JOC The Journal of Organic Chemistry

ARTICLE

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Synthesis and Preliminary Evaluation of pro-RNA 2′-*O*-Masked with Biolabile Pivaloyloxymethyl Groups in an RNA Interference Assay

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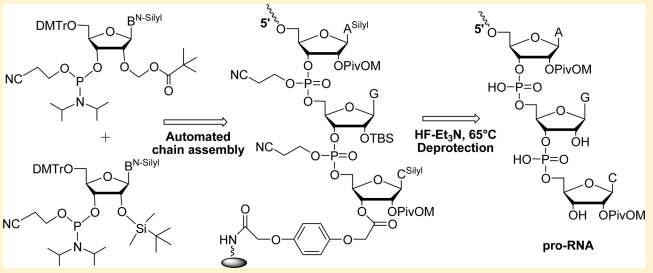
Supporting Information

ABSTRACT: The cellular delivery of bioactive nucleic acid-based drugs such as small interfering RNA (siRNA) represents a major technical hurdle for their pharmaceutical application. Prodrug-like approaches provide an attractive concept to address the delivery problem. With the aim to prepare RNA-based prodrugs bearing biolabile protections which facilitate cellular uptake and are prone to be removed enzymatically inside cells in order to release functional RNA, we synthesized pro-RNA totally or partially masked in 2′-OH position with pivaloyloxymethyl (PivOM) groups. A suitable strategy has been developed to synthesize and to purify base-sensitive mixed 2′-OH/2′-*O*-PivOM oligoribonucleotides, and to include them in siRNA. In this strategy, the fluoride labile [(triisopropylsilyl)oxy]-benzyloxycarbonyl group (tboc) as nucleobase protection (for A and C), the TBS group as 2′-OH protection and the Q-linker to solid-support were compatible with the PivOM groups masking some 2′-OH. We have taken advantage of the specific stability of the PivOM group to apply selected acidic, basic, and fluoride ions treatment for the deprotection and release of pro-RNA. This kind of pro-siRNA was studied in a human cell culture-based RNAi assay and preliminary promising data are discussed.



INTRODUCTION

Since 1978 when the first antisense DNA oligonucleotide was employed to prevent translation of Rous Sarcoma virus,[1] synthetic nucleic acids have been used as tools to regulate gene expression through different mechanisms of action. These methods include antisense nucleic acids, ribozymes, aptamers, and more recently small interfering RNAs (siRNA), one of the key effectors in natural gene regulation processes termed RNA interference (RNAi), wherein double-stranded RNAs (dsRNA) interfere with the expression of complementary genes. For more than one decade from its discovery,[2] RNAi has had a broad impact on biology as attested by the thousands of reports describing the use of synthetic siRNA to study gene function, identify drug targets, and develop more specific therapeutics.[3,4] Indeed, several siRNA-based drugs are currently under clinical evaluation.[3] However, applications of this gene silencing technology need to overcome quite a few hurdles. Limitations of unmodified siRNA are their susceptibility to nuclease degradation, off-target effects,[5] toxicity due to saturation of the endogenous RNAi functions, and, most importantly, effective targeted delivery. To circumvent these problems, chemical modifications can provide solutions to the challenges facing siRNA therapeutics.[6]

Many authors described the benefits of chemical modifications of siRNA but no accurate rationale could be ascertained for the best way to produce efficient and potent modified siRNA.[6,7] Most of the existing modifications in RNA are permanent and may not be introduced in any position to maintain the biological effect. This drawback would be circumvented with RNA prodrugs bearing temporary modifications as biolabile groups. Indeed, we chemically substituted RNA in 2′-OH position with acetalester groups which should be cleaved by intracellular esterases to liberate the native RNA inside cells and potentially lead to RNAi applications (Figure 1). First results demonstrated that 2′-*O*-acyloxymethyled uridylates fulfilled the criteria of nuclease resistance, esterase hydrolysis and ability to form stable dsRNA.[8] Among the different evaluated acyloxymethyl groups, the pivaloyloxymethyl (PivOM) successfully completes the requirements to functionalize a potential siRNA prodrug.[8,9] Moreover, siRNA may exhibit an improved cellular uptake as a consequence of the lipophilic character of the PivOM group.

In the perspective of in vivo RNA applications, its features support the ambition of synthesizing mixed-nucleobase RNA (21 nucleotides long) 2′-*O*-masked with PivOM groups. Therefore,

Received: April 21, 2011
Published: May 27, 2011

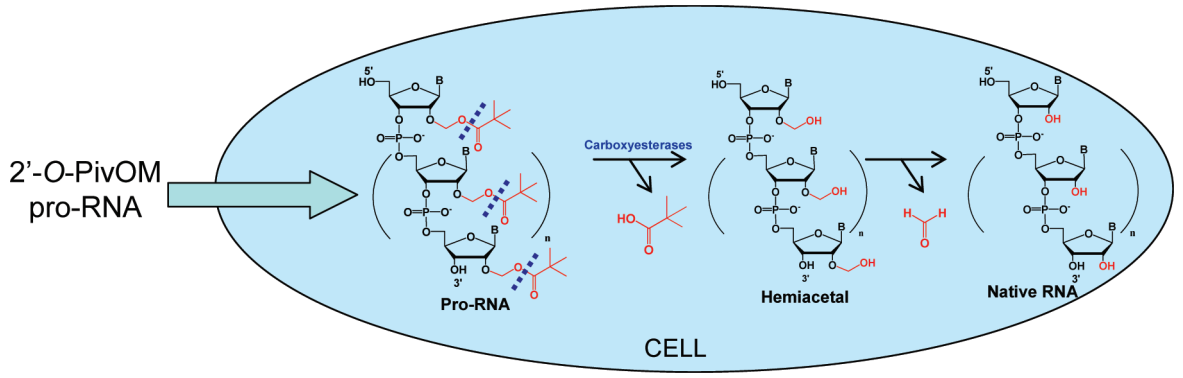


Figure 1. Demasking of 2′-*O*-PivOM pro-RNA inside the cells into native RNA.

our goal was to synthesize pro-RNA (for RNA prodrugs) which were totally or partially 2′-modified with PivOM. It is noteworthy that very recently, Lönnberg et al. reported on the use of the biolabile PivOM for the synthesis of a 2′,5′-dinucleoside in a prodrug strategy to enhance the cellular uptake.[10] With the same objective, Damha et al. developed a new method for the synthesis of 2′-*O*-acetalester oligonucleotides with levulinic ester or amino acid moieties to make siRNA prodrugs.[11]

Generally, RNA oligonucleotides are synthesized on solid support by the standard phosphoramidite approach following a coupling-oxidation-capping-detritylation cycle. The deprotection process is commonly performed by an ammonia treatment to remove base-labile protective groups from both nucleobases and phosphates and to release RNA from the solid support. Obviously, since basic conditions cannot be applied on RNA bearing base-sensitive modifications such as 2′-*O*-PivOM groups,[12] pro-RNA cannot be obtained through the standard method. This drove us to work out a different synthetic strategy avoiding ammonia, the final deprotection of modified RNAs being achieved with fluoride ions.

In a previous study of pro-RNA uridylates, the fluoride-labile 2-(trimethylsilyl)ethyl group was chosen as the phosphate protection, but we found that it was prematurely removed during the RNA assembly by treatment with the solution of iodine during the normal oxidation protocol leading to low coupling yields.[8] Consequently, we preferred to protect phosphates with the standard 2-cyanoethyl (CNE) group eliminated by a non-nucleophilic strong organic base, DBU or piperidine, which does not affect the PivOM and allows high coupling yields.[12] Likewise, we recently investigated an appropriate linker that could be cleaved under conditions suitable for the preservation of PivOM. The accessible hydroquinone-*O*,*O*′-diacetic acid (Q-linker)[13] was chosen and a fluoride ions treatment was optimized to efficiently release base-sensitive RNAs from solid supports.[14] Thus, removal of nucleobase protections and TBS groups from 2′-OH in partially modified 2′-*O*-PivOM pro-RNA, was performed with the same final fluoride treatment.

In this work, we describe the first synthesis of pro-RNA heteropolymers, suited to the preservation of PivOM groups, involving silyl-based protections on the exocyclic amino functions of the nucleobases combined to CNE on phosphates and the Q-linker between pro-RNA and the solid support. Several pro-RNAs (21 nucleotides in length) totally or partially modified with 2′-PivOM groups were successfully prepared and one of them was evaluated in a human cell culture-based RNA interference assay.

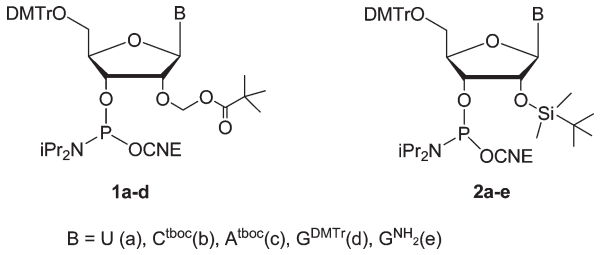


Figure 2. 2′-*O*-PivOM and 2′-*O*-TBS ribonucleoside phosphoramidites **1a**−**d** and **2a**−**e**.

RESULTS AND DISCUSSION

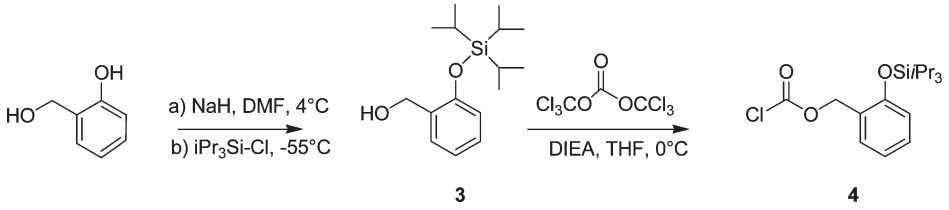
Synthesis of 2′-*O*-PivOM or 2′-*O*-TBS Ribonucleosides Amidites 1a−d or 2a−e. First, our investigations began with the preparation of both 2′-*O*-PivOM **1a**−**d** and 2′-*O*-TBS **2a**−**e** ribonucleoside phosphoramidites appropriate for pro-RNA synthesis on solid support (Figure 2).

For the incorporation of uridine, we used the regular 2′-*O*-TBS phosphoramidite **2a** and we prepared the 2′-*O*-PivOM uridine amidite building block **1a** as early described.[12] For the synthesis of cytidine and adenosine building blocks **1b**−**c** and **2b**−**c**, we protected the exocyclic amino functions of cytosine (C) and adenine (A) with a silyl group previously used in RNA synthesis for the preparation of a base-sensitive aminoacylated RNA sequence.[15] This fluoride-labile [(triisopropylsilyl)oxy]-benzyloxycarbonyl group (tboc) was introduced differently on C and A related to the disparity of their amino function reactivity. Initially, the reagent 2-[(triisopropylsilyl)oxy]-benzyl alcohol **3** was obtained by selective silylation of the phenol function of the commercially available hydroxybenzylalcohol (Scheme 1).[15]

