH_2Cl_2, rt.

Table 1
Synthesised GP modified oligonucleotides

Name	Sequence
GP2 siRNA3	5-UU$_{GP}$ G AAG UAU GCC UCA AGG UCG-3′
GP3 siRNA3	5-UUG$_{GP}$ AAG UAU GCC UCA AGG UCG-3′
GP4 siRNA3	5-UUG A$_{GP}$ AG UAU GCC UCA AGG UCG-3′
GP5 siRNA3	5-UUG AA$_{GP}$ G UAU GCC UCA AGG UCG-3′
GP6 siRNA3	5-UUG AAG$_{GP}$ UAU GCC UCA AGG UCG-3′
GP7 siRNA3	5-UUG AAG U$_{GP}$ AU GCC UCA AGG UCG-3′
GP8 siRNA3	5-UUG AAG UA$_{GP}$ U GCC UCA AGG UCG-3′
GP13 siRNA3	5-UUG AAG UAU GCC U$_{GP}$ CA AGG UCG-3′

Table 2
Effect of GP modification on duplex stability with complementary RNA (5′-GGCAU-ACUUCAA-3′)

Oligo	Sequence	T_m [°C]	ΔT_m [°C]
ON 1	5′-UUG AAG UAU GCC-3′	54.9	–
ON 2	5′-UUG$_{GP}$ AAG UAU GCC-3′	54.5	−0.4
ON 3	5′-UUG AAG$_{GP}$ UAU GCC-3′	52.5	−2.4
ON 4	5′-UUG AAG UAU$_{GP}$ GCC-3′	54.4	−0.5
ON 5	5′-UUG$_{GP}$ AAG$_{GP}$ UAU GCC-3′	54.6	−0.3
ON 6	5′-UUG$_{GP}$ AAG$_{GP}$ UAU$_{GP}$ GCC-3′	54.4	−0.5

confirmed by the observation that part of the amino compound was not released from the catalyst during filtration, despite being subjected to several washes with methanol. To minimise losses the crude unpurified 2′-*O*-aminopropyl compounds were used to introduce the guanidino groups. *N*,*N*′-di-Boc-*N*″-triflylguanidine was employed as guanidinylation agent. The procedure we employed was initially reported by Goodman et al. in 1998 and the reagent is now commercially available.[25] Our previous studies showed that the boc groups are cleaved under the repetitive deprotection conditions during oligonucleotide synthesis when employing the TBDMS-phosphoramidite method. Also, the guanidino group undergoes no side reaction during the solid phase synthesis.[26] The guanidinylation took place with good yields (70% for **1c** (A), 60% for **2c** (C), around 60% for **3c** (U) and approximately 90% for **4c** (G)).

After successful guanidinylation, established reaction conditions were applied to synthesize the desired phosphoramidites (**1d**– **4d**). This entailed use of protection groups that were suitable for the TBDMS method of oligoribonucleotide synthesis. The A derivative was protected with dimethylaminomethylene at the N^6-position, and the exocyclic amino function of the C derivative was protected with a benzoyl group. The N^2-position of the G derivative was protected with an isobutyryl group. However, under the reaction conditions we employed, a mixture of the desired G derivative product, as well as a compound with an additional isobutyryl group on the non-boc-protected nitrogen of the guanidino group, were obtained. It was very difficult to separate this double isobutyryl modified compound using chromatography. However, since it would be cleaved during the ammonia deprotection step at the completion of oligonucleotide synthesis, we utilised this mixture of **4d** and **4d*** for solid phase oligonucleotide synthesis. To synthesise U derivatives, no further protection was necessary. For synthesis of all of the 2′-*O*-guanidinopropyl phosphoramidites, removal of silyl protecting groups was achieved with $Et_3N{\cdot}3HF$. The 5′-OH-group was protected with a 4,4′-dimethoxytrityl group and in a last step the 3′-OH group was converted to a phosphoramidite using 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylamino phosphane and 4,5-dicyanoimidazole as activator. Starting with the adenosine, cytidine and guanosine nucleosides, synthesis of the 2′-*O*-guanidinopropyl phosphoramidites took place in 10 steps and provided overall yields of 15.4% (**1d**), 6.3% (**2d**) and 7.5% (**4d**). Synthesis of the 2′-*O*-guanidinopropyl uridine phosphoramidite was performed in eight steps with an overall yield of 11.8% (**3d**).

The obtained phosphoramidites were used for synthesis of the GP-modified siRNA antisense strands depicted in Table 1 and the GP-modified oligonucleotides for melting point studies depicted in Table 2. For the modified phosphoramidites a prolonged coupling step of 25 min was used. To ensure complete cleavage of the boc groups, an additional deprotection step of 30 min with 3% trichloroacetic acid in dichloromethane was applied after completion of oligonucleotide synthesis. The boc group was chosen for protection of the guanidine moiety due to its compatibility concerning the protecting group strategy that was used for synthesis of the monomers. Although partial cleavage of the boc groups during RNA-synthesis is expected in consequence of the acidic deprotection step of each cycle, no side reaction of the free guanidino groups was observed. According to the anion-exchange chromatograms of the deprotected RNA (an exemplary chromatogram is shown in the Supplementary data), only one main product has formed. After purification the oligonucleotides were obtained with 10% to 40% yield (1 μmol synthesis scale).

2.2. Hybridization studies

The influence of GP-modified nucleosides on thermal stability of different RNA duplexes was examined. For this purpose, the G_{GP} and U_{GP} modified phosphoramidites were inserted into 12mer RNA (ON2–ON6) and the duplex melting point measured. As shown in Table 2, the presence of GP group in oligoribonucleotides did not significantly affect the stability of duplexes, although slightly destabilized them. Guanidinopropyl modified building blocks give almost the same Tm value for single, double and triple substituted oligonucleotides.

Interestingly, in the case when a GP modification of G was placed in a central position, the T_m decreased more significantly ($\Delta T_m = -2.4$ °C).

The results indicate that the thermodynamic effect of GP group is more or less independent on the placement of the modification and modified nucleoside. While more modifications were incorporated, substitutions were not adjacent, additional destabilizing effect was not observed (ON5 and ON6, Table 2). Moreover, for the modified oligonucleotides bearing more than one GP residue, high binding affinity to the complementary strand was unaffected.

2.3. Inhibition of Firefly luciferase activity in transfected cells

Initially, to measure knockdown efficiency of GP-modified siRNAs in situ, HEK293 cells were co-transfected with RNAi activators together with a reporter gene plasmid (psiCHECK-*HBx*)[20] (Fig. 1). The siRNAs targeted a single sequence of the X open reading frame (ORF) of HBV (*HBx*) that has previously been shown to be an effective cognate for RNAi-based silencing.[27] Each of the siRNAs differed with respect to location of the GP modification, and these were within the seed region or at nucleotide 13 of the antisense strand of the siRNA duplex. siRNAs have been named according to the positioning of the GP modifications from the 5′ end of the intended guide strand. In psiCHECK-*HBx*, the viral target sequence was located in the *Renilla* transcript but downstream of the reporter ORF (Fig. 1A). Expression of Firefly luciferase is constitutively active to enable correction for variations in transfection efficiency. The ratio of *Renilla* to Firefly luciferase activity was used to assess knockdown efficacy. Compared to a scrambled siRNA control, analysis showed that the Firefly luciferase activity was diminished by approximately 70% when co-transfected with the unmodified siRNA (Fig. 1B). There was some variation in the efficacy of the inhibition of reporter gene activity that was dependent on the position of the chemically modified siRNAs. Knockdown efficacy was weakest

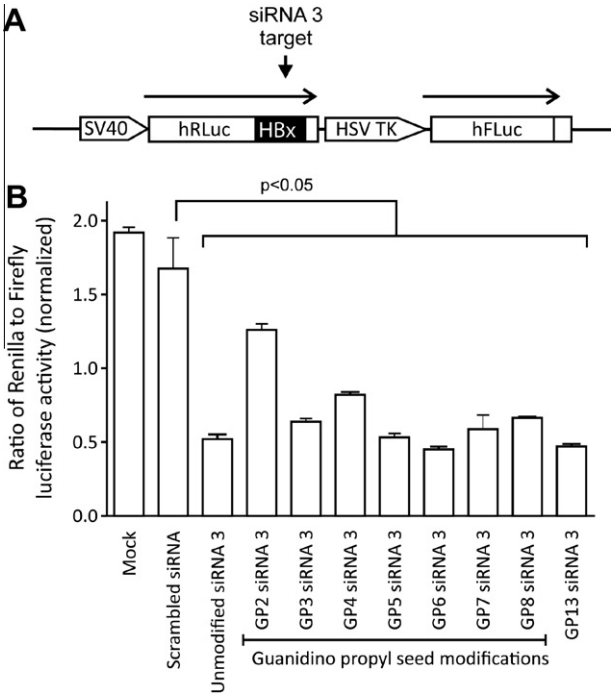


Figure 1. Dual luciferase assay to determine efficacy of GP antiHBV siRNAs. (A) Schematic illustration of dual luciferase reporter plasmid. The HBx target sequence was inserted downstream of the hRLuc ORF. *Renilla* luciferase activity was used as an indicator of target silencing and efficacy was determined relative to activity of constitutively expressed Firefly luciferase. (B) Ratio of *Renilla* to Firefly luciferase activity following cotransfection with indicated siRNAs together with dual luciferase reporter plasmid. Controls included a mock transfection in which inert plasmid DNA was substituted for siRNA as well as a scrambled siRNA that did not have complementary sequences to the HBx target. Data are represented as mean ratios of *Renilla* to Firefly luciferase activity (±SEM) and are normalised relative to the mock treated cells. Differences were considered statistical significant when the *p* value, determined according to the Student's 2 tailed paired *t*-test, was less than 0.05.

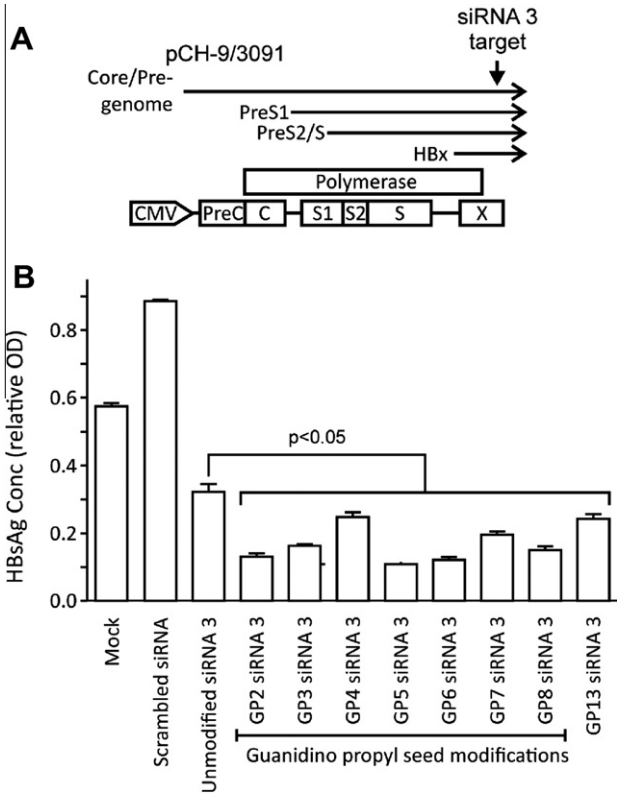


Figure 2. Inhibition of HBV replication by antiHBV siRNAs in cultured cells. A. Illustration of the HBV replication competent plasmid, pCH-9/3091, which was used to transfect liver-derived Huh7 cells in culture. (B) The concentration of HBsAg was measured in cell culture supernatants following co-transfection GP-modified siRNAs. Values are given as relative OD readings from the ELISA assay. Unmodified siRNA did not include GP residues. Controls included a mock transfection in which inert plasmid DNA was substituted for siRNA as well as a scrambled siRNA that did not have complementary sequences to the *HBx* target. Data are represented as mean ratios of *Renilla* to Firefly luciferase activity (±SEM) and are normalised relative to the mock treated cells. Differences were considered statistical significant when the *p* value, determined according to the Student's 2 tailed paired *t*-test, was less than 0.05.

with GP2 siRNA3, when the GP modification was placed at nucleotide 2 of the siRNA antisense sequence. siRNAs with the modification at positions 5 and 6 (GP5 siRNA3 and GP6 siRNA3) achieved most effective knockdown of reporter gene expression which was similar to that of the unmodified siRNA. A siRNA with the GP modification placed outside of the seed region at nucleotide 13 also achieved knockdown of 75%. GP modifications in antiHBV siRNA sequences are therefore compatible with effective target silencing, but position within the seed of the antisense guide influences efficacy.

