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Supporting Information

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Modulation of RNA Tertiary Folding by Incorporation of Caged Nucleotides

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Solid-phase RNA synthesis

Oligoribonucleotides that incorporate an NPE-modified nucleotide were synthesized at the UIUC W. M. Keck Center for Comparative and Functional Genomics on the 1 μmol scale on an ABI/PerkinElmer 394 DNA Synthesizer. Standard methods were followed with only minor modifications. The following reagents were purchased from Glen Research: 2′-*O*-TOM nucleoside phosphoramidites of adenosine, uridine, cytidine, and guanosine;[1] UltraMild phosphoramidites of N^6-phenoxyacetyl-2′-*O*-tBDMS-adenosine and N^2-(4-isopropylphenoxy)acetyl-2′-*O*-tBDMS-guanosine; the corresponding 1000-Å CPG supports; 5-ethylthio-1*H*-tetrazole; and phenoxyacetic anhydride cap reagent A. The coupling time for the NPE-modified phosphoramidites was 10 min. For the preparation of oligoribonucleotides that incorporate O^6-NPE-guanosine or O^4-NPE-uridine, phenoxyacetic anhydride was used instead of acetic anhydride during the capping step. Synthesis of O^4-NPE-uridine modified RNA employed the UltraMild adenosine and guanosine phosphoramidites indicated above along with the standard 2′-*O*-TOM uridine and cytidine phosphoramidites.

Oligoribonucleotide deprotection for RNA that incorporates either N^6-NPE-adenosine, N^4-NPE-cytidine, O^6-NPE-guanosine, or N^3-NPE-uridine followed the standard protocol for deprotection of 2′-*O*-TOM-protected RNA, using treatment with $MeNH_2$ (1:1 mixture of 40% $MeNH_2$ in water and 33% $MeNH_2$ in ethanol; 10–14 h at room temperature or 7–10 h at 37 °C) followed by treatment with TBAF (1 M in THF; 11–13 h at 37 °C). Oligoribonucleotides that incorporate O^4-NPE-uridine were deprotected with 29% aqueous ammonia/ethanol (3:1) at room temperature for 21 h, followed by standard TBAF deprotection. All oligoribonucleotides were desalted using NAP-25 columns (Amersham Biosciences) and purified by denaturing (7 M urea) 20% polyacrylamide gel electrophoresis with running buffer of 1× TBE (89 mM each Tris and boric acid, 2 mM EDTA, pH 8.3). Oligoribonucleotides were extracted from the gel with two portions of TEN (10 mM Tris•HCl, pH 8.0, 1 mM EDTA, 300 mM NaCl) at 4 °C for 8–12 h for each extraction and precipitated with 3 volumes of ethanol at –20 °C.

Decaging of NPE-caged oligoribonucleotides analyzed by PAGE, HPLC, and ESI-MS

Photolysis of NPE-caged RNA oligonucleotides and P4-P6 derivatives was performed in 1× TB buffer (89 mM each Tris and boric acid, pH 8.3) containing 5 mM DTT to scavenge decaging byproducts. The arc lamp source was a 300-W Xe arc lamp (Varian Eimac) with a Schott UG-1 filter that passes only 300–400 nm light. A liquid light guide was used to direct the light beam into the 0.7-mL eppendorf tube that held the RNA sample.

The sequence of the 15-mer RNA oligonucleotide with an NPE-caged nucleotide (Figure 2) was 5′-GGAAUUGCGGGAAAG-3′, where one of the four underlined nucleotides has the NPE caging group. This 15-mer corresponds to nucleotides 102-116 of the P4-P6 RNA sequence,[2] with ^{NPE}U, ^{NPE}C, ^{NPE}G, or ^{NPE}A at position U107, C109, G110, or A114, respectively. Gel analyses of photolyzed oligonucleotide samples were performed by 20% denaturing PAGE of 5′-^{32}P-radiolabeled oligonucleotides; gels were visualized with a PhosphorImager. For anion-exchange HPLC analysis, samples were separated on a Dionex DNAPac PA100 column (4 × 250 mm) using a gradient over 45 min of 0–60% buffer B in buffer A at 80 °C with UV detection at 260 nm. Buffer A was 25 mM Tris•HCl, pH 8.0 with 6 M urea; buffer B was the same as buffer A with inclusion of 0.5 M $NaClO_4$. Shown in Figure S1 are HPLC traces for decaging of the ^{NPE}U, ^{NPE}C, and ^{NPE}G 15-mer oligonucleotides. These traces are analogous to that shown in Figure 2b for decaging of the ^{NPE}A oligonucleotide.

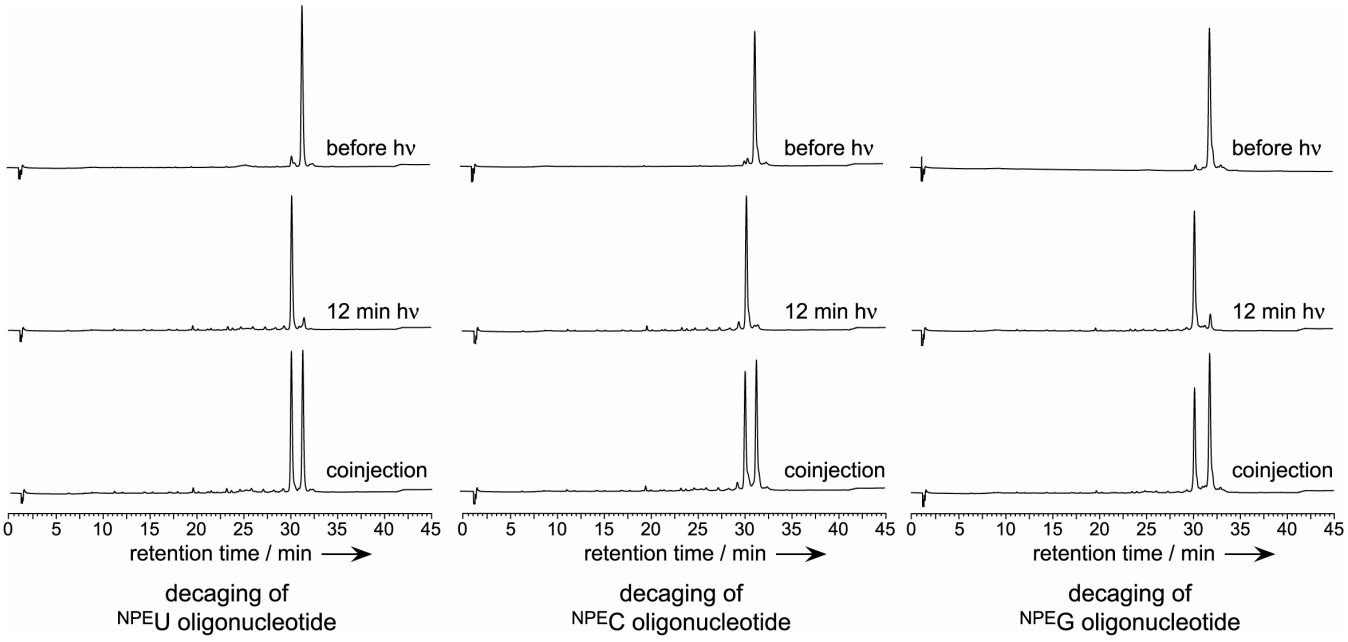


Figure S1. HPLC traces analogous to that shown in Figure 2b for decaging of three NPE-caged oligonucleotides (photolysis with 300-W Xe arc lamp).

Photochemical decaging of the four 15-mer oligonucleotides was also monitored by electrospray ionization mass spectrometry (ESI-MS). In all four cases, the MS data clearly showed the expected 149-amu loss of mass due to photocleavage of the NPE group (Figure S2).

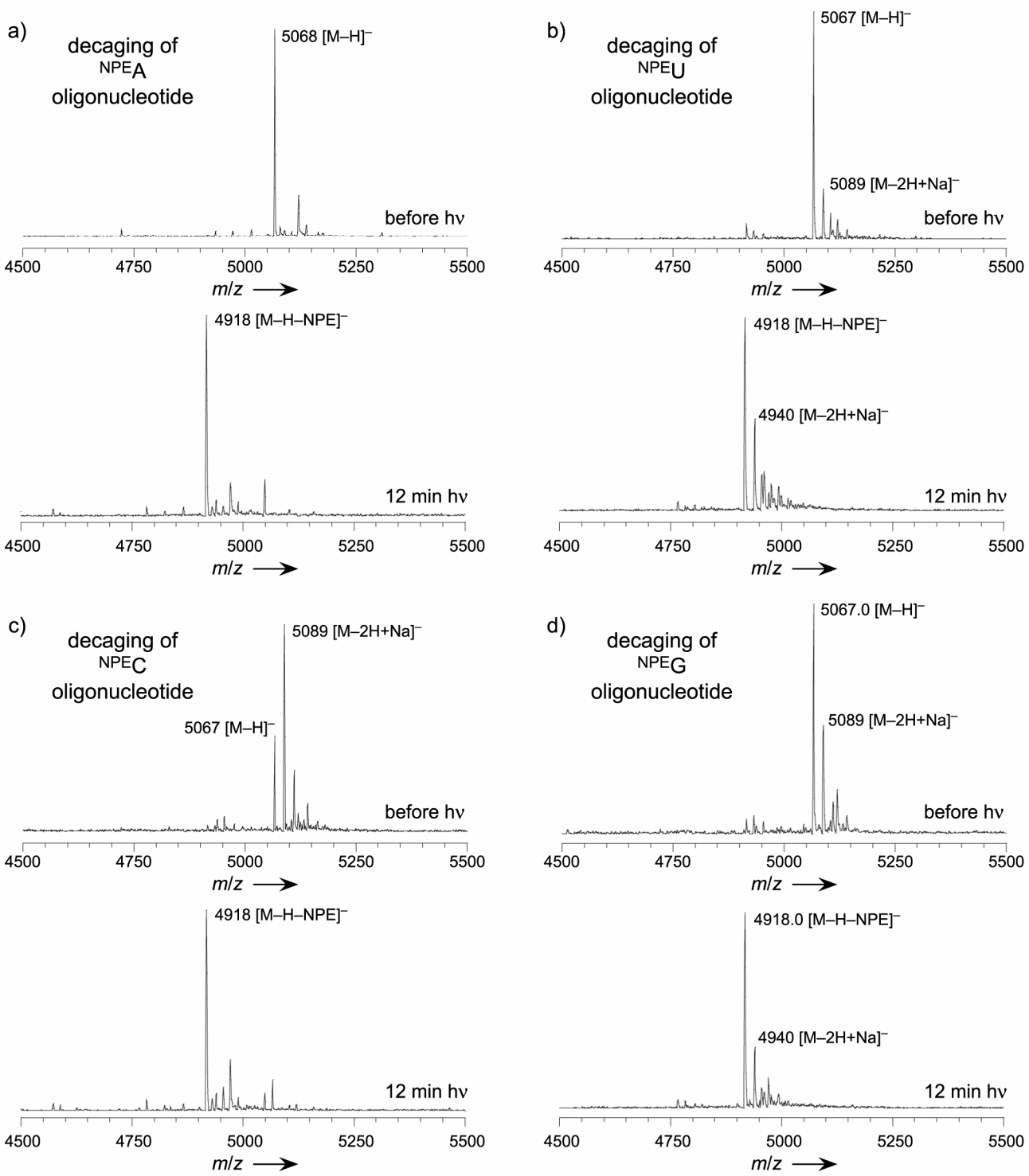


Figure S2. ESI-MS data that demonstrate decaging of the four NPE-caged oligonucleotides. Photolysis used the 300-W Xe arc lamp. The calculated $[M–H]^-$ *m*/*z* values for the caged and uncaged 15-mer oligonucleotides are 5066.2 and 4917.1, respectively.

Decaging of an RNA oligonucleotide that incorporates N^3-NPE-caged uridine

For caging of RNA at a uridine nucleotide, it was desirable to append the NPE caging group at N^3 of the pyrimidine ring for two reasons: (1) The caging group would be present at the Watson-Crick face of uridine and therefore interfere with base pairing. (2) The preparation of N^3-NPE-uridine modified RNA oligonucleotides was anticipated not to require changes of the standard RNA solid-phase synthesis and deprotection protocols (unlike O^4-NPE-uridine, which required UltraMild adenosine and guanosine phosphoramidites in combination with ammonia deprotection rather than methylamine deprotection). However, the main requirement for a useful caging group—efficient photolytic cleavage—was not fulfilled for N^3-NPE-caged uridine. Photolysis of an N^3-NPE-uridine modified 15-mer RNA oligonucleotide was relatively inefficient under all tested conditions and resulted in the formation of two products. The mixture of reaction products was analyzed by denaturing PAGE, anion-exchange HPLC, and ESI-MS (Figure S3). In addition to the desired decaged 15-nt RNA, which was produced as the minor product, a byproduct was observed as the major product with a mass 18 amu lower than that of the caged RNA (corresponding to net loss of H_2O from the caged RNA).

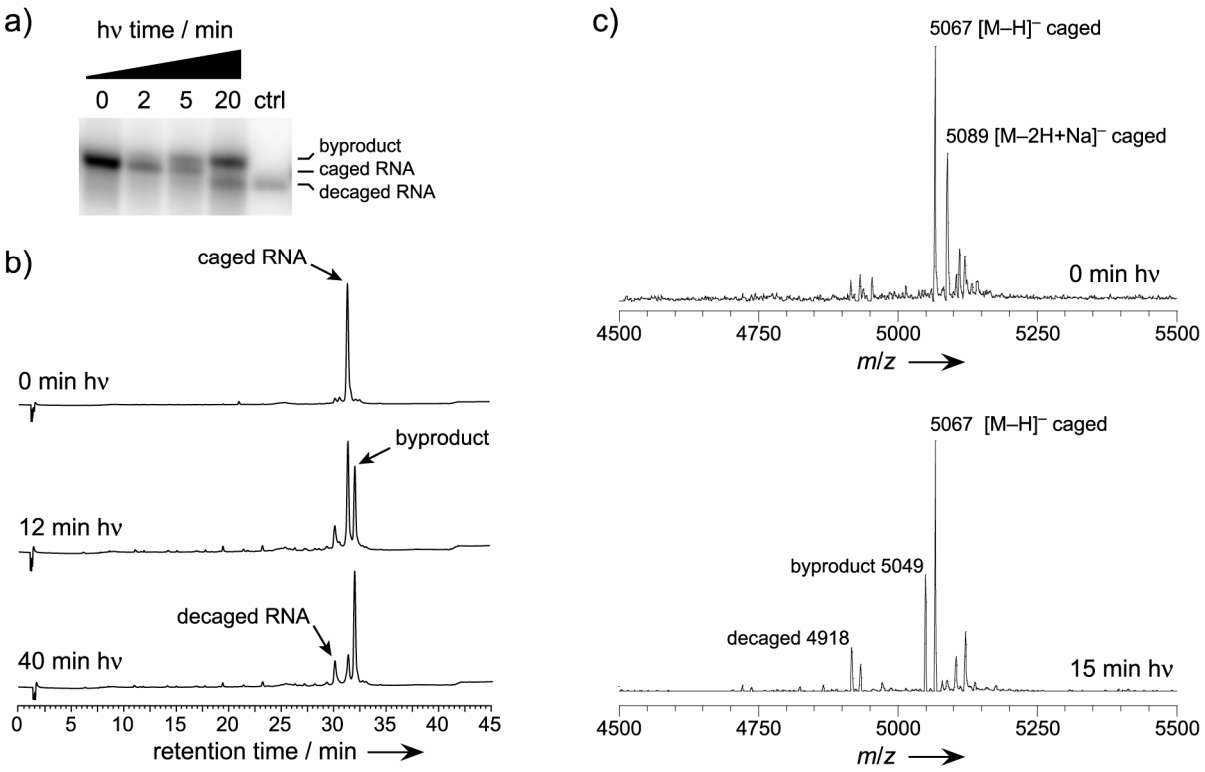


Figure S3. Decaging of the 15-mer RNA oligonucleotide with N^3-NPE-caged uridine. The caging group was at the same nucleotide position (analogous to U107) as for the O^4-NPE-caged uridine described on page S3. a) 20% PAGE. Photolysis used a 300-W Xe arc lamp filtered to provide 300-400 nm light. The sample was in 50 mM triethylammonium acetate, pH 7.0. Using a handheld UV lamp (365 nm), photolysis was complete in <60 min, and approximately equal amounts of the decaged RNA and the byproduct were observed (data not shown). b) HPLC traces for photolysis using the arc lamp. The assignment of the HPLC peak for decaged RNA is based on the data shown in Figure S1. c) ESI-MS data for the N^3-NPE-caged uridine RNA before and after 15 min photolysis with the arc lamp.

As one possible structure for the observed byproduct we propose **G**, which has a nitrosostyrene moiety attached to the N^3 position of the pyrimidine ring (Figure S4). Formation of **G** can be rationalized by considering mechanistic aspects of the photocleavage reaction, which have been reviewed recently.[3] The photolytic process consists of transfer of the benzylic proton of starting material **A** to an oxygen of the nitro group, forming a *Z*-nitronic acid which is deprotonated to form an *aci*-nitro anion. Experimental and computational studies[4] have indicated that reprotonation of the *aci*-nitro anion to form the *E*-nitronic acid (comparable to **B**) is followed by cyclization to the *N*-hydroxybenzisoxazoline derivative (comparable to **C**), which can decompose directly to the desired decaged product (**D**) and nitrosoacetophenone **E**. However, for NPE-caged alcohols it has been shown that a hemiacetal intermediate is formed and that decomposition of this hemiacetal is rate-limiting for product formation.[5] Analogous rearrangement of **C** would lead to the hemiaminal intermediate **F**,[6] which has the same mass as the caged starting material **A**. Hemiaminal **F** could fragment to form the decaged RNA **D** and nitrosoacetophenone **E,** or alternatively, **F** could eliminate water to form the byproduct **G**.

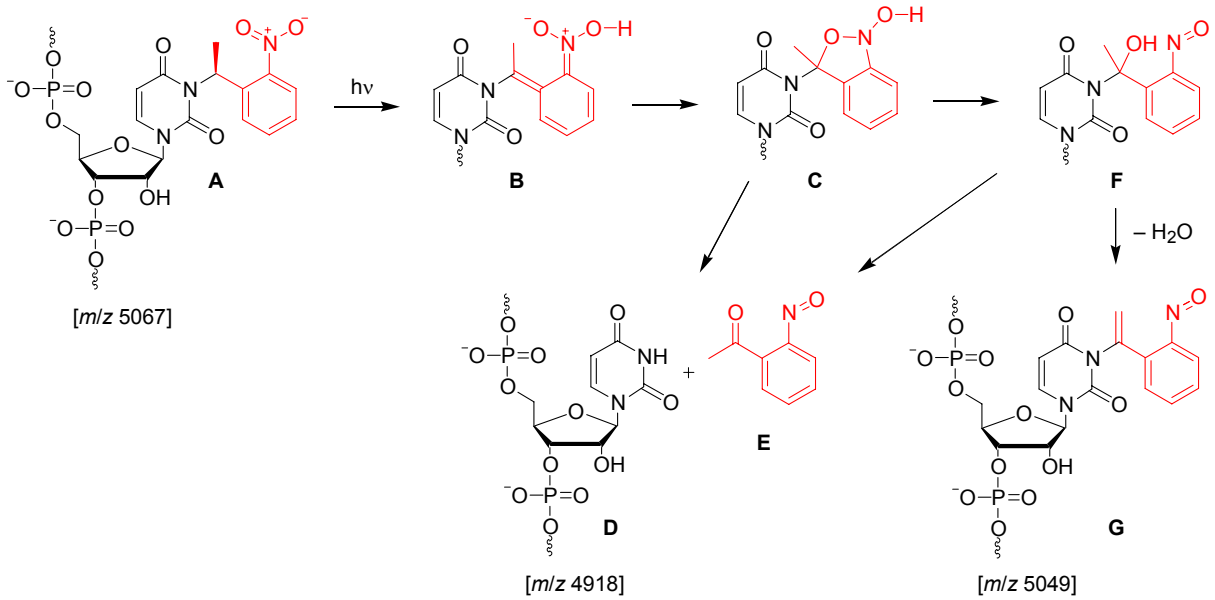


Figure S4. Proposed intermediates and products formed upon photolysis of N^3-NPE-caged RNA. Masses of the corresponding 15-mer RNA oligonucleotides are shown below **A**, **E**, and **G**, which correspond to caged RNA, decaged RNA, and the byproduct, respectively (see Figure S3).