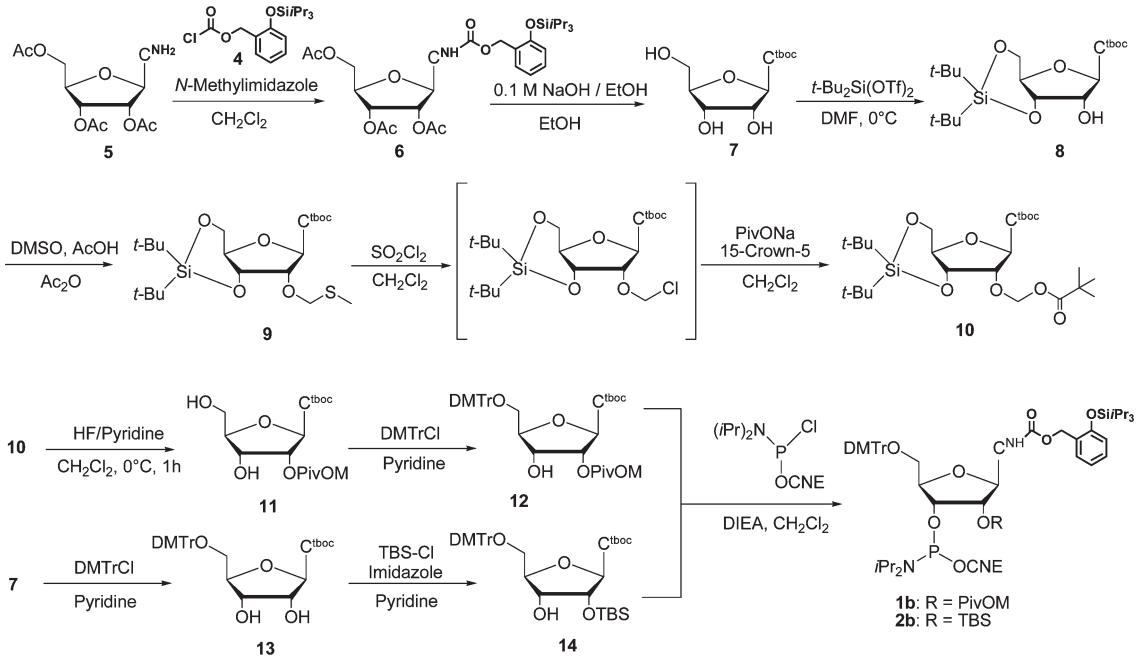
In the case of C, the tboc group was introduced via a reactive carbonochloridate **4** formed on the primary benzyl alcohol by addition of triphosgene in THF (Scheme 1). This intermediate **4** was directly reacted with 2′,3′,5′-tri-*O*-acetyl-cytidine **5**[16] in the presence of *N*-methylimidazole to give N^4-tboc-protected cytidine **6** with 64% yield (Scheme 2). Then the ribose hydroxyls were deacetylated with a sodium hydroxide solution in EtOH without loss of the tboc group. For the introduction of the PivOM group, we chose the site-specific manner involving the di-*tert*-butylsilylene group which simultaneously blocks 5′-OH and 3′-OH of the N^4-tboc-cytidine **7** leaving the 2′-OH free to accept the PivOM in compound **8**.[17] This silyl group takes advantage on the widely used tetraisopropyldisiloxane protection since it could be cleaved with fluoride ions under mild conditions (HF/pyridine)

dx.doi.org/10.1021/jo200826h |*J. Org. Chem.* 2011, 76, 5719–5731

Scheme 1. Synthesis of 2-[(Triisopropylsilyl)oxy]-benzyl Carbonochloridate 4



Scheme 2. Synthesis of 2′-*O*-PivOM and 2′-*O*-TBS Cytidine Amidites 1b and 2b



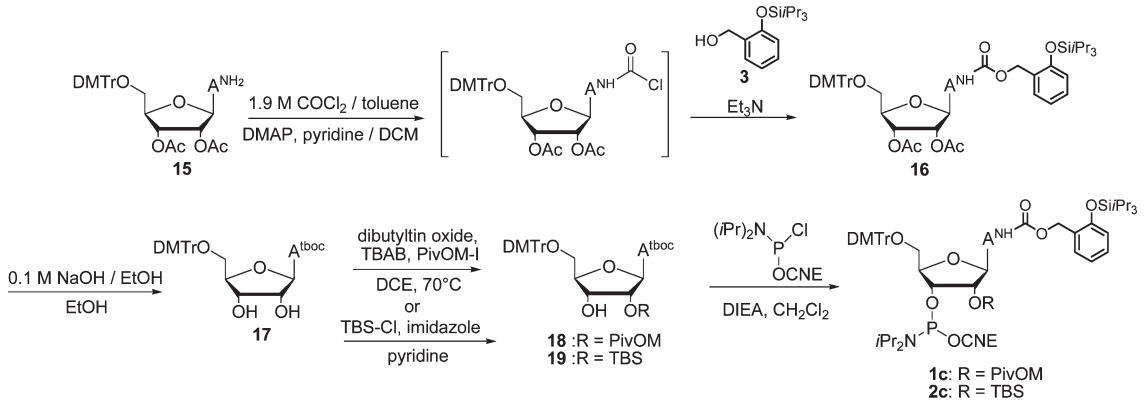
without affecting the fluoride labile tboc group, whereas TBAF or Et_3N-3HF removed it. Furthermore, the regioselective route was preferred to the direct alkylation in 2′-position with PivOM chloride[12] which leads to a mixture of 2′- and 3′-isomers rather difficult to separate by chromatography in this case. Thus, compound **8** was converted in its methylthiomethyl ether derivative **9** which was cleaved with sulfuryl chloride to give the chloromethyl ether.[8] This intermediate was reacted with the pivalate sodium salt in the presence of 15-crown-5 to afford the 2′-*O*-PivOM derivative **10**. Removal of di-*tert*-butylsilylene protection using HF-pyridine in DCM at 0 °C furnished **11** in a sufficient purity to proceed to the tritylation without additional purification. Next, the dimethoxytrityl (DMTr) group was typically introduced in the 5′-OH to give **12**. In the same way, to obtain the 2′-*O*-TBS cytidine derivative **14**, compound **7** was first 5′-*O*-tritylated to give **13** and then 2′-*O*-protected with TBS following standard procedures. Compound **14** was obtained with 70% yield after several conversions of 3′-*O*-TBS into 2′-*O*-TBS in MeOH. Finally, subsequent phosphitylation of **12** and **14** was conducted with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite under standard conditions to give the N^4-tboc cytidine amidites **1b** and **2b**, respectively.

Concerning the preparation of the 2′-*O*-PivOM and 2′-*O*-TBS adenosine amidites **1c** and **2c**, the lack of nucleophilicity of the exocyclic amino function in adenine prevents its protection by tboc following the same procedure as for C. Indeed, the solubility of tri-*O*-Ac adenosine in the mixture pyridine/DCM was too low to give the N^6-tboc derivative with good and reproducible yield. Therefore, 5′-*O*-DMTr-2′,3′-di-*O*-Ac adenosine **15** was preferred as starting material (Scheme 3). It was activated as a N^6-carbamoyl derivative by treatment with carbonyl chloride and DMAP to react next with 2-[(triisopropylsilyl)oxy]-benzyl alcohol **3** in the presence of Et_3N resulting in the N^6-tboc protected adenosine **16** with 87% yield. Then, ribose 3′-OH and 2′-OH were deacetylated in the same conditions as for C. The PivOM group was introduced via a 2′,3′-*O*-dibutylstannylidene intermediate using the PivOM iodide more efficient than PivOM chloride with adenosines.[18] The 2′-*O*-PivOM derivative **18** was obtained with 28% yield. The 2′-*O*-TBS N^6-tboc adenosine **19** was isolated with 71% yield. Both 2′-*O*-protected N^6-tboc adenosine **18** and **19** were phosphitylated to afford the amidites **1c** and **2c** with 77% and 73% yield, respectively.

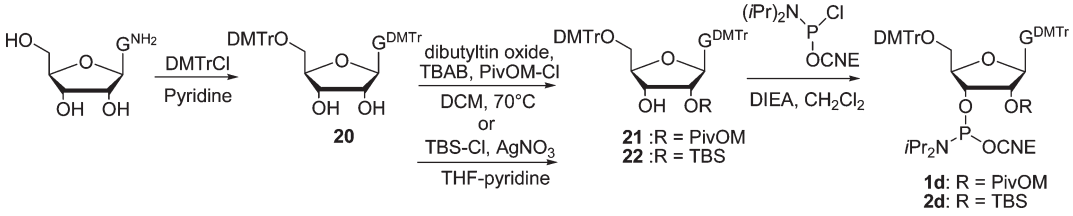
In contrast, several attempts to protect the guanine with tboc group following the same procedures were unsuccessful, which led us to consider the use of DMTr as protective group of the 5′-OH and the exocyclic amino function. The two acid-labile groups will be simultaneously removed during the elongation cycle in the detritylation step. Indeed, it has been reported that unprotected guanines do not react under coupling conditions because

dx.doi.org/10.1021/jo200826h |*J. Org. Chem.* 2011, 76, 5719–5731

Scheme 3. Synthesis of 2′-*O*-PivOM and 2′-*O*-TBS Adenosine Amidites 1c and 2c



Scheme 4. Synthesis of 2′-*O*-PivOM and 2′-*O*-TBS Guanosine Amidites 1d and 2d



of their low nucleophilicity.[19−21] Bis-protection of guanosine with DMTr was achieved with acceptable yield (66%) in reacting 2.2 equiv of DMTr chloride (Scheme 4).

PivOM group was not introduced in 2′-position of N^2,5′-*O*-bis-DMTr-guanosine **20** in the same conditions as previously described for N^2-*tert*-butylphenoxyacetyl-5′-*O*-DMTr-guanosine.[12] Actually, these conditions were not efficient since the reaction did not go to completion (50% starting material left) and the ratio between 2′- and 3′-*O*-PivOM isomers was 1/1 instead of 9/1 obtained for the preparation of N^2-*tert*-butylphenoxyacetyl-5′-*O*-DMTr-2′-*O*-PivOM-guanosine. In order to improve the PivOM introduction, the equivalents of each reagent (dibutyltin oxide, TBAB and PivOM-Cl) were increased 2-fold and the compound N^2,5′-*O*-bis-DMTr-2′-*O*-PivOM guanosine **21** could be obtained with 25% isolated yield. The same difficulty was observed for the 2′-OH protection by TBS of bis-DMTr G. The standard conditions used for C and A were not convenient for TBS introduction in guanosine since the reaction progress did not reach more than 50% after several hours. The addition of silver nitrate to the reaction mixture allowed the total conversion of the starting material in the mixture of 2′-*O*- or 3′-*O*-TBS derivatives without inducing the predominance of the 2′-isomer.[22] It is noteworthy that N^2-DMTr also slowed down the TBS isomerization from 3′- to 2′-position since several days in a mixture pyridine/MeOH with imidazole were required to obtain the two isomers in a 1/1 ratio. However, N^2,5′-*O*-bis-DMTr-2′-*O*-TBS guanosine **22** was isolated with 69% yield. Subsequent phosphitylation of **21** and **22** afforded the guanosine amidites **1d** and **2d** in good yields (88% and 71% respectively). An alternative to this amidite derivative **2d** was investigated with the preparation of the 5′-*O*-DMTr-2′-*O*-TBS guanosine amidite without nucleobase protection. The treatment of the commercial 5′-*O*-DMTr-3′-*O*-amidite-2′-*O*-TBS N^2-phenoxyacetyl-guanosine with $MeNH_2$ gave the unprotected-base derivative **2e** (84%).[23]

Evaluation of the Coupling Efficiency of N^2-DMTr Guanosine Amidites 1d and 2d, and N^2-Unprotected Guanosine Amidite 2e during Oligonucleotides Synthesis. At first, we applied the conditions used for standard automated synthesis of oligonucleotides (ON) to the elongation of a mixed deoxyribonucleotide sequence TGTGTTT using N^2-DMTr G amidites **1d** and **2d**. It was observed that the usual acidic treatment with 3% di- or trichloroacetic acid (DCA or TCA) in DCM for 60s (6 × 10s) did not completely remove the trityl group from the amino function of guanosine at each detritylation step. Consequently, the incorporation of the deoxythymidine synthon into the growing chain with partially N^2-DMTr G at 5′-end was achieved with only 95% coupling yield estimated by DMTr cation assay, possibly because of the steric hindrance of the DMTr. Thus the detritylation step was increased to 180s (18 × 10s) using 3% TCA in DCM to get rid of the DMTr *N*-protection. We performed the synthesis of three small ON with the sequence TGTGTTT incorporating either N^2-DMTr-2′-*O*-PivOM, or N^2-DMTr-2′-*O*-TBS, or N^2-unprotected-2′-*O*-TBS guanosine amidites **1d**, **2d**, **2e**, respectively. The coupling yields of the amidites **1d** and **2e** were 97.5% whereas the amidite **2d** was incorporated with 96.5% yield within the same 300s coupling time. Although these yields have been lower than those obtained in standard RNA synthesis, we considered that the amidites **1d** and **2e**, preferred to **2d**, could be used to give modified pro-RNA. The crude ON were analyzed after deprotection with DBU then aqueous ammonia and their HPLC purity was similar and estimated at 90%.

dx.doi.org/10.1021/jo200826h |*J. Org. Chem.* 2011, 76, 5719–5731

Table 1. Fully or Partially 2′-*O*-PivOM pro-RNA Synthesized by Use of 2′-*O*-PivOM and 2′-*O*-TBS Ribonucleoside Amidites 1a−d and 2a−e[a]

no.	oligonucleotides sequence 5′-3′	RNA amidites	Maldi-TOF mass	
			calcd	found
23	UU***CUUC***UUTT	1a, 1b, 2a	3449.41	3450.95
24	***UUUGUAUUCAGCCCAUAUC***TT	1a−d	8696.75	8696.32
25	***GAUAUGGGCUGAAUACAAA***TT	1a−d	8908.99	8907.43
26	***GAUAU***GGGCUGAAUACAAATT	1a, 1c−d, 2a−c, 2e	7310.95	7311.28
27	GGGUUCUGCUGCCC***GUAGC***TT	1a−d, 2a−b, 2e	7223.81	7224.04
28	GCUACGGGCAGCAG***AACCC***TT	1b−c, 2a−c, 2e	7275.94	7277.85
29	***GCUAC***GGGCAGCAGAACCCTT	1a−d, 2b−c, 2e	7275.94	7273.48

[a] Italic and bold characters: 2′-*O*-PivOM ribonucleosides.