2.4. Inhibition of HBV surface antigen (HBsAg) secretion from transfected cells by GP-modified siRNAs

To assess efficacy against HBV replication in vitro, Huh7 liver-derived cells were co-transfected with siRNAs together with the pCH-9/3091 HBV replication competent target plasmid (Fig. 2A).[28] Compared to HBsAg concentration in the culture supernatant of cells treated with scrambled siRNA, knockdown of up to 85% of viral antigen secretion was achieved by GP-modified siRNAs (Fig. 2B). The unmodified siRNA was slightly less effective than the siRNAs containing GP moieties. Of the modified siRNAs, positioning of the GP residue at nucleotides 5 or 6 (GP5 siRNA3 and GP6 siRNA3) resulted in the most effective suppression of HBsAg secretion (approximately 90%). These data correlate with observations using the reporter gene knockdown assay. Interestingly, GP2 siRNA3 inhibited HBsAg secretion from transfected cells more effectively than it did *Renilla* luciferase activity. The reason for this difference is unclear but may result from better GP2 siRNA3 target accessibility in the context of the natural HBV transcripts. Overall, these data support the notion that seed region GP modifications are compatible with target silencing that is similar or more effective than unmodified siRNAs.

2.5. Stability of GP-modified siRNAs in 80% FCS

siRNAs containing GP modifications were incubated in the presence or absence of 80% foetal calf serum (FCS) for time intervals of 0–24 h to assess their stability (Fig. 3). siRNAs were detected using polyacrylamide gel electrophoresis and staining with ethidium bromide. Bands corresponding to siRNAs were quantified to determine stability and FCS resistance. Analysis revealed that unmodified siRNA3 was stable for 24 h when maintained in DMEM tissue culture medium that did not include FCS. However, rapid degradation of siRNA occurred in the presence of FCS, and approximately 10% of the input siRNA remained at 5 h. Analysis of stability of GP2 siRNA3, GP3 siRNA3, GP4 siRNA3, GP5 siRNA3 and GP6 siRNA3 showed a similar rapid degradation. When the GP modifications were placed further from the 5′ end of the antisense strand of the siRNA (GP7 siRNA3, GP8 siRNA3 and GP13 siRNA3) slower degradation of the siRNAs was observed. With these siRNAs, at the time point of 5 h approximately 40% of the input siRNA remained intact. Stability is therefore improved by including GP

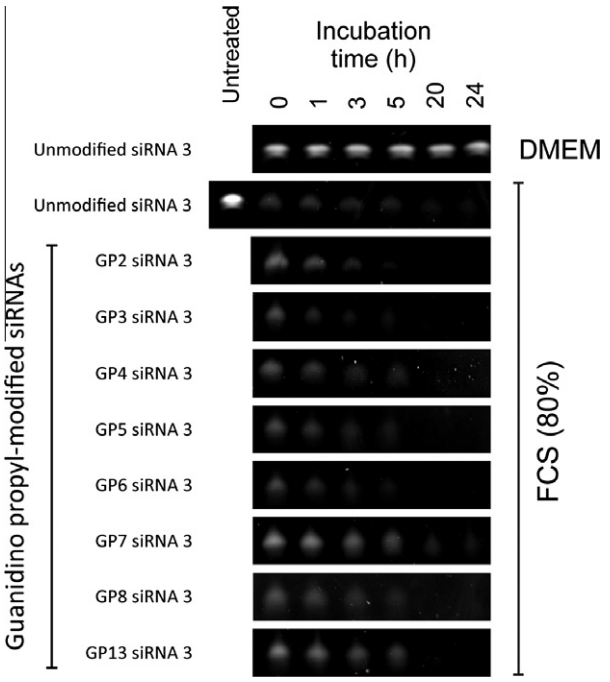


Figure 3. Assessment of stability of GP-modified siRNAs. The panel of GP-modified siRNAs was incubated with DMEM alone, or DMEM with 80% foetal calf serum, for times ranging from 0 to 24 h. Thereafter degradation of siRNAs was assessed using polyacrylamide gel electrophoresis with ethidium bromide staining.

modifications, but location of these moieties to central regions of the siRNAs is important to confer this property.

3. Discussion

Successful preparation of GP phosphoramidite nucleosides for incorporation into siRNAs presents a novel approach to utilising chemical modification for enhancement of efficacy of synthetic siRNAs. Ideally, for gene silencing sequences to be therapeutically applicable they should be stable, amenable to efficient delivery to target tissues, effective and specific to their targets. Alterations to siRNAs have typically involved 2′-OH modification of the ribose groups ([11] and reviewed in Refs. 4,5). Changes at this site are useful for improving overall siRNA efficacy for several reasons. The 2′-OH may cause nucleophilic attack and removal of the hydroxyl group limits susceptibility of siRNAs to degradation. Alterations at the 2′-OH of ribose may also attenuate toxic innate immune responses of duplex RNA by preventing interaction with intracellular Toll Like Receptors (TLRs). Furthermore, 2′-OH modification may influence nucleotide stacking to enhance interaction of the siRNA guide with its target. Alterations to the 2′-OH that have been reported to date have included 2′-F, 2′-methoxy ribosyl (2′-OMe) and locked nucleic acid modifications.[5,11] Recently a 2′-azido modification was reported, that not only replaces the 2′-OH group but can also be used for postsynthetic labelling of RNA.[29] Although improvements in siRNA stability, efficacy and immunostimulatory effects have been reported, difficulties with efficient regulation of nucleic acid delivery remain. The GP modification reported here presents a novel approach that has distinct properties that potentially have advantages over previously described 2′-OH modifications. The three amino groups of the GP moiety together with the three carbon atom propyl linker provide a flexible charge-neutralising group.

Chemical synthesis of GP-modified nucleosides is straightforward and comprises the following crucial steps. Cyanoethylation based on Michael addition of suitably protected nucleosides with acrylonitrile. The following Raney-Ni reduction results in the propylamino derivatives that are finally guanidinylated by triflylguanidine. Different chemical structures of each of the bases require that the protecting group strategies differ slightly between the four nucleosides. Standard phosphitylation provides the appropriate phosphoramidites **1d–4d**, which are ready for solid phase oligonucleotide synthesis (see Tables 1 and 2). Using this reaction sequence, this is the first time that a full set of phosphoramidites modified with guanidino groups at the 2′ position is provided for incorporation in synthetic oligonucleotides. Synthesis of modified oligonucleotides was carried out under standard conditions of phosphoramidite chemistry. Moreover coupling efficiency of the GP-modified phosphoramidites was as good as for the unmodified phosphoramidites.

An additional advantage of the synthetic procedures described here is that it is possible to introduce diversification at the 2′-*O*-aminopropyl site of our compounds. With common peptide-coupling reagents, such as carbodiimides and 1-hydroxybenzotriazoles, the 2′-*O*-aminopropyl group can readily be modified with carboxylic acid derivatives. These include amino acids, fatty acids or carboxy-modified spermine to obtain more cationic or more lipophilic oligonucleotides.[24] Also, protection of the amino group with a trifluoroacetyl group during oligonucleotide solid phase synthesis would enable postsynthetic labelling with amino-reactive fluorophore derivatives (e.g., NHS-esters or isothiocyanates) or reaction with cross linkers to connect nucleic acids to carrier molecules, for example, cell penetrating peptides.

The melting behaviour of the duplex oligonucleotides showed that the melting temperature was independent of the placement and number of GP modifications (Table 2). For this analysis, we chose a 12-mer with a sequence that was identical to the seed sequence of our siRNA. This shorter sequence, with a T_m of approximately 55 °C, was better for demonstrating smaller changes in melting properties. The results concur with hybridization analysis of oligonucleotides containing 2′-O-aminopropyl (AP) groups.[30] Incorporation of one AP unit at the 3′-end or in the middle of an oligomer reduces the Tm of a RNA duplex. Duplex stabilization was observed in RNA containing AP groups at adjacent sites or within an entire strand. Molecular dynamic and NMR data indicate that no strong electrostatic interaction or hydrogen bonding is formed as a result of flexibility of aminoalkyl chains.[30] Another possibility that has been proposed is that some stabilizing effect might be conferred by hydration of the AP.[31,32] For the guanidino group reported here, there are three nitrogen atoms in a plane which are protonated over a wide pH range. The individual contributions of GP-RNA to thermodynamic stability are not clear yet but nanosecond molecular modelling points to high flexibility in water. (Villa and Stock, unpublished)

When investigating the potential therapeutic utility of a new chemical modification to siRNAs, it is desirable to verify that silencing is maintained in a clinically relevant model. In this study, we assessed the efficacy in a model of HBV infection. Persistence of HBV is an important global cause of public health problems (reviewed in Ref. 33). Cirrhosis and liver cancer, which are potentially fatal complications of HBV persistence, occur frequently. There is a need to develop more effective antiHBV treatments to eliminate these problems and harnessing the RNAi pathway to counter the infection has shown promise.[34] To assess silencing by GP-modified siRNAs, a panel of silencing sequences was tested against a previously described susceptible HBV target.[27] Evidence from this study shows that incorporation of GP groups at the 2′ ribose position enhances HBV silencing, while also improving stability. Interaction of sequences within the guide seed region and the target are critically important to effect silencing. Chemical modification in the guide seed potentially compromises the interaction of the guide with

its cognate. Our data demonstrate that silencing efficacy of the intended target is retained after incorporating the GP residues into each of the nucleotides comprising the guide strand seed. This observation is in accordance with previous reports showing minimal attenuation of intended target silencing caused by modification in the seed region.[35,36] Complete base pair matching within the remainder of the siRNA guide and intended target compensates for disruption that may occur in the seed.

The novel 2′-GP modification that is described here potentially has several advantages for therapeutic application. Our data demonstrate that changes in single nucleotides achieve enhanced silencing efficacy and stability of siRNAs. Modification of more than one nucleotide in a siRNA is feasible and multiple PG moieties could further augment the efficacy of therapeutic sequences. Current investigations are addressing this as well as the role of GP modifications on off target seed interaction and improvement in in vivo delivery of GP-modified siRNA by non-viral vectors.

4. Experimental

4.1. Material and methods

All reagents were of analytical reagent grade, obtained from commercial resources and used without further purification. For synthesis, solvents with quality *pro analysis* were used. Dry solvents were kept over molecular sieve and for column chromatography technical solvents were distiled before use.

All NMR spectra were measured on *Bruker* AM250 (^{1}H: 250 MHz, ^{13}C: 63 MHz), AV300 (^{1}H: 300 MHz, ^{13}C: 75 MHz, ^{31}P: 121 MHz) and AV400 (^{1}H: 400 MHz, ^{13}C: 101 MHz, ^{31}P: 162 MHz) instruments. Chemical shifts (δ) are reported in parts per million (ppm). The following annotations were used with peak multiplicity: 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. High resolution mass spectra were acquired on a *Thermo* MALDI Orbitrap XL.