Preparation of NPE-caged P4-P6

For the preparation of NPE-caged P4-P6 RNA, two different ligation sites within the 160-nucleotide RNA were chosen. For incorporation of a caging group in the 5′-portion of P4-P6 (ligation site 1, Figure S5; modifications at nucleotide positions U107, G108, C109, G110 and A114), a 15-mer oligoribonucleotide corresponding to P4-P6 nucleotides 102–116 was prepared by solid-phase synthesis. This oligonucleotide was ligated to the remaining 145 nt of P4-P6 (nt 117–261) using a complementary 51-nt DNA splint and T4 DNA ligase essentially as described previously.[7,8] The 145-nt RNA is the 3′-partner (donor) in the ligation reaction and was prepared by in vitro transcription using T7 RNA polymerase, with inclusion of GMP to incorporate a 5′-monophosphate in the majority of the transcripts. For incorporation of a caging group in the 3′-portion of P4-P6 RNA (ligation site 2; modifications at positions C240, G245, A246, U247, A248, U249, G250, C255, and A256), a 24-mer oligoribonucleotide corresponding to P4-P6 nucleotides 238–261 was prepared by solid-phase synthesis. This oligonucleotide was 5′-phosphorylated with ATP and T4 polynucleotide kinase (PNK) and then ligated to the remaining 136 nt of P4-P6 (nt 102-237). The 136-nt 5′-partner (acceptor) for the ligation reaction was prepared by in vitro transcription using T7 RNA polymerase and in cis hammerhead processing, resulting in an unwanted 2′,3′-cyclic phosphate at its 3′-end.[9] The cyclic phosphate was removed with T4 PNK in the absence of ATP prior to ligation. Ligation site 2 is located in a loop region connecting two single-stranded RNA ends that can be joined by T4 RNA ligase (without a splint).

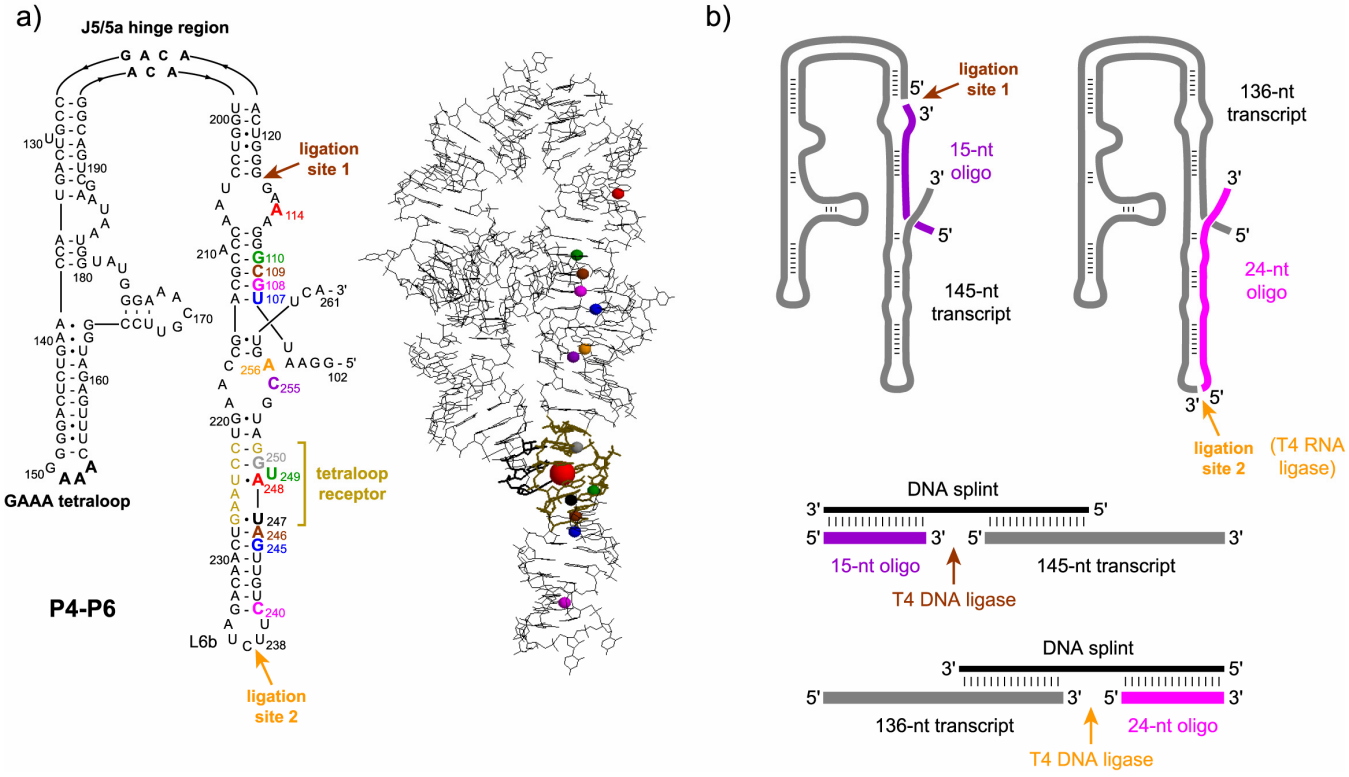


Figure S5. Secondary structure and synthetic strategies to prepare P4-P6 by ligation. a) Secondary structure of P4-P6 with caging sites highlighted. The adjacent tertiary structure is shown in the same orientation as in Figure 3a. b) Ligation strategies. For ligation at site 1, the splint ligation strategy was used (T4 DNA ligase). For ligation at site 2, either an analogous splint ligation strategy was used (T4 DNA ligase), or ligation without a splint was performed (T4 RNA ligase). In the latter case, the secondary structure of P4-P6 itself holds the two RNA partners together.

Due to the presence in the 24-nt donor RNA of an NPE caging group, which possibly interferes with proper annealing of the two RNA substrates, yields for ligation at site 2 with T4 RNA ligase were typically lower than for ligations mediated by a complementary 51-nt DNA splint and T4 DNA ligase. As tabulated in Table S1, isolated yields of full-length caged P4-P6 RNAs for ligation site 1 were 30–60% using T4 DNA ligase. For ligation site 2, yields were 40–53% using T4 DNA ligase and 16–34% using T4 RNA ligase.

P4-P6 derivative	enzyme for ligation[a]	isolated yield (pmol)	isolated yield (%)
Ligation site 1			
^{NPE}U107	D	306	61
^{NPE}G108	D	214	43
^{NPE}C109	D	304	61
^{NPE}G110	D	219	44
^{NPE}A114	D	157	31
Ligation site 2			
^{NPE}C240	R	226	45
^{NPE}G245	D	240	48
^{NPE}A246	D	245	49
	R	78	16
^{NPE}U247	D	255	51
	R	130	26
^{NPE}A248	D	231	46
	R	113	23
^{NPE}U249	D	266	53
	R	168	34
^{NPE}G250	D	193	39
	R	18	4
^{NPE}C255	R	87	17
^{NPE}A256	D	257	51
	R	140	28

Table S1. Ligation yields for the P4-P6 derivatives. All reactions were performed using 500 pmol of the limiting RNA substrate (see procedures in text). [a] D = T4 DNA ligase (along with the complementary DNA splint); R = T4 RNA ligase.

Ligation using T4 DNA ligase and a complementary DNA splint (ligation site 1).[7,8] The 15-mer NPE-caged oligoribonucleotide (corresponding to P4-P6 nt 102–116; 500 pmol) was mixed with the 145-nt transcript (nt 117–261; 900 pmol) and a 51-nt DNA splint (complementary to nt 104–154; 750 pmol) in a total volume of 24 µL containing 5 mM Tris•HCl, pH 7.5 and 0.1 mM EDTA. The sample was annealed by heating at 95 °C for 3 min followed by slow cooling to room temperature over 20 min. Then, 3 µL of 10× ligase buffer (500 mM Tris•HCl, pH 8.0, 100 mM $MgCl_2$, 75 mM DTT, 10 mM ATP) and 3 µL of T4 DNA ligase (prepared from a His_6-tagged construct provided by S. Strobel, Yale University) were added, providing a total volume of 30 µL. The sample was incubated at 37 °C for 4–5 h and quenched with 25 µL of stop solution (80% formamide, 1× TB, 50 mM EDTA, and 0.025% each bromophenol blue and xylene cyanol). The ligation product was purified by 12% denaturing PAGE.

Ligation using T4 DNA ligase and a complementary DNA splint (ligation site 2). The 5′-phosphorylated 24-mer NPE-caged oligoribonucleotide (corresponding to P4-P6 nt 238–261; 550 pmol)

was mixed with the 2′,3′-dephosphorylated 136-nt transcript (nt 102–237; 500 pmol) and a 51-nt DNA splint (complementary to nt 211–261; 750 pmol) in a total volume of 24 μL containing 5 mM Tris•HCl, pH 7.5 and 0.1 mM EDTA. The sample was annealed by heating at 95 °C for 3 min followed by slow cooling to room temperature over 20 min. Then, 3 μL of 10× ligase buffer (500 mM Tris•HCl, pH 8.0, 100 mM $MgCl_2$, 75 mM DTT, 10 mM ATP) and 3 μL of T4 DNA ligase (prepared from a His_6-tagged construct provided by S. Strobel, Yale University) were added, providing a total volume of 30 μL. The sample was incubated at 37 °C for 4–5 h and quenched with 25 μL of stop solution. The ligation product was purified by 12% denaturing PAGE.

Ligation using T4 RNA ligase (ligation site 2). The 5′-phosphorylated 24-mer NPE-caged oligoribonucleotide (nt 238–261; 550 pmol) was mixed with the 2′,3′-dephosphorylated 136-nt transcript (nt 102–237; 500 pmol) in a total volume of 13 μL containing 5 mM Tris•HCl, pH 7.5, 15 mM NaCl and 0.1 mM EDTA. The sample was annealed by heating at 95 °C for 3 min followed by slow cooling to room temperature over 20 min. Then, 2 μL of 10× ligase buffer (500 mM Tris•HCl, pH 7.5, 50 mM $MgCl_2$, 100 mM DTT, 0.5 mM ATP) and 5 μL of T4 RNA ligase (20 U/μL, Fermentas) were added, providing a total volume of 20 μL. The sample was incubated at 37 °C for 4–5 h and quenched with 30 μL of stop solution. The ligation product was purified by 12% denaturing PAGE.

5′-Phosphorylation of oligoribonucleotides prepared by solid-phase synthesis (in preparation for ligation at site 2). Typically, 2.5 nmol of the NPE-modified 24-mer oligoribonucleotide was incubated with 40 units of T4 polynucleotide kinase (PNK, Fermentas) in a total volume of 50 μL containing 50 mM Tris•HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA and 1 mM ATP. The sample was incubated at 37 °C for 4–5 h and quenched by the addition of 45 μL stop solution. The phosphorylated oligonucleotide was purified by 20% denaturing PAGE.

Cleavage and removal of 2′,3′-cyclic phosphate from RNA transcript (in preparation for ligation at site 2). The 136-nt transcript with a 2′,3′-cyclic phosphate was incubated with T4 PNK in the absence of ATP. The incubation conditions were 15 μM RNA in 70 mM Tris•HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, and 0.1 mM spermidine with 0.4 U/μL T4 PNK. The sample was incubated at 37 °C for 1.5 h, and the enzyme was removed by phenol-chloroform extraction and ethanol precipitation of the RNA.

5′-Dephosphorylation of P4-P6 (removal of 5′-triphosphate, for products of ligation using site 2). Typically, 50 pmol of 5′-triphosphate RNA was incubated in a total volume of 10 μL containing 50 mM Tris•HCl, pH 8.5, 0.1 mM EDTA and 5 units of calf intestinal phosphatase (CIP, Roche, 20 U/μL). The sample was incubated at 37 °C for 1.5 h, and the enzyme was removed by phenol-chloroform extraction and ethanol precipitation of the RNA.

5′-^{32}P-Radiolabeling of P4-P6. Typically, 25 pmol of RNA was incubated with 25 pmol of γ-^{32}P-ATP (6000 Ci/mmol, PerkinElmer) in a total volume of 10 μL containing 50 mM Tris•HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, and 0.1 mM spermidine with 10 units of T4 PNK (Fermentas) at 37 °C for 15 min. The sample was quenched with 15 μL of stop solution and purified by 6% denaturing PAGE.

Structural context of caging sites within P4-P6

On the basis of the P4-P6 X-ray crystal structure,[2] the sites of NPE caging were examined using the RasMol program. At four nucleotide positions within the tetraloop receptor (A246, U247, A248, and G250; Figure S6), caging of the appropriate nucleobase atom is predicted to disrupt at least one tertiary hydrogen bond. In the case of N^6-NPE-caged A248, the bulky NPE group is additionally predicted to introduce steric clashes within the tetraloop-receptor interaction, because the caging site is completely enclosed within the RNA. For the remaining nucleotide within the tetraloop receptor (U249; Figure S7c), the uridine nucleobase makes no tertiary hydrogen bonds, and appending a bulky group does not appear to induce any steric clash. For all other nucleotide positions shown in Figure S7 and for the positions remote from the tetraloop-receptor interaction shown in Figure S8, introduction of the caging group is not expected to cause a substantial steric clash. However, in many cases (U107, G108, C109, G110, C240, and G245), the NPE caging group should disrupt canonical Watson-Crick hydrogen bonding. In two other cases (A114 and A256), the caging site atom is involved in a noncanonical interaction that should be disrupted by the NPE group. In one final case (C255), the caging site atom does not appear to act with any other RNA functional groups.

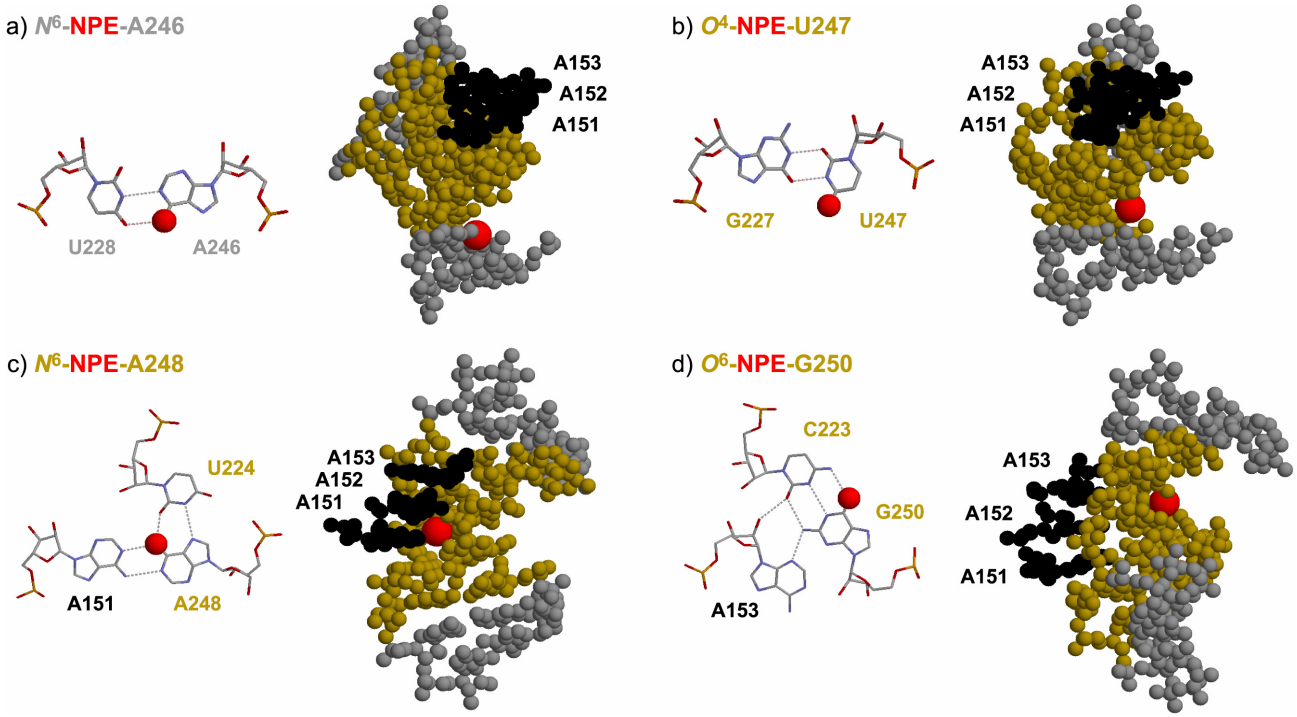


Figure S6. Structural context of caging sites for the four P4-P6 nucleotide positions for which unambiguous structural disruptions were observed by native PAGE (Figure 4b, red). All four sites are located within or near the tetraloop receptor. Images were rendered in RasMol using coordinates from the X-ray structure.[2] As in Figure 3, the adenosine nucleotides of the tetraloop are colored black (A151–A153); the receptor nucleotides are gold (222–227 and 247–251); and other P4-P6 nucleotides are grey (220–221, 228–229, 245–246, and 252–253). For N^6-NPE-A248 (panel c), the caging site is buried within the tetraloop-receptor interface; therefore, appending the NPE group should introduce severe steric clashes as well as disrupt several hydrogen bonds. For the other three nucleotides (panels a, b, and d), the NPE group should disrupt at least one tertiary hydrogen bond, whereas there appears to be sufficient space to accommodate the NPE group in terms of its steric interactions. In all cases, the effects of caging upon stacking interactions are difficult to predict from these molecular models.

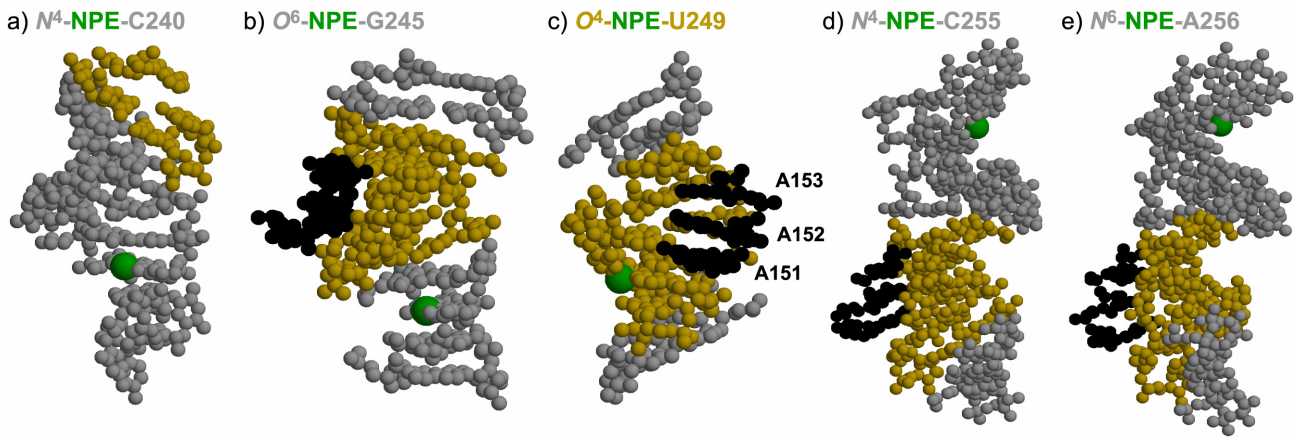


Figure S7. Structural context of caging sites for nucleotide positions in the lower portion of P4-P6, within or near the tetraloop receptor (gold; nucleotides 222–227 and 247–251). Adenosine nucleotides of the tetraloop are black (A151–A153). Other nucleotides of P4-P6 are grey. In all of these cases, only small effects due to NPE-caging of the indicated nucleobase were observed by native PAGE (Figure 4 and Figure S9).