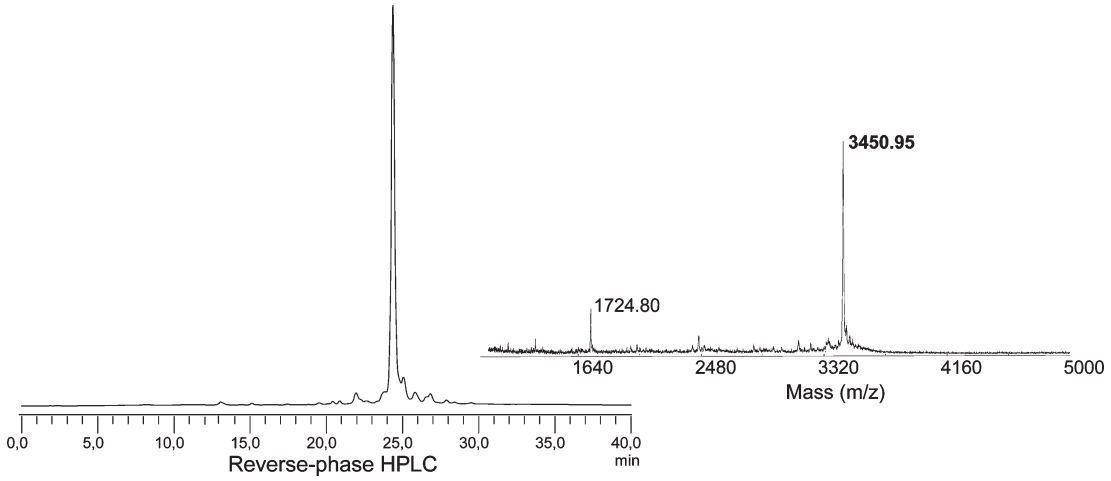


Figure 3. HPLC and MALDI-TOF mass spectrum of the crude pro-RNA **23**.

Evaluation of the Deprotection Efficiency of pro-RNA with the Treatment aq HF-Et_3N (1:3) at 65 °C. To check the removal of tboc groups from A and C in pro-RNA with the final treatment aq HF-Et_3N (1:3),[14] we prepared a decamer pro-RNA model $UUC_{PivOM}U_{PivOM}U_{PivOM}C_{PivOM}UUTT$ **23** with the 2′-*O*-PivOM and 2′-*O*-TBS uridine building blocks **1a** and **2a**, and with the 2′-*O*-PivOM cytidine amidite **1b** (Table 1). After assembly, the CNE were removed with DBU and the fluoride treatment was applied for 10 h at 65 °C to release deprotected **23** with the PivOM maintained in the pro-RNA. The crude **23** was passed through a short reversed-phase silica column to get rid of the salts and the desired pro-RNA **23** was obtained with 90% purity based on HPLC analysis (Figure 3). Fifty O.D. (λ = 260 nm) were recovered corresponding to 55% yield which is comparable with the amount of ON obtained by RNA standard synthesis using succinyl linker to the solid support.

Synthesis of Fully 2′-*O*-PivOM pro-RNA (21-mers). Once the amidites **1a**−**d** and the deprotection method in hands to achieve the pro-RNA, we synthesized two modified 2′-*O*-PivOM 21-mers with complementary sequences **24** and **25** to evaluate the RNA duplex forming ability in the silencing of *firefly luciferase* gene (Table 1). The stepwise coupling yields during the pro-RNA assembly varied from 98% (for amidites **1b** and **1c**) to 99% (**1a**). In contrast, the guanosine amidite **1d** was incorporated in RNA chain with lower yields depending on the nucleotide at the 5′-end of the ON. Interestingly, the yield was higher (97.5%) when G amidite **1d** reacted with 5′-OH of cytosine or uridine than when the coupling occurred between **1d** and adenosine (95.5%) or guanosine (93%). Finally, pro-RNA **24** and **25** were assembled with 65% and 49% overall coupling yield, respectively. After deprotection by a treatment with DBU for 3 min then with the mixture aq HF-Et_3N (1:3) at 65 °C for 10 h, followed by a desalting through a reverse-phase C_8-silica column, the crude **24** and **25** were purified by C_{18}-RP-HPLC but only few O.D. were recovered (8 O.D for **25**) (Figure 4). The low yield was due to the high absorption of these 2′-*O*-PivOM 21-mers on silica and essentially to their precipitation in water because of the high lipophilicity of completely modified pro-RNA. These data prompted us to synthesize partially modified 2′-*O*-PivOM pro-RNA leaving some free 2′-OH to circumvent the solubility problem.

Synthesis of Partially Modified pro-RNA (21-mers) with 2′-*O*-PivOM. The synthesis of partially 2′-*O*-PivOM modified oligoribonucleotides was performed with the phosphoramidite 2′-*O*-PivOM **1a**−**d** and 2′-*O*-TBS **2a**−**c**, **e** building blocks. It is noteworthy that the amidites **2b**, **2c**, and **2e** were not soluble in neat CH_3CN and DCM (from 20% to 50%) should be added to obtain a clear homogeneous solution. The reactivity of such amidites **2b**−**c** bearing both tboc and TBS protections decreased and the coupling yields were lower (95%). Therefore, to improve

dx.doi.org/10.1021/jo200826h |*J. Org. Chem.* 2011, 76, 5719–5731

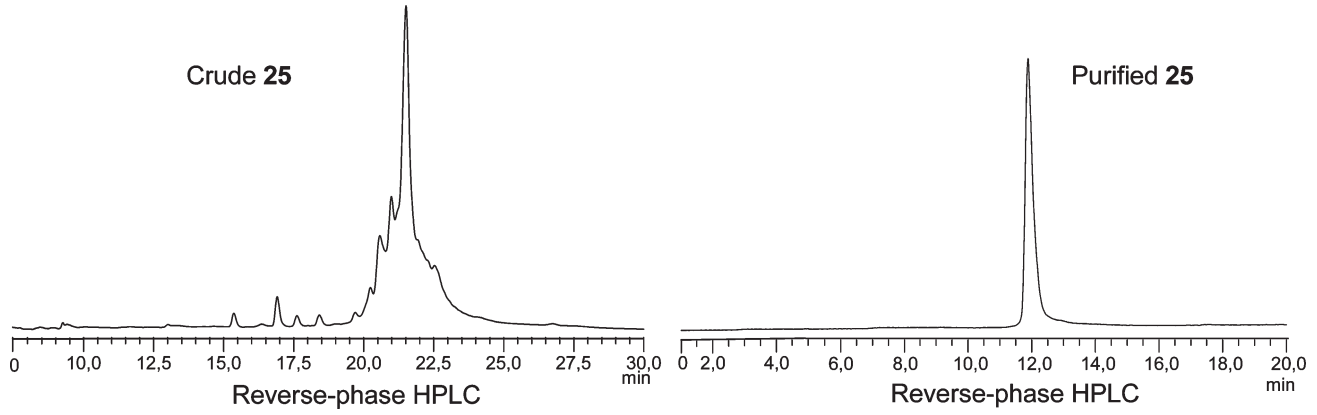


Figure 4. RP-HPLC of crude (left) and purified (right) fully 2′-*O*-PivOM RNA **25**.

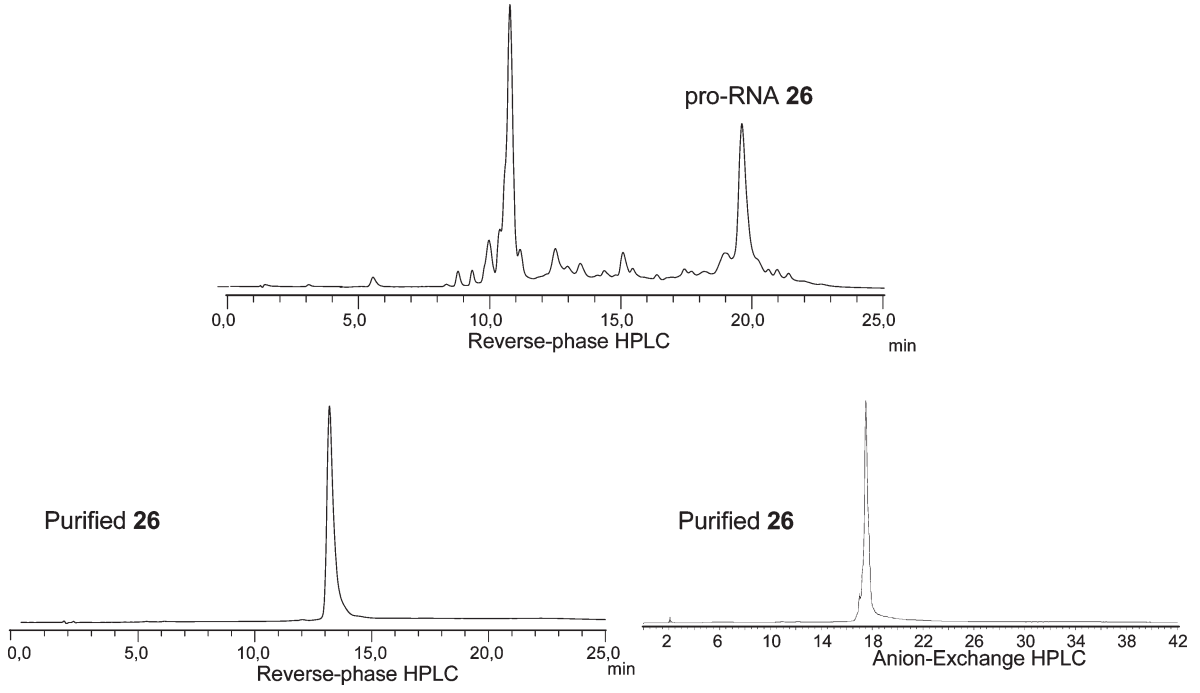


Figure 5. RP-HPLC profile of crude partially 2′-*O*-PivOM RNA **26** (top) and RP-HPLC (left) and anion-exchange HPLC (right) profiles of purified partially 2′-*O*-PivOM RNA **26**.

the yield of the elongation, the concentration of the amidites solutions was increased from 0.1 to 0.15 M, and the coupling time was up to 500 s in the case of A and G amidites **2c** and **1d**. With these changes, several pro-RNA 21-mers **26**−**29** were successfully synthesized with five PivOM groups located at their 5′-or 3′-end. Despite the presence of numerous shorter sequences which made difficult the RP-HPLC purification of the crude mixture, the partially 2′-*O*-PivOM pro-RNA **26**−**29** were obtained in high purity (Figure 5 and Supporting Information).