UV-melting curves were measured on a *JASCO* V-650 spectrophotometer. Melting profiles of the RNA duplexes were recorded in a phosphate buffer containing NaCl (100 mM, pH 7) at oligonucleotide concentrations 2 μM for each strand at wavelength 260 nm. Each melting curve was determined triply. The temperature range was 5–95 °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.

Statistical analysis: Data have been expressed as the mean ± standard error of the mean. Statistical difference was considered significant when $P < 0.05$ and was determined according to the student's *t*-test and calculated with the GraphPad Prism software package (GraphPad Software Inc., CA, USA).

4.2. Chemistry

4.2.1. Synthesis of the 2′-*O*-guanidinopropyl adenosine phosphoramidite

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-N^6-dimethylaminomethylene adenosine (**1a**) was synthesised as previously described.[16]

4.2.1.1. N^6 -Dimethylaminomethylene-2′-*O*-cyanoethyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-adenosine (1e). To a solution of compound **1a** (3.0 g, 5.31 mmol) in *tert*-butanol (25 mL), freshly distiled acrylonitrile (6.7 mL, 102 mmol) and cesium carbonate (1.6 g, 4.9 mmol) were added. The mixture was stirred vigorously at room temperature for 3 h. The reaction mixture was filtered and the residue was washed with dichloromethane. The filtrate was evaporated and the residue was purified using column chromatography with ethyl acetate/methanol (99:1–95:5, v/v) to give 3.28 g (87%) of the product. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 8.90 (s, 1H, admidine-*H*), 8.34 (s, 1H, *H*2 or *H*8), 8.32 (s, 1H, *H*2 or *H*8), 6.02–6.01 (m, 1H, *H*1′), 5.05–5.01 (m, 1H, *H*3′), 4.64–4.62 (m, 1H, *H*2′), 4.08–3.84 (m, 5H, *H*4′, 2 × *H*5′, O–CH_2–CH_2–CN), 3.20 (s, 3H, N–CH_3), 3.13 (s, 3H, N–CH_3), 2.83–2.80 (m, 2H, O–CH_2–CH_2–CN), 1.10–1.00 (m, 28H, tetraisopropyl-C*H* and -CH_3); MS (ESI) was calculated to be 618.3 for $C_{28}H_{48}N_7O_5Si_2$ (M+H$^+$), and found to be 618.8.

4.2.1.2. 2′-*O*-Cyanoethyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-adenosine (1b). N^6-Dimethylaminomethylene-2′-*O*-cyanoethyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-adenosine (**1e**) (1.0 g, 1.62 mmol) was dissolved in methanol (20 mL) then hydrazine hydrate (H_2N–NH_2·H_2O; 500 μL, 10.3 mmol) was added. The reaction solution was stirred at room temperature for 3 h. The solvents were evaporated and the residue was purified using a silica gel column with ethylacetate as eluent to give 773 mg (87%) of **1b**. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 8.21 (s, 1H, *H*2 or *H*8), 8.07 (s, 1H, *H*2 or *H*8), 7.33 (bs, 2H, NH_2), 5.98–5.96 (m, 1H, *H*1′), 5.03–4.99 (m, 1H, *H*3′), 4.59–4.57 (m, 1H, *H*2′), 4.08–3.83 (m, 5H, *H*4′, 2 × *H*5′, O–CH_2–CH_2–CN), 2.84–2.80 (m, 2H, O–CH_2–CH_2–CN), 1.09–0.97 (m, 28H, tetraisopropyl-C*H* and -CH_3); ^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] 156.01, 152.41 (*C*2 or *C*8), 148.46, 139.26 (*C*2 or *C*8), 119.20, 118.83, 87.47 (*C*1′), 81.11 (*C*2′), 80.45 (*C*4′), 70.04 (*C*3′), 65.62 (O–CH_2–CH_2–CN), 60.09 (*C*5′), 18.38 (O–CH_2–CH_2–CN), {17.20, 17.06, 17.05, 17.01, 16.98, 16.85, 16.81, 16.71} (tetraisopropyl-CH_3), {12.60, 12.28, 12.09, 12.04} (tetraisopropyl-*C*H); MS (MALDI) was calculated to be 563.8 for $C_{25}H_{43}N_6O_5Si_2$ (M+H$^+$) and found to be 564.0.

4.2.1.3. 2′-*O*-Aminopropyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-adenosine (1f). Compound **1b** (1.0 g, 1.78 mmol) was dissolved in 10 mL of methanol in a glass tube suitable for use in an autoclave. Approximately 0.5 mL of the Raney-nickel slurry was rinsed thoroughly with dry methanol and then washed into the glass tube with the solution of **1b**. After addition of 5 mL methanol saturated with ammonia, the mixture was stirred for 1 h at room temperature under a hydrogen atmosphere (30 bar). The reaction mixture was filtered and the catalyst was washed several times with methanol. The filtrate was evaporated and the residue was purified using column chromatography with ethyl acetate/methanol/triethylamine (70:25:5, v/v/v) to yield 503 mg (50%) of the desired compound. When this reaction was repeated, the crude product was used for the next step without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 8.20 (s, 1H, *H*2 or *H*8), 8.07 (s, 1H, *H*2 or *H*8), 7.32 (bs, 2H, NH_2), 5.95–5.94 (m, 1H, *H*1′), 4.95–4.90 (m, 1H, *H*3′), 4.41–4.39 (m, 1H, *H*2′), 4.08–3.90 (m, 3H, *H*4′, 2 × *H*5′), 3.86–3.70 (m, 2H, O–CH_2–CH_2–CH_2–NH_2), 2.66–2.61 (m, 2H, O–CH_2–CH_2–CH_2–NH_2), 1.65–1.58 (m, 2H, O–CH_2–CH_2–CH_2–NH_2), 1.08–0.96 (m, 28H, tetraisopropyl-C*H* and -CH_3); MS (MALDI) was calculated to be 567.9 for $C_{25}H_{47}N_6O_5Si_2$ (M+H$^+$), and found to be 567.9.

4.2.1.4. 2′-*O*-(*N*,*N*′-Di-boc-guanidinopropyl)-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-adenosine (1c). *N*,*N*′-Di-boc-*N*″-triflyl guanidine (280 mg, 0.72 mmol) was dissolved in 5 mL dichloromethane then triethylamine (100 μL) was added. After cooling to 0 °C, 2′-*O*-aminopropyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-adenosine (**1f**) (400 mg, 0.71 mmol) was added and the mixture was stirred for 1 h at 0 °C then for 1 h at room temperature. The reaction was diluted with dichloromethane and washed with saturated sodium bicarbonate solution and brine. The organic

layer was dried over Na_2SO_4 and the solvent was evaporated. The residue was purified using column chromatography with dichloromethane/methanol (98:2, v/v) to give a yield of 402 mg (70%) of **1c**. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 11.50 (s, 1H, N*H*-boc), 8.45–8.41 (m, 1H, N*H*–CH_2–), 8.17 (s, 1H, *H*2 or *H*8), 8.06 (s, 1H, *H*2 or *H*8), 7.31 (bs, 2H, NH_2), 6.02–5.99 (m, 1H, *H*1′), 4.96–4.91 (m, 1H, *H*3′), 4.43–4.40 (m, 1H, *H*2′), 4.06–3.70 (m, 5H, *H*4′, 2 × *H*5′, O–CH_2–CH_2–CH_2–NH–), 3.51–3.32 (m, 2H, O–CH_2–CH_2–CH_2–NH–), 1.84–1.78 (m, 2H, O–CH_2–CH_2–CH_2–NH–), 1.44 (s, 9H, C(CH_3)$_3$), 1.37 (s, 9H, C(CH_3)$_3$), 1.07–0.99 (m, 28H, tetraisopropyl-*CH* and -CH_3); ^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] 163.00, 156.00, 155.07, 152.40 (*C*2 or *C*8), 151.96, 148.48, 139.04 (*C*2 or *C*8), 119.21, 87.69 (*C*1′), 82.70, 81.26 (*C*2′), 80.41 (*C*4′), 77.87, 69.99 (*C*3′), 69.63 (O–CH_2–CH_2–CH_2–NH–), 60.13 (*C*5′), 38.41 (O–CH_2–CH_2–CH_2–NH–), 28.71 (O–CH_2–CH_2–CH_2–NH–), 27.85 (C(CH_3)$_3$), 27.44 (C(CH_3)$_3$), {17.19, 17.05, 17.03, 17.00, 16.95, 16.82, 16.74, 16.68} (tetraisopropyl-CH_3), {12.59, 12.28, 12.09, 12.01} (tetraisopropyl-*CH*); MS (MALDI) was calculated to be 810.1 for $C_{36}H_{65}N_8O_9Si_2$ (M+H$^+$), and found to be 808.3.

4.2.1.5. N^6 -Dimethylaminomethylene-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-adenosine (1g).

Compound **1c** (500 mg, 0.61 mmol) was dissolved in methanol (5 mL) and *N*,*N*-dimethylformamide dimethyl acetale (500 μL, 3.7 mmol) was added. The reaction was stirred at room temperature overnight and the solvents were evaporated. The crude product was used for further reactions without purification.

4.2.1.6. N^6 -Dimethylaminomethylene-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-adenosine (1h).

Compound **1g** (500 mg, 0.58 mmol) was dissolved in tetrahydrofurane (5 mL) and triethylammonium trihydrofluoride ($Et_3N{\cdot}3HF$; 330 μL, 2.0 mmol) was added. The mixture was stirred at room temperature for 1.5 h, then the solvent was evaporated. The residue was purified by column chromatography with ethyl acetate/methanol (98:2–9:1, v/v) giving 300 mg (83%) of the desired product. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 11.47 (s, 1H, N*H*-boc), 8.92 (s, 1H, N^6 = C*H*–NMe_2), 8.50 (s, 1H, *H*2 or *H*8), 8.41 (s, 1H, *H*2 or *H*8), 8.33–8.29 (m, 1H, N*H*–CH_2–), 6.11–6.09 (m, 1H, *H*1′), 5.28–5.24 (m, 1H, 5′-O*H*), 5.18–5.16 (m, 1H, 3′-O*H*), 4.46–4.43 (m, 1H, *H*2′), 4.36–4.32 (m, 1H, *H*3′), 4.01–3.98 (m, 1H, *H*4′), 3.72–3.46 (4H, 2 × *H*5′, O–CH_2–CH_2–CH_2–NH–), 3.33–3.28 (m, 2H, O–CH_2–CH_2–CH_2–NH–), 3.20 (s, 3H, N–CH_3), 3.13 (s, 3H, N–CH_3), 1.74–1.68 (m, 2H, O–CH_2–CH_2–CH_2–NH–), 1.45 (s, 9H, C(CH_3)$_3$), 1.37 (s, 9H, C(CH_3)$_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] 162.97, 159.22, 157.97 (N^6 = *C*H–NMe_2), 155.09, 151.89, 151.77 (*C*2 or *C*8), 151.00, 141.08 (*C*2 or *C*8), 125.70, 85.91 (*C*1′), 85.74 (*C*4′), 82.72, 81.02 (*C*2′), 77.99, 68.72 (*C*3′), 67.88 (O–CH_2–CH_2–CH_2–NH–), 61.04 (*C*5′), 40.56 (N–CH_3), 37.86 (O–CH_2–CH_2–CH_2–NH–), 34.45 (N–CH_3), 28.57 (O–CH_2–CH_2–CH_2–NH–), 27.87 (C(CH_3)$_3$), 27.51 (C(CH_3)$_3$); MS (MALDI) was calculated to be 622.7 for $C_{27}H_{44}N_9O_8$ (M+H$^+$), and found to be 624.6.

4.2.1.7. N^6 -Dimethylaminomethylene-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-5′-*O*-(4,4′-dimethoxytrityl)-adenosine (1i).