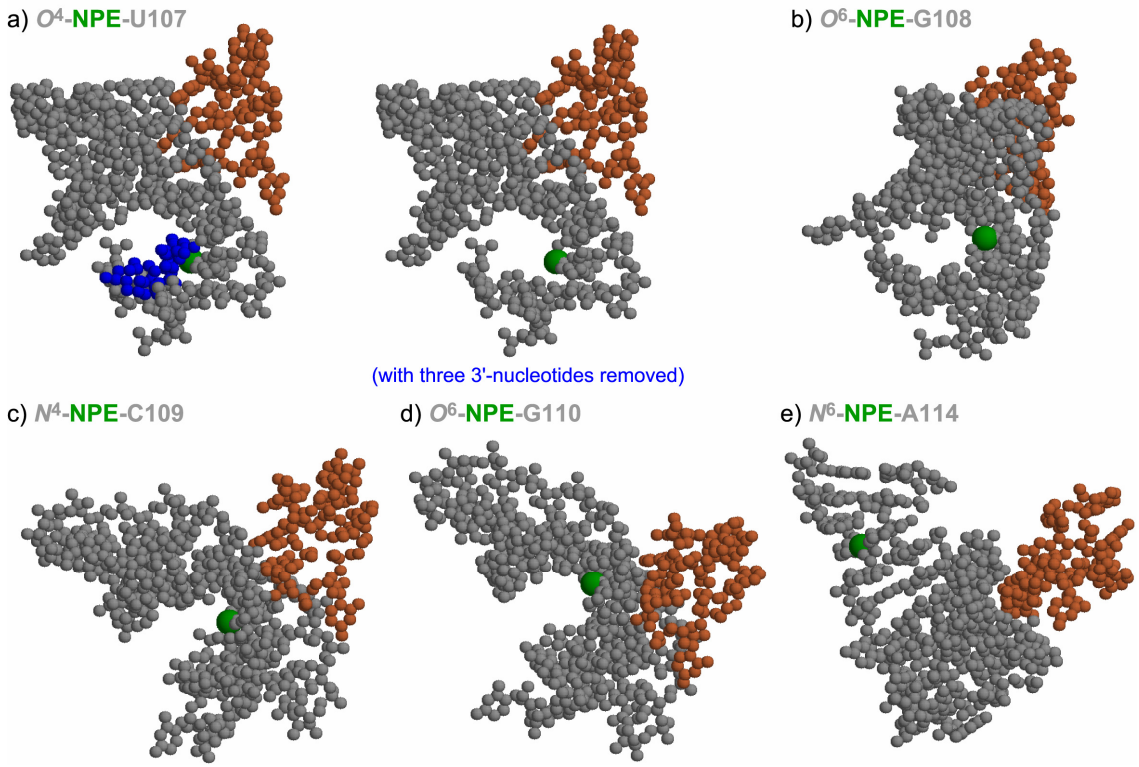


Figure S8. Structural context of caging sites for nucleotide positions in the upper portion of P4-P6, far from the tetraloop-receptor tertiary interaction. Nucleotides 182–188 of the A-rich bulge are colored brown; other P4-P6 nucleotides are grey (105–117, 204–216, and 257–261). In all of these cases, only small effects due to NPE-caging of the indicated nucleobase were observed by native PAGE (Figure 4 and Figure S9). In panel a for the NPE-caged U107 derivative, the three nucleotides U259–A261 at the 3′-end of the P4-P6 molecule are colored blue instead of grey. When these three nucleotides are removed from the model (right structure within panel), the site of caging is accessible to derivatization with a bulky group without appearing to induce a steric clash. It is likely that these three terminal nucleotides of P4-P6 can adopt a conformation other than that observed in the crystal structure without inducing a substantial thermodynamic penalty.

Native PAGE assays: procedures and data tabulation

The native PAGE experiments were performed essentially as described.[7] Such experiments have been used many times both by us[7,8] and by others[10] for assaying the thermodynamic effects of modifications to P4-P6. Briefly, samples were prepared by mixing 2 µL of a radiolabeled P4-P6 stock solution in 1× TB and 5 mM DTT with 2 µL of 2× native gel loading buffer (1× TB [note that the RNA stock solution also includes 1× TB], 10% glycerol, and twice the final desired $MgCl_2$ concentration). The samples were heated at 50 °C for 5 min and equilibrated at 35 °C for 5–10 min before loading into gel lanes. The samples were electrophoresed at 150–200 V and 35 °C for 5–6 h on 8% polyacrylamide gels containing 1× TB (made using a 40% 29:1 acrylamide:bis-acrylamide stock solution) along with the appropriate concentration of $MgCl_2$. Gels were dried at 60 °C for 20 min and exposed to a PhosphorImager screen. As described,[7] the mobility of each P4-P6 derivative relative to an unfoldable P4-P6 mutant was measured. The data were fit to the titration equation

$$M_{obs} = (M_{low} + M_{high} \bullet K \bullet [Mg^{2+}]^n)/(1 + K \bullet [Mg^{2+}]^n)$$

where M_{obs} is the observed relative mobility as a function of $[Mg^{2+}]$; M_{low} and M_{high} are the limiting low and high values of relative mobility; and K and n are the equilibrium constant and Mg^{2+} Hill coefficient for the simple model equation $U + nMg^{2+} = F \bullet nMg^{2+}$ (U = unfolded state, F = folded state). The Mg^{2+} midpoint ($[Mg^{2+}]_{1/2}$ value) is $K^{-1/n}$. Values of ΔΔG°′ were calculated as described,[7] with ΔG°′ for each RNA equal to $+nRT \bullet \ln [Mg^{2+}]_{1/2}$. The ΔΔG°′ value is defined as zero for wild-type (uncaged) P4-P6. Regardless of the fit value of n that was used along with K to compute $[Mg^{2+}]_{1/2}$, $n = 4$ was assumed in calculating all ΔG°′ values, as described.[7] Values for $[Mg^{2+}]_{1/2}$, the Hill coefficient n, and the free energy perturbation ΔΔG°′ are tabulated in Table S2.

In Figure 4 are shown the native PAGE titration data for most of the NPE-caged P4-P6 derivatives studied in this report. In Figure S9 are shown the native PAGE titration data for NPE-caged P4-P6 derivatives with the NPE group at the three remaining nucleotide positions G108, G245, and C255.

P4-P6 derivative	$[Mg^{2+}]_{1/2}$, mM	Hill coefficient *n*	ΔΔG°′, kcal/mol
wild-type	0.70 ± 0.03	4.2 ± 0.3	(0)
^{NPE}U107	0.77 ± 0.02	3.9 ± 0.2	0.2 ± 0.1
^{NPE}G108	0.70 ± 0.04	3.9 ± 0.4	0.0 ± 0.1
^{NPE}C109	0.42 ± 0.05	3.9 ± 0.4	–1.3 ± 0.3
^{NPE}G110	0.88 ± 0.02	3.7 ± 0.3	0.6 ± 0.1
^{NPE}A114	0.87 ± 0.03	3.8 ± 0.3	0.5 ± 0.1
^{NPE}C240	0.84 ± 0.03	3.9 ± 0.3	0.4 ± 0.1
^{NPE}G245	0.71 ± 0.04	4.4 ± 0.4	0.0 ± 0.1
^{NPE}A246	5.1 ± 1.3	2.0 ± 0.3	4.9 ± 0.7
^{NPE}U247	4.0 ± 0.7	2.4 ± 0.2	4.3 ± 0.5
^{NPE}A248	8.5 ± 2.6	1.7 ± 0.2	6.1 ± 0.7
^{NPE}U249	0.88 ± 0.04	3.3 ± 0.3	0.6 ± 0.1
^{NPE}G250	>10	–	>6
^{NPE}C255	0.71 ± 0.03	4.7 ± 0.4	0.1 ± 0.1
^{NPE}A256	0.76 ± 0.04	3.8 ± 0.4	0.2 ± 0.1

Table S2. Values for the $[Mg^{2+}]_{1/2}$, the Hill coefficient n, and the free energy perturbation ΔΔG°′ determined from the native PAGE titration curves of Figure 4 and Figure S9. Entries are colored red or green to match these figures.

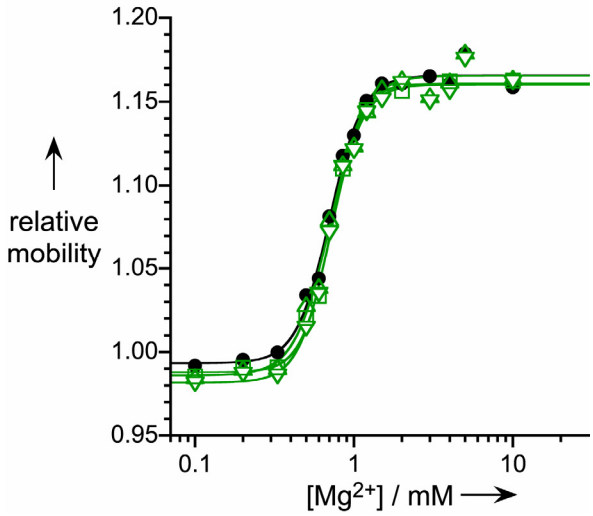


Figure S9. Native PAGE titration data for the NPE-caged G108, G245, and C255 P4-P6 derivatives not shown in Figure 4. In all three cases, the thermodynamic effect due to the caging group was very small.

DMS probing assays: procedures and photolysis to remove the NPE caging group

A 100-µL sample containing 5 pmol of P4-P6 RNA in 35 mM Tris•HCl, pH 7.5 and the appropriate concentration of $MgCl_2$ was incubated at 50 °C for 5 min and then at 42 °C for 1 min. To this sample was added 1.25 µL of a freshly prepared dilution of DMS in ethanol (1:4), and the solution was incubated at 42 °C for 30 min. The reaction was quenched by the addition of 25 µL of 1 M β-mercaptoethanol followed by 15 µL of 3 M NaCl, 12 µg of carrier yeast tRNA (Sigma) and water to give a final volume of 150 µL. The DMS-modified RNA was precipitated with 450 µL of ethanol at –80 °C. The pellet was dissolved in 100 µL of TEN (10 mM Tris•HCl, pH 8.0, 1 mM EDTA, 300 mM NaCl) and reprecipitated with ethanol followed by washing with 75% ethanol. The sample was dried and dissolved in 10 µL of water.

For reverse transcription, a 2.5-µL sample containing 0.5 pmol of DMS-treated P4-P6 RNA and 1.5 pmol of 5′-^{32}P-radiolabeled DNA primer (complementary to P4-P6 nucleotides 195-225) in 5 mM Tris•HCl, pH 7.5 and 0.1 mM EDTA was heated at 95 °C for 2.5 min and then cooled slowly to room temperature over 20 min. The sample was combined with 2.25 µL of a solution containing 110 mM Tris•HCl, pH 8.3, 165 mM KCl, 6.6 mM $MgCl_2$, 22 mM DTT and 0.55 mM of each dNTP. Then, 50 units of reverse transcriptase (Superscript II RNase H^-, Invitrogen, 200 U/µL) were added. The sample was incubated at 42 °C for 2 h and then quenched with 10 µL of stop solution (80% formamide, 1× TB, and 50 mM EDTA plus 0.025% each bromophenol blue and xylene cyanol). The sample was heated at 95 °C for 3 min and then cooled on ice for 5 min before loading onto a 12% denaturing polyacrylamide gel. Dideoxy sequencing ladders (not shown) were generated using wild-type P4-P6 along with 0.25 mM of one ddNTP and the corresponding dNTP at 0.05 mM.

The Mg^{2+}-dependent intensity of the reverse transcription blockage band corresponding to A152 in each gel lane was normalized to the intensity in the same lane of the A171 band, for which DMS modification is nearly independent of [Mg^{2+}]. The fraction of methylated RNA relative to the intensity of the reverse transcription abort band for A152 at 0.2 mM Mg^{2+} was plotted versus [Mg^{2+}] and fit to a standard titration curve.

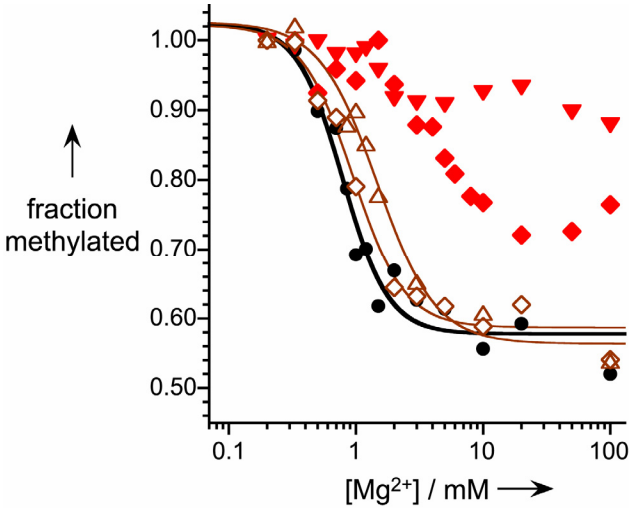
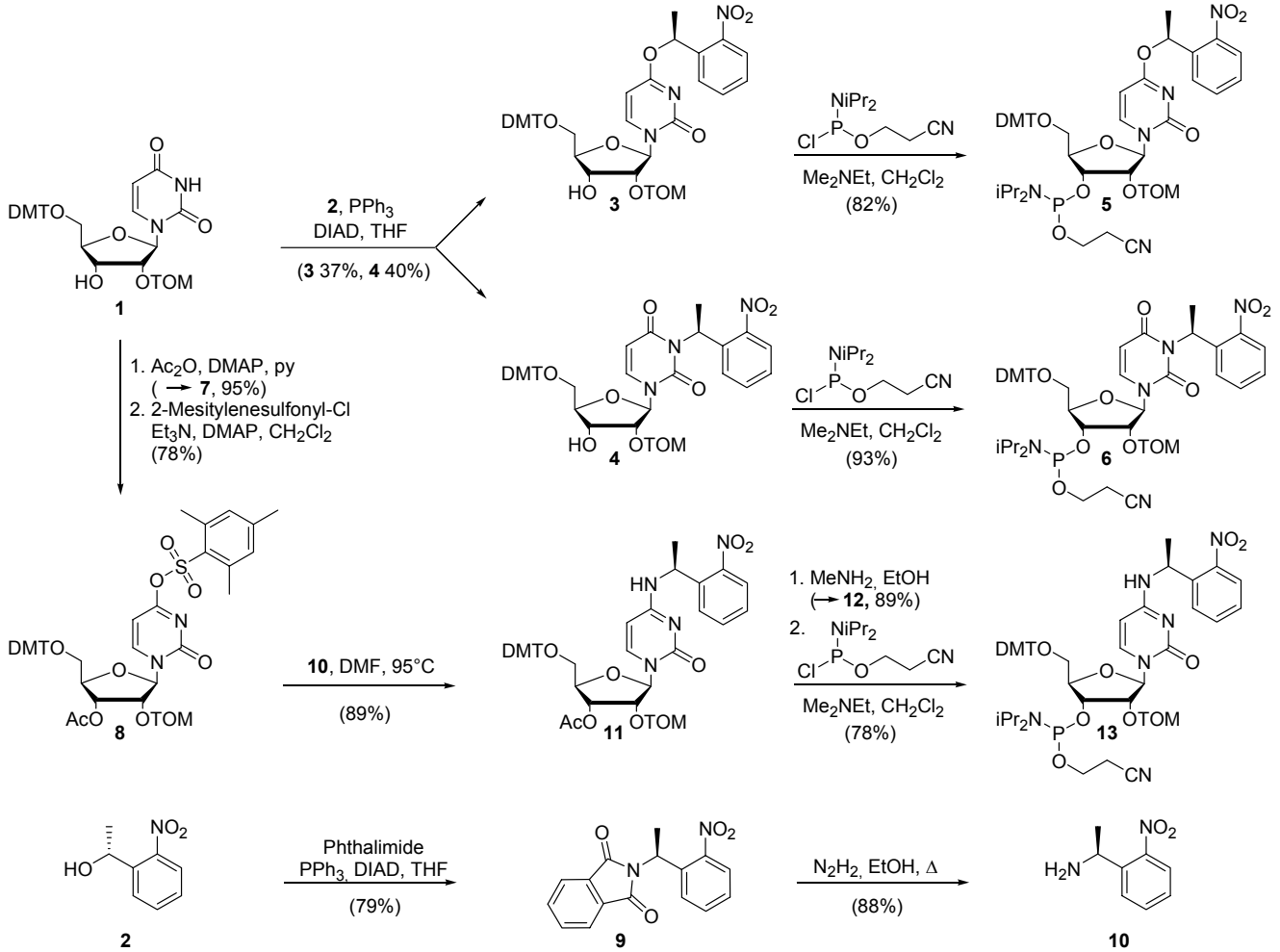


Figure S10. DMS probing experiments with caged P4-P6 RNAs before and after photolysis to remove the caging group. The U247 (◆) and G250 (▼) NPE-caged P4-P6 derivatives were examined by DMS probing before and after 10 min photolysis with the arc lamp as described on page S3. Closed symbols (*red*) are before photolysis, and open symbols (*brown*) are after photolysis. Wild-type P4-P6 RNA (●) was also examined.

Organic synthesis procedures

Reagents were commercial grade and used without purification unless otherwise indicated. Dry solvents were obtained from Aldrich Sure/Seal or Acros Acroseal bottles or by drying over freshly activated 4Å molecular sieves overnight. All reactions were performed under argon or nitrogen unless otherwise noted. Thin-layer chromatography (TLC) was performed on silica gel plates pre-coated with fluorescent indicator with visualization by UV light (254 nm) and/or by dipping into a solution of anisaldehyde (10 mL) in ethanol (420 mL), conc. H_2SO_4 (15 mL), and glacial acetic acid (5 mL) or a solution of vanillin (6.0 g) in ethanol (400 mL) and conc. H_2SO_4 (8 mL), and subsequently heating on a heat block or with a heat gun. Flash column chromatography was performed with silica gel (230-400 mesh). For all compounds that contain the 4,4′-dimethoxytrityl (DMT) group, silica gel columns were packed with the initial solvent additionally containing 1% triethylamine unless otherwise noted, and solvents without triethylamine were used for chromatography unless otherwise noted. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Varian Unity 400, Varian Unity 500, or Varian Unity Inova 500NB instrument. The chemical shifts in parts per million (δ) are reported downfield from TMS (0 ppm) and referenced to the residual proton signal of the deuterated solvent, as follows: $CDCl_3$ (7.26 ppm), DMSO-d_6 (2.49 ppm), acetone-d_6 (2.04 ppm) for ^{1}H NMR spectra; $CDCl_3$ (77.0 ppm), DMSO-d_6 (39.5 ppm), acetone-d_6 (29.8 ppm) for ^{13}C NMR spectra. ^{31}P shifts are relative to external 85% phosphoric acid at 0 ppm. The assignments of ^{1}H NMR and ^{13}C NMR resonances are based on correlations observed in two-dimensional NMR experiments (COSY, HMQC, and HMBC). Multiplicities of ^{1}H NMR spin couplings are reported as s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), or m (multiplet and overlapping spin systems). Values for apparent coupling constants (*J*, Hz) are reported. Mass spectrometry data were obtained at the UIUC School of Chemical Sciences mass spectrometry laboratory using a Micromass ZAB-SE or 70-SE-4F instrument (EI), a Micromass Quattro instrument (LR-ESI), a Micromass Q-Tof Ultima instrument (HR-ESI), or a Voyager SE-DTR instrument (MALDI).

Synthesis of (*S*)-NPE-caged pyrimidine nucleoside phosphoramidites



Scheme S1. Syntheses of (*S*)-NPE-caged pyrimidine nucleoside phosphoramidites **5**, **6**, and **13** and (*S*)-1-(2-nitrophenyl)ethylamine **10**.