Digestion of the Partially 2′-*O*-PivOM Modified RNA 26 by Nuclease P1 and Alkaline Phosphatase. To check the absence of 2′-5′ internucleotide linkages and modified nucleobases, the purified 21-mer **26** was digested by nuclease P1 and alkaline phosphatase and gave the four natural ribonucleosides and thymidine. These data indicated the absence of any nucleobase modification or unnatural internucleoside linkages, such as *N*-branched oligonucleotide, in the 3′-part of the RNA constituted with 13 2′-OH ribonucleosides and two thymidines (Figure 6). Furthermore, a peak of higher retention time in the RP-HPLC chromatogram was assigned to the hexamer $G_{PivOM}A_{PivOM}U_{PivOM}A_{PivOM}U_{PivOM}G$ formed with five 2′-*O*-PivOM ribonucleosides and a guanosine residue. The observation of this 5′-fragment of the pro-RNA strengthened the data already obtained on the RNA protection by the PivOM modifications against nucleases degradation.[8]

Hybridization properties of 2′-*O*-PivOM pro-RNA 25 and 26. Hybridization properties of fully 2′-*O*-PivOM pro-RNA **25** with its complementary strand which is similarly 2′-*O*-PivOM modified **24**, and of pro-RNA **26** containing five PivOM groups with the complementary unmodified RNA strand were studied in comparison with the stability of the natural RNA duplex (Table 2). As a result it turned out that the thermal stability of the fully 2′-*O*-PivOM modified RNA duplex (T_m 86 °C) was considerably higher by 19 °C than that of the natural duplex

dx.doi.org/10.1021/jo200826h |*J. Org. Chem.* 2011, 76, 5719–5731

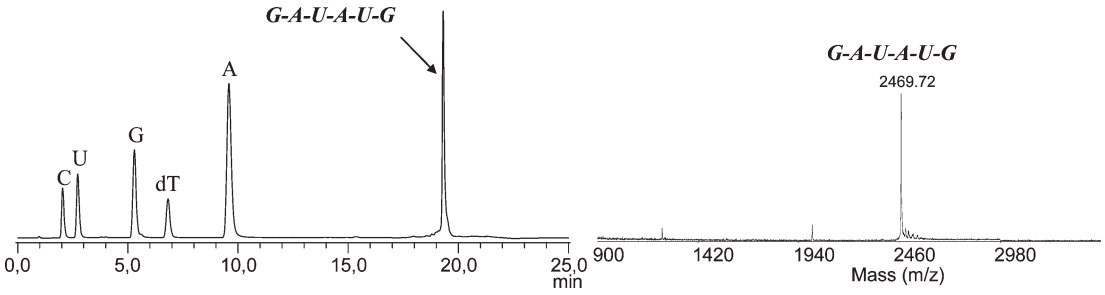


Figure 6. RP-HPLC profile and MALDI-TOF mass spectrum of the digest of 21-mer **26** by nuclease P1 and phosphatase alkaline.

Table 2. Hybridization Properties of 2′-*O*-PivOM pro-RNA

no.	sequence 5′-3′	complementary RNA strand	T_m[a]	ΔT_m	ΔT_m/mod.
	GAUAUGGGCUGAAUACAAATT	UUUGUAUUCAGCCCAUAUCTT	67		
25	***GAUAUGGGCUGAAUACAAA*TT**	***UUUGUAUUCAGCCCAUAUC*TT**	86	19.0	1.0
26	***GAUAUG*GGCUGAAUACAAATT**	UUUGUAUUCAGCCCAUAUCTT	68	1.0	0.2

[a] T_m (°C). Conditions: 10 mM sodium cacodylate buffer (pH 7.0), 100 mM NaCl, and 3 μM duplex. Italic and bold characters: 2′-*O*-PivOM ribonucleosides

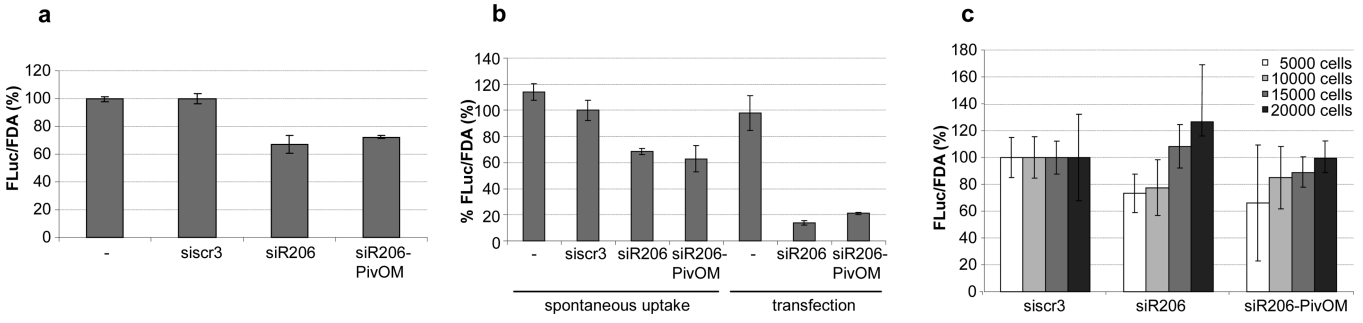


Figure 7. (a) Delivery of siRNA to ECV-GL3 cells by electroporation at 10 nM siRNA. (b) ECV-GL3 cells were incubated with 1 μM naked siRNA for spontaneous uptake (left panel) or transfected with 1 nM siRNAs (right panel). (c) ECV-GL3 cells were incubated with 1 μM naked siRNA at various cell densities. The expression levels of FLuc *(firefly luciferase)* were normalized to FDA, an indicator of viable cells. Control siRNA (siscr3) has no cellular target and was set to 100 in all panels.

(T_m 67 °C). In contrast, stability of the duplex formed between partially 2′-*O*-modified RNA **26** with five consecutive PivOM at 5′-end and the unmodified RNA target (T_m 68 °C), was similar to the natural one. Although the average stabilizing effect of one PivOM modification was weak, these data suggested that pro-RNA would form duplex stable enough to be investigated as siRNA prodrugs.

Biological Evaluation. First, we assured that the 2′-*O*-PivOM modification does not interfere with duplex formation with complementary RNA to yield siRNA and that this modification allows extraction of 2′-*O*-PivOM-derived siRNA (**26** and its complementary strand), termed siR206-PivOM, from cell culture supernatants as well as from cells by phenol/chloroform extraction (data not shown). In order to investigate the biological effectiveness of partially 2′-*O*-PivOM-modified siRNA, we used an established luciferase-based read-out system.[24] Briefly, unmodified or 2′-*O*-PivOM-modified siR206 was delivered to human ECV304 cells constitutively expressing a chromosomally integrated *firefly luciferase* gene (ECV304-GL3). Delivery was facilitated as described below or cells were incubated with unmodified naked luciferase-directed siRNAs. First, siRNAs were delivered by electroporation at 10 nM in ECV304-GL3 cells which bypasses the cellular uptake step. This experiment indicated an approximately 6% decrease of the potency of siR206-PivOM versus its unmodified control siR206 (Figure 7a). Similarly, delivery of siR206-PivOM at 1 nM by Lipofectamine 2000 showed a 1.5 fold decreased target suppression compared to siR206-PivOM (Figure 7b, right panel). Together both control experiments indicate a decreased intracellular potency of the 2′-*O*-PivOM-modified version of siR206. Notably, the addition of 1 μM of siRNA to cell culture medium of ECV304-GL3 cells showed stronger down-regulation of *luciferase* expression in the use of siR206-PivOM versus siR206 approaching 60% of *luciferase* expression of untreated cells whereby siR206-PivOM was more potent than siR206 by 7.5% to 20% (Figure 7b, left panel). It is important to note that this cell culture-based test system strongest target suppression at low cell densities of 5.000 to 10.000 cells per dish of 96 well plates. At higher cell density, the spontaneous cellular uptake of siRNA is substantially reduced (Figure 7c).

In summary, these studies indicate that 2′-*O*-PivOM-derived siRNA is biologically active and it exerts improved cellular uptake. Considering the reduced quasi intrinsic potency, increased cellular delivery is approximately 2-fold over conventional siRNA.

dx.doi.org/10.1021/jo200826h |*J. Org. Chem.* 2011, 76, 5719–5731

CONCLUSIONS

This work describes a feasible method to synthesize 2′-*O*-PivOM-modified siRNA. We have prepared 2′-*O*-PivOM and 2′-*O*-TBS ribonucleoside amidites bearing a silyl group as the protecting group of adenine and cytosine and without protection for G. We were able to synthesize fully 2′-*O*-PivOM RNA oligomers which were difficult to handle and to isolate because they were too lipophile and insoluble in water. We succeeded in applying conditions suitable for selective deprotection of the phosphates, nucleobases and some 2′-OH, and for Q-linker cleavage while the PivOM groups remained completely intact to obtain very pure partially 2′-*O*-PivOM modified RNA. One of them with five PivOM groups at the 5′-end was evaluated in a siRNA assay and it turned out that the delivery of such modified siRNA was more effective. These preliminary and promising data provide a proof-of-concept for a prodrug-based approach for the delivery of siRNA to living human cells. Further studies on the influence of the number or the position of the PivOM in the RNA sequence are ongoing.

EXPERIMENTAL SECTION

General Remarks. NMR spectra were obtained on 300 and 400 MHz spectrometers. ^{1}H and ^{13}C assignments were done using both COSY and $^{1}H-^{13}C$ HSQC experiments. Flash column chromatography was performed using silica gel (0.040−0.063 mm). TLC were performed on silica gel 60 F_{254} and compounds were visualized by illumination under UV light (254 nm) or by spraying with 10% ethanolic sulfuric acid.

General Procedure for Phosphoramidite Synthesis. A nucleoside derivative was dried by 3 coevaporations with anhydrous CH_3CN. Then the residue was dissolved in anhydrous CH_2Cl_2 and a mixture of *N*,*N*-diisopropylethylamine (1.5 equiv), 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (1.5 equiv) and CH_2Cl_2 was added dropwise. The mixture was stirred under argon, at room temperature for 3 h. After completion, ethyl acetate containing 1% pyridine was added, the reaction mixture was poured into saturated $NaHCO_3$ solution and ethyl acetate extractions were carried out. The mixture obtained after drying of the extract over Na_2SO_4 and removal of the solvent was purified by silica gel column chromatography with an isocratic elution of CH_2Cl_2/ethyl acetate, 1:1 (v,v) containing 1% pyridine.

The desired phosphoramidite was obtained as white foam after evaporation of the solvent.

Compounds **1a**,[8,12] **3**,[15] **5**[16] and **20**[25] were synthesized according to the literature.

***N*⁴-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-2′,3′,5′-tri-*O*-acetyl-cytidine 6.** To a solution of **3** (10.30 g, 36.90 mmol) in dry THF (250 mL), under argon at 0 °C, was added *N*,*N*-diisopropylethylamine (6.4 mL, 36.90 mmol) and triphosgene (6.8 g, 22.91 mmol) in small portions over 10 min. The resulting mixture was stirred for 10 min at 0 °C then for 30 min at room temperature. The mixture was filtered and concentrated. The 2-[(triisopropylsilyl)oxy]-benzyl carbonochloridate **4** was directly used in the next step. To a stirred solution of **5** (15 g, 40.7 mmol, 1 equiv) in dry CH_2Cl_2 (750 mL), under nitrogen at 20 °C, was added *N*-methylimidazole (4.68 mL, 56.9 mmol, 1.4 equiv) and **4** (20 g, 56.9 mmol, 1.4 equiv). The resulting mixture was stirred at room temperature for 2 h and then diluted with CH_2Cl_2. The organic layer was quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient with MeOH (0−4%) in CH_2Cl_2. The desired compound **6** was obtained as white foam (17.6 g, 26 mmol, 64%). R_f = 0.85 (CH_2Cl_2/MeOH 90:10 v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (d, J = 7.6 Hz, 1H, H-6), 7.54 (s, 1H, N*H*), 7.37−6.72 (m, 5H, H ar, H-5), 6.15 (d, J = 4.2 Hz, 1H, H-1′), 5.42 (m, 1H, H-2′), 5.33 (m, 1H, H-3′), 5.29 (s, 2H, OCH_2Ar, tboc); 4.41 (m, 3H, H-5′, H-5″, H-4′), 2.2−2 (3s, 9H, 3 C(O)CH_3), 1.32 (m, 3H, 3 CH(CH_3)$_2$), 1.09 (d, J = 7.4 Hz, 18H, CH(CH_3)$_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.1, 169.6, 169.5 (3 O*C*═O), 162.6 (C_4), 154.8 (Cq ar-O), 154.7 (C_2), 152.2 (NHCO), 143.5 (C_6), 130.6−118.2 (4 CH, Car), 124.8 (Cq Car-CH_2), 95.4 (C_5), 88.8 ($C_{1'}$), 79.8 ($C_{4'}$), 73.8 ($C_{2'}$), 69.7 ($C_{3'}$), 64.5 (OCH_2Ar), 62.6 ($C_{5'}$), 20.8−20.5 (3 OCOCH_3), 18.0 (3 CH(*C*H_3)$_2$), 13.0 (3 *C*H(CH_3)$_2$). HRMS (ESI^+) m/z calcd for $C_{32}H_{46}N_3O_{11}Si$ (M + H)$^+$ 676.2902, found 676.2920.