Compound **1h** (1.0 g, 1.6 mmol) was dissolved in dry pyridine (20 mL). 4,4′-Dimethoxytrityl chloride (660 mg, 1.95 mmol) was added and the reaction was stirred at room temperature overnight. The solution was diluted with dichloromethane and washed with saturated sodium bicarbonate solution. After evaporation of the solvents the residue was purified on a silica gel column with dichloromethane/methanol (98:2, v/v) containing 0.5% triethylamine, and 1.32 g (90%) of the tritylated compound was obtained. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 11.48 (s, 1H, N*H*-boc), 8.90 (s, 1H, N^6 = C*H*–NMe_2), 8.38–8.34 (m, 3H, *H*2, *H*3, N*H*–CH_2–), 7.37–7.34 (m, 2H, DMTr), 7.27–7.17 (m, 7H, DMTr), 6.84–6.79 (m, 4H, DMTr), 6.14–6.13 (m, 1H, *H*1′), 5.18–5.15 (m, 1H, 3′-O*H*), 4.57–4.54 (m, 1H, *H*2′), 4.47–4.42 (m, 1H, *H*3′), 4.14–4.08 (m, 1H, *H*4′), 3.72–3.71 (m, 6H, 2 × OCH_3), 3.70–3.56 (m, 2H, O–CH_2–CH_2–CH_2–NH–), 3.37–3.32 (m, 2H, O–CH_2–CH_2–CH_2–NH–), 3.24–3.21 (m, 2H, 2 × *H*5′), 3.19 (s, 3H, N–CH_3), 3.12 (s, 3H, N–CH_3), 1.77–1.70 (m, 2H, O–CH_2–CH_2–CH_2–NH–), 1.44 (s, 9H, C(CH_3)$_3$), 1.35 (s, 9H, C(CH_3)$_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] 162.98, 159.15, 157.97, 157.94, 157.91, 157.85 (N^6 = *C*H–NMe_2), 155.09, 151.88 (*C*2 or *C*8), 151.06, 144.73, 141.18 (*C*2 or *C*8), 135.44, 135.37, {129.60, 129.56, 127.64, 127.59, 126.53} (DMTr), 125.70, 112.99 (DMTr), 86.14 (*C*1′), 85.34, 82.97 (*C*4′), 82.70, 80.36 (*C*2′), 77.96, 69.08 (*C*3′), 68.21 (O–CH_2–CH_2–CH_2–NH–), 63.40 (*C*5′), 54.88 (OCH_3), 40.54 (N–CH_3), 37.97 (O–CH_2–CH_2–CH_2–NH–), 34.44 (N–CH_3), 28.56 (O–CH_2–CH_2–CH_2–NH–), 27.84 (C(CH_3)$_3$), 27.50 (C(CH_3)$_3$); MS (MALDI) was calculated to be 925.1 for $C_{48}H_{62}N_9O_{10}$ (M+H$^+$), and found to be 924.9.

4.2.1.8. N^6 -Dimethylaminomethylene-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-5′-*O*-(4,4′-dimethoxytrityl)-adenosine 3′-(cyanoethyl)-N,N-diisopropyl phosphoramidite (1d).

N^6-Dimethylaminomethylene-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-5′-*O*-(4,4′-dimethoxytrityl)-adenosine (**1i**) (320 mg, 346 μmol) was dissolved in dichloromethane (8 mL). 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylamino phosphane (132 μL, 415 μmol) and 4,5-dicyanoimidazole (47 mg, 398 μmol) were added. The mixture was stirred at room temperature. After 3 h, TLC revealed that some starting material did not react. An additional 0.6 equiv of the phosphitylating agent as well as the catalyst were therefore added. After 4 h the reaction was complete. The mixture was diluted with dichloromethane, washed with saturated sodium bicarbonate solution and the organic layer was dried over $MgSO_4$. The solvent was evaporated and the residue dissolved in a small amount of dichloromethane (ca. 5 mL). This solution was added dropwise into a flask with hexane (500 mL) to form a white precipitate. Two thirds of the solvent were evaporated and the remaining solvent was decanted from the solid. The precipitated product was redissolved in benzene and lyophilised to give 329 mg (84%) of **1d** as a white powder. ^{1}H NMR (300 MHz, acetone-d_6) δ [ppm] 11.65 (s, 1H, N*H*-boc) 8.95–8.93 (m, 1H, N^6 = C*H*–NMe_2), 8.42–8.27 (m, 3H, *H*2, *H*3, N*H*–CH_2–), 7.50–7.46 (m, 2H, DMTr), 7.38–7.17 (m, 7H, DMTr), 6.87–6.80 (m, 4H, DMTr), 6.28–6.26 (m, 1H, *H*1′), 4.96–4.79 (m, 2H, *H*2′, *H*3′) 4.45–4.37 (m, 1H, *H*4′), 4.05–3.35 (m, 16H), 3.25 (s, 3H, N–CH_3), 3.18 (s, 3H, N–CH_3), 2.85 (m, 1H, cyanoethyl), 2.64–2.60 (m, 1H, cyanoethyl), 1.90–1.82 (m, 2H, O–CH_2–CH_2–CH_2–NH–), 1.50–1.49 (m, 9H, C(CH_3)$_3$), 1.42–1.40 (m, 9H, C(CH_3)$_3$), 1.25–1.10 (m, 12H, *i*Pr-CH_3); ^{31}P NMR (121 MHz, acetone-d_6) δ [ppm] 149.6, 149.3; MS (ESI) was calculated to be 1125.3 for $C_{57}H_{79}N_{11}O_{11}P$ (M+H$^+$), and found to be 1125.7.

4.2.2. Synthesis of the 2′-*O*-guanidinopropyl cytidine phosphoramidite

N^4-Dimethylaminomethylene-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-cytidine (**2a**) was synthesised according to a previously described procedure.[37]

4.2.2.1. N^4 -Dimethylaminomethylene-2′-*O*-cyanoethyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-cytidine (2e).

Compound **2a** (4 g, 7.39 mmol) was dissolved in acrylonitrile (8 mL, 122 mmol) and *tert*-Butanol (35 mL). Cesium carbonate (1.8 g, 5.52 mmol) was added and the reaction was stirred for 2.5 h at room temperature. The mixture was filtered over celite, the solvents evaporated, and then the residue was purified using column chromatography. Ethyl acetate was initially used as solvent then changed to ethyl acetate/methanol (9:1, v/v) after the unpolar impurities had passed through the column. A yield of 3.78 g (86%) of the product were obtained. ^{1}H NMR (400 MHz,

DMSO-d_6) δ [ppm] 8.62 (s, 1H, N^4 = C*H*–NMe_2), 7.88 (d, 1H, *J* = 7.3 Hz, *H*6), 5.90 (d, 1H, *J* = 7.3 Hz, *H*5), 5.65 (s, 1H, *H*1′), 4.22–3.91 (m, 7H), 3.17 (s, 3H, N–C*H*$_3$), 3.04 (s, 3H, N–C*H*$_3$), 2.86–2.82 (m, 2H, O–CH_2–C*H*$_2$–CN), 1.07–0.96 (m, 28H, tetraisopropyl-C*H* and -C*H*$_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] 171.21 (*C*4), 157.77 (N^4 = *C*H–NMe_2), 154.57 (*C*2), 140.61 (*C*6), 118.86 (O–CH_2–CH_2–*C*N), 101.14 (*C*5), 88.99 (*C*1′), 81.42, 80.69, 67.83, 65.22 (O–*C*H_2–CH_2–CN), 59.39 (*C*5′), 40.79 (N–*C*H_3),34.71 (N–*C*H_3), 18.18 (O–CH_2–*C*H_2–CN), {17.22, 17.11, 17.04, 16.97, 16.84, 16.72, 16.69, 16.61} (tetraisopropyl-*C*H_3), {12.60, 12.20, 11.88} (tetraisopropyl-*C*H); MS (ESI) was calculated to be 594.9 for $C_{27}H_{48}N_5O_6Si_2$ ($M+H^+$) and found to be 594.9.

4.2.2.2. 2′-*O*-Cyanoethyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-cytidine (2b). N^4-Dimethylaminomethylene-2′-*O*-cyanoethyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-cytidine (**2e**) (1.0 g, 1.68 mmol) was dissolved in methanol (10 mL) and hydrazine hydrate (500 μL, 10.3 mmol) was added. The mixture was stirred for 1 h at room temperature and then the solvents were evaporated. The residue was purified on a silica gel column with ethyl acetate/methanol (95:5, v/v) to give 745 mg (82%) of **2b**. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 7.69 (d, 1H, *J* = 7.4 Hz, *H*6), 7.21 (s, 2H, N*H*$_2$), 5.69 (d, 1H, *J* = 7.4 Hz, *H*5), 5.61 (s, 1H, *H*1′); 4.19–3.90 (m, 7H), 2.90–2.76 (m, 2H, O–CH_2–C*H*$_2$–CN), 1.07–0.97 (m, 28 H, tetraisopropyl-C*H* and -C*H*$_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] 165.70, 154.60, 139.36 (*C*6), 118.89, 93.30 (*C*5), 88.66 (*C*1′), 81.55 (*C*2′), 80.49, 67.92, 65.19 (O–*C*H_2–CH_2–CN), 59.44 (*C*5′), 18.20 (O–CH_2–*C*H_2–CN), {17.23, 17.11, 17.05, 16.98, 16.85, 16.73, 16.72, 16.63} (tetraisopropyl-*C*H_3), {12.62, 12.28, 12.21, 11.88} (tetraisopropyl-*C*H); MS (ESI) was calculated to be 539.8 for $C_{24}H_{43}N_4O_6Si_2$ ($M+H^+$) and found to be 540.0.

4.2.2.3. 2′-*O*-Aminopropyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-cytidine (2f). Compound **2b** (500 mg, 928 μmol) was dissolved in 10 mL of methanol in a glass tube. Approximately 0.5 mL of the Raney-nickel sediment was washed thoroughly with dry methanol and was rinsed into the glass tube with the solution of **2b**. After addition of 5 mL methanol saturated with ammonia, the mixture was stirred for 1 h at room temperature under a hydrogen atmosphere (30 bar). The reaction mixture was filtered through celite and the catalyst was washed several times with methanol. The solvent was evaporated and the residue was purified on a silica gel column using ethyl acetate/methanol/triethylamine (60:35:5) to give 251 mg (50%) of the product. When this procedure was repeated, the crude material after filtration and evaporation was used in further reactions without purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 7.69 (d, 1H, *J* = 7.2 Hz, *H*6), 7.18 (bs, 2H, ar. N*H*$_2$), 5.68 (d, 1H, *J* = 7.5 Hz, *H*5), 5.60 (s, 1H, *H*1′), 4.18–3.76 (m, 7H), 2.70–2.66 (m, 2H, O–CH_2–CH_2–C*H*$_2$–NH_2), 1.68–1.61 (m, 2H, O–CH_2–C*H*$_2$–CH_2–NH_2), 1.07–0.95 (m, 28 H, tetraisopropyl-C*H* and -C*H*$_3$); MS (MALDI) was calculated to be 643.8 for $C_{24}H_{47}N_4O_6Si_2$ ($M+H^+$) and found to be 544.6.