5′-*O*-(4,4′-Dimethoxytrityl)-*O*^{4}-[(*S*)-1-(2-nitrophenyl)ethyl]-2′-*O*-[[(triisopropylsilyl)oxy]methyl]uridine (3) and 5′-*O*-(4,4′-Dimethoxytrityl)-3-[(*S*)-1-(2-nitrophenyl)ethyl]-2′-*O*-[[(triisopropylsilyl)oxy]methyl]uridine (4)

A solution of 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-[[(triisopropylsilyl)oxy]methyl]uridine (**1**) (150 mg, 0.20 mmol, prepared as described[1a]) and triphenylphosphine (70 mg, 0.31 mmol) in dry THF (1 mL) was cooled in an ice-water bath. (*R*)-1-(2-Nitrophenyl)ethanol (**2**) (41 mg, 0.24 mmol, prepared as described[11]) was dissolved in dry THF (1 mL) and added via cannula. The solution was treated with diisopropyl azodicarboxylate (60 µL, 0.30 mmol) within 5 min, then stirred at 0 °C for 20 min and then at room temperature for 5 h. The yellow solution was concentrated under vacuum. The resulting viscous oil was dissolved in dichloromethane and concentrated to give a yellow foam. The isomers **3** and **4** were separated by chromatography with 2–6% diethyl ether in dichloromethane.

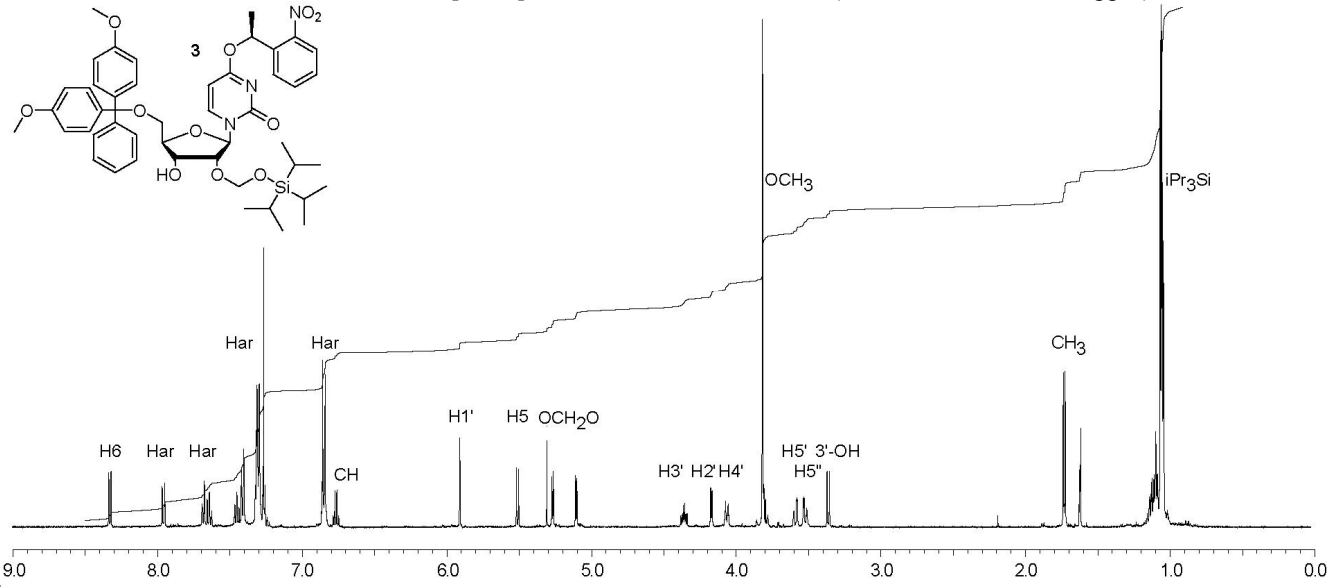
Yield: 65 mg of **3** (37%) and 70 mg of **4** (40%) as colorless foams.

Data of **3**: TLC (dichloromethane/diethyl ether 9:1): R_f 0.6.

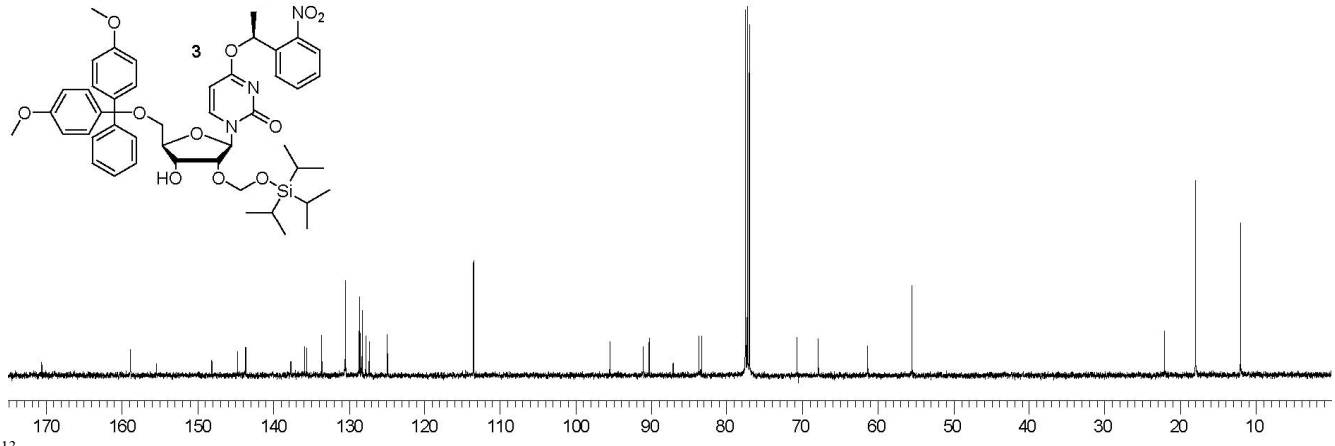
^{1}H NMR (500 MHz, $CDCl_3$): δ 1.03-1.13 (m, 21H, iPr_3Si); 1.72 (d, J = 6.5 Hz, 3H, OCHC$\underline{H}_3$); 3.34 (d, J = 8.5 Hz, 1H, HO-C(3′)); 3.51 (dd, J = 2.7, 11.1 Hz, 1H, H^1-C(5′)); 3.58 (dd, J = 1.7, 11.1 Hz, 1H, H^2-C(5′)); 3.81 (s, 6H, 2 OCH_3); 4.06 (m, 1H, H-C(4′)); 4.16 (d, $J \approx$ 4.8 Hz, 1H, H-C(2′)); 4.35 (m, 1H, H-C(3′)); 5.10, 5.26 (2d, J = 4.7 Hz, 2H, OCH_2O); 5.51 (d, J = 7.4 Hz, 1H, H-C(5)); 5.91 (d, J < 1 Hz, 1H, H-C(1′)); 6.77 (q, J = 6.5 Hz, 1H, OC$\underline{H}CH_3$); 6.85 (m, 4H, H-C(ar-DMT)); 7.26-7.32 (m, 7H, H-C(ar-DMT)); 7.40-7.42 (m, 2H, H-C(ar-DMT)); 7.45, 7.64 (2t, $J \approx$ 7 Hz, 2H, H-C(ar-NPE)); 7.68, 7.96 (2d, $J \approx$ 7 Hz, 2H, H-C(ar-NPE)); 8.33 (d, J = 7.4 Hz, 1H, H-C(6)) ppm.

^{13}C NMR (125 MHz, $CDCl_3$): δ 11.76 (Si(C$\underline{H}$(CH$_3$)$_2$)$_3$); 17.72 (Si(CH(C$\underline{H}_3$)$_2$)$_3$); 21.81 (OCH$\underline{C}H_3$); 55.23 (OCH_3); 61.10 (C(5′)); 67.61 (C(3′)); 70.42 (O$\underline{C}$HCH$_3$); 83.06 (C(2′)); 83.43 (C(4′)); 86.83; 90.00 (C(1′)); 90.81 (OCH_2O); 95.19 (C(5)); 113.23, 124.63, 127.03, 127.48, 127.95, 128.26, 128.39, 130.18 (8 C(ar)); 133.31, 135.33, 135.60, 137.41; 143.38 (C(6)); 144.42, 147.86; 155.17 (C(2)); 158.60, 170.35 (C(4)) ppm.

ESI-HRMS: *m*/*z* calcd. for $C_{48}H_{59}N_3O_{11}Si$ $[M+H]^+$ 882.3997, found 882.3392 (Δm –0.0005, error –0.5 ppm).

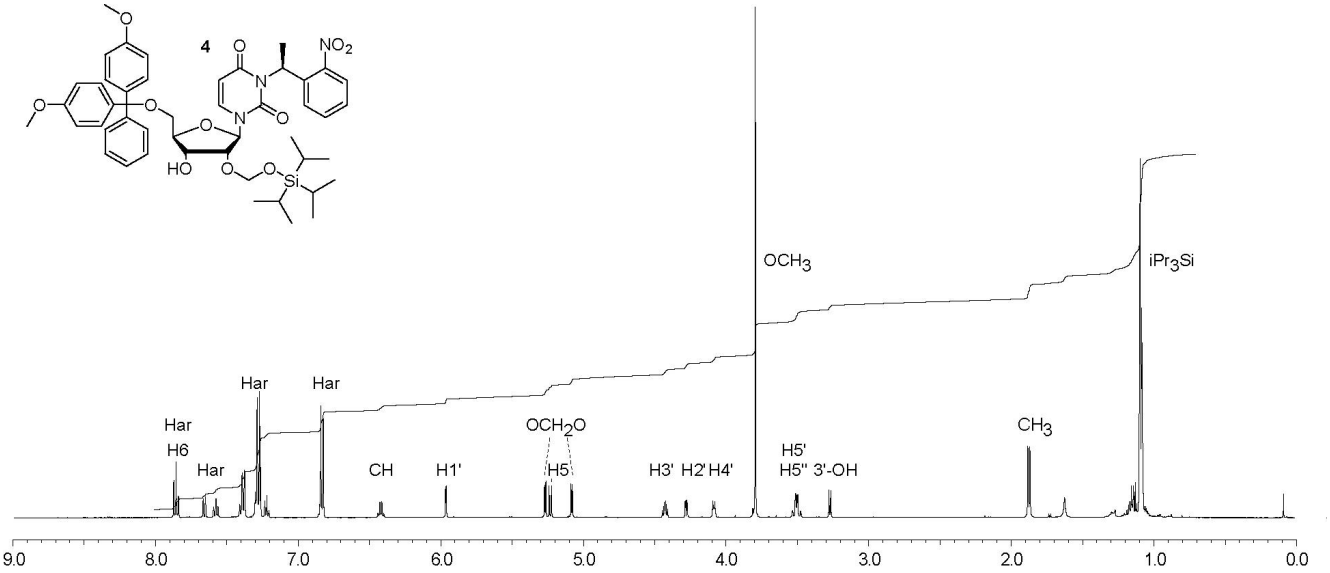


^{1}H NMR (500 MHz, $CDCl_3$) of **3**.

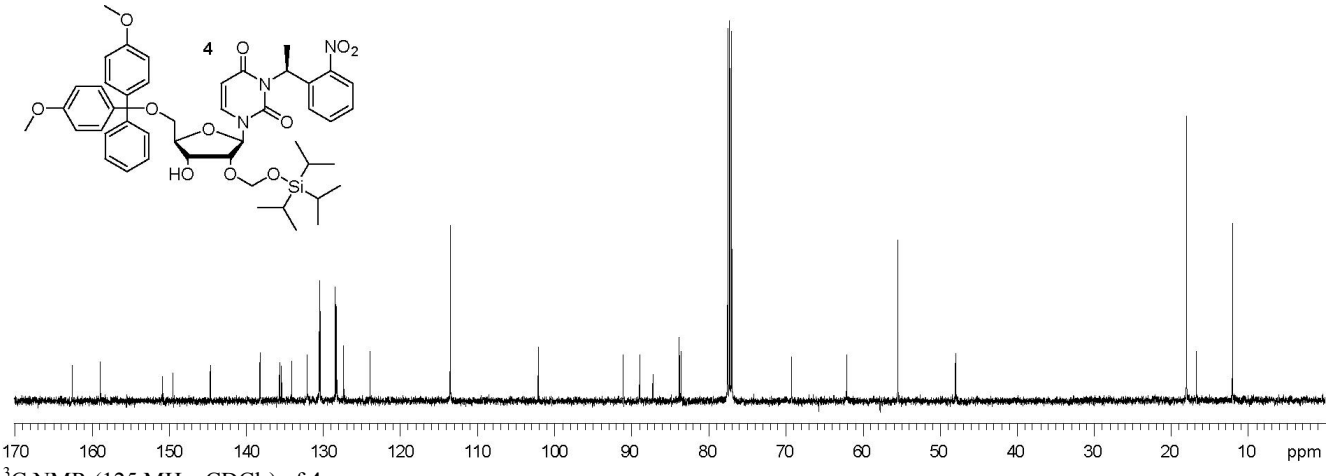


^{13}C NMR (125 MHz, $CDCl_3$) of **3**.

Data of **4**: TLC (dichloromethane/diethyl ether 9:1): R_f 0.7.
^{1}H NMR (500 MHz, $CDCl_3$): δ 1.04-1.18 (m, 21H, iPr_3Si); 1.87 (d, J = 7.0 Hz, 3H, NCHC$\underline{H}_3$); 3.27 (d, J = 6.3 Hz, 1H, HO-C(3′)); 3.49 (dd, J = 2.6, 11.0 Hz, 1H, H^1-C(5′)); 3.51 (dd, J = 2.1, 11.0 Hz, 1H, H^2-C(5′)); 3.79 (s, 6H, 2 OCH_3); 4.08 (m, 1H, H-C(4′)); 4.28 (dd, J = 4.9, 2.7 Hz, 1H, H-C(2′)); 4.43 (m, 1H, H-C(3′)); 5.08, 5.27 (2d, J = 4.7 Hz, 2H, OCH_2O); 5.23 (d, J = 8.1 Hz, 1H, H-C(5)); 5.97 (d, J = 2.7 Hz, 1H, H-C(1′)); 6.42 (q, J = 7.0 Hz, 1H, NC$\underline{H}CH_3$); 6.84 (m, 4H, H-C(ar-DMT)); 7.21-7.31 (m, 7H, H-C(ar-DMT)); 7.38-7.41 (m, 3H, H-C(ar-DMT), H-C(ar-NPE)); 7.58 (ddd, J = 1.1, 8.1, 8.3 Hz, 1H, H-C(ar-NPE)); 7.67 (dd, J = 1.1, 8.1 Hz, 1H, H-C(ar-NPE)); 7.85 (d, J = 8.3 Hz, 1H, H-C(ar-NPE)); 7.87 (d, J = 8.1 Hz, 1H, H-C(6)) ppm.
^{13}C NMR (125 MHz, $CDCl_3$): δ 11.80 (Si($\underline{C}$H(CH_3)$_2$)$_3$); 16.48 (NCH$\underline{C}H_3$); 17.75 (Si(CH($\underline{C}H_3$)$_2$)$_3$); 47.72 (N$\underline{C}$HCH$_3$); 55.21 (OCH_3); 61.84 (C(5′)); 68.96 (C(3′)); 83.31 (C(2′)); 83.54 (C(4′)); 86.96; 88.67 (C(1′)); 90.83 (OCH_2O); 101.82 (C(5)); 113.23, 123.65, 127.6, 127.97, 128.10, 128.13, 130.12, 130.16, 130.28, 131.78 (10 C(ar)); 133.83, 135.12, 135.38; 137.92 (C(6)); 144.35, 149.22; 150.54 (C(2)); 158.61, 158.64; 162.25 (C(4)) ppm.
ESI-HRMS: m/z calcd. for $C_{48}H_{59}N_3O_{11}Si$ $[M+H]^+$ 882.3997, found 882.4006 (Δm +0.0009, error +1.0 ppm).



^{1}H NMR (500 MHz, $CDCl_3$) of **4**.



^{13}C NMR (125 MHz, $CDCl_3$) of **4**.

5′-*O*-(4,4′-Dimethoxytrityl)-*O*^{4}-[(*S*)-1-(2-nitrophenyl)ethyl]-2′-*O*-[[(triisopropylsilyl)oxy]methyl]uridine 3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (5)

A solution of compound **3** (140 mg, 158 µmol) in a dry dichloromethane (1.5 mL) containing ethyldimethylamine (172 µL, 1.58 mmol) was treated with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (39 mg, 166 µmol). After stirring for 1 h at room temperature, the solution was quenched with methanol (100 µL), diluted with dichloromethane, extracted with semi-saturated aqueous sodium bicarbonate solution, dried over Na_2SO_4, and evaporated. The crude product was chromatographed with ethyl acetate/hexane 1:4–1:3 containing 2% triethylamine.

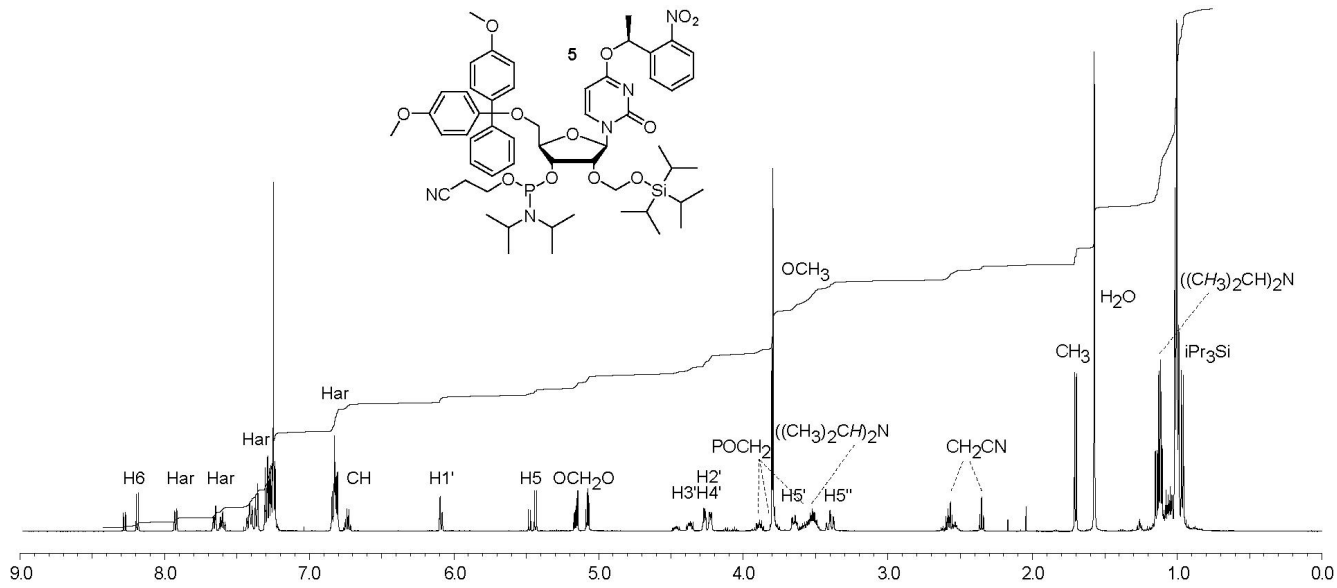
Yield: 140 mg of **5** as a colorless foam (5:9 mixture of diastereoisomers, 82%).

TLC (dichloromethane/diethyl ether 9:1): R_f 0.7.