***N*⁴-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-cytidine 7.** To a stirred solution of **6** (24 g, 35.6 mmol, 1 equiv) in EtOH (210 mL) was added a solution of sodium hydroxide 0.1 M in EtOH (356 mL, 35.6 mmol, 1 equiv). The resulting mixture was stirred at room temperature for 2 h and neutralized by addition of Dowex 50WX8 under pyridinium form. The resin was removed by filtration and the solvent was evaporated. The residue was subjected to silica gel column chromatography with a step gradient with MeOH (0−6%) in CH_2Cl_2. The desired compound **7** was obtained as white foam (17.6 g, 32 mmol, 95%). R_f = 0.21 (CH_2Cl_2/MeOH 90:10 v/v). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.81 (br, 1H, N*H*), 8.42 (d, J = 7.6 Hz, 1H, H-6), 7.37−6.72 (m, 5H, H ar, H-5), 5.78 (d, J = 2.5 Hz, 1H, H-1′), 5.50 (d, J = 2.8 Hz, 1H, OH-2′), 5.18 (m, 4H, OH-5′, OH-3′, OCH_2Ar), 3.99−3.90 (m, 5H, H-2′, H-3′, H-4′, H-5′, H-5″), 1.25 (m, 3H, 3 CH(CH_3)$_2$), 1,09 (d, J = 7.4 Hz, 18H, CH(CH_3)$_2$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.7 (C_4), 154.4 (Cq ar-O), 153.5 (C_2), 153.2 (NHCO), 144.9 (C_6), 129.8−117.8 (4 CH, Car), 125.4 (Cq ar-CH_2), 94.1 (C_5), 90.1 ($C_{1'}$), 84.2 ($C_{4'}$), 74.4 ($C_{2'}$), 68.7 ($C_{3'}$), 62.6 (OCH_2Ar), 59.9 ($C_{5'}$), 17.6 (3 CH(*C*H_3)$_2$), 12.0 (3 *C*H(CH_3)$_2$). HRMS (ESI^+) m/z calcd for $C_{26}H_{40}N_3O_8Si$ (M + H)$^+$ 550.2585, found 550.2587.

***N*⁴-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-3′,5′-*O*-di-*tert*-butylsilanediyl-cytidine 8.** To a solution of **7** (4.00 g, 7.28 mmol, 1eq) and silver nitrate (2.72 g, 16.00 mmol, 2.2eq) in dry DMF (72 mL) at 0 °C, was added dropwise di-*tert*-butylchlorosilane (2.6 mL, 8,00 mmol, 1.1 equiv). The mixture was stirred 10 min at 0 °C, then warmed to room temperature and stirred for 30 min. Et_3N (2.25 mL, 16.00 mmol, 2.2eq) was added and the mixture was stirred for an additional 10 min. The reaction mixture was quenched with water and extracted with ethyl acetate. The organic layer was washed with water, then brine, dried (Na_2SO_4), filtered and concentrated. The residue was subjected to silica gel column chromatography with a step gradient with MeOH (0−1%) in CH_2Cl_2. The desired compound **8** was obtained as white foam (4.48 g, 6.50 mmol, 89%). R_f = 0.58 (CH_2Cl_2/MeOH 90:10 v/v).). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.83 (s, 1H, NH), 8.00 (d, J = 7.5 Hz, 1H, H-6), 7.39−6.85 (m, 4H, H ar), 7.06 (d, J = 7.5 Hz, 1H, H-5), 5.75 (d, J = 4.5 Hz, 1H, OH-2′), 5.68 (s, 1H, H-1′), 5.18 (s, 2H, OCH_2Ar, tboc), 4.40 (m, 1H, H-5′), 4.16 (m, 2H, H-2′, H-5″), 4.03 (m, 1H, H-3′), 3.94 (m, 1H, H-4′), 1.30 (m, 3H, 3 CH(CH_3)$_2$), 1.08−0.96 (m, 36H, CH(CH_3)$_2$, tbu). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.9, 162.2, 153.9, 153.5, 153.1, 145.1, 129.8, 129.6, 125.4, 120.8, 117.8, 94.6, 93.0, 75.4, 73.9, 72.7, 66.6, 62.6, 27.2, 26.9, 17.7, 12.3, 11.9. HRMS (ESI^+) m/z calcd for $C_{34}H_{55}N_3O_8Si_2$ (M + H)$^+$ 690.3528, found 690.3599.

***N*⁴-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-3′,5′-*O*-di-*tert*-butylsilanediyl-2′-*O*-methylthiomethyl-cytidine 9.** To a stirred solution of **8** (4.30 g, 6.24 mmol) in dry DMSO (9.4 mL), acetic anhydride (9.4 mL) and acetic acid (12.5 mL) were added. The resulting mixture was stirred at 35 °C until completion (24 h). Then the mixture was added slowly to a solution of potassium carbonate (17 g) in water (85 mL) and extracted with ethyl acetate. The organic layer was washed with water (3 times) then brine, dried (Na_2SO_4), filtered and concentrated. The residue was subjected to silica gel column chromatography with a step gradient with ethyl

dx.doi.org/10.1021/jo200826h |*J. Org. Chem.* 2011, 76, 5719–5731

acetate (10−20%) in cyclohexane. The desired compound **9** was obtained as white foam (2.87 g, 3.83 mmol, 61%). R_f = 0.23 (cyclohexane/EtOAc 80:20 v/v). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.65 (d, J = 7.5 Hz, 1H, H-6), 7.27−6.76 (m, 5H, H ar, H-5), 5.76 (s, 1H, H-1′), 5.21 (s, 2H, OCH_2Ar, tboc), 4.95 (J = 11.4 Hz, 2H, OCH_2S), 4.48 (dd, J = 9.3 Hz, J = 4.8 Hz, 1H, H-5′), 4.42 (d, J = 4.5 Hz, 1H, H-2′), 4.16 (m, 1H, H-4′), 3.95 (t, J = 9.3 Hz, 1H, H-5″), 3.80 (dd, J = 9.3 Hz, J = 4.5 Hz, 1H, H-3′), 1.28 (m, 3H, 3 $CH(CH_3)_2$), 1.18−1.03 (m, 36H, $CH(CH_3)_2$, tbu). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.3, 153.6, 151.3, 142.1, 129.6, 129.2, 123.9, 119.9, 117.3, 93.8, 90.2, 76.3, 75.3, 74.2, 73.6, 66.6, 63.6, 26.4, 26.0, 25.5, 21.9, 19.4, 17.1, 12.3, 12.0. HRMS (ESI$^+$) m/z calcd for $C_{36}H_{59}N_3O_8SSi_2$ $(M + H)^+$ 750.3561, found 750.3612.

***N*^{4}-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-3′,5′-*O*-di-*tert*-butylsilanediyl-2′-*O*-pivaloyloxymethyl-cytidine 10.** To a solution of **9** (500 mg, 0.67 mmol, 1eq) in dry CH_2Cl_2 (6.7 mL) was added dropwise under argon a 1.0 M sulfuryl chloride solution in CH_2Cl_2 (0.8 mL, 0.80 mmol, 1.2eq). The reaction mixture was stirred at room temperature for 2 h. After completion, the solvent were removed and the obtained chloromethyl ether derivative was directly used in the next step. A solution of chloromethyl ether derivative in dry CH_2Cl_2 (6.7 mL) was added to a suspension of sodium pivalate (158 mg, 1.27 mmol, 1.9 equiv) and 15-crown-5 (0.1 mL, 0.50 mmol, 0.75eq) in dry CH_2Cl_2 (6.7 mL). After stirring at room temperature for 2 h, the mixture was diluted in ethyl acetate and filtered to eliminate the salt. The organic layer was washed with water, then brine, dried (Na_2SO_4), filtered and concentrated. The residue was subjected to silica gel column chromatography with a step gradient with ethyl acetate (10−30%) in cyclohexane. The desired compound **10** was obtained as white foam (467 mg, 0.58 mmol, 87%). R_f = 0.23 (cyclohexane/EtOAc 80:20 v/v). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.63 (d, J = 7.5 Hz, 1H, H-6), 7.29 (m, 1H, Har), 7.23 (d, J = 7.5 Hz, 1H, H-5), 7.14 (m, 1H, Har), 6.86 (m, 1H, Har), 6.77 (m, 1H, Har), 5.69 (s, 1H, H-1′), 4.95 (J = 6.3 Hz, 2H, OCH_2O), 5.22 (s, 2H, OCH_2Ar, tboc), 4.46 (dd, J = 9.3 Hz, J = 5.1 Hz, 1H, H-5′), 4.37 (d, J = 4.5 Hz, 1H, H-2′), 4.14 (m, 1H, H-4′), 3.99 (t, J = 9.3 Hz, 1H, H-5″), 3.80 (dd, J = 9.3 Hz, J = 4.5 Hz, 1H, H-3′), 2.15 (s, 3H, CH_3S), 1.23 (m, 3H, 3 $CH(CH_3)_2$), 1.18−1.03 (m, 36H, $CH(CH_3)_2$, tbu). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.9, 162.5, 154.7, 154.3, 152.3, 152.2, 143.0, 130.7, 130.2, 124.9, 118.3, 113.2, 94.9, 92.3, 87, 79.5, 75.7, 74.9, 67.5, 64.5, 27.3, 43.6, 30.3, 27.3, 27.0, 26.5, 22.8, 20.5, 18.1, 13.0. HRMS (ESI$^+$) m/z calcd for $C_{40}H_{65}N_3O_{10}Si_2$ $(M + H)^+$ 804.4208, found 804.4278.