4.2.2.4. 2′-*O*-(*N*,*N*′-Di-boc-guanidinopropyl)-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-cytidine (2c). *N*,*N*′-Di-boc-*N*″-triflyl guanidine (360 mg, 920 μmol) was dissolved in 5 mL dichloromethane and triethylamine (125 μL) then added. After cooling to 0 °C, compound **2f** (500 mg, 922 μmol) was added and the solution was stirred for 1 h at 0 °C and then 1 h at room temperature. The reaction was diluted with dichloromethane and washed with saturated sodium bicarbonate solution and brine. The combined organic layers were dried over Na_2SO_4 and after evaporating the solvent the residue was purified using column chromatography with dichloromethane/methanol (98:2–95:5, v/v) to give 434 mg (60%) of **2c**. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 11.48 (s, 1H, N*H*-boc), 8.38–8.35 (m, 1H, N*H*–CH_2–), 7.67 (d, 1H, *J* = 7.4 Hz, *H*6), 7.19 (bs, 2H, N*H*$_2$), 5.68 (d, 1H, *J* = 7.4 Hz, *H5*), 5.63 (s, 1H, *H*1′), 4.17–3.78 (m, 7H), 3.49–3.33 (m, 2H, O–CH_2–CH_2–C*H*$_2$–NH–), 1.84–1.77 (m, 2H, O–CH_2–C*H*$_2$–CH_2–NH–), 1.45 (m, 9H, C(C*H*$_3$)$_3$), 1.38 (m, 9H, C(C*H*$_3$)$_3$), 1.06–0.96 (m, 28 H, tetraisopropyl-C*H* and -C*H*$_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] 165.61, 162.99, 155.04, 154.52, 151.94, 139.45 (*C*6), 93.21 (*C*5), 88.97 (*C*1′), 82.66, 81.76 (*C*2′), 80.36 (*C*4′), 77.86, 69.11 (O–*C*H_2–CH_2–CH_2–NH–), 68.27 (*C*3′), 59.51 (*C*5′), 38.28 (O–CH_2–CH_2–*C*H_2–NH–), 28.61 (O–CH_2–*C*H_2–CH_2–NH–), 27.86 (C(*C*H_3)$_3$), 27.44 (C(*C*H_3)$_3$), {17.22, 17.10, 17.03, 16.96, 16.83, 16.70, 16.68, 16.60} (tetraisopropyl-*C*H_3), {12.59, 12.26, 12.21, 11.87} (tetraisopropyl-*C*H); MS (MALDI) was calculated to be 786.1 for $C_{35}H_{65}N_6O_{10}Si_2$ ($M+H^+$) and found to be 786.4.

4.2.2.5. N^4-Benzoyl-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-cytidine (2g). Compound **2c** (1.0 g, 1.27 mmol) was dissolved in dry pyridine (10 mL) and the solution was cooled in an ice bath. Benzoyl chloride (240 μL, 2.06 mmol) was added and the reaction solution was stirred at 0 °C for 1 h. The reaction was quenched with water and ammonia (25% in water; 3 mL) was added. The mixture was then stirred for 30 min at room temperature. The solvents were evaporated and the residue was dissolved in dichloromethane and washed with saturated sodium bicarbonate solution. The organic layer was dried over Na_2SO_4 and after evaporating the solvent, the residue was purified by column chromatography using dichloromethane/methanol (98:2, v/v) and 950 mg (84%) of the product were obtained. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 11.50 (s, 1H, N*H*), 11.31 (s, 1H, N*H*), 8.40–8.37 (m, 1H, N*H*–CH_2–), 8.15 (d, 1H, *J* = 7.3 Hz, *H*6), 8.03–7.99 (m, 2H, benzoyl), 7.65–7.60 (m, 1H, benzoyl), 7.53–7.49 (m, 2H, benzoyl), 7.38 (d, 1H, *J* = 7.3 Hz, *H*5), 5.73 (s, 1H, *H*1′), 4.24–4.13 (m, 3H, *H*3′, *H*4′, *H*5′), 4.02–4.03 (m, 1H, *H*2′), 3.97–3.92 (m, 1H, *H*5′), 3.87–3.83 (m, 2H, O–C*H*$_2$–CH_2–CH_2–NH–), 3.52–3.35 (m, 2H, O–CH_2–CH_2–C*H*$_2$–NH–), 1.87–1.80 (m, 2H, O–CH_2–C*H*$_2$–CH_2–NH–), 1.45 (m, 9H, C(C*H*$_3$)$_3$), 1.38 (m, 9H, C(C*H*$_3$)$_3$), 1.08–0.95 (m, 28H, tetraisopropyl-C*H* and -C*H*$_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] 167.21, 163.14, 163.00, 155.06, 154.01, 151.97, 143.37 (*C*6), 132.97, 132.64, 128.31, 95.61 (*C*5), 89.46 (*C*1′), 82.67, 81.30 (*C*2′), 80.86 (*C*4′), 77.86, 69.26 (O–*C*H_2–CH_2–CH_2–NH–), 67.95 (*C*3′), 59.38 (*C*5′), 38.28 (O–CH_2–CH_2–*C*H_2–NH–), 28.62 (O–CH_2–*C*H_2–CH_2–NH–), 27.86 (C(*C*H_3)$_3$), 27.43 (C(*C*H_3)$_3$), {17.22, 17.11, 17.04, 16.97, 16.89, 16.73, 16.71, 16.66} (tetraisopropyl-*C*H_3), {12.56, 12.29, 12.22, 11.86} (tetraisopropyl-*C*H); MS (ESI) was calculated to be 890.2 for $C_{42}H_{69}N_6O_{11}Si_2$ ($M+H^+$), and found to be 890.4.

4.2.2.6. N^4-Benzoyl-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-cytidine (2h). N^4-Benzoyl-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-cytidine (**2g**) (900 mg, 1.01 mmol) was dissolved in tetrahydrofurane (20 mL). Triethylamine trihydrofluoride ($Et_3N{\cdot}3HF$; 560 μL, 3.54 mmol) was added and the solution was stirred at room temperature for 2 h. The solvent was evaporated and the residue was purified using column chromatography with dichloromethane/methanol (98:2–97:3, v/v) to give 607 mg (93%) of the product as a pale yellow foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] 11.50 (s, 1H, N*H*), 11.28 (bs, 1H, N*H*), 8.57 (d, 1H, *J* = 7.5 Hz, *H*6), 8.40–8.35 (m, 1H, N*H*–CH_2–), 8.02–7.98 (m, 2H, benzoyl), 7.66–7.60 (m, 1H, benzoyl), 7.54–7.48 (m, 2H, benzoyl), 7.34 (d, 1H, *J* = 7.2 Hz, *H*5), 5.86–5.85 (m, 1H, *H*1′), 5.24 (t, 1H, *J* = 5.0 Hz, 5′-O*H*), 4.98 (d, 1H, *J* = 6.8 Hz, 3′-O*H*), 4.12–3.60 (m, 7H), 3.44–3.37 (m, 2H, O–CH_2–CH_2–C*H*$_2$–NH–), 1.85–1.76 (m, 2H, O–CH_2–C*H*$_2$–CH_2–NH–), 1.46 (m, 9H, C(C*H*$_3$)$_3$), 1.38 (m, 9H, C(C*H*$_3$)$_3$); ^{13}C NMR (75 MHz, acetone-d_6) δ [ppm] 169.22, 165.59, 164.82, 157.83, 156.15, 154.80, 147.10 (*C*6), 135.58, 134.60, 130.46, 130.05, 97.67 (*C*5), 91.39 (*C*1′), 86.19 (*C*4′), 84.69 (*C*(CH$_3$)$_3$), 84.62 (*C*2′), 79.88 (*C*(CH$_3$)$_3$), 70.71

(O–CH_2–CH_2–CH_2–NH–), 69.63 (*C*3′), 61.46 (*C*5′), 40.29 (O–CH_2–CH_2–CH_2–NH–), 30.86 (O–CH_2–CH_2–CH_2–NH–), 29.46 (C(CH_3)$_3$), 29.14 (C(CH_3)$_3$); HRMS (MALDI) was calculated to be 647.3035 for $C_{30}H_{43}N_6O_{10}$ (M+H$^+$), and found to be 647.3031.

4.2.2.7. N^4-Benzoyl-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-5′-*O*-(4,4′-dimethoxytrityl)-cytidine (2i). N^4-Benzoyl-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-cytidine (**2h**) (516 mg, 798 μmol) was dissolved in dry pyridine (20 mL) and the solution was cooled in an ice bath. 4,4′-Dimethoxytrityl chloride (515 mg, 1.52 mmol) was added and the mixture was stirred overnight while the bath came up to room temperature. The reaction was quenched with methanol (10 mL) and the solvents were evaporated. The residue was purified by column chromatography using dichloromethane/methanol (99:1–98:2, v/v). The column was packed with solvent containing 1% triethylamine to yield 715 mg (94%) of the product as a pale yellow foam. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 11.50 (s, 1H, N*H*), 11.29 (bs, 1H, N*H*), 8.43–8.37 (m, 2H, *H*6, N*H*–CH_2–), 8.02–7.99 (m, 2H, benzoyl), 7.65–7.60 (m, 1H, benzoyl), 7.54–7.50 (m, 2H, benzoyl), 7.43–7.25 (m, 9H, DMTr), 7.18–7.15 (m, 1H, *H*5), 6.94–6.91 (m, 4H, DMTr), 5.88 (s, 1H, *H*1′), 5.04 (d, 1H, *J* = 7.3 Hz, 3′-O*H*), 4.34–4.28 (m, 1H, *H*3′), 4.13–4.10 (m, 1H, *H*4′), 3.94–3.87 (m, 2H, *H*2′, 1 × O–CH_2–CH_2–CH_2–NH–), 3.76 (s, 6H, 2 × OCH_3), 3.76–3.70 (m, 1H, 1 × O–CH_2–CH_2–CH_2–NH–), 3.46–3.36 (m, 4H, 2 × *H*5′, O–CH_2–CH_2–CH_2–NH–), 1.86–1.80 (m, 2H, O–CH_2–CH_2–CH_2–NH–), 1.42 (m, 9H, C(CH_3)$_3$), 1.36 (m, 9H, C(CH_3)$_3$); ^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] 167.19, 163.02, 158.11, 158.08, 155.12, 154.07, 151.93, 144.24 (*C*6), 135.47, 135.11, 133.06, 132.62, 129.70, 129.55, 128.35, 127.85, 127.73, 126.78, 113.19, 95.93 (*C*5), 88.99 (*C*1′), 85.90, 82.67 (*C*(CH$_3$)$_3$), 81.93 (*C*2′), 81.44 (*C*4′), 77.94 (*C*(CH$_3$)$_3$), 68.44 (O–CH_2–CH_2–CH_2–NH–), 67.62 (*C*3′), 61.36 (*C*5′), 54.91 (OCH_3), 54.90 (OCH_3), 38.17 (O–CH_2–CH_2–*C*H_2–NH–), 28.59 (O–CH_2–*C*H_2–CH_2–NH–), 27.87 (C(*C*H_3)$_3$), 27.48 (C(*C*H_3)$_3$); HRMS (MALDI) was calculated to be 971.4161for $C_{51}H_{60}N_6O_{12}$Na (M+Na$^+$), and found to be 971.4181.