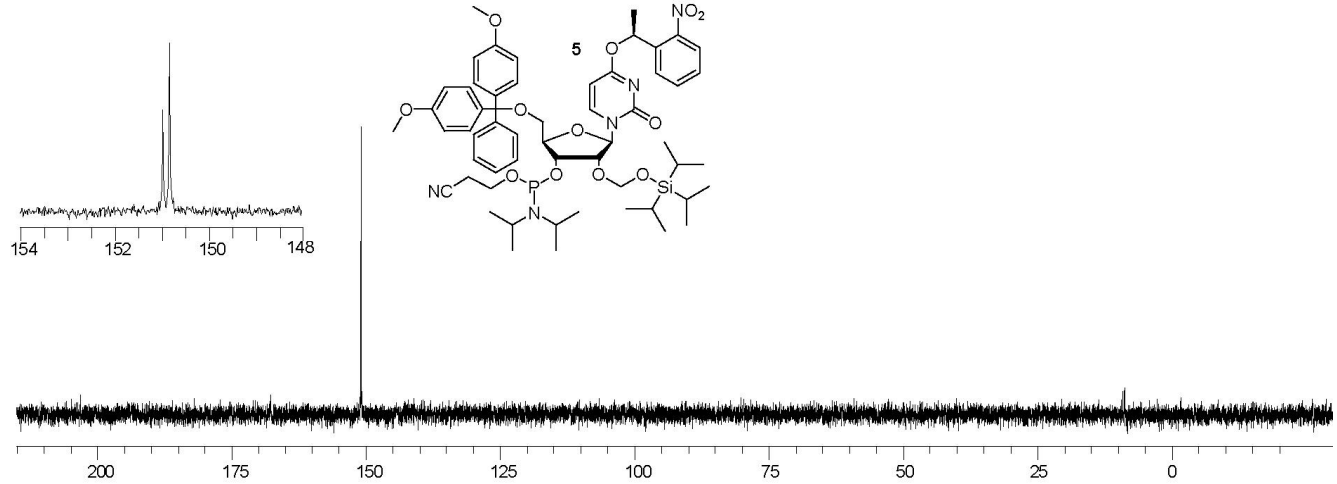
^{1}H NMR (500 MHz, $CDCl_3$): δ 0.95-1.09 (m, 66H, iPr_3Si, $((C\underline{H}_3)_2CH)_2N$); 1.70 (2d, J = 6.3 Hz, 6H, $OCHC\underline{H}_3$); 2.35 (m, 2H, CH_2CN); 2.58 (m, 2H, CH_2CN); 3.38-3.43 (m, 2H, H^1-C(5′)); 3.49-3.67 (m, 6H, H^2-C(5′), $((CH_3)_2C\underline{H})_2N$); 3.80-3.82 (m, 14H, OCH_3, $POCH_2$); 3.88-3.90 (m, 2H, $POCH_2$); 4.23-4.28 (2m, 4H, H-C(2′), H-C(4′)); 4.35-4.40, 4.45-4.50 (2m, 2H, H-C(3′)); 5.08, 5.09, 5.16, 5.17 (4d, 4H, J = 4.2 Hz, OCH_2O); 5.45, 5.49 (2d, J = 7.3, 7.6 Hz, 2H, H-C(5)); 6.09, 6.11 (2d, J = 2.0, 2.5 Hz, 2H, H-C(1′)); 6.75 (2q, J = 6.3 Hz, 2H, $OC\underline{H}CH_3$); 6.80-6.86 (m, 8H, H-C(ar-DMT)); 7.25-7.32 (m, 14H, H-C(ar-DMT)); 7.36-7.45 (m, 6H, H-C(ar-DMT), H-C(ar-NPE)); 7.60-7.69 (2m, 4H, H-C(ar-NPE)); 7.93-7.95 (m, 2H, H-C(ar-NPE)); 8.21, 8.29 (2d, J = 7.3, 7.6 Hz, 2H, H-C(6)) ppm.

^{31}P NMR (162 MHz, $CDCl_3$): δ 150.85, 150.99 ppm.

ESI-HRMS: *m*/*z* calcd. for $C_{57}H_{76}N_5O_{12}PSi$ $[M+H]^+$ 1082.5076, found 1082.5082 (Δm +0.0006, error +0.6 ppm).



^{1}H NMR (500 MHz, $CDCl_3$) of **5**.



^{31}P NMR (162 MHz, $CDCl_3$) of **5**.

5′-*O*-(4,4′-Dimethoxytrityl)-3-[(*S*)-1-(2-nitrophenyl)ethyl]-2′-*O*-[[(triisopropylsilyl)oxy]methyl]uridine 3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (6)

A solution of compound **4** (110 mg, 124 µmol) in a dry dichloromethane (1.5 mL) containing ethyldimethylamine (135 µL, 1.24 mmol) was treated with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (31 mg, 130 µmol). After stirring for 1.5 h at room temperature, the sample was quenched with methanol (100 µL), diluted with dichloromethane, extracted with semi-saturated aqueous sodium bicarbonate solution, dried over Na_2SO_4, and evaporated. The crude product was chromatographed with ethyl acetate/hexane 1:4 containing 2% triethylamine.

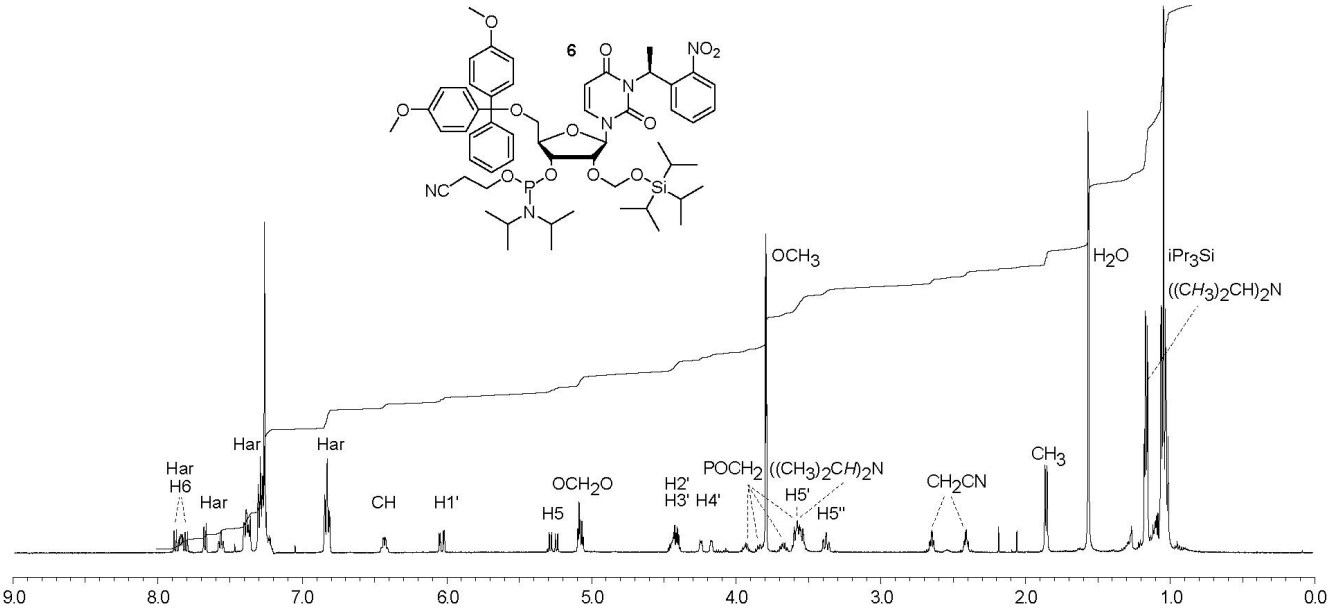
Yield: 125 mg of **6** as a colorless foam (1:1 mixture of diastereoisomers, 93%).

TLC (dichloromethane/diethyl ether 9:1): R_f 0.8.

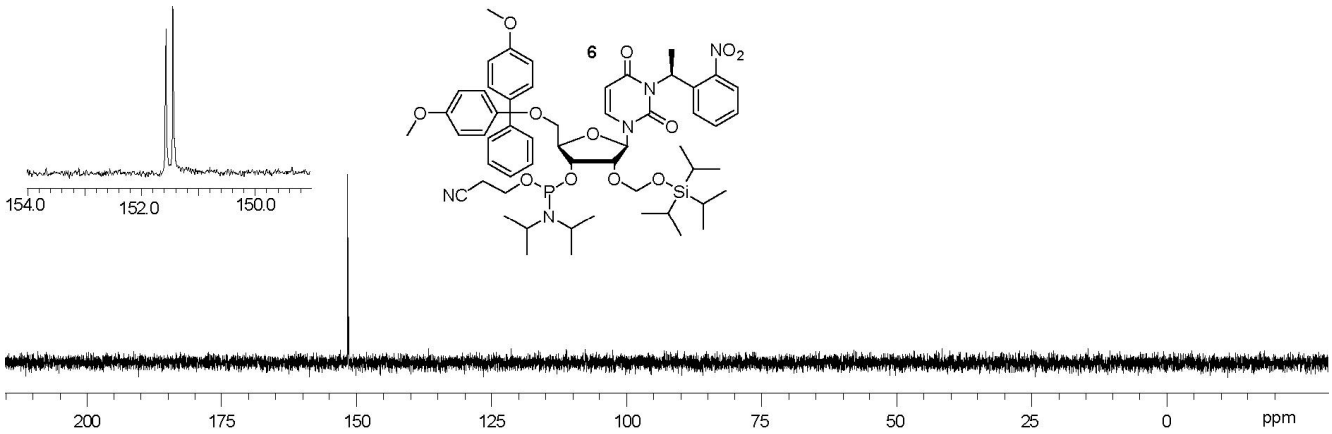
^{1}H NMR (500 MHz, $CDCl_3$): δ 1.00-1.18 (m, 66H, iPr_3Si, $((CH_3)_2CH)_2N$); 1.85 (2d, J = 7.0 Hz, 6H, $OCHCH_3$); 2.40 (m, 2H, CH_2CN); 2.64 (m, 2H, CH_2CN); 3.35-3.40 (m, 2H, H^1-C(5′)); 3.51-3.61 (m, 7H, H^2-C(5′), $POCH_2$, $((CH_3)_2CH)_2N$); 3.63-3.71 (m, 1H, $POCH_2$); 3.78-3.86 (m, 13H, OCH_3, $POCH_2$); 3.89-3.96 (m, 1H, $POCH_2$); 4.15-4.18, 4.23-4.25 (2m, 2H, H-C(4′)); 4.38-4.45 (m, 4H, H-C(2′), H-C(3′)); 5.05-5.09 (m, 4H, OCH_2O); 5.24, 5.28 (2d, J = 8.0 Hz, 2H, H-C(5)); 6.02, 6.05 (2d, J = 3.8, 3.5 Hz, 2H, H-C(1′)); 6.43, 6.44 (2q, J = 7.0 Hz, 2H, $OCHCH_3$); 6.81-6.85 (m, 8H, H-C(ar-DMT)); 7.24-7.31 (m, 14H, H-C(ar-DMT)); 7.36-7.40 (m, 6H, 4 H-C(ar-DMT), 2 H-C(ar-NPE)); 7.57 (t, $J \approx$ 7.5 Hz, 2H, H-C(ar-NPE)); 7.62 (d, J = 7.7 Hz, 2H, H-C(ar-NPE)); 7.81 (d, J = 8.0 Hz, 1H, H-C(6)); 7.83-7.85 (m, 2H, H-C(ar-NPE)); 7.88 (d, J = 8.0 Hz, 1H, H-C(6)) ppm.

^{31}P NMR (202 MHz, $CDCl_3$): δ 151.58, 151.61 ppm.

ESI-HRMS: *m*/*z* calcd. for $C_{57}H_{76}N_5O_{12}PSi$ $[M+H]^+$ 1082.5076, found 1082.5073 (Δm –0.0003, error –0.3 ppm).



^{1}H NMR (500 MHz, $CDCl_3$) of **6**.



^{31}P NMR (202 MHz, $CDCl_3$) of **6**.

3′-*O*-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-[[(triisopropylsilyl)oxy]methyl]uridine (7)

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[[(triisopropylsilyl)oxy]methyl]uridine (**1**)[a] (650 mg, 0.89 mmol) was dissolved in pyridine (6 mL). At room temperature, DMAP (12 mg, 0.1 mmol) was added, followed by acetic anhydride (135 µL, 1.33 mmol). After stirring for 50 min, TLC showed complete consumption of the starting material. Methanol (0.1 mL) was added and the solvents were evaporated. The residue was dissolved in dichloromethane, and the solution was extracted with 5% aqueous citric acid, water, and saturated aqueous sodium bicarbonate solution. The solution was dried over Na_2SO_4 and the solvent was evaporated. The crude product was used in the next transformation without further purification. For characterization, 25 mg were purified by chromatography with 1–2% methanol in dichloromethane.

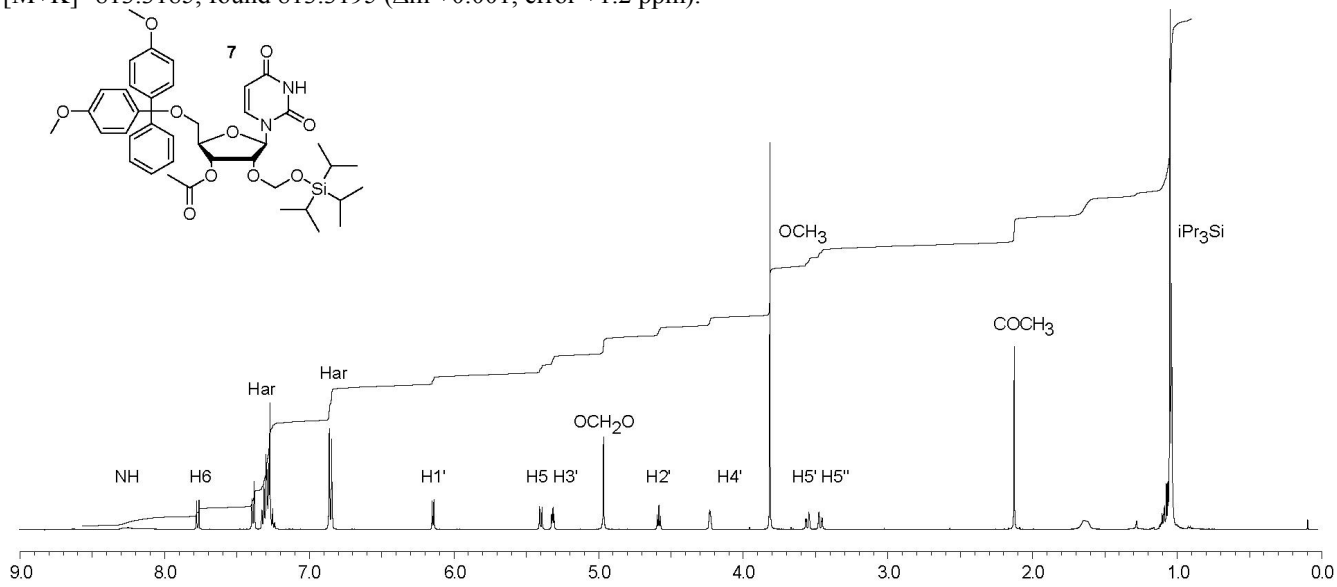
Yield: 654 mg of crude **7** as a colorless foam (95%).

TLC (dichloromethane/methanol 96:4): R_f 0.5.

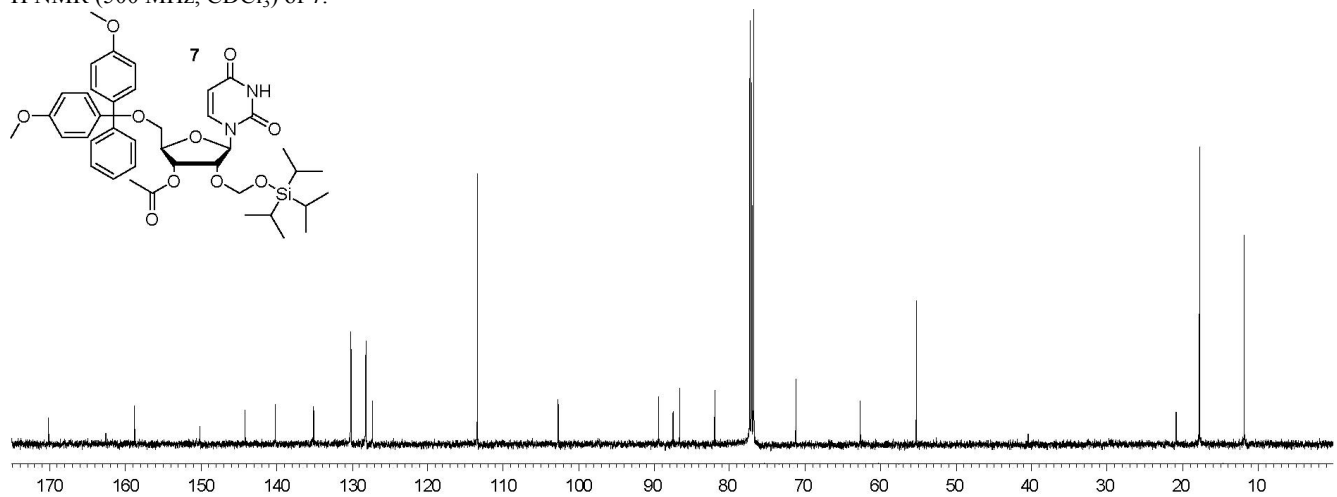
^{1}H NMR (500 MHz, $CDCl_3$): δ 1.01-1.10 (m, 21H, iPr_3Si); 2.11 (s, 3H, $COCH_3$); 3.45, 3.54 (2dd, J = 2.3, 11.0 Hz, 2H, H_2-C(5′)); 3.80 (s, 6H, 2 OCH_3); 4.21 (m, 1H, H-C(4′)); 4.57 (m, 1H, H-C(2′)); 4.95 (s, 2H, OCH_2O); 5.30 (m, 1H, H-C(3′)); 5.39 (d, J = 8.1 Hz, 1H, H-C(5)); 6.13 (d, J = 6.0 Hz, 1H, H-C(1′)); 6.84 (m, 4H, H-C(ar)); 7.23-7.32 (m, 7H, H-C(ar)); 7.37-7.39 (m, 2H, H-C(ar)); 7.76 (d, J = 8.1 Hz, 1H, H-C(6)); 8.2 (br., 1H, NH) ppm.

^{13}C NMR (125 MHz, $CDCl_3$): δ 11.80 (Si($\underline{C}H(CH_3)_2)_3$); 17.72 (Si($CH(\underline{C}H_3)_2)_3$); 20.81 ($CO\underline{C}H_3$); 55.213 ($OCH_3$); 62.64 (C(5′)); 71.19 (C(3′)); 77.25 (C(2′)); 81.93 (C(4′)); 86.56 (C(1′)); 87.44; 89.35 (OCH_2O); 102.65 (C(5)); 113.34, 127.23, 128.08, 128.12, 130.08, 130.13 (6 C(ar)); 135.00, 135.11; 140.11 (C(6)); 144.10; 150.13 (C(2)); 158.76; 162.57 (C(4)); 170.13 ($\underline{C}OCH_3$) ppm.

ESI-HRMS: *m/z* calcd. for $C_{42}H_{54}N_2O_{10}Si$ $[M+Na]^+$ 797.3445, found 797.3456 (Δm +0.0011, error +1.4 ppm); calcd. for $[M+K]^+$ 813.3185, found 813.3195 (Δm +0.001, error +1.2 ppm).



^{1}H NMR (500 MHz, $CDCl_3$) of **7**.



^{13}C NMR (125 MHz, $CDCl_3$) of **7**.

3′-*O*-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-*O*^{4}-(2-mesitylene)sulfonyl-2′-*O*-[[(triisopropylsilyl)oxy]methyl]uridine (8)

Compound **7** (600 mg, 0.77 mmol) was dissolved in dichloromethane (5 mL). Triethylamine (540 µL, 3.85 mmol) and DMAP (11 mg, 0.09 mmol) were added, followed by 2-mesitylenesulfonyl chloride (219 mg, 1.0 mmol). The solution was stirred for 45 min at room temperature. A precipitate of small colorless needles was observed. The reaction mixture was diluted with dichloromethane, and the clear solution was extracted with saturated aqueous sodium bicarbonate solution, dried over Na_2SO_4, and evaporated. The crude product was purified by chromatography with 0–0.5% methanol in dichloromethane.