***N*^{4}-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-pivaloyloxymethyl-cytidine 12.** To a solution of **10** (467 mg, 0.58 mmol, 1eq) in dry CH_2Cl_2 (5 mL), under argon at 0 °C, was added dropwise a solution HF·pyridine (70%, 60 μL, 2.20 mmol, 3.8 equiv) diluted in dry pyridine (2.5 mL). The mixture was stirred at 0 °C for 1 h. The reaction was quenched with saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2 (twice). The organic layer was washed with brine, dried (Na_2SO_4), filtered and concentrated. The compound **11** was obtained as white foam and was directly used in the next step. R_f = 0.36 (CH_2Cl_2/MeOH 95:5 v/v). Compound **11** was dried by 3 coevaporations with dry pyridine. Then to a stirred solution of **11** in dry pyridine (2 mL), under nitrogen at 20 °C, was added dimethoxytrityl chloride (180 mg, 0.53 mmol, 1.1 equiv) in small portions over 20 min. The resulting mixture was stirred at room temperature overnight. After completion, the reaction was quenched with saturated aqueous $NaHCO_3$ and extracted with ethyl acetate. The organic layer was washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient with ethyl acetate (20−40%) in cyclohexane containing 1% pyridine. The desired compound **12** was obtained as white foam (290 mg, 0.30 mmol, 63%). R_f = 0.5 (CH_2Cl_2/EtOAc 30:70 v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.41 (d, J = 7.5 Hz, 1H, H-6), 7.35 (br, 1H, N*H*), 7.34−6.76 (m, 18H, H ar, H-5), 5.86 (s, 1H, H-1′), 5.63 and 5.20 (J = 5.3 Hz, 2H, OCH_2O), 5.18 (s, 2H, OCH_2Ar, tboc), 4.32 (m, 1H, H-3′), 4.21 (d, J = 5.3 Hz, 1H, H-2′), 3.96 (m, 1H, H-4′), 3.62 (s, 6H, 2 OCH_3), 3.35 (m, H-5′, H-5″), 2.30 (d, J = 10.9 Hz, 1H, OH-3′), 1.28 (m, 3H, 3 $CH(CH_3)_2$), 1.09 (m, 27H, $CH(CH_3)_2$, $C(O)(CH_3)_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.2 (C═O), 162.2 (C_4), 158.7 (Cq, Car), 154.9 (Cq ar-O), 154.5 (C_2), 152.1 (NHCO), 144.3 (Cq, Car), 144.1 (C_6), 135.6−113.3 (17 CH, Car), 125.0 (Cq ar-CH_2), 94.9 (C_5), 89.7 ($C_{1'}$), 87.6 (OCH_2O), 87.0 (OCq, DMTr), 83.1 ($C_{4'}$), 81.6 ($C_{2'}$), 67.5 ($C_{3'}$), 64.2 (OCH_2Ar), 60.7 ($C_{5'}$), 55.2 (OCH_3, DMTr), 38.9 (Cq, $OCOC(CH_3)_3$), 26.9 ($OCOC(CH_3)_3$), 18.0 (3 $CH(CH_3)_2$), 13.0 (3 $CH(CH_3)_2$). HRMS (ESI$^+$) m/z calcd for $C_{47}H_{58}N_3O_{10}Si$ $(M + H)^+$ 966.4572, found 966.4561.

***N*^{4}-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-5′-*O*-(4,4′-dimethoxytrityl)-cytidine 13.** Compound **7** (250 mg, 0.46 mmol, 1eq.) was dried by 3 coevaporations with dry pyridine. Then to a stirred solution of **7** in dry pyridine (4 mL), under nitrogen at 20 °C, was added dimethoxytrityl chloride (190 mg, 0.55 mmol, 1.2 equiv) in small portions over 20 min. The resulting mixture was stirred at room temperature for 2 h. After completion, the mixture was concentrated and CH_2Cl_2 was added. The organic layer was washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient with MeOH (0−3%) in CH_2Cl_2 containing 1% pyridine. The desired compound **13** was obtained as white foam (325 mg, 0.38 mmol, 83%). R_f = 0.5 (CH_2Cl_2/MeOH 94:6 v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, J = 7.5 Hz, 1H, H-6), 7.67 (br, 1H, N*H*), 7.26−6.74 (m, 18H, H ar, H-5), 5.77 (d, J = 3.8 Hz, 1H, H-1′), 5.20 (s, 2H, OCH_2Ar, tboc), 4.32 (m, 4H, OH-2′, H-2′, H-3′, H-4′), 3.70 (s, 6H, 2 OCH_3), 3.35 (m, 3H, OH-3′, H-5′, H-5″), 1.28 (m, 3H, 3 $CH(CH_3)_2$), 1.09 (d, J = 7.4 Hz, 18H, $CH(CH_3)_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.6 (C_4), 158.6 (Cq, Car), 156.6 (Cq ar-O), 154.4 (C_2), 152.2 (NHCO), 144.3 (Cq, Car), 144.1 (C_6), 130.3- 113.3 (17 CH, Car), 125.0 (Cq ar-CH_2), 95.1 (C_5), 93.4 ($C_{1'}$), 87.0 ($C_{4'}$), 85.8 (OCq, DMTr), 76.7 ($C_{2'}$), 69.7 ($C_{3'}$), 64.2 (OCH_2Ar), 62.7 ($C_{5'}$), 55.2 (OCH_3, DMTr), 18.0 (3 $CH(CH_3)_2$), 13.0 (3 $CH(CH_3)_2$). HRMS (ESI$^+$) m/z calcd for $C_{47}H_{58}N_3O_{10}Si$ $(M + H)^+$ 852.3891, found 852.3893.

***N*^{4}-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-cytidine 14.** To a stirred solution of **13** (9.73 g, 11.42 mmol, 1 equiv) in dry pyridine (70 mL), under nitrogen at 20 °C, was added imidazole (1.74 g, 29.96 mmol, 2.6 equiv) and *tert*-butyldimethylsilyl chloride (2.28 g, 15.13 mmol, 1.3 equiv). The resulting mixture was stirred at room temperature for 48 h and then diluted with CH_2Cl_2. The organic layer was quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient with acetone (0−10%) in CH_2Cl_2 containing 1% pyridine. The compound **14** was collected and 3′-*O*-TBS derivative was isomerized in MeOH for 24 h. Purification by chromatography furnished more **14** as white foam, after three isomerisations, pure **14** is obtained (7.76 g, 8.03 mmol, 70%). R_f = 0.51 (CH_2Cl_2/MeOH 98:2 v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (d, J = 7.4 Hz, 1H, *H*-6), 7.38 (br, 1H, N*H*), 7.25−6.64 (m, 18H, H ar, *H*-5), 5.70 (s, 1H, *H*-1′), 5.07 (s, 2H, OCH_2Ar, tboc), 4.17 (m, 1H, *H*-3′), 4.10 (m, 1H, *H*-2′), 3.92 (m, 1H, *H*-4′), 3.61 (s, 6H, 2 OCH_3), 3.47 (m, 2H, *H*-5′, *H*-5″), 2.22 (d, J = 10.9 Hz, 1H, O*H*-3′), 1.14 (m, 3H, 3 $CH(CH_3)_2$), 0.95 (m, 27H, $C(O)C(CH_3)_3$, $CH(CH_3)_2$), 0.74 (s, 9H, $SiC(CH_3)_3$), 0.12, 0.00 (2s, 6H, 2 $SiCH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.3 (C_4), 158.7 (Cq, Car), 154.9 (Cq ar-O), 154.5 (C_2), 152.2 (NHCO), 144.6 (Cq, Car), 144.1 (C_6), 135.6−113.3 (17 CH, Car), 125.0 (Cq ar-CH_2), 94.7 (C_5), 90.7 ($C_{1'}$), 87.1 (OCq, DMTr), 83.1 ($C_{4'}$), 76.6 ($C_{2'}$), 69.1 ($C_{3'}$), 64.1 (OCH_2Ar), 61.4 ($C_{5'}$), 55.2 (OCH_3, DMTr), 25.8 ($SiC(CH_3)_3$), 18.0 (3 $CH(CH_3)_2$), 13.0

dx.doi.org/10.1021/jo200826h |*J. Org. Chem.* 2011, 76, 5719–5731

(3 $CH(CH_3)_2$), −4.3 and −5.4. (2 $SiCH_3$). HRMS (ESI^+) m/z calcd for $C_{53}H_{72}N_3O_{10}Si_2$ $(M + H)^+$ 966.4756, found 966.4770.

***N*⁴-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-pivaloyloxymethyl-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-cytidine 1b.** Using general procedure starting from **12** (1.7 g, 1.8 mmol) afforded **1b** (1.73 g, 1.48 mmol, 82% yield). ^{31}P NMR (121 MHz, CD_3CN) δ 150.24, 148.54. HRMS (ESI^+) m/z calcd for $C_{62}H_{85}N_5O_{13}PSi$ $(M + H)^+$ 1166.5651, found 1166.5664.

***N*⁴-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-cytidine 2b.** Using general procedure starting from **14** (2.58 g, 2.67 mmol) afforded **2b** (2.7 g, 2.31 mmol, 86% yield). ^{31}P NMR (121 MHz, CD_3CN) δ 149.58, 148.88. HRMS (ESI^+) m/z calcd for $C_{62}H_{89}N_5O_{11}PSi_2$ $(M + H)^+$ 1166.5835, found 1166.5848.

5-*O*-(4,4′-Dimethoxytrityl)-2′,3′-bis-*O*-acetyl-adenosine 15. Adenosine (2.7 g, 10 mmol, 1 equiv) was dried by 3 coevaporations with dry pyridine. Then to a stirred solution of adenosine in dry pyridine (50 mL), under nitrogen at 20 °C, was added DMAP (60 mg, 0.5 mmol, 0.05 equiv) and dimethoxytrityl chloride (4 g, 12 mmol, 1.2 equiv) in small portions over 20 min. The mixture was stirred at room temperature for 3 h. To the resulting solution of 5′-*O*-(4,4′-dimethoxytrityl)-adenosine in pyridine was added DMAP (120 mg, 1 mmol, 0.1 equiv), Et_3N (2.28 mL, 20 mmol, 2 equiv) and acetic anhydride (2.26 mL, 24 mmol, 2.4 equiv). The resulting mixture was stirred at room temperature for 30 min and then diluted with CH_2Cl_2. The organic layer was quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient with MeOH (0−10%) in CH_2Cl_2 containing 1% pyridine. The desired compound **15** was obtained as yellow foam (3.92 g, 6 mmol, 60%). R_f = 0.22 (CH_2Cl_2/MeOH 94:6 v/v). ^1H NMR (400 MHz, $CDCl_3$) δ 8.33 (s, 1H, *H*-2), 7.99 (s, 1H, *H*-8), 7.46−6.81 (m, 13H, H-ar, DMTr), 6.34 (d, J = 6.9 Hz, 1H, *H*-1′), 6.18−6.08 (m, 3H, *H*-2′,NH_2), 5.71 (dd, J = 5.2 Hz; J = 2.8 Hz, 1H, *H*-3′), 4.36 (m, 1H, *H*-4′), 3.79 (s, 6H, 2 OCH_3), 3.53 (dd, J = 10.5 Hz, J = 3 Hz, 1H, *H*-5′), 3.46 (dd, J = 10.5 Hz, J = 3.5 Hz, 1H, *H*-5″), 2.16−2.10 (2s, 6H, C(O)CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.6, 169.4 (OC═O), 158.6 (Cq, Car), 155.7 (C_6), 153.4 (C_2), 150.2 (C_4), 144.1, 141.2 (Cq, Car), 138.5 (C_8), 130.2, 129, 128.2, 128, 127.1, 113.3 (CH, Car), 119.7 (C_5), 87.0 (OCq, DMTr), 86.5 ($C_{4'}$), 84.5 ($C_{1'}$), 73.2 ($C_{2'}$), 70.6 ($C_{3'}$), 63.1 ($C_{5'}$), 55.3 (OCH_3, DMTr), 20.7, 20.5 (OC(O)CH_3). HRMS (ESI^+) m/z calcd for $C_{35}H_{36}N_5O_8$ $(M + H)^+$ 654.2564, found 654.2580.