4.2.2.8. N^4-Benzoyl-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-5′-*O*-(4,4′-dimethoxytrityl)-cytidine 3′-(cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite (2d). Compound **2i** (683 mg, 720 μmol) was dissolved in dichloromethane (15 mL). 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylamino phosphane (274 μL, 864 μmol) and 4,5-dicyanoimidazole (98 mg, 828 μmol) were added. After stirring at room temperature for 5 h, TLC revealed that some starting material had not reacted. Therefore 10 mg of 4,5-dicyanoimidazole and 30 μL of the phosphitylation agent were added and the reaction was stirred at room temperature overnight. The solution was diluted with dichloromethane and washed with saturated sodium bicarbonate solution. After drying the organic layer over $MgSO_4$ the solvent was evaporated and the residue was dissolved in a small amount (5 mL) of dichloromethane. This solution was dripped into a flask with hexane (500 mL) to form a white precipitate. Two thirds of the solvent was evaporated and the residual solvent was decanted carefully. The precipitate was redissolved in benzene and lyophilised to give 738 mg (89%) of **2d**. According to ^{31}P NMR spectrum the product was still containing a small amount of the hydrolysed phosphitylation reagent but this did not interfere with the oligonucleotide synthesis. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 11.50–11.48 (m, 1H, N*H*), 11.25 (bs, 1H, N*H*), 8.52–8.45 (m, 1H, *H*6), 8.39–8.34 (m, 1H, N*H*–CH_2–), 8.01–7.98 (m, 2H, benzoyl), 7.66–7.61 (m, 1H, benzoyl), 7.53–7.49 (m, 2H, benzoyl), 7.45–7.25 (m, 9H, DMTr), 7.13–7.09 (m, 1H, *H*5), 6.93–6.89 (m, 4H, DMTr), 5.95–5.92 (m, 1H, *H*1′), 4.56–4.38 (m, 1H, *H*3′), 4.31–4.28 (m, 1H, *H*4′), 4.07–3.29 (m, 17H), 2.90–2.57 (m, 2H, cyanoethyl), 1.86–1.78 (m, 2H, O–CH_2–CH_2–CH_2–NH–), 1.40–1.35 (m, 18H, 2 × C(CH_3)$_3$), 1.20–0.93 (m, 12H, *i*Pr-CH_3); ^{31}P NMR (162 MHz, DMSO-d_6) δ [ppm] 148.4, 148.0 (The signal of the hydrolysed phosphitylation reagent appears at 13.9 ppm); HRMS (MALDI) was calculated to be 1149.5421 for $C_{60}H_{78}N_8O_{13}P$ (M+H$^+$), was found to be 1149.5447.

4.2.3. Synthesis of the 2′-*O*-guanidinopropyl uridine phosphoramidite

N^3-Benzoyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-uridine (**3a**) was synthesised according to a previously described procedure.[22]

4.2.3.1. N^3-Benzoyl-2′-*O*-cyanoethyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-uridine (3b). Compound **3a** (1.14 g, 1.93 mmol) was dissolved in 9.6 mL of *tert*-butanol. Freshly distiled acrylonitrile (2.5 mL, 38.6 mmol) was added. After addition of cesium carbonate (645 mg, 1.98 mmol) the reaction was stirred for 4 h at room temperature. The reaction solution was filtered over celite. The residue was washed with 100 mL of dichloromethane. The filtrate was evaporated in vacuum. Purification via column chromatography in dichloromethane/ethyl acetate (99:1–95:5, v/v) yielded 746 mg (60%) of the desired product as a white powder. ^{1}H NMR (250 MHz, acetone-d_6) δ [ppm] 8.03–7.99 (m, 3H, *H*6, benzoyl), 7.79–7.72 (m, 1H, benzoyl), 7.61–7.55 (m, 2H, benzoyl), 5.80–5.74 (m, 2H, *H*5, *H*1′), 4.50–3.94 (m, 7H), 2.80–2.75 (m, 2H, O–CH_2–CH_2–CN), 1.17–1.07 (m, 28H, tetraisopropyl-C*H* and -CH_3); ^{13}C NMR (63 MHz, acetone-d_6) δ [ppm] 171.11, 163.78, 151.06, 141.48, 136.94, 133.92, 132.20, 131.13, 119.80, 102.75, 91.47, 84.05, 83.69, 70.65, 68.08, 61.60, 20.35, 18.92, 18.91, 18.75, 18.73, 18.64, 18.50, 18.46, 18.40, 15.23, 14.79, 14.72, 14.43; HRMS (XXX) was calculated to be 666.2637 for $C_{31}H_{45}N_3O_8Si_2$Na (M+Na$^+$) and found to be 666.2647.

4.2.3.2. 2′-*O*-(Aminopropyl)-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-uridine (3e). Compound **3b** (500 mg, 0.78 mmol) was dissolved in 10 mL of methanol in a glass tube suitable for the applied autoclave. Approximately 0.5 mL of the Raney-nickel slurry was put on a glass filter, washed thoroughly with dry methanol and rinsed into the glass tube with the solution of **3b**. After addition of 5 mL methanol saturated with ammonia, the mixture was stirred for 1 h at room temperature in an autoclave under a hydrogen atmosphere (30 bar). The reaction solution was decanted from the catalyst into a glass filter. The catalyst was washed several times with methanol and the solvent was removed from the combined filtrates under reduced pressure. The product was purified on a silica gel column initially using dichloromethane/ethyl acetate (7:3–0:1, v/v) and thereafter ethyl acetate/methanol/triethylamine (6: 3.5: 0.5, v/v/v) to obtain 253 mg (60%) of a white powder. When we repeated the reduction we used the crude product after filtration and evaporation for further derivatisation. ^{1}H NMR (250 MHz, acetone-d_6) δ [ppm] 7.81 (d, 1H, *J* = 8.1 Hz, *H*6), 5.71 (s, 1H, *H*1′), 5.53 (d, 1H, *J* = 8.1 Hz, *H*5), 4.39–4.34 (m, 1H, *H*3′), 4.28–4.23 (m, 1H, *H*5′), 4.14–4.03 (m, 3H, *H*2′, *H*4′, *H*5′), 3.97–3.81 (m, 2H, O–CH_2–CH_2–CH_2–NH_2), 3.37–3.25 (m, 2H, O–CH_2–CH_2–CH_2–NH_2), 1.92–1.82 (m, 2H, O–CH_2–CH_2–CH_2–NH_2), 1.14–1.05 (m, 28H, tetraisopropyl-C*H* and -CH_3); ^{13}C NMR (63 MHz, acetone-d_6) δ [ppm] 167.18, 164.59, 151.93, 141.033, 102.82, 91.15, 83.77, 83.50, 70.88, 70.85, 61.78, 49.36, 33.03, 18.91, 18.90, 18.74, 18.61, 18.49, 18.47, 18.40, 15.19, 14.82, 14.71, 14.41; HRMS (MALDI) was calculated to be 544.2869 for $C_{24}H_{46}N_3O_7Si_2$ (M+H$^+$), and found to be 544.2880.

4.2.3.3. 2′-*O*-(*N*,*N*′-Di-boc-guanidinopropyl)-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-uridine (3c). *N*,*N*′-Di-boc-*N*″-triflyl guanidine (320 mg, 0.82 mmol) was dissolved in 3.6 mL dichloromethane and triethylamine (150 μL) was added. The solution was cooled in an ice bath and 2′-*O*-(aminopropyl)-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-uridine (**3e**) (490 mg, 0.9 mmol)

was added. After 15 min the reaction mixture was removed from the ice bath was and stirred for 2.5 h at room temperature. The reaction solution was washed with saturated sodium bicarbonate solution and brine. After drying over Na_2SO_4 the solvent was evaporated in vacuum. The crude product was purified using column chromatography with dichloromethane/methanol (96:4–94:6, v/v). 410 mg (58%) of compound **3c** were obtained. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 11.49 (s, 1H, N*H*), 11.37 (m, 1H, N$H_{uridine}$), 8.40–8.37 (m, 1H, N*H*–CH_2–), 7.64 (d, 1H, *J* = 7.9 Hz, *H*6), 5.64 (s, 1H, *H*1′), 5.53 (d, 1H, *J* = 7.9 Hz, *H*5), 4.25–4.22 (*H*3′), 4.13–4.09 (m, 1H, *H*5′), 4.06–4.05 (m, 1H, *H*2′), 4.03–4.00 (m, 1H, *H*4′), 3.93–3.89 (m, 1H, *H*5′), 3.84–3.70 (m, 2H, O–C*H*$_2$–CH_2–CH_2–NH–), 3.49–3.32 (m, 2H, O–CH_2–CH_2–C*H*$_2$–NH–), 1.83–1.77 (m, 2H, O–CH_2–C*H*$_2$–CH_2–NH–), 1.45 (s, 9H, C(C*H*$_3$)$_3$), 1.38 (s, 9H, C(C*H*$_3$)$_3$), 1.06–0.97 (m, 28H, tetraisopropyl-C*H* and -C*H*$_3$); HRMS (MALDI) was calculated to be 808.3955 for $C_{35}H_{63}N_5O_{11}Si_2Na$ (M+Na$^+$), and found to be 808.3991.

4.2.3.4. 2′-*O*-(*N*,*N*′-Di-boc-guanidinopropyl)-uridine (3f). To a solution of compound **3c** (910 mg, 1.16 mmol) and triethylamine (240 μL) in 13 mL tetrahydrofurane NEt_3·3HF (700 μL, 4.3 mmol) was added. The reaction mixture was stirred for 1 h at room temperature. The solvents were evaporated and the residue was purified on a silica gel column using dichloromethane/methanol (93:7–92:8, v/v) to give 629 mg (97%) of a white foam. ^{1}H NMR (250 MHz, acetone-d_6) δ [ppm] 11.67 (bs, 1H, N*H*), 10.03 (bs, 1H, N*H*), 8.46–8.41 (m, 1H, N*H*–CH_2–), 8.10 (d, 1H, *J* = 8.2 Hz, *H*6), 5.99–5.97 (m, 1H, *H*1′), 5.58 (d, 1H, *J* = 8.2 Hz, *H*5), 4.39–3.46 (m, 11H), 1.95–1.85 (m, 2H, O–CH_2–C*H*$_2$–CH_2–NH–), 1.51 (s, 9H, C(C*H*$_3$)$_3$), 1.43 (s, 9H, C(C*H*$_3$)$_3$); ^{13}C NMR (63 MHz, acetone-d_6) δ [ppm] 165.64, 164.59, 157.88, 154.86, 152.37, 142.33, 103.31, 89.82, 86.67, 84.77, 84.74, 84.39, 79.91, 70.73, 70.69, 62.45, 40.20, 30.96, 29.51, 29.19; MS (ESI) was calculated to be 566.2 for $C_{23}H_{37}N_5O_{10}Na$ (M+Na$^+$), and found to be 567.0.

4.2.3.5. 2′-*O*-(*N*,*N*′-Di-boc-guanidinopropyl)-5′-*O*-(4,4′-dimethoxytrityl)-uridine (3g). 2′-*O*-(*N*,*N*′-Di-boc-guanidinopropyl)-uridine (**3f**) (588 mg, 1.08 mmol) was dissolved in 11.4 mL of dry pyridine and 4,4′-dimethoxytrityl chloride (460 mg, 1.36 mmol) was added. The reaction solution was stirred at room temperature for 5 h. The reaction mixture was quenched with water and the solvents were evaporated. The residue was dissolved in dichloromethane, washed twice with saturated sodium bicarbonate solution (2 × 50 mL) and then twice with brine (2 × 50 mL). The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. After purification using column chromatography with dichloromethane/methanol (97:3, v/v) containing 0.5% triethylamine, 785 mg (86%) of a yellow powder was obtained. The yellow impurity could not be separated on the column. ^{1}H NMR (250 MHz, DMSO-d_6) δ [ppm] 11.49 (s, 1H, N*H*), 11.37 (m, 1H, N*H*), 8.41–8.36 (m, 1H, N*H*–CH_2–), 7.75 (d, 1H, *J* = 8.1 Hz, *H*6), 7.40–7.23 (m, 9H, DMTr), 6.92–6.88 (m, 4H, DMTr), 5.83–5.82 (m, 1H, *H*1′), 5.29–5.25 (m, 1H, *H*5), 5.09–5.06 (m, 1H, 3′-O*H*), 4.23–3.88 (m, 3H), 3.74 (s, 6H, 2 × O–C*H*$_3$), 3.68–3.63 (m, 2H), 3.43–3.20 (m, 4H), 1.82–1.72 (m, 2H, O–CH_2–C*H*$_2$–CH_2–NH–), 1.44 (s, 9H, C(C*H*$_3$)$_3$), 1.37 (s, 9H, C(C*H*$_3$)$_3$); HRMS (MALDI) was calculated to be 846.3920 for $C_{44}H_{56}N_5O_{12}$ (M+H$^+$), and found to be 846.3946.