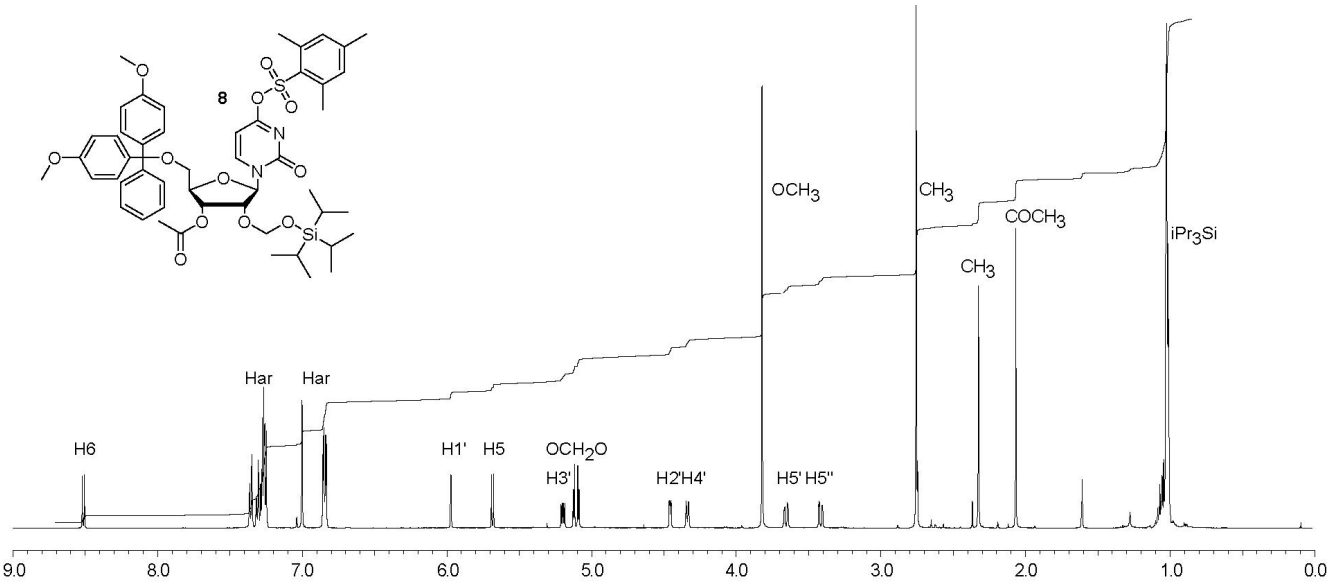
Yield: 577 mg of **8** as a colorless foam (78%).

TLC (dichloromethane/methanol 98:2): R_f 0.7.

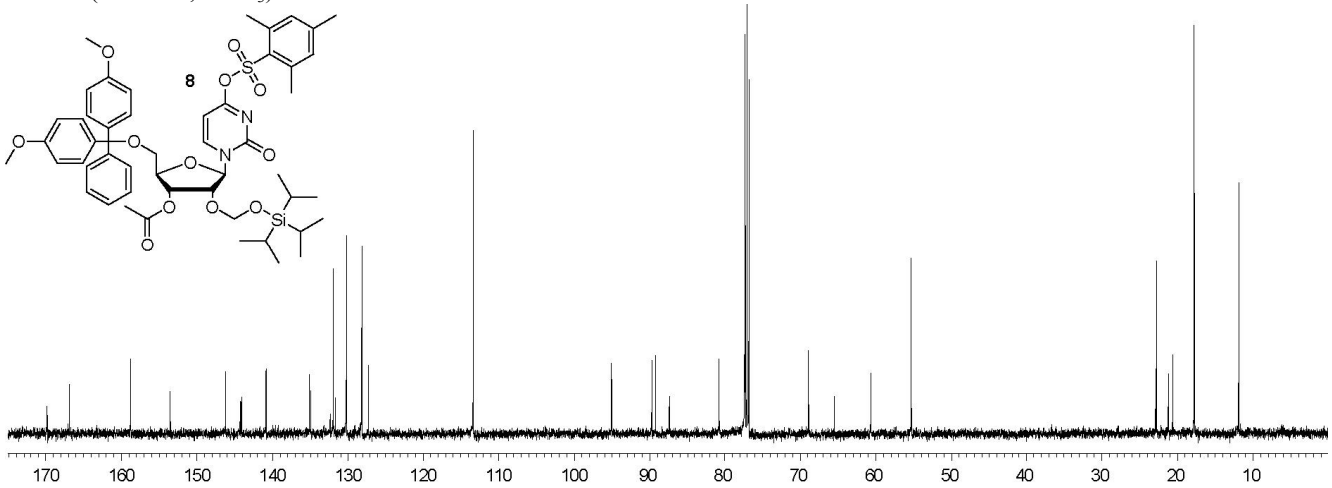
^{1}H NMR (500 MHz, $CDCl_3$): δ 1.00-1.08 (m, 21H, iPr_3Si); 2.05 (s, 3H, $COCH_3$); 2.31 (s, 3H, CH_3); 2.74 (s, 6H, 2 CH_3); 3.40, 3.64 (2dd, J = 1.9, 11.3 Hz, 2H, H_2-C(5′)); 3.81 (s, 6H, 2 OCH_3); 4.33 (m, 1H, H-C(4′)); 4.45 (dd, J = 1.8, 4.8 Hz, 1H, H-C(2′)); 5.08, 5.12 (2d, J = 4.6 Hz, 2H, OCH_2O); 5.19 (dd, J = 5.0, 8.0 Hz, 1H, H-C(3′)); 5.68 (d, J = 7.3 Hz, 1H, H-C(5)); 5.97 (d, J = 1.8 Hz, 1H, H-C(1′)); 6.84 (m, 4H, H-C(ar)); 7.00 (s, 2H, H-C(ar)); 7.25-7.36 (m, 9H, H-C(ar)); 8.51 (d, J = 7.3 Hz, 1H, H-C(6)) ppm.

^{13}C NMR (125 MHz, $CDCl_3$): δ 11.82 (Si(CH(CH_3)$_2$)$_3$); 17.71 (Si(CH(CH_3)$_2$)$_3$); 20.59 (COCH$_3$); 21.15 (CH_3); 22.74 (2 CH_3); 55.24 (OCH_3); 60.60 (C(5′)); 68.87 (C(3′)); 77.34 (C(2′)); 80.72 (C(4′)); 87.32; 89.15 (OCH_2O); 89.64 (C(1′)); 94.99 (C(5)); 113.33, 127.24, 128.07, 128.10, 130.12, 130.14; 131.89 (7 C(ar)); 134.86, 135.04, 140.81, 144.06, 144.23; 146.16 (C(6)); 153.50 (C(2)); 158.75; 166.90 (C(4)); 169.84 (COCH$_3$) ppm.

ESI-HRMS: *m*/*z* calcd. for $C_{51}H_{64}N_2O_{12}SSi$ $[M+H]^+$ 957.4028, found 957.4051 (Δm +0.0023, error +2.4 ppm).



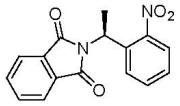
^{1}H NMR (500 MHz, $CDCl_3$) of **8**.



^{13}C NMR (125 MHz, $CDCl_3$) of **8**.

***N*-[(*S*)-1-(2-Nitrophenyl)ethyl]phthalimide (9)**

Phthalimide (520 mg, 3.5 mmol) was added to a solution of (*R*)-1-(2-nitrophenyl)ethanol[b] (**2**) (590 mg, 3.5 mmol) and triphenylphosphine (1.10 g, 4.2 mmol) in THF (10 mL). The suspension was cooled to 0 °C and treated with diisopropyl azodicarboxylate (0.83 mL, 4.2 mmol), which was added dropwise over 8 min. After stirring at room temperature for 3 h, the yellow solution was concentrated under vacuum. The residual sticky foam was chromatographed with dichloromethane.
Yield: 702 mg of **9** as a pale yellow solid (68%); TLC (dichloromethane): R_f 0.7.
^{1}H NMR (500 MHz, $CDCl_3$): δ 1.97 (d, J = 7.3 Hz, 3H, CH_3); 6.08 (q, J = 7.3 Hz, 1H, CH); 7.43, 7.60 (2t, $J \approx 8$ Hz, 2H, H-C(ar)); 7.68-7.72, 7.79-7.82 (2m, 5H, H-C(ar)); 7.92 (d, J = 7.5 Hz, 1H, H-C(ar)) ppm.
^{13}C NMR (125 MHz, $CDCl_3$): δ 18.38 (CH_3); 45.76 (CH); 123.38, 124.28, 128.62, 129.65, 131.64, 132.90, 134.16, 135.12, 148.94; 168.08 (CO) ppm.
EI-MS: m/z $[M+H]^+$ 297.2.

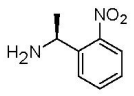


9

^{1}H NMR (500 MHz, $CDCl_3$) of **9**.

(*S*)-1-(2-Nitrophenyl)ethylamine (10)

The phthalimide derivative **9** (645 mg, 2.17 mmol) was dissolved in ethanol (10 mL) upon heating to ~50 °C. The nearly colorless solution was allowed to cool to room temperature and treated with hydrazine hydrate (263 µL, 5.44 mmol). The reaction mixture was then heated under reflux. After 10 min, the formation of a white precipitate was observed. After 50 min, the suspension was cooled on ice and diluted with diethyl ether (20 mL). The precipitate was separated by filtration and washed with diethyl ether. The combined filtrate and diethyl ether wash solutions were extracted with water (2 × 20 mL) and washed with brine. The organic layer was dried over Na_2SO_4 and the solvents were removed under vacuum. The crude product was used in the following transformation without further purification.
Yield: 323 mg of **10** as a pale yellow oil (89%); TLC (dichloromethane/methanol 97:3): R_f 0.3.
^{1}H NMR (500 MHz, $CDCl_3$): δ 1.45 (d, J = 6.6 Hz, 3H, CH_3); 4.59 (q, J = 6.6 Hz, 1H, CH); 7.36, 7.60 (2m, 2H, H-C(ar)); 7.78 (m, 2H, H-C(ar)) ppm.
^{13}C NMR (125 MHz, $CDCl_3$): δ 24.66 (CH_3); 45.95 (CH); 123.98, 127.48, 127.51, 133.08, 141.93, 148.96 ppm.
EI-MS: m/z $[M+H]^+$ 166.17.



10

^{1}H NMR (400 MHz, $CDCl_3$) of **10**.

3′-*O*-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-N^4-[(*S*)-1-(2-nitrophenyl)ethyl]-2′-*O*-[[(triisopropylsilyl)oxy]methyl]cytidine (11)

A solution of compound **8** (280 mg, 0.29 mmol) in dry DMF (1.5 mL) was treated with a solution of (*S*)-1-(2-nitrophenyl)ethylamine (**10**) (121.5 mg, 0.73 mmol) in dry DMF (1.5 mL) by addition via cannula; the flask was rinsed with DMF (1 mL). The solution was heated to 90 °C for 45 min. The solvent was removed under vacuum; the oily residue was dissolved in dichloromethane, extracted with 5% aqueous citric acid, and washed with water and saturated aqueous sodium bicarbonate solution. The organic layer was dried over Na_2SO_4 and concentrated under vacuum. The crude product was purified by chromatography with 0.25–1.5% methanol in dichloromethane.

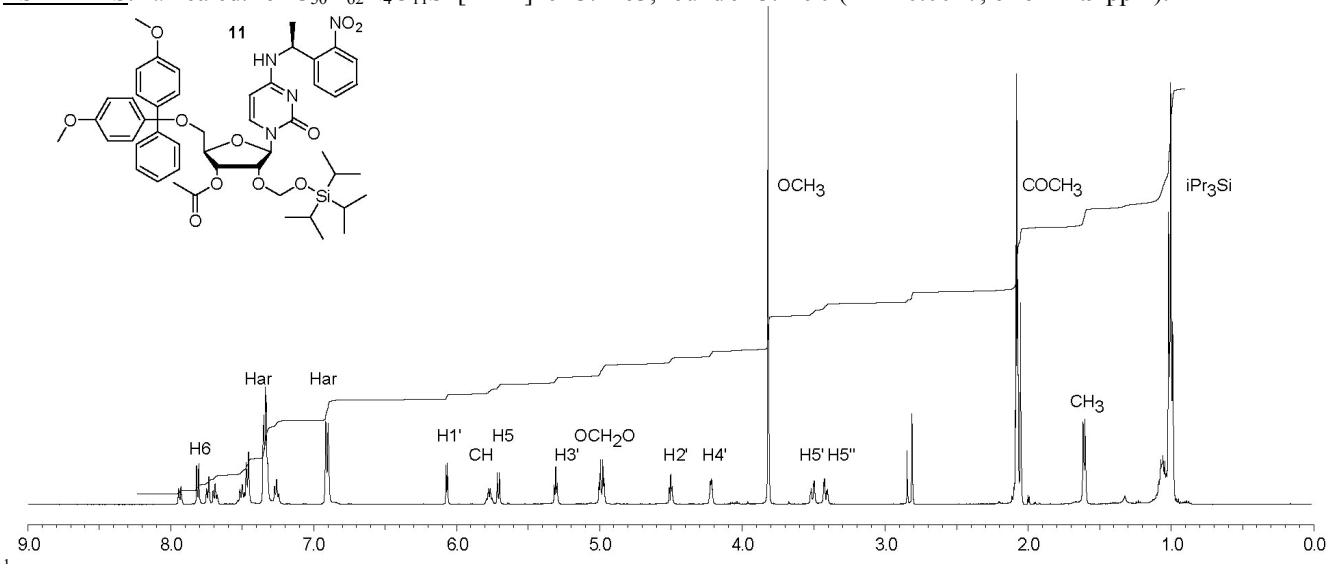
Yield: 215 mg of **11** as a colorless foam (79%)

TLC (dichloromethane/methanol 96:4): R_f 0.3.

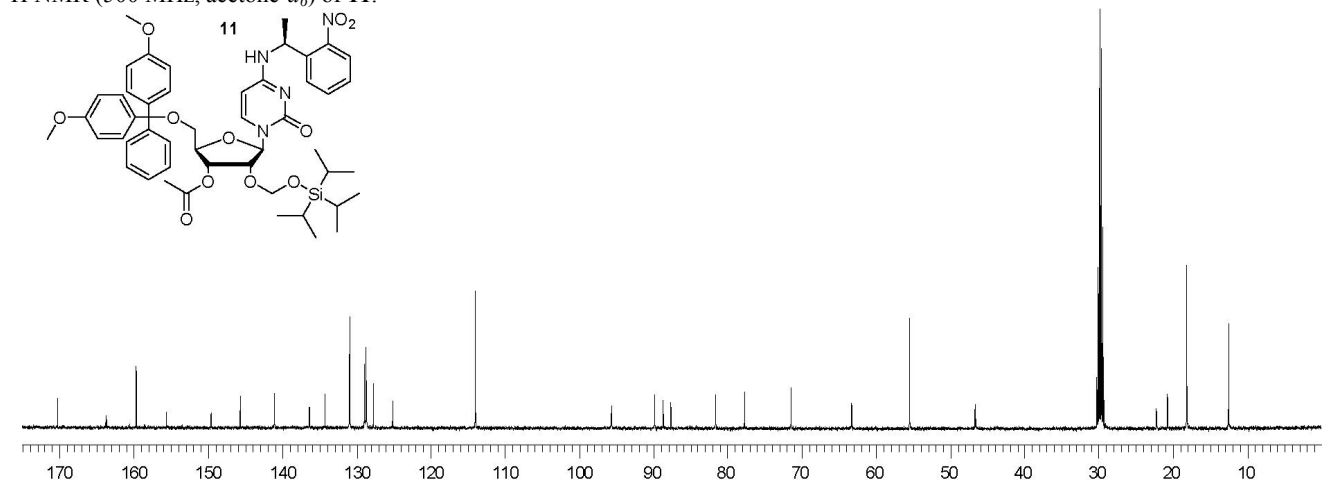
^{1}H NMR (500 MHz, acetone-d_6): δ 0.96 -1.08 (m, 21H, iPr_3Si); 1.59 (d, J = 6.9 Hz, 3H, NCHC$\underline{H}_3$); 2.03 (s, 3H, $COCH_3$); 3.39 (dd, J = 2.7, 11.0 Hz, 1H, H^1-C(5′)); 3.48 (dd, J = 3.0, 11.0 Hz, 1H, H^2-C(5′)); 3.81 (s, 6H, 2 OCH_3); 4.21 (m, 1H, H-C(4′)); 4.49 (t, $J \approx 5$ Hz, 1H, H-C(2′)); 4.96, 4.98 (2d, J = 4.9 Hz, 2H, OCH_2O); 5.30 (t, $J \approx 5$Hz, 1H, H-C(3′)); 5.70 (d, J = 7.4 Hz, 1H, H-C(5)); 5.76 (m, 1H, NC$\underline{H}$CH$_3$); 6.06 (d, J = 4.7 Hz, 1H, H-C(1′)); 6.90 (m, 4H, H-C(ar-DMT)); 7.26 (t, $J \approx 7.2$ Hz, 1H, H-C(ar-DMT)); 7.32-7.35 (m, 6H, H-C(ar-DMT)); 7.45-7.52 (m, 4H, 2 H-C(ar-DMT), 2H-C(ar-NPE)); 7.69 (t, $J \approx 7.5$ Hz, 1H, H-C(ar-NPE)); 7.74 (d, J = 7.7 Hz, 1H, H-C(ar-NPE)); 7.81 (d, J = 7.4 Hz, 1H, H-C(6)); 7.94 (d, J = 7.9 Hz, 1H, H-C(ar-NPE)) ppm.

^{13}C NMR (125 MHz, acetone-d_6): δ 12.50 (Si($\underline{C}$H(CH$_3$)$_2$)$_3$); 18.16 (Si(CH($\underline{C}$H$_3$)$_2$)$_3$); 20.74 (CO$\underline{C}$H$_3$); 22.24 (NCH$\underline{C}$H$_3$); 46.67 (N$\underline{C}$HCH$_3$); 55.49 (OCH_3); 63.30 (C(5′)); 71.45 (C(3′)); 77.72 (C(2′)); 81.62 (C(4′)); 87.64; 88.68 (C(1′)); 89.83 (OCH_2O); 95.66 (C(5)); 113.97, 125.13, 127.72, 128.71, 128.92, 130.93, 134.26 (7 C(ar)); 136.33, 136.39; 141.06 (C(6)); 145.67, 149.60; 155.60 (C(2)); 159.68; 163.75 (C(4)); 163.68; 170.28 ($\underline{C}OCH_3$) ppm.

ESI-HRMS: *m/z* calcd. for $C_{50}H_{62}N_4O_{11}Si$ $[M+H]^+$ 923.4263, found 923.4290 (Δm +0.0027, error +2.9 ppm).



^{1}H NMR (500 MHz, acetone-d_6) of **11**.



^{13}C NMR (125 MHz, acetone-d_6) of **11**.

5′-*O*-(4,4′-Dimethoxytrityl)-N^4-[(*S*)-1-(2-nitrophenyl)ethyl]-2′-*O*-[[(triisopropylsilyl)oxy]methyl]cytidine (12)

Compound **11** (200 mg, 0.21 mmol) was dissolved in a solution of 33% methylamine in ethanol (2 mL). The flask was tightly closed with a PE caplug and the solution was stirred at room temperature for 45 min. The sample was concentrated under vacuum, and the crude foam was chromatographed with 0.5–1.5% methanol in dichloromethane.

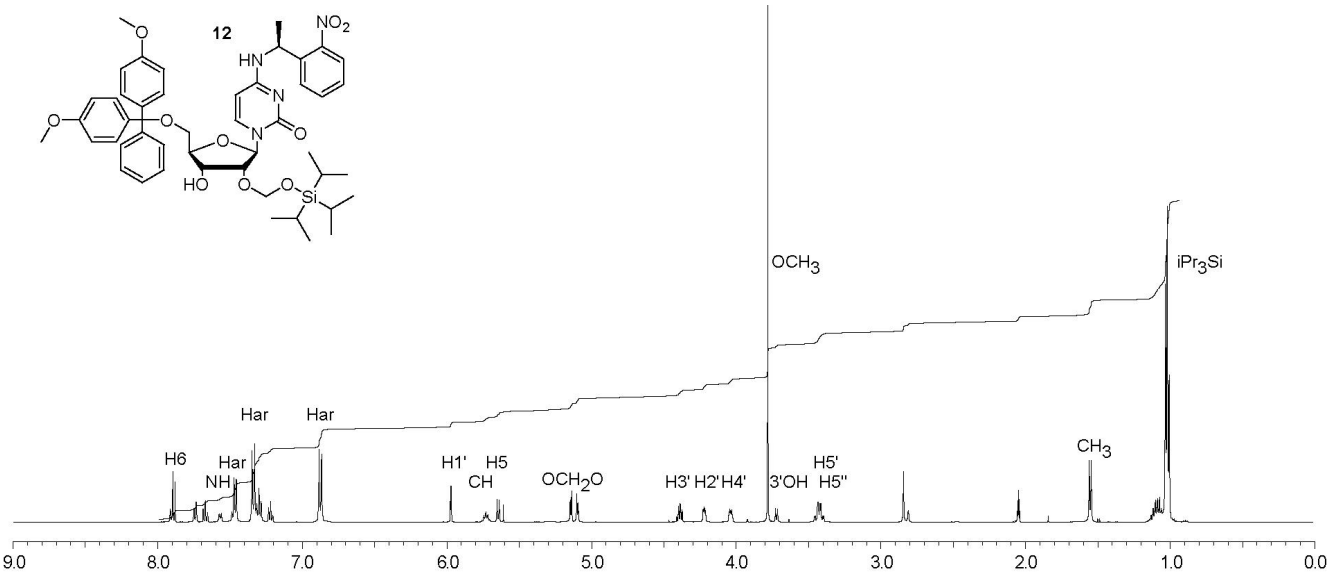
Yield: 168 mg of **12** as a colorless foam (88%).