***N*⁶-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-5′-*O*-(4,4′-dimethoxytrityl)-2′,3′-bis-*O*-acetyl-adenosine 16.** A solution of **15** (17 g, 26 mmol, 1 equiv) in dry CH_2Cl_2 (78 mL) was added during 20 min to a suspension obtained from 1.9 M $COCl_2$ in toluene (20.6 mL, 39 mmol, 1.5 equiv) and DMAP (317 mg, 2.6 mmol, 0.1 equiv) in dry pyridine (52 mL) and dry CH_2Cl_2 (104 mL). After 20 min at 20 °C, a solution of **3** (22 g, 78 mmol, 3 equiv) in triethylamine (14.5 mL, 104 mmol, 4 equiv) was added dropwise. The resulting mixture was stirred at room temperature for 36 h and then diluted with CH_2Cl_2. The organic layer was quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient with MeOH (0−2%) in CH_2Cl_2. The desired compound **16** was obtained as white foam (21.72 g, 22.6 mmol, 87%). R_f = 0.19 (CH_2Cl_2/MeOH 97:3 v/v). ^1H NMR (400 MHz, $CDCl_3$) δ 8.73 (s, 1H, *H*-2), 8.15 (br, 1H, N*H*), 8.12 (s, 1H, *H*-8), 7.45−6.75 (m, 17H, H ar, DMTr-tboc), 6.36 (d, J = 6.8 Hz, 1H, *H*-1′), 6.14 (dd, 1H, J = 6.8 Hz, J = 5.5 Hz, *H*-2′), 5.72 (dd, J = 5.2 Hz, J = 2.8 Hz, 1H, *H*-3′), 5.4 (s, 2H, OCH_2Ar, tboc), 4.38 (m, 1H, *H*-4′), 3.79 (s, 6H, 2 OCH_3), 3.51 (m, 2H, *H*-5′, *H*-5″), 2.16 and 2.07 (2s, 6H, C(O)CH_3), 1.37 (m, 3H, 3 $CH(CH_3)_2$), 1.13 (d, J = 7.4 Hz, 18H, $CH(CH_3)_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.7, 169.3 (OC═O), 158.6 (C, Car), 154.3 (C ar-O), 153.1 (C_2), 151.4 (NHCO), 150.8 (C_4), 149.5 (C_6), 144.1, 140.8 (Cq, Car), 135.2 (C_8), 130.2−113.3 (17 CH, Car), 125.5 (Cq ar-CH_2), 122.1 (C_5), 87.1 (OCq, DMTr), 84.8 ($C_{1'}$), 82.6 ($C_{4'}$), 73.2 ($C_{2'}$), 71.7 ($C_{3'}$), 63.8 (OCH_2Ar), 63.0 ($C_{5'}$), 55.3 (OCH_3, DMTr), 20.7, 20.4 (OC(O)CH_3), 18.0 (3 $CH(CH_3)_2$), 12.7 (3 $CH(CH_3)_2$). HRMS (ESI^+) m/z calcd for $C_{52}H_{62}N_5O_{11}Si$ $(M + H)^+$ 960.4225, found 960.4215.

***N*⁶-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-5′-*O*-(4,4′-dimethoxytrityl)-adenosine 17.** To a stirred solution of **16** (16 g, 16.7 mmol, 1 equiv) in EtOH (120 mL) was added a solution of sodium hydroxide 0.1 M in EtOH (200 mL, 20 mmol, 1.2 equiv). The resulting mixture was stirred at room temperature for 1 h and neutralized by addition of Dowex 50WX8 under pyridinium form. The resin was removed by filtration and the solvent was evaporated. The residue was subjected to silica gel column chromatography with a step gradient with MeOH (0−6%) in CH_2Cl_2. The desired compound **17** was obtained as white foam (13.46 g, 15.36 mmol, 92%). R_f = 0.45 (CH_2Cl_2/MeOH 94:6 v/v). ^1H NMR (400 MHz, $CDCl_3$) δ 8.58 (s, 1H, *H*-2), 8.42 (br, 1H, N*H*), 8.08 (s, 1H, *H*-8), 7.32−6.61 (m, 17H, H ar, DMTr-tboc), 5.91 (d, J = 5.8 Hz, 1H, *H*-1′), 5.74 (d, J = 3.1 Hz, 1H, O*H*-2′), 5.29 (s, 2H, OCH_2Ar, tboc), 4.77 (m, 1H, *H*-2′), 4.35 (m, 2H, *H*-4′, *H*-3′), 3.65 (s, 6H, 2 OCH_3), 3.46 (d, J = 2 Hz, 1H, O*H*-3′), 3.23 (m, 2H, *H*-5′, *H*-5″), 1.28 (m, 3H, 3 $CH(CH_3)_2$), 1.02 (d, J = 7,3 Hz, 18H, CH-$(CH_3)_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.6 (Cq, Car), 154.3 (Cq ar-O), 152.4 (C_2), 150.8 (NHCO), 150.3 (C_4), 149.6 (C_6), 144.3, 141.2 (Cq, Car), 135.3 (C_8), 130.2−113.1 (17 CH, Car), 125.5 (Cq ar-CH_2), 122.2 (C_5), 90.7 ($C_{1'}$), 86.6 (OCq, DMTr), 86.1 ($C_{4'}$), 75.8 ($C_{2'}$), 72.7 ($C_{3'}$), 63.9 (OCH_2Ar), 63.5 ($C_{5'}$), 55.2 (OCH_3, DMTr), 18.0 (3 $CH(CH_3)_2$), 13.0 (3 $CH(CH_3)_2$). HRMS (ESI^+) m/z calcd for $C_{48}H_{58}N_5O_9Si$ $(M + H)^+$ 876.4004, found 876.3996.

***N*⁶-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-pivaloyloxymethyl-adenosine 18.** To a solution of **17** (700 mg, 0.8 mmol, 1 equiv) in DCE (4 mL) were added TBAB (335 mg, 1.04 mmol, 1.3 equiv), dibutyltin oxide (260 mg, 1.04 mmol, 1.3 equiv) and iodomethyl pivalate (484 μL, 2 mmol, 2.5 equiv). The reaction mixture was stirred under nitrogen at 75 °C for 2 h, cooled and the solvent was evaporated. The reaction mixture was subjected to silica gel column chromatography with a step gradient with acetone (0−30%) in CH_2Cl_2. The first-eluted isomer was the desired compound **18** and was obtained as white foam (221 mg, 0.22 mmol, 28%). R_f = 0.6 (CH_2Cl_2/MeOH 97:3 v/v). ^1H NMR (400 MHz, $CDCl_3$) δ 8.59 (s, 1H, *H*-2), 8.42 (br, 1H, N*H*), 8.06 (s, 1H, *H*-8), 7.36−6.70 (m, 17H, H ar, DMTr-tboc), 6.08 (d, J = 4.6 Hz, 1H, *H*-1′), 5.43 and 5.30 (2d, J = 5.4 Hz, 1H + 1H, OCH_2O), 5.29 (s, 2H, OCH_2Ar, tboc), 4.98 (t, J = 4.6 Hz, 1H, *H*-2′), 4.45 (m, 1H, *H*-3′), 4.18 (m, 1H, *H*-4′), 3.65 (s, 6H, 2 OCH_3), 3.23 (m, 2H, *H*-5′, *H*-5″), 2.54 (d, J = 5.3 Hz, 1H, O*H*-3′), 1.28 (m, 3H, 3 $CH(CH_3)_2$), 1.05 (m, 27H, C(O)$C(CH_3)_3$, $CH(CH_3)_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.9 (OC═O), 158.6 (Cq, Car), 154.3 (Cq ar-O), 152.9 (C_2), 150.8 (NHCO), 150.8 (C_4), 149.5 (C_6), 144.4, 141.5 (Cq, Car), 141.3 (C_8), 130.2- 113.2 (17 CH, Car), 125.5 (Cq ar-CH_2), 122.8 (C_5), 89.0 (OCH_2O), 87.3 ($C_{1'}$), 86.6 (OCq, DMTr), 84.1 ($C_{4'}$), 81.7 ($C_{2'}$), 70.5 ($C_{3'}$), 63.8 (OCH_2Ar), 62.9 ($C_{5'}$), 55.2 (OCH_3, DMTr), 38.9 (Cq, $OCOC(CH_3)_3$), 26.9 ($OCOC(CH_3)_3$), 18.0 (3 $CH(CH_3)_2$), 13.0 (3 $CH(CH_3)_2$). HRMS (ESI^+) m/z calcd for $C_{54}H_{68}N_5O_{11}Si$ $(M + H)^+$ 990.4685, found 990.4678.

***N*⁶-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-adenosine 19.** To a stirred solution of **17** (1.5 g, 1.71 mmol, 1 equiv) in dry pyridine (10 mL), under nitrogen at 20 °C, were added imidazole (350 mg, 5.1 mmol, 3 equiv) and *tert*-butyldimethylsilyl chloride (370 mg, 5.1 mmol, 3 equiv). The resulting mixture was stirred at room

dx.doi.org/10.1021/jo200826h |*J. Org. Chem.* 2011, 76, 5719–5731

temperature for 12 h and then diluted with CH_2Cl_2. The organic layer was quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient with ethyl acetate (0−10%) in cyclohexane containing 1% pyridine. The compound **19** was collected and 3′-*O*-TBS derivative was isomerized in MeOH for 24 h. Purification by chromatography furnished **19** as white foam, after three isomerisations (1.2 g, 1.21 mmol, 71%). R_f = 0.48 (EtOAc/Cyclohexane 40:60 v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.72 (s, 1H, *H*-2), 8.18 (s, 1H, *H*-8), 8.42 (s, 1H, N*H*), 7.49−6.81 (m, 17H, H ar, DMTr-tboc), 6.09 (d, J = 5.4 Hz, 1H, *H*-1′), 5.40 (s, 2H, OCH_2Ar, tboc), 5.02 (t, J = 5.2 Hz, 1H, *H*-2′), 4.35 (m, 1H, *H*-3′), 4.25 (m, 1H, *H*-4′), 3.80 (s, 6H, 2 OCH_3), 3.45 (m, 2H, *H*-5′, *H*-5″), 2.73 (d, J = 3.9 Hz, 1H, O*H*-3′), 1.28 (m, 3H, 3 CH(CH_3)$_2$), 1.05 (m, 18H, 3 CH(CH_3)$_2$), 0.82 (s, 9H, SiC(CH_3)$_3$), (−)0.05-(−)0.18 (2s, 6H, 2 SiCH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.9 (OC═O), 158.6 (Cq, Car), 154.2 (Cq ar-O), 152.9 (C_2), 151.1 (NHCO), 150.9 (C_4), 149.5 (C_6), 144.6, 141.5 (Cq, Car), 141.4 (C_8), 130.2- 113.2 (17 CH, Car), 125.6 (Cq ar-CH_2), 122.3 (C_5), 88.4 ($C_{1'}$), 86.7 (OCq, DMTr), 84.3 ($C_{4'}$), 75.6 ($C_{2'}$), 71.6 ($C_{3'}$), 63.8 (OCH_2Ar), 63.3 ($C_{5'}$), 55.2 (OCH_3, DMTr), 25.6 (SiC(CH_3)$_3$), 18.0 (3 CH(CH_3)$_2$), 13.0 (3 CH(CH$_3$)$_2$), −5.0 and −5.1. (2 SiCH_3). HRMS (ESI$^+$) m/z calcd for $C_{54}H_{72}N_5O_9Si_2$ $(M + H)^+$ 990.4869, found 990.4863.

***N*^{6}-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-pivaloyloxymethyl-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-adenosine 1c.** Using general procedure starting from **18** (4.3 g, 4.35 mmol) afforded **1c** (4 g, 3.36 mmol, 77% yield). ^{31}P NMR (121 MHz, CD_3CN) δ 150.71, 149.76. HRMS (ESI$^+$) m/z calcd for $C_{63}H_{85}N_7O_{12}PSi$ $(M + H)^+$ 1190.5763, found 1190.5771.

***N*^{6}-{{{2-[(Triisopropylsilyl)oxy]benzyl}oxy}carbonyl}-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-adenosine 2c.** Using general procedure starting from **19** (1.5 g, 1.55 mmol) afforded **2c** (1.3 g, 1.09 mmol, 73% yield). ^{31}P NMR (121 MHz, CD_3CN) δ 150.18, 148.63. HRMS (ESI$^+$) m/z calcd for $C_{63}H_{89}N_7O_{10}PSi_2$ $(M + H)^+$ 1190.5947, found 1190.5952.