4.2.3.6. 2′-*O*-(*N*,*N*′-Di-boc-guanidinopropyl)-5′-*O*-(4,4′-dimethoxytrityl)-uridine 3′-(cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite (3d). 2′-*O*-(*N*,*N*′-Di-boc-guanidinopropyl)-5′-*O*-(4,4′-dimethoxytrityl)-uridine (**3g**) (770 mg, 0.9 mmol) was dissolved in dichloromethane (11 mL). To this solution, 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylamino phosphane (400 μL, 1.26 mmol) and 4,5-dicyanoimidazole (130 mg, 1.1 mmol) were added. The reaction progress was observed with TLC (dichloromethane/ethyl acetate 1:1 (v:v), containing 0.5% triethylamine). Because the reaction was not complete after 2 h, an additional 0.3 equiv of the reagents were added and the reaction was completed after additional 40 min. The resulting solution was washed twice with saturated sodium bicarbonate solution (2 × 100 mL) and once with brine (200 mL). After drying over Na_2SO_4, the solvent was evaporated and the residue was purified on a silica gel column with dichloromethane/ethyl acetate (6:4–1:1, v/v) containing 0.5% triethylamine. The mixture of the two diastereomers was obtained as a light yellow foam (762 mg, 83%). ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 11.50–11.48 (m, 1H, N*H*), 11.35 (bs, 1H, N*H*), 8.39–8.33 (m, 1H, N*H*–CH_2–), 7.87–7.80 (m, 1H, *H*6), 7.41–7.22 (m, 9H, DMTr), 6.91–6.86 (m, 4H, DMTr), 5.86–5.84 (m, 1H, *H*1′), 5.23–5.18 (m, 1H, *H*5), 4.46–4.32 (m, 1H), 4.21–4.16 (m, 1H), 4.09–4.03 (m, 1H), 3.83–3.26 (m, 16H), 2.80–2.59 (m, 2H, –O–CH_2–C*H*$_2$–CN), 1.81–1.74 (m, 2H, O–CH_2–C*H*$_2$–CH_2–NH–), 1.42–1.36 (m, 18H, C(C*H*$_3$)$_3$), 1.13–0.94 (m, 12H, *i*Pr-C*H*$_3$); ^{31}P NMR (121 MHz, DMSO-d_6) δ [ppm] 150.0, 148.6; HRMS (MALDI) was calculated to be 1046,4999 for $C_{53}H_{73}N_7O_{13}P$ (M+H$^+$), and found to be 1046,5021.

4.2.4. Synthesis of the 2′-*O*-guanidinopropyl guanosine phosphoramidite

O^6-(2,4,6-Triisopropylbenzenesulfonyl)-3′,5′-*O*-di-*tert*-butylsilanediyl guanosine (**4a**) was synthesised according to a previously described procedure.[21]

4.2.4.1. 2′-*O*-(2-Cyanoethyl)-3′,5′-*O*-di-tert-butylsilanediyl guanosine (4b). Compound **4a** (2.28 g, 3.3 mmol) was dissolved in *tert*-butanol (17 mL). Freshly distiled acrylonitrile (4.25 mL, 66 mmol) and cesium carbonate (1.16 g, 3.3 mmol) were added to the solution. After vigorous stirring at room temperature for 2–3 h, the mixture was filtered through celite. The solvent and excess reagents were removed in vacuum. The crude material was used for the next reaction without further purification. The residue was dissolved in 4 mL of a mixture of formic acid/dioxane/water (70:24:6, v/v/v). After stirring at room temperature for 1 h, water (150 mL) was added to the mixture and the solution extracted with dichloromethane. The organic layer was dried over Na_2SO_4 and the solvent was evaporated. The residue was purified using column chromatography with dichloromethane/methanol (9:1, v/v) to give 1.1 g (70% over two steps) of **4b** as a colourless foam. ^{1}H NMR (250 MHz, DMSO-d_6) δ [ppm] 10.71 (bs, 1H, N*H*), 7.89 (s, 1H, *H*8), 6.45 (bs, 2H, NH_2), 5.81 (s, 1H, *H*1′), 4.45–4.33 (m, 3H), 4.05–3.81 (m, 4H), 2.83–2.76 (m, 2H, O–CH_2–C*H*$_2$–CN), 1.06 (s, 9H, C(C*H*$_3$)$_3$), 1.01 (s, 9H, C(C*H*$_3$)$_3$); ^{13}C NMR (63 MHz, DMSO-d_6) δ [ppm] 156.51, 153.69, 150.50, 135.36, 118.71, 116.53, 87.25, 80.31, 76.35, 73.80, 66.64, 65.14, 27.12, 26.80, 22.07, 19.82, 18.29; MS (ESI) was calculated to be 477.2 for $C_{21}H_{33}N_6O_5Si$ (M+H$^+$), and found to be 477.5.

4.2.4.2. 2′-*O*-(2-Aminopropyl)-3′,5′-*O*-di-tert-butylsilanediyl guanosine (4e). Compound **4b** (500 mg, 1.06 mmol) was dissolved in dry methanol (5 mL). Raney nickel (ca. 0.5 mL of the methanol-washed sediment) and methanol (5 mL) saturated with ammonia were then added. The mixture was hydrogenated at 30 bar hydrogen-pressure for 1 h at room temperature. Thereafter the mixture was filtered through a glass filter and the catalyst was washed several times with methanol and a methanol/water mixture. The solvents were evaporated from the filtrate and the residue was used without further purification for the next reaction. MS (ESI) was calculated to be 481.3 for $C_{21}H_{37}N_6O_5Si$ (M+H$^+$), and found to be 481.8.

4.2.4.3. 2′-*O*-(*N*,*N*′-Di-boc-guanidinopropyl)-3′,5′-*O*-di-tert-butylsilanediyl guanosine (4c). *N*,*N*′-Di-boc-*N*″-triflyl guanidine (163 mg, 0.415 mmol) was dissolved in dichloromethane (2.1 mL)

and triethylamine (54 μL) was then added. The solution was cooled in an ice bath and then 2′-*O*-(2-Aminopropyl)-3′,5′-*O*-di-*tert*-butylsilanediyl guanosine (**4e**) (200 mg, 0.42 mmol) was added. After 30 min the reaction mixture was removed from the ice bath then stirred for an additional 30 min at room temperature. The reaction solution was washed with saturated sodium bicarbonate solution and brine. After drying over Na_2SO_4 the solvent was evaporated. The residue was purified by column chromatography using dichloromethane/methanol (9:1, v/v) to give 270 mg (89%) of **4c**. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 11.49 (bs, 1H, N*H*), 10.66 (bs, 1H, N*H*), 8.56–8.53 (m, 1H, N*H*–CH_2–), 7.87 (s, 1H, *H*8), 6.39 (bs, 2H, NH_2), 5.86 (s, 1H, *H*1′), 4.42–4.38 (m, 1H, *H*3′), 4.30–4.27 (m, 2H, *H*2′, *H*5′), 4.06–3.93 (m, 3H, *H*4′, *H*5′, ½ × O–CH_2–CH_2–CH_2–NH–), 3.72–3.67 (m, 1H, ½ × O–CH_2–CH_2–CH_2–NH–), 3.51–3.30 (m, 2H, O–CH_2–CH_2–CH_2–NH–), 1.84–1.77 (m, 2H, O–CH_2–CH_2–CH_2–NH–), 1.46 (s, 9H, –CO–C(CH_3)$_3$), 1.39 (s, 9H, –CO–C(CH_3)$_3$), 1.06 (s, 9H, –Si–C(CH_3)$_3$), 0.97 (s, 9H, –Si–C(CH_3)$_3$); HRMS (MALDI) was calculated to be 723.3856 for $C_{32}H_{55}N_8O_9Si$ (M+H$^+$), and found to be 723.3880.

4.2.4.4. N^2-Isobutyryl-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-3′,5′-*O*-di-*tert*-butylsilanediyl guanosine (4f) and N^2-Isobutyryl-2′-*O*-(*N*,*N*′-di-boc-*N*″-isobutyryl-guanidinopropyl)-3′,5′-*O*-di-*tert*-butylsilanediyl guanosine (4f*).

A solution of compound **4c** (400 mg, 0.55 mmol) in 3.6 mL of pyridine was cooled in an ice bath and isobutyryl chloride (145 μL, 1.37 mmol) was then added dropwise. The mixture was stirred at 0 °C for 1 h, then at room temperature for 1 h and evaporated to dryness. The residue was dissolved in 40 mL dichloromethane and washed twice with saturated sodium bicarbonate solution (2 × 60 mL) and once with brine (60 mL). The organic phase was dried over Na_2SO_4 and the solvent was evaporated. The residue was purified by column chromatography using dichloromethane/methanol (95:5–90:10, v/v) to give 318 mg (ca. 70%) of a mixture of mono- and di-isobutyryl derivative. ^{1}H NMR (250 MHz, DMSO-d_6) δ [ppm] 12.12 (s, 1H, N*H*), 11.57–11.51 (m, N*H*, N*H*-boc), 10.53 (s, N*H*-boc*), 8.54–8.49 (m, 2′-O–CH_2–CH_2–CH_2–N*H*–), 8.25–8.22 (m, 1H, *H*8), 5.90–5.88 (m, 1H, *H*1′), 4.42–3.42 (m, 9H), 2.85–2.72 (m, 1.5H, –C*H*(CH_3)$_2$), 1.99–1.73 (m, 2H, 2′-O–CH_2–C*H*$_2$–CH_2–NH–), 1.47–1.33 (m, 18H, 2 × –CO–C(CH_3)$_3$), 1.15–0.99 (m, 27H, 2 × –Si–C(CH_3)$_3$, –CH(CH_3)$_2$, –CH(CH_3)$_2$*). As a result of the mixture comprising mono- and diisobutyryl derivatives, some of the integrals could not be given as whole numbers. Thus, signals that depend only on the diisobutyryl compound are marked with an asterisk. MS (ESI) was calculated to be 793.4 for $C_{36}H_{61}N_8O_{10}Si$ (M+H$^+$), and found to be 794.6.

4.2.4.5. N^2-Isobutyryl-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-guanosine (4g) and N^2-Isobutyryl-2′-*O*-(N,N′-di-boc-*N*″-isobutyryl-guanidinopropyl)-guanosine (4g*).