TLC (dichloromethane/methanol 96:4): R_f 0.3.

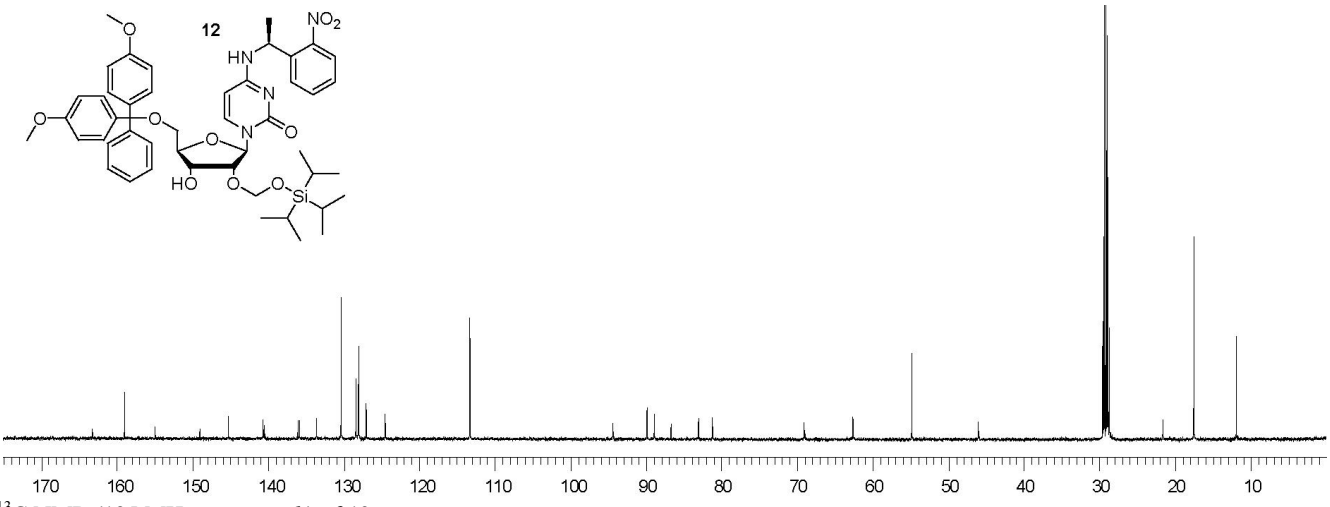
^{1}H NMR (500 MHz, acetone-d_6): δ 0.99 -1.10 (m, 21H, iPr_3Si); 1.54 (d, J = 7.0 Hz, 3H, $NCHCH_3$); 3.41 (dd, J = 3.7, 10.7 Hz, 1H, H^1-C(5′)); 3.42 (dd, J = 2.4, 10.7 Hz, 1H, H^2-C(5′)); 3.71 (d, J = 7.0 Hz, 1H, HO-C(3′)); 3.78 (s, 6H, 2 OCH_3); 4.03 (m, 1H, H-C(4′)); 4.22 (m, 1H, H-C(2′)); 4.38 (m, 1H, H-C(3′));, 5.10, 5.14 (2d, J = 4.9 Hz, 2H, OCH_2O); 5.64 (d, J = 7.3 Hz, 1H, H-C(5)); 5.73 (m, 1H, $NCHCH_3$); 5.97 (d, J = 2.8 Hz, 1H, H-C(1′)); 6.87 (m, 4H, H-C(ar-DMT)); 7.22 (t, $J \approx$ 7.2 Hz, 1H, H-C(ar-DMT)); 7.28-7.35 (m, 6H, H-C(ar-DMT)); 7.46-7.50 (m, 4H, 2 H-C(ar-DMT), 1 H-C(ar-NPE)); 7.57 (d, J = 7.0 Hz, 1H, NH); 7.67 (t, $J \approx$ 7.5 Hz, 1H, H-C(ar-NPE)); 7.75 (d, $J \approx$ 7.5 Hz, 1H, H-C(ar-NPE)); 7.88-7.92 (m, 2H, H-C(6), H-C(ar-NPE)) ppm.

^{13}C NMR (125 MHz, acetone-d_6): δ 12.55 ($Si(CH(CH_3)_2)_3$); 18.15 ($Si(CH(CH_3)_2)_3$); 22.26 ($NCHCH_3$); 46.66 ($NCHCH_3$); 55.47 (OCH_3); 63.25 (C(5′)); 69.71 (C(3′)); 81.84 (C(2′)); 83.68 (C(4′)); 87.32; 89.54 (C(1′)); 90.50 (OCH_2O); 95.04 (C(5)); 113.93, 125.14, 127.65, 128.66, 128.71, 129.02, 130.98, 134.22 (8 C(ar)); 136.47, 136.68, 141.11; 141.29 (C(6)); 145.89, 149.66; 155.58 (C(2)); 159.64; 163.88 (C(4)) ppm.

ESI-HRMS: m/z calcd. for $C_{48}H_{60}N_4O_{10}Si$ $[M+H]^+$ 881.4157, found 881.4168 (Δm +0.0011, error +1.3 ppm).



^{1}H NMR (500 MHz, acetone-d_6) of **12**.



^{13}C NMR (125 MHz, acetone-d_6) of **12**.

5′-*O*-(4,4′-dimethoxytrityl)-N^4-[(*S*)-1-(2-nitrophenyl)ethyl]-2′-*O*-[[(triisopropylsilyl)oxy]methyl]cytidine 3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (13)

A solution of compound **12** (145 mg, 164 µmol) in dry dichloromethane (2 mL) containing ethyldimethylamine (180 µL, 1.64 mmol) was treated with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (46 mg, 197 µmol). After stirring for 1.5 h at room temperature, the sample was quenched with methanol (100 µL), diluted with dichloromethane, extracted with semi-saturated sodium bicarbonate solution, dried over Na_2SO_4, and evaporated. The crude product was chromatographed with ethyl acetate/hexane 1:4–3:2 containing 2% triethylamine. For further purification (separation of a phosphonate impurity originating from hydrolyzed chlorophosphoramidite reagent), the product was dissolved in dichloromethane (1 mL) and the solution was added dropwise with stirring to hexane previously cooled to –20 °C. The precipitate was separated by centrifugation and the solvent was decanted. The purified phosphoramidite was dried under vacuum.

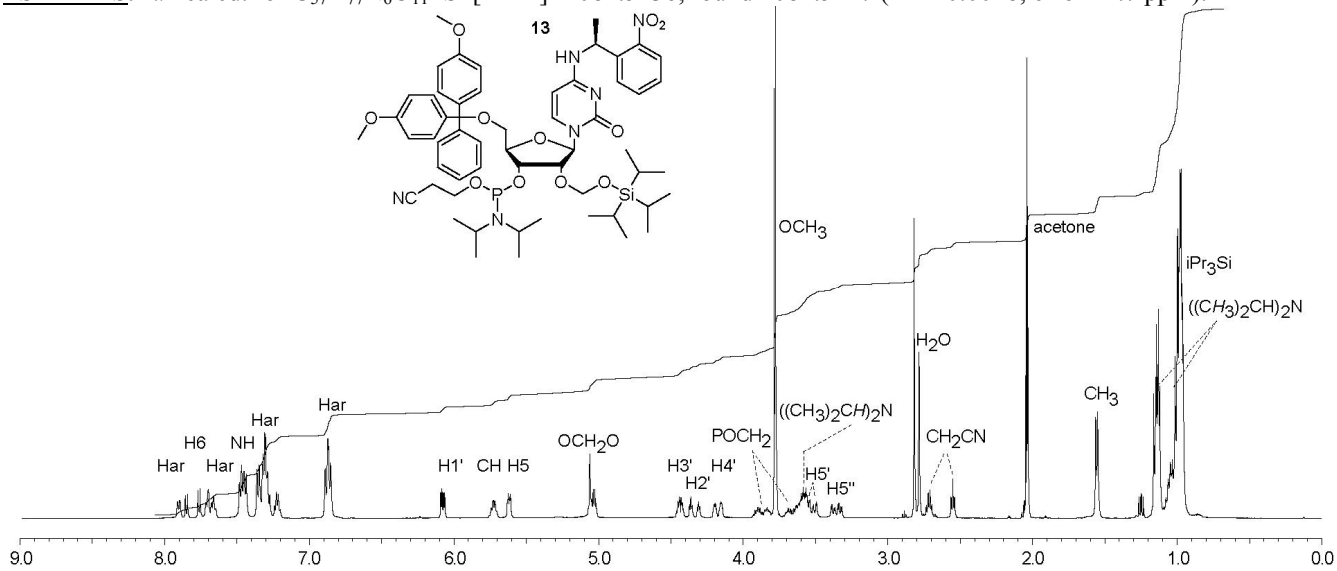
Yield: 153 mg of **13** as a colorless foam (1:1 mixture of diastereoisomers, 86 %).

TLC (ethyl acetate/hexanes 6:4): R_f 0.6.

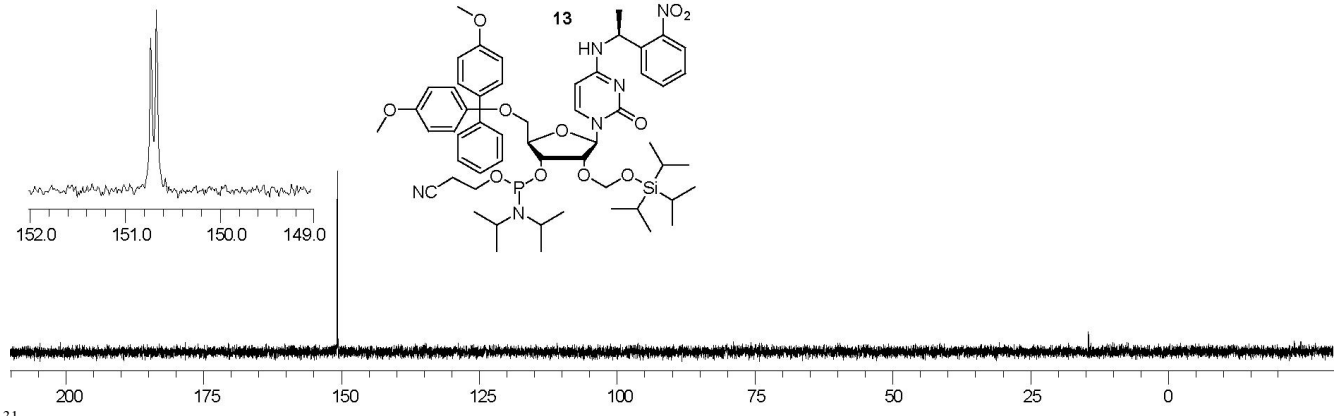
^{1}H NMR (500 MHz, acetone-d_6): δ 0.97-1.16 (m, 66H, iPr_3Si, $((CH_3)_2CH)_2N$); 1.55 (d, J = 7.0 Hz, 6H, $NCHCH_3$); 2.55 (m, 2H, CH_2CN); 2.72 (m, 2H, CH_2CN); 3.34 (dd, J = 3.8, 10.6 Hz, 1H, H^1-C(5′)); 3.38 (dd, J = 3.3, 10.9 Hz, 1H, H^1-C(5′)); 3.49-3.73 (m, 8H, H^2-C(5′), $POCH_2$, $((CH_3)_2CH)_2N$); 3.78, 3.79 (2s, 12H, OCH_3); 3.82-3.92 (m, 2H, $POCH_2$); 4.16, 4.20 (2m, 2H, H-C(4′)); 4.31, 4.37 (2m, 2H, H-C(2′)); 4.42-4.47 (m, 2H, H-C(3′)); 5.02-5.09 (m, 4H, OCH_2O); 5.62, 5.63 (2d, J = 7.4 Hz, 2H, H-C(5)); 5.71-5.76 (m, 2H, $NCHCH_3$); 6.08, 6.10 (2d, J = 4.1, 4.4 Hz, 2H, H-C(1′)); 6.86-6.91 (m, 8H, H-C(ar-DMT)); 7.21-7.25 (m, 2H, H-C(ar-DMT)); 7.28-7.38 (m, 14H, H-C(ar-DMT), H-C(ar-NPE), NH); 7.43-7.51 (m, 8H, H-C(ar-DMT), H-C(ar-NPE)); 7.66-7.73 (m, 3H, H-C(ar-NPE)); 7.78, 7.86 (2d, J = 7.4 Hz, 1H, H-C(6)); 7.91 (br.d, 1H, H-C(ar-NPE)) ppm.

^{31}P NMR (202 MHz, acetone-d_6): δ 150.66, 150.72 ppm.

ESI-HRMS: *m*/*z* calcd. for $C_{57}H_{77}N_6O_{11}PSi$ $[M+H]^+$ 1081.5236, found 1081.5217 (Δm –0.0018, error –1.7 ppm).

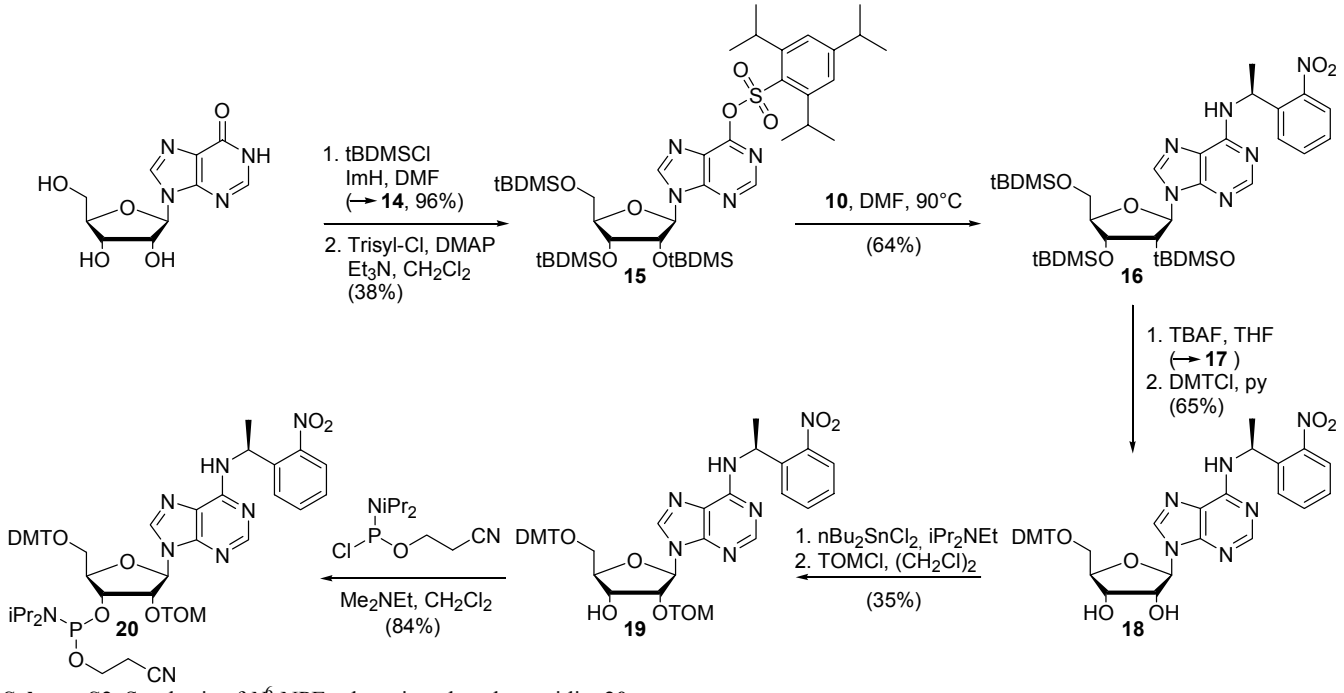


^{1}H NMR (500 MHz, acetone-d_6) of **13**.



^{31}P NMR (202 MHz, acetone-d_6) of **13**.

Synthesis of (*S*)-NPE-caged adenosine nucleoside phosphoramidite



Scheme S2. Synthesis of N^6-NPE-adenosine phosphoramidite **20**.

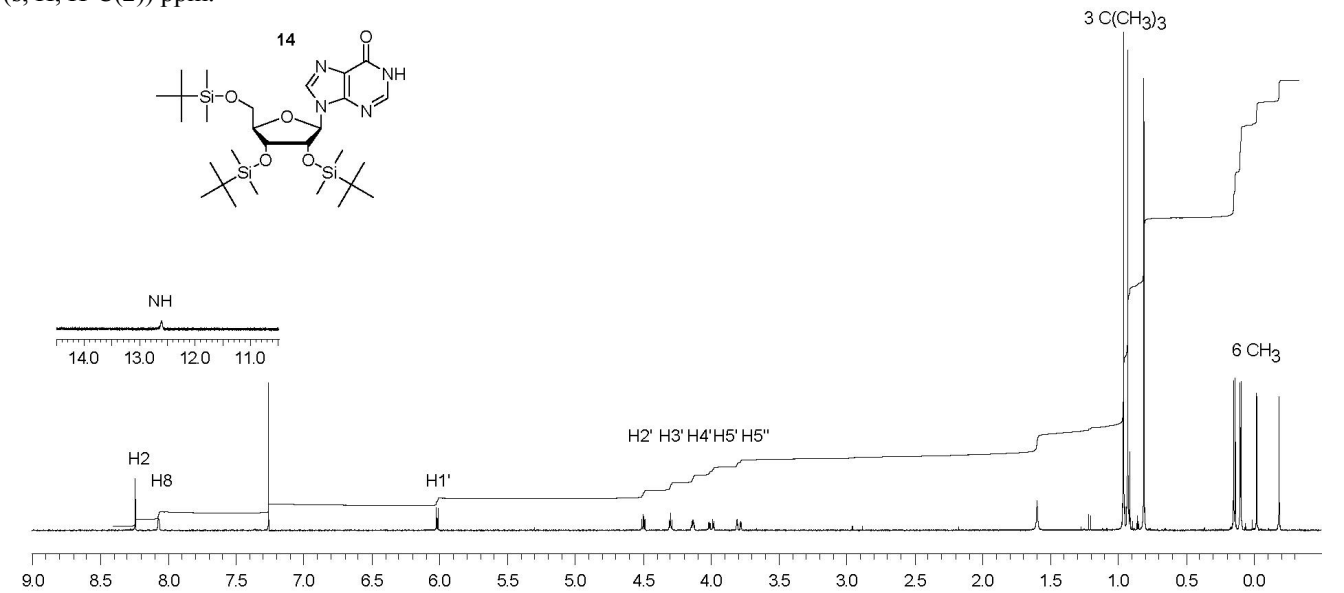
2′,3′,5′-Tris-*O*-(*tert*-butyldimethylsilyl)inosine (14)

tert-Butyldimethylsilyl chloride (2.8 g, 18.5 mmol) was added to a solution of inosine (1.0 g, 3.7 mmol) and imidazole (1.7 g, 25.0 mmol) in DMF (4 mL). The solution was stirred at room temperature for 12 h. The solvent was removed under vacuum. The residue was dissolved in dichloromethane and extracted with water and saturated aqueous sodium bicarbonate solution. The organic solution was dried over Na_2SO_4 and evaporated. The product was dried under vacuum and used for the following transformation without further purification.