***N*^{2},5′-*O*-Bis-(4,4′-dimethoxytrityl)-2′-*O*-pivaloyloxymethyl-guanosine 21.** To a solution of **20** (2.1 g, 2.34 mmol, 1 equiv) in DCE (13 mL) were added TBAB (1.88 g, 5.85 mmol, 2.5 equiv), dibutyltin oxide (1.46 g, 5.85 mmol, 2.5 equiv) and chloromethyl pivalate (1.76 mL, 11.7 mmol, 5 equiv). The reaction mixture was stirred under nitrogen at 75 °C for 6 h, cooled and the solvent was evaporated. The reaction mixture was subjected to silica gel column chromatography with a step gradient with acetone (0−30%) in CH_2Cl_2. The first-eluted isomer was the desired compound **21** and was obtained as white foam after evaporation of the solvent (582 mg, 0.58 mmol, 25%). R_f = 0.45 (CH_2Cl_2/MeOH 95:5 v/v). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.68 (br, 1H, N*H*-ar), 7.77 (s, 1H, *H*-8), 7.58 (br, 1H, N*H*), 7.38−6.78 (m, 26H, H-ar, DMTr), 5.32 (d, J = 3.9 Hz, 1H, *H*-1′), 5.11 and 4.83 (2d, J = 6.4 Hz, 1H + 1H, OCH_2O), 5.01 (d, J = 7.4 Hz, 1H, O*H*-3′), 4.05 (m, 1H, H-2′), 3.81 (m, 1H, H-4′), 3.72−3.62 (2s, 12H, 4 OCH_3), 3.58 (m, 1H, H-3′), 3.09 (m, 2H, *H*-5′, *H*-5″), 0.96 (s, 9H, C(O)C(CH_3)$_3$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.0 (OC═O), 158.6 (Cq, Car), 158.1 (Cq, Car), 156.9 (C_6), 151.6 (C_4), 149.7 (C_2), 145.3−113.4 (CH, Cq, Ar), 136.0 (C_8), 118.2 (C_5), 88.1 (OCH_2O), 86.9 ($C_{1'}$), 86.1 (OCq, DMTr), 82.5 ($C_{4'}$), 79.8 ($C_{2'}$), 69.9 ($C_{3'}$), 69.6 (NHCq, DMTr), 63.5 ($C_{5'}$), 55.4 (OCH_3, DMTr), 38.5 (Cq, OCOC(CH$_3$)$_3$), 26.8 (OCOC(CH_3)$_3$). HRMS (ESI$^+$) m/z calcd for $C_{58}H_{60}N_5O_{11}$ $(M + H)^+$ 1002.4289, found 1002.4308.

***N*^{2},5′-*O*-Bis-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-guanosine 22.** To a stirred solution of **20** (6 g, 6.75 mmol, 1 equiv) in a mixture of dry THF (50 mL) and dry pyridine (7 mL), under nitrogen at 20 °C, were added silver nitrate (1.6 g, 9.45 mmol, 1.4 equiv) and *tert*-butyldimethylsilyl chloride (2.55 g, 16.9 mmol, 2.5 equiv). The resulting mixture was stirred at room temperature for 5 h and then diluted with CH_2Cl_2. The organic layer was quenched with saturated aqueous $NaHCO_3$, washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was subjected to silica gel column chromatography with a step gradient with MeOH (0−5%) in CH_2Cl_2 containing 1% pyridine. The compound **22** was collected and 3′-*O*-TBS derivative was isomerized in MeOH in the presence of imidazole for 24 h. Purification by chromatography furnished **22** as white foam, after three isomerisations (4.7 g, 4.68 mmol, 69%). R_f = 0.52 (CH_2Cl_2/MeOH 95:5 v/v). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.77 (br, 1H, N*H*-ar), 7.84 (s, 1H, *H*-8), 7.58 (br, 1H, N*H*), 7.44−6.85 (m, 26H, H-ar, DMTr), 5.25 (d, J = 4.6 Hz, 1H, *H*-1′), 4.80 (d, J = 5.5 Hz, 1H, O*H*-3′), 4.32 (m, 1H, H-2′), 3.94 (m, 2H, H-3′, H-4′), 3.92−3.71 (2s, 12H, 4 OCH_3), 3.17 (m, 2H, *H*-5′, *H*-5″), 0.82 (s, 9H, SiC(CH_3)$_3$), 0-(−)0.15 (2s, 6H, 2 SiCH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 158.0 (Cq, Car), 157.6 (Cq, Car), 156.5 (C_6), 151.1 (C_4), 150.1 (C_2), 145.1−112.9 (CH, Cq, Ar), 136.1 (C_8), 117.0 (C_5), 86.5 ($C_{1'}$), 85.5 (OCq, DMTr), 82.6 ($C_{3'}$), 74.9 ($C_{2'}$), 70.2 ($C_{4'}$), 69.4 (NHCq, DMTr), 64.1 ($C_{5'}$), 54.9 (OCH_3, DMTr); 25.7 (SiC(CH_3)$_3$), (−)4.8, (−)5.2 (2 SiCH_3). HRMS (ESI$^+$) m/z calcd for $C_{58}H_{64}N_5O_9Si$ $(M + H)^+$ 1002.4473, found 1002.4376.

***N*^{2},5′-*O*-Bis-(4,4′-dimethoxytrityl)-2′-*O*-pivaloyloxymethyl-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-guanosine 1d.** Using general procedure starting from **21** (1.17 g, 1.17 mmol) afforded **1d** (1.2 g, 1 mmol, 88% yield). ^{31}P NMR (121 MHz, CD_3CN) δ 150.16, 149.90. HRMS (ESI$^+$) m/z calcd for $C_{67}H_{77}N_7O_{12}P$ $(M+H)^+$ 1202.5368, found 1202.5376.

***N*^{2},5′-*O*-Bis-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-guanosine 2d.** Using general procedure starting from **22** (1.14 g, 1.14 mmol) afforded **2d** (1 g, 0.83 mmol, 71% yield). ^{31}P NMR (121 MHz, CD_3CN) δ 151.01, 147.96. HRMS (ESI$^+$) m/z calcd for $C_{67}H_{81}N_7O_{10}PSi$ $(M + H)^+$ 1202.5552, found 1202.5573.

5-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-guanosine 2e. A solution of the commercial 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-TBS-*N*^{2}-(*tert*-butylphenoxyacetyl)-guanosine (0.9 g, 0.83 mmol, 1equiv) in 2 M $MeNH_2$ (40% aqueous solution) (0.7 mL) in THF (10 mL) was stirred at room temperature for 20 min. The reaction mixture was concentrated and coevaporated twice with CH_3CN. The residue was subjected to silica gel column chromatography with a step gradient with MeOH (0−3%) in CH_2Cl_2 containing 1% pyridine. The fractions containing pure **2e** were pooled and concentrated to give white foam (0.63 g, 0.70 mmol, 84% yield). ^{31}P NMR (121 MHz, CD_3CN) δ 151.03, 149.15. HRMS (ESI$^+$) m/z calcd for $C_{46}H_{63}N_7O_8PSi$ $(M + H)^+$ 900.4245, found 900.4230.

Oligonucleotide Synthesis. The oligonucleotides were synthesized at 1-μmol scale on DNA synthesizer (ABI 381A) by using phosphoramidite building blocks **1a**−**d**, **2a**−**e**, and **thymidine**. Syntheses were carried out on thymidine-Q-linker CPG support, 5-benzylmercaptotetrazole (BMT) was used as the activator for coupling, the capping step was performed with phenoxyacetic anhydride, using a commercial solution (Cap A: Pac_2O/pyridine/THF 10:10:80 v/v/v and Cap B: 10% *N*-methylimidazole in THF). Oxidation was performed with a commercial solution of iodide (0.1 M I_2, THF, pyridine/water 90:5:5, v/v/v). Detritylation was performed with 3% TCA in CH_2Cl_2. See Supporting Information for detailed oligonucleotides automated synthesis conditions.

General Procedure for Deprotection of Solid-Supported Oligonucleotides. CPG beads were treated with an anhydrous 1 M DBU solution in CH_3CN (3 mL) at room temperature for 5 min to get rid of cyanoethyl groups. The solution was removed by filtration and the support was washed with CH_3CN (5 mL) then dried to be transferred

into a screw-capped O-ring eppendorf. The support containing 1 μmol of the oligonucleotide was suspended in a mixture (pH 8.3) of HF 48 wt.% in H_2O (0.5 mL) and Et_3N (1.5 mL) at 65 °C for 10 h. After centrifugation, the supernatant was transferred in a round flask, the support was washed with water (0.5 mL × 2). After centrifugation, the combined supernatants were concentrated under reduced pressure. The crude material diluted in water (1 mL) was loaded onto a home-made RP C_8 column (3 × 1 cm) equilibrated in water. The column was first flushed with water (20 mL) to remove the salts. Then the desalted ON was eluted with 50% acetonitrile (10 mL). Further purification was performed using RP-HPLC. ON purity was confirmed by RP-HPLC, anion-exchange HPLC and MALDI-TOF MS.

Melting Experiments. T_m experiments were performed on an UV spectrophotometer equipped with a Peltier temperature controller and a thermal analysis software. The samples were prepared by mixing each strand together to give 3 μM final concentration in 10 mM sodium cacodylate, 100 mM NaCl, pH 7. A heating−cooling cycle in the 0−95 °C temperature range with a gradient of 0.5 °C.min^{-1} was applied. T_m values were determined from the maxima of the first derivative plots of absorbance versus temperature.

Enzymatic Digestion. pro-RNA **26** (1 OD unit at 260 nm) was incubated with nuclease P1 (0.125 units) at 37 °C for 48 h. Then alkaline phosphatase (1.25 units) and buffer (50 mM Tris-HCl, pH 9.3, containing 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine; final concentrations) were added to give a total volume of 100 μL and the mixture was incubated at 37 °C for a further 24 h. The reaction mixture was then analyzed by HPLC.

Oligonucleotides for Cell Culture Studies. All ON used in this study were synthesized as described above or purchased from IBA (Göttingen, Germany). Before use, the integrity and purity of the single-stranded siRNAs were checked by electrophoresis on 20% denaturing polyacrylamide gels. For annealing of complementary RNA strands, equimolar amounts of sense- and antisense strands respectively were incubated in 20 mM Tris/HCl pH 7.4 and 100 mM NaCl for 2 min at 95 °C followed by slow cooling to room temperature. Oligoribonucleotides sequences are as follows: siR206, sense strand, 5′-GAUAUGGGCUGAAUACAAAdTdT-3′; antisense strand, 5′-UUUGUAUUCAGCCCAUAUCdTdT-3′; siR206-PivOM, sense strand **26** 5′-***GAUAU***GGGCUGAAUACAAAdTdT-3′ (PivOM-modified nucleotides indicated in bold and italic); antisense strand, 5′-UUUGUAUUCAGCCCAUAUCdTdT-3′; siscr3, sense strand, 5′-CGGACGCACUGGUCUGACCGGdTdT-3′; antisense strand, 5′-CCGGUCAGACCAGUGCGUCCGdTdT-3′.

Cell Culture. ECV304 cells constitutively expressing a chromosomally integrated *firefly luciferase* gene (ECV304-GL3)[24] were cultured in Medium 199 (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (PAA, Pasching, Austria) and routinely split twice a week after trypsin treatment. Cells were incubated at 37 °C in an atmosphere of 5% CO_2.

Transfection of Cells. ECV304-GL3 cells were seeded into culture plates (Greiner, Frickenhausen, Germany), 18 h prior to treatment with siRNA. Then, cells were washed twice with Phosphate Buffered Saline (PBS). Transfection in the use of Lipofectamin 2000 (Invitrogen, Karlsruhe, Germany) was performed according to the manufacturer's instructions. Briefly, transfection mixtures were prepared with Opti-MEM (Invitrogen, Karlsruhe, Germany) containing 1nM siRNA and 5 μg/mL Lipofectamin 2000. Cells were incubated for 4 h, before the transfection medium was replaced by Medium 199 supplemented with 10% fetal calf serum. After 20 h incubation, cells were harvested by trypsin treatment, washed once with 500 μL PBS, and resuspended in 100 μL PBS. A 25-μL portion of cell suspension was transferred to a 96-well plate (Greiner, Frickenhausen, Germany) and subsequently cell viability (FDA viability assay) and *firefly luciferase* activity were measured with a fluorimeter as previously described.[24] The spontaneous uptake of naked siRNA was performed as described above in the absence of lipofectant and using 1 μM instead of 1nM siRNA. For electroporation, we used 1 × 10^6 ECV304-GL3 cells in 200 μL PBS and preincubated with 10nM siRNA for 1 min on ice. Subsequently, cells were transferred to electroporation cuvettes with 4 mm interelectrode distance. Cells were pulsed once at 180 V and 950 μF ($t_{1/2} \approx 50$ ms) and cultured in 12-well culture dishes. After 24 h, cells were harvested and viability and *firefly luciferase* activity were measured as described above.

■ ASSOCIATED CONTENT

Supporting Information. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR of all new compounds. HPLC profiles and MALDI-TOF spectra of synthesized oligonucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

This work was supported by the Association pour la Recherche contre le Cancer (ARC) and Ministère de l'Enseignement supérieur et de la Recherche.

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