A mixture of N^2-Isobutyryl-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-3′,5′-*O*-di-*tert*-butylsilanediyl guanosine (**4f**) and N^2-Isobutyryl-2′-*O*-(*N*,*N*′-di-boc-*N*″-isobutyryl-guanidinopropyl)-3′,5′-*O*-di-*tert*-butylsilanediyl guanosine (**4f***) (490 mg, ca. 592 μmol) was dissolved in dry tetrahydrofurane (7 mL). Triethylamine (165 μL, 1.11 mmol) and $Et_3N{\cdot}3HF$ (352 μL, 2.16 mmol) were then added. After stirring at room temperature for 1 h the solvent was evaporated. The residue was purified using column chromatography with dichloromethane/methanol (9:1, v/v) to give 322 mg (ca. 79%) of a mixture of N^2-isobutyryl-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-guanosine and N^2-Isobutyryl-2′-*O*-(*N*,*N*′-di-boc-*N*″-isobutyryl-guanidinopropyl)-guanosine as white foam. A small sample of the mixture was separated for NMR spectroscopy. NMR data is given for the mono-isobutyryl compound. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 12.08 (s, 1H, N*H*), 11.65 (s, 1H, N*H*), 11.46 (s, 1H, N*H*), 8.29 (s, 1H, *H*8), 8.28–8.25 (m, 1H, N*H*–CH_2–), 5.91 (d, 1H, *J* = 6.0 Hz, *H*1′), 5.16 (d, 1H, *J* = 4.8 Hz, 3′-O*H*), 5.06–5.03 (m, 1H, 5′-O*H*), 4.36–4.29 (m, 2H, *H*2′, *H*3′), 3.95–3.93 (m, 1H, *H*4′), 3.67–3.46 (m, 4H, 2 × *H*5′, O–CH_2–CH_2–CH_2–NH–), 3.33–3.28 (m, 2H, O–CH_2–CH_2–C*H*$_2$–NH–), 2.77 (sep, 1H, *J* = 6.8 Hz, –C*H*(CH_3)$_2$), 1.75–1.67 (m, 2H, O–CH_2–C*H*$_2$–CH_2–NH–), 1.45 (s, 9H, –CO–C(C*H*$_3$)$_3$), 1.37 (s, 9H, –CO–C(C*H*$_3$)$_3$), 1.12 (d, 6H, *J* = 6.8 Hz, –CH(C*H*$_3$)$_2$); ^{13}C NMR (63 MHz, $CDCl_3$) δ [ppm] 178.72, 163.52, 156.12, 155.16, 153.39, 147.73, 147.05, 138.81, 122.49, 88.47, 86.74, 83.65, 82.28, 79.58, 70.69, 69.87, 62.66, 38.87, 36.39, 29.32, 28.28, 28.11, 18.96, 18.89; HRMS (MALDI) was calculated to be 653.3253 for $C_{28}H_{45}N_8O_{10}$ (M+H$^+$), and found to be 653.3274.

4.2.4.6. N^2-Isobutyryl-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-5′-*O*-(4,4′-dimethoxytrityl)-guanosine (4h) and N2-Isobutyryl-2′-*O*-(*N*,*N*′-di-boc-N″-isobutyryl-guanidinopropyl)-5′-*O*-(4,4′-dimethoxytrityl)-guanosine (4h*).

A mixture of N^2-Isobutyryl-2′-*O*-(*N*,*N*′-di-boc-guanidinopropyl)-guanosine (**4g**) and N^2-Isobutyryl-2′-*O*-(*N*,*N*′-di-boc-*N*″-isobutyryl-guanidinopropyl)-guanosine (**4g***) (400 mg, ca. 583 μmol) was dissolved in dry pyridine (11 mL). 4,4′-Dimethoxytrityl chloride (280 mg, 0.82 mmol) was added and the solution was stirred for 3 h at room temperature. TLC revealed that some starting material remained at this time and an additional 0.3 equiv of 4,4′-Dimethoxytrityl chloride were therefore added. When TLC demonstrated that the starting material had been consumed, the reaction was quenched with water and the solvents evaporated. The residue was purified by column chromatography using dichloromethane/methanol (98:2, v/v) containing 0.5% triethylamine to give 427 mg (ca. 74%) of the desired products. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 12.09 (s, 1H, N*H*), 11.58 (s, 1H, N*H*), 11.47 (s, 0.5H, N*H*-boc), 10.50 (s, 0.5H, N*H*–boc*), 8.33–8.30 (m, 0.5H, 2′-O–CH_2–CH_2–CH_2–N*H*–), 8.15–8.12 (m, 1H, *H*8), 7.35–7.18 (m, 9H, DMTr), 6.84–6.80 (m, 4H, DMTr), 5.97–5.94 (m, 1H, *H*1′), 5.15–5.13 (m, 1H, 3′-O*H*), 4.42–4.37 (m, 1H, *H*2′), 4.35–4.30 (m, 1H, *H*3′), 4.09–4.03 (m, 1H, *H*4′), 3.72 (s, 6H, 2 × O–C*H*$_3$), 3.69–3.47 (m, 2H, 2′-O–C*H*$_2$–CH_2–CH_2–NH–), 3.37–3.26 (m, 3H, 2′-O–CH_2–CH_2–C*H*$_2$–NH–, *H*5′), 3.17–3.13 (m, 1H, *H*5′), 2.79–2.73 (m, 1.5H, –C*H*(CH_3)$_2$), 1.77–1.67 (m, 2H, 2′-O–CH_2–C*H*$_2$–CH_2–NH–), 1.43–1.35 (m, 18H, 2 × –CO–C(C*H*$_3$)$_3$), 1.13–1.10 (m, 6H, –CH(C*H*$_3$)$_2$), 1.00–0.98 (m, 3H, –CH(C*H*$_3$)$_2$*). As a result of the mixture comprising mono- and diisobutyryl derivatives, some of the integrals could not be given as whole numbers. Thus, signals that depend only on the diisobutyryl compound are marked with an asterisk. MS (ESI) was calculated to be 955.5 for $C_{49}H_{63}N_8O_{12}$ (M+H$^+$), and found to be 956.5.

4.2.4.7. N^2-Isobutyryl-2′-*O*-(N,N′-di-boc-guanidinopropyl)-5′-*O*-(4,4′-dimethoxytrityl)-guanosine 3′-(cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite (4d) and N2-Isobutyryl-2′-*O*-(*N*,*N*′-di-boc-N″-isobutyryl-guanidinopropyl)-5′-*O*-(4,4′-dimethoxytrityl)-guanosine 3′-(cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite (4d*).

A mixture of compounds **4h** and **4h*** (380 mg, ca. 384 μmol) was dissolved in dichloromethane (8 mL), then 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylamino phosphane (160 μL, 0.52 mmol) and 4,5-dicyanoimidazole (57 mg, 0.5 mmol) were added. After 2 h TLC showed complete consumption of the starting material. The reaction solution was then washed twice with saturated sodium bicarbonate solution (2 × 50 mL) and once with brine (100 mL). After drying over Na_2SO_4 the solvent was evaporated and the residue was purified using column chromatography with dichloromethane/ethyl acetate (8:2, v/v) containing 0.5% triethylamine to give 350 mg (ca. 76%) of the two diastereomers of **4d** and **4d***. ^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] 12.11 (bs, 1H, N*H*), 11.61–11.57 (m, 1H, N*H*), 11.46–11.44 (m, 0.5H, N*H*-boc), 10.50–10.46 (m, 0.5H, N*H*-boc*), 8.31–8.27 (m, 0.5H, 2′-O–CH_2–CH_2–CH_2–N*H*–), 8.18–8.14 (m, 1H, *H*8), 7.36–7.19 (m, 9H, DMTr), 6.85–6.80 (m, 4H, DMTr), 5.97–5.88 (m, 1H, *H*1′), 4.64–4.61 (m,

1H, *H2′*), 4.44–4.37 (m, 1H, *H3′*), 4.27–4.12 (m, 1H, *H4′*), 3.79–3.18 (m, 10H), 3.72 (s, 6H, 2 × OCH_3), 2.81–2.70 (m, 2.5H, –$CH(CH_3)_2$), 2.60–2.47 (m, 2H, –P–O–CH_2–CH_2–CN), 1.75–1.65 (m, 2H, 2′-O–CH_2–CH_2–CH_2–NH–), 1.41–1.34 (m, 18H, 2 × –CO–C(CH_3)$_3$), 1.15–1.10 (m, 18H, –N(($CH(CH_3)_2$)$_2$, –CO–$CH(CH_3)_2$), 1.00–0.96 (m, 3H, –$CH(CH_3)_2$*); ^{31}P NMR (162 MHz, DMSO-d_6) δ [ppm] 149.59, 149.44, 149.52, 149.19. As a result of the mixture comprising mono- and diisobutyryl derivatives, some of the integrals could not be given as whole numbers. Thus, signals that depend only on the diisobutyryl compound are marked with an asterisk. MS (ESI) was calculated to be 1155.6 for $C_{58}H_{80}N_{10}O_{13}P$ ($M+H^+$), and found to be 1157.3.

4.3. Oligonucleotide synthesis

Modified oligonucleotides were synthesised on 500 Å CPG material on an *Expedite* 8909 synthesiser using phosphoramidite chemistry. The GP modified nucleosides were inserted into the HBV antisense strand (intended guide, 5′ UUG AAG UAU GCC UCA AGG UCG 3′) at each of positions 2, 3, 4, 5, 6, 7 and 13 from the 5′ end. The sense strand oligonucleotide (5′ ACC UUG AAG CAU ACU UCA ATT 3′) did not include modifications. The duplex HBV siRNA3 targeted HBV genotype A coordinates 1693 to 1711. Control siRNA with scrambled unmodified sequences comprised 5′-UAUUGGGUGUGCGGUCACGGT-3′ (antisense) and 5′-CGUGACCGCACACCCAAUATT-3′ (sense). 5-Ethylthio-1H-tetrazole (0.25 M in acetonitrile) was used as activator. Unmodified 2′-TBDMS-phorphoramidites were benzoyl- (A), isobutyryl- (G) or acetyl- (C) protected. Coupling time for the modified phosphoramidites was 25 min. After completion of synthesis, 30 min of deprotection in 3% trichloroacetic acid in dichloromethane was carried out to ensure complete cleavage of the boc groups. The RNA oligomers were cleaved from the controlled-pore-glass (CPG) support by incubation at 40 °C for 24 h using an ethanol:ammonia solution (1:3). The 2′-TBDMS groups were deprotected by incubation for 90 min at 65 °C with a triethylamine, *N*-methylpyrrolidinone and $Et_3N{\cdot}3HF$ mixture. RNA oligomers were precipitated with BuOH at 80 °C for 30 min and purified by anion exchange HPLC using a *Dionex* DNA-Pac 100 column(1 M LiCl, water, gradient: 0–70% LiCl solution in 40 min, flow: 1 mL/min). Oligonucleotides were desalted in a subsequent reverse phase HPLC step (column: *phenomenex* Jupiter 4u Proteo 90A 250 mm × 15 mm, 0.1 M triethylammonium acetate pH 7, acetonitrile, gradient: 0% acetonitrile for 2 min, 0–37% acetonitrile in 28 min). Identity was confirmed by mass spectroscopy on a *Bruker* micrOTOF-Q.

4.4. Biological experiments

4.4.1. Cell culture, transfection, dual luciferase assay and measurement of HBV surface antigen (HBsAg) concentrations

Huh7 and HEK293 cells were cultured in DMEM (Lonza, Basel, Switzerland) supplemented with 5% foetal calf serum (Gibco BRL, UK). Cells were seeded in 24-well plates at a confluency of 40% on the day before transfection, and were then maintained in antibiotic-free medium for at least an hour prior to transfection. To assess target knockdown when using the luciferase reporter assay, Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was employed to transfect HEK293 cells with 100 ng psiCHECK-HBx[20] and 32.5 ng siRNA (5 nM final concentration) at ratios of 1:1 and 1:3 (ml:mg), respectively. The psiCHECK-HBx reporter vector contains the *HBx* target sequence downstream of the *Renilla* ORF within the psiCHECK 2.2 (Promega, WI, USA) and has been described previously.[20] Forty-eight hours after transfection, cells were assayed for luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega, WI, USA) and the ratio of *Renilla* luciferase to Firefly luciferase activity was calculated. Similarly, to assess knockdown of HBV replication in a liver-derived line, Huh7 cells were transfected with 100 ng pCH-9/3091[28] and 32.5 ng siRNA. Forty-eight hours after transfection, growth medium was harvested and HBsAg concentration was measured by ELISA using the MONOLISA® HBs Ag ULTRA kit (Bio-Rad, CA, USA). Each experiment was repeated at least in triplicate.

4.4.2. siRNA serum stability assessment

Annealed siRNAs were diluted in 80% FCS to a final concentration of 5 μM and incubated at 37 °C. At time points ranging from 1 to 24 h, aliquots were removed and snap frozen using liquid nitrogen. Twenty picomoles of the samples were subjected to electrophoresis through a 10% denaturing polyacrylamide gel then stained with ethidium bromide.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.12.024.

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