Yield: 2.2 g of **14** as white powder (96%).

TLC (dichloromethane/methanol 96:4): R_f 0.3.

^{1}H NMR (400 MHz, $CDCl_3$): δ –0.19, –0.02, 0.09, 0.10, 0.14, 0.15 (6s, 18H, 6 $SiCH_3$); 0.81, 0.93, 0.96 (3s, 27H, 3 $SiC(CH_3)_3$); 3.80 (dd, J = 2.7, 11.4 Hz, 1H, H^1-C(5′)); 3.99 (dd, J = 3.8, 11.4 Hz, 1H, H^2-C(5′)); 4.13 (m, 1H, H-C(4′)); 4.29 (t, $J \approx$ 4.0 Hz, 1H, H-C(3′)); 4.49 (t, $J \approx$ 4.6 Hz, 1H, H-C(2′)); 6.01 (d, J = 4.9 Hz, 1H, H-C(1′)); 8.07 (s, 1H, H-C(8)); 8.24 (s, H, H-C(2)) ppm.



^{1}H NMR (400 MHz, $CDCl_3$) of **14**.

2′,3′,5′-Tris-*O*-(*tert*-butyldimethylsilyl)-O^6-[(2,4,6-triisopropylbenzene)sulfonyl]inosine (15)

A stirred solution of compound **15** (1.70 g, 2.78 mmol) and DMAP (34 mg, 0.28 mmol) in anhydrous dichloromethane (20 mL) was cooled to 0 °C and treated with triethylamine (1.9 mL, 13.9 mmol) then with (2,4,6-triisopropylbenzene)sulfonyl chloride (1.51 g, 5.0 mmol). The solution was stirred in the ice bath for 10 min and then for 45 min at room temperature. The solution was concentrated under vacuum; the residue was re-dissolved in dichloromethane and applied to a short flash filtration column of silica gel, eluting with dichloromethane. The O^6- and N^1-substituted isomers were separated by chromatography with 0–1% methanol in dichloromethane.

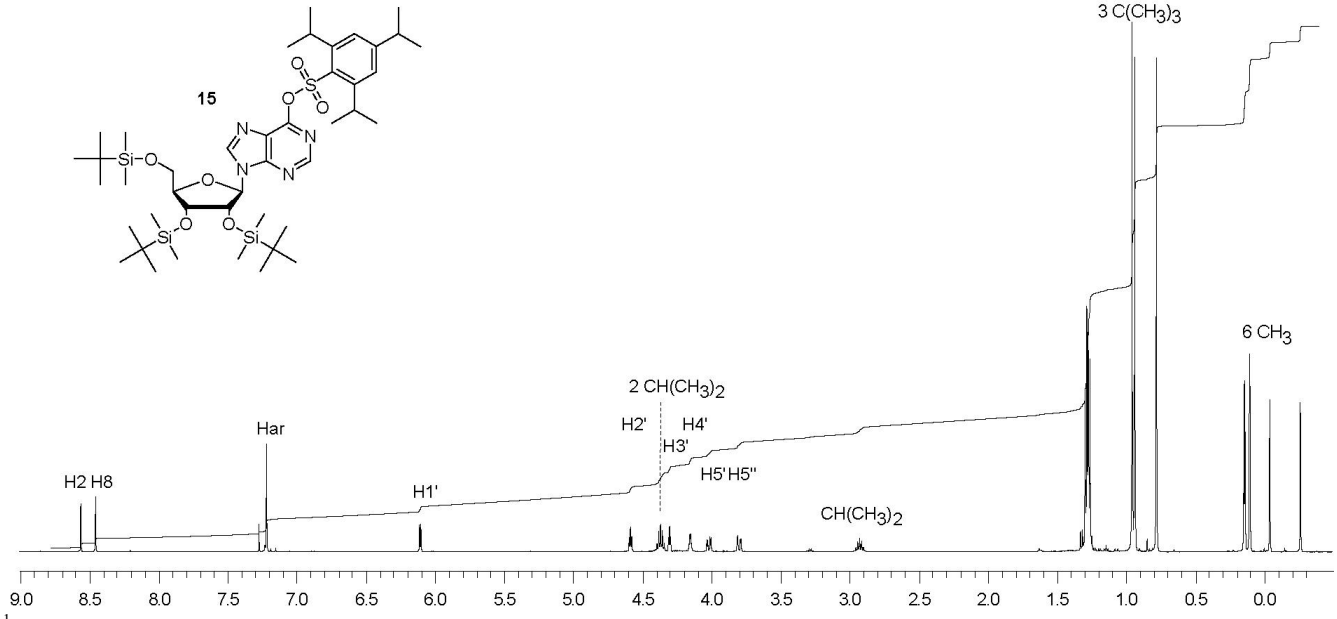
Yield: 0.92 g of **15** as a colorless foam (38%) and 1.33 g of N^1-sulfonylated isomer as a colorless foam (54%).

TLC (dichloromethane/methanol 99:1): R_f 0.8, N^1-sulfonylated isomer R_f 0.7.

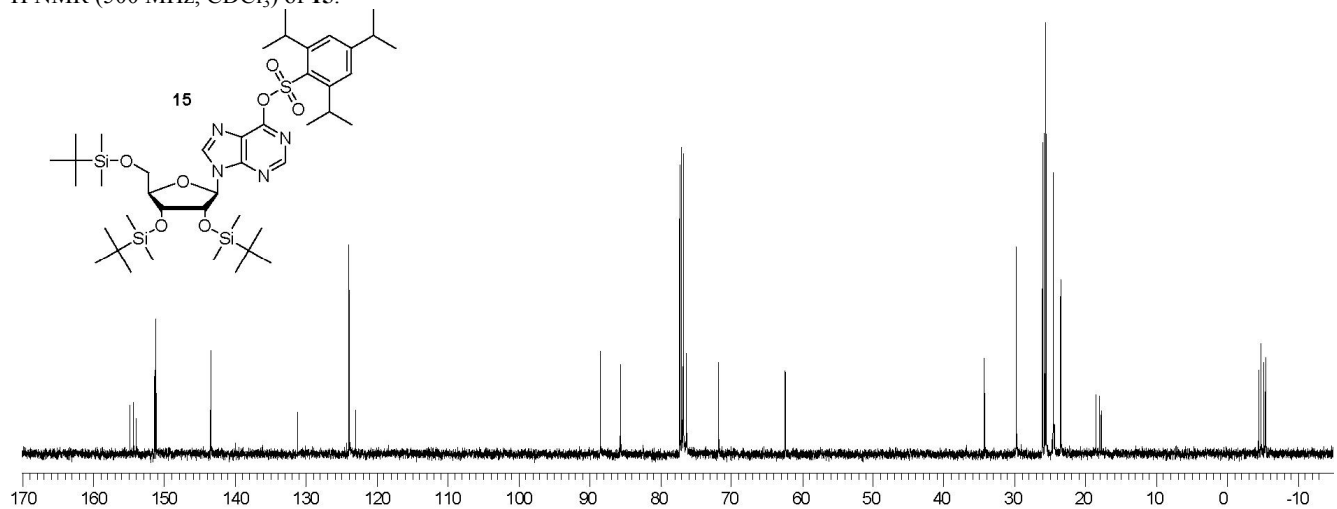
^{1}H NMR (500 MHz, $CDCl_3$): δ –0.27, –0.05, 0.09, 0.10, 0.13, 0.14 (6s, 18H, 6 $SiCH_3$); 0.77, 0.93, 0.95 (3s, 27H, 3 $SiC(CH_3)_3$); 1.24-1.29 (m, 18H, 3 $CH(CH_3)_2$); 2.92 (septet, J = 6.8 Hz, 1H, $CH(CH_3)_2$); 3.79 (dd, J = 2.4, 11.2 Hz, 1H, H^1-C(5′)); 4.01 (dd, J = 3.5, 11.2 Hz, 1H, H^2-C(5′)); 4.14 (m, 1H, H-C(4′)); 4.29 (t, $J \approx$ 4.1 Hz, 1H, H-C(3′)); 4.36 (septet, J = 6.8 Hz, 2H, 2 $CH(CH_3)_2$); 4.58 (t, $J \approx$ 4.7 Hz, 1H, H-C(2′)); 6.10 (d, J = 4.8 Hz, 1H, H-C(1′)); 8.45 (s, 1H, H-C(8)); 8.56 (s, H, H-C(2)) ppm.

^{13}C NMR (125 MHz, $CDCl_3$): δ –5.45, –5.42, –5.17, –4.79, –4.75, –4.45 (6 $SiCH_3$); 17.76, 18.02, 18.49 (3 $SiC(CH_3)_3$); 23.48, 24.51, 24.55, 25.57, 25.78, 26.05 (3 $SiC(CH_3)_3$); 29.74, 34.26 (3 $CH(CH_3)_2$); 62.35 (C(5′)); 71.76 (C(3′)); 76.30 (C(2′)); 85.62 (C(4′)); 88.46 (C(1′)); 122.96 (C(5)); 123.93 (C(ar)); 131.17 (C(ar)); 143.45 (C(8)); 151.17, 151.35 (C(2), C(ar)); 153.95, 154.36, 154.87 (C(4), C(6), C(ar)) ppm.

ESI-HRMS: *m*/*z* calcd. for $C_{43}H_{76}N_4O_7SSi_3$ $[M+H]^+$ 877.4821, found 877.4813 (Δm –0.0008, error –0.9 ppm).



^{1}H NMR (500 MHz, $CDCl_3$) of **15**.



^{13}C NMR (125 MHz, $CDCl_3$) of **15**.

2′,3′,5′-Tris-*O*-(*tert*-butyldimethylsilyl)-N^6-[(*S*)-1-(2-nitrophenyl)ethyl]adenosine (16)

A solution of compound **15** (445 mg, 0.51 mmol) and (*S*)-1-(2-nitrophenyl)ethylamine (**10**) (200 mg, 1.20 mmol) in dry DMF (5 mL) was heated to 85–90 °C for 16 h. The solvent was removed under vacuum; the oily residue was dissolved in dichloromethane, extracted with 5% aqueous citric acid, washed with water and with saturated aqueous sodium chloride solution. The organic layer was dried over Na_2SO_4 and concentrated under vacuum. The crude product was chromatographed with 0–2% methanol in dichloromethane.

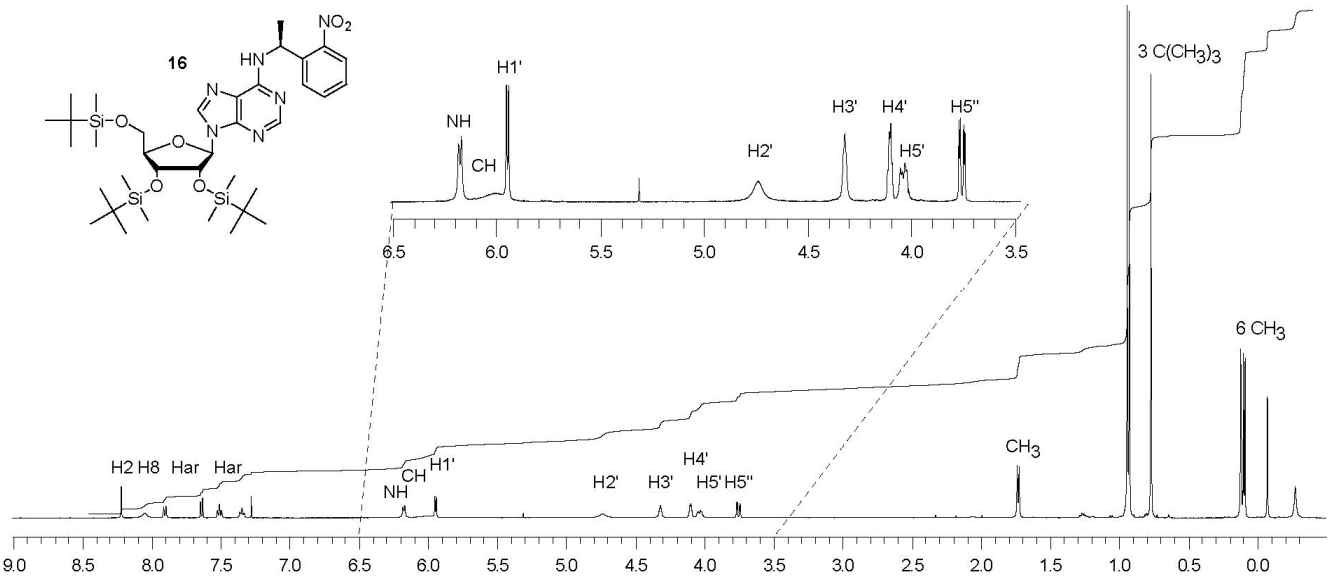
Yield: 246 mg of **16** as a colorless foam (64%).

TLC (dichloromethane/methanol 99:1): R_f 0.5.

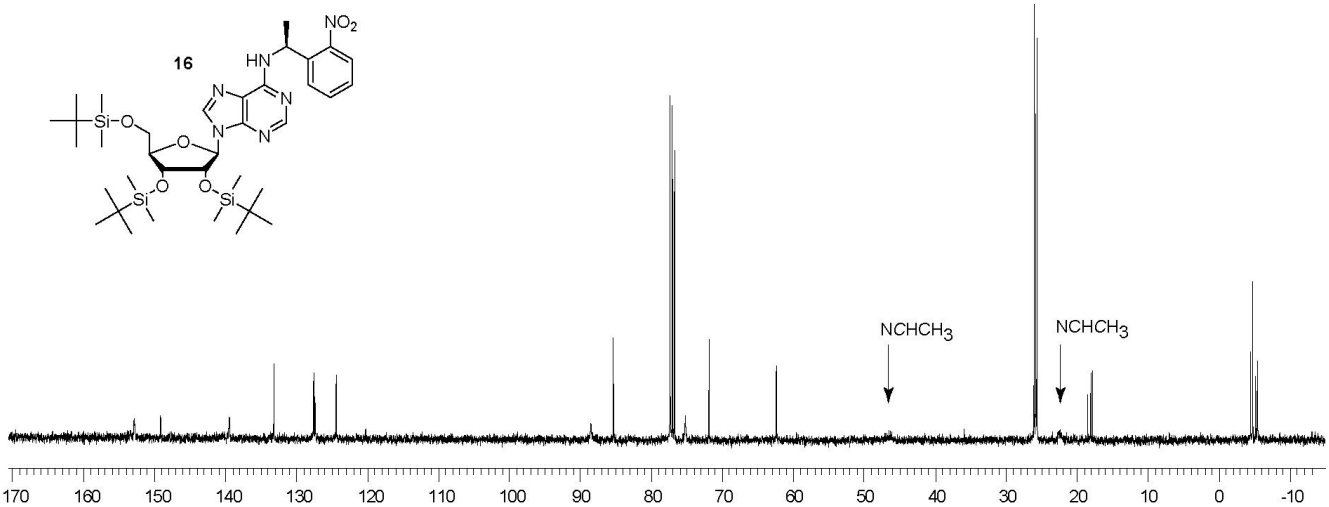
^{1}H NMR (500 MHz, $CDCl_3$): δ –0.28, –0.08, 0.08, 0.10, 0.11, 0.12 (6s, 18H, 6 $SiCH_3$); 0.76, 0.92, 0.94 (3s, 27H, 3 $SiC(CH_3)_3$); 1.72 (d, J = 6.7 Hz, 3H, $NCHCH_3$); 3.75 (dd, J = 3.0, 11.3 Hz, 1H, H^1-C(5′)); 4.02 (m, 1H, H^2-C(5′)); 4.09 (m, 1H, H-C(4′)); 4.31 (m, 1H, H-C(3′)); 4.72 (m, 1H, H-C(2′)); 5.93 (d, J = 5.2 Hz, 1H, H-C(1′)); 6.0 (br., 1H, $NCHCH_3$); 6.16 (m., 1H, NH); 7.33, 7.50 (2t, $J \approx$ 7.6 Hz, 2H, H-C(ar)); 7.62, 7.89 (2d, J = 7.6 Hz, 2H, H-C(ar)); 8.04 (br.s, 1H, H-C(8)); 8.21 (s, 1H, H-C(2)) ppm.

^{13}C NMR (125 MHz, $CDCl_3$): δ –5.45, –5.41, –5.17, –4.76, –4.67 (6 $SiCH_3$); 17.81, 18.04, 18.48 (3 $SiC(CH_3)_3$); 22.5 ($NCHCH_3$); 25.63, 25.81, 26.03 (3 $SiC(CH_3)_3$); 46.5 ($NCHCH_3$); 62.35 (C(5′)); 71.84 (C(3′)); 75.17 (C(2′)); 85.31 (C(4′)); 88.47 (C(1′)); 120.2 (C(5)); 124.36, 127.34, 127.51, 133.13 (C(ar)); 139.40, 139.49 (C(8), C(ar)); 149.1 (C(4), C(ar)); 152.8, 152.9 (C(2), C(6)) ppm.

ESI-HRMS: m/z calcd. for $C_{36}H_{62}N_6O_6Si_3$ $[M+H]^+$ 759.4117, found 759.4140 (Δm +0.0023, error +3.0 ppm).



^{1}H NMR (500 MHz, $CDCl_3$) of **16**.



^{13}C NMR (100 MHz, $CDCl_3$) of **16**. Assignments of spectral features for the $NCHCH_3$ moiety is based on HMQC and HMBC NMR.

N^6-[(*S*)-1-(2-nitrophenyl)ethyl]adenosine (17)

Compound **16** (225 mg, 0.299 mmol) was dissolved in THF (9 mL) and treated with 1 M TBAF in THF (0.9 mL). The solution was stirred at room temperature for 30 min. The solvent was evaporated and the residue was chromatographed with 0-8% methanol in dichloromethane. The product contained minor amounts of a tetrabutylammonium impurity, which did not interfere with the following transformation and was removed in the subsequent purification.

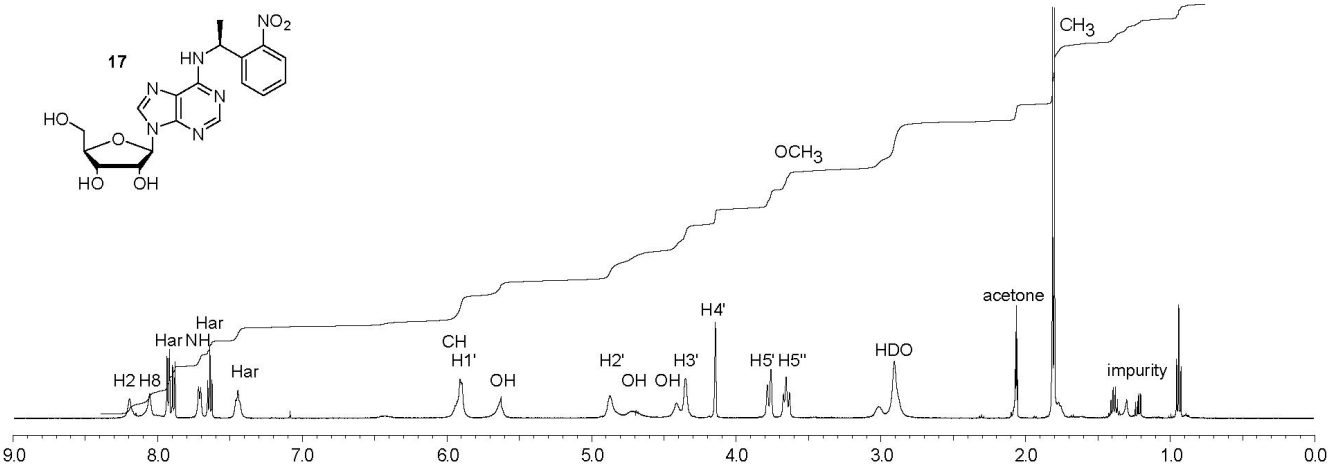
Yield: 125 mg of **17** as a white solid (purity ~90%).

TLC (dichloromethane/methanol 9:1): R_f 0.4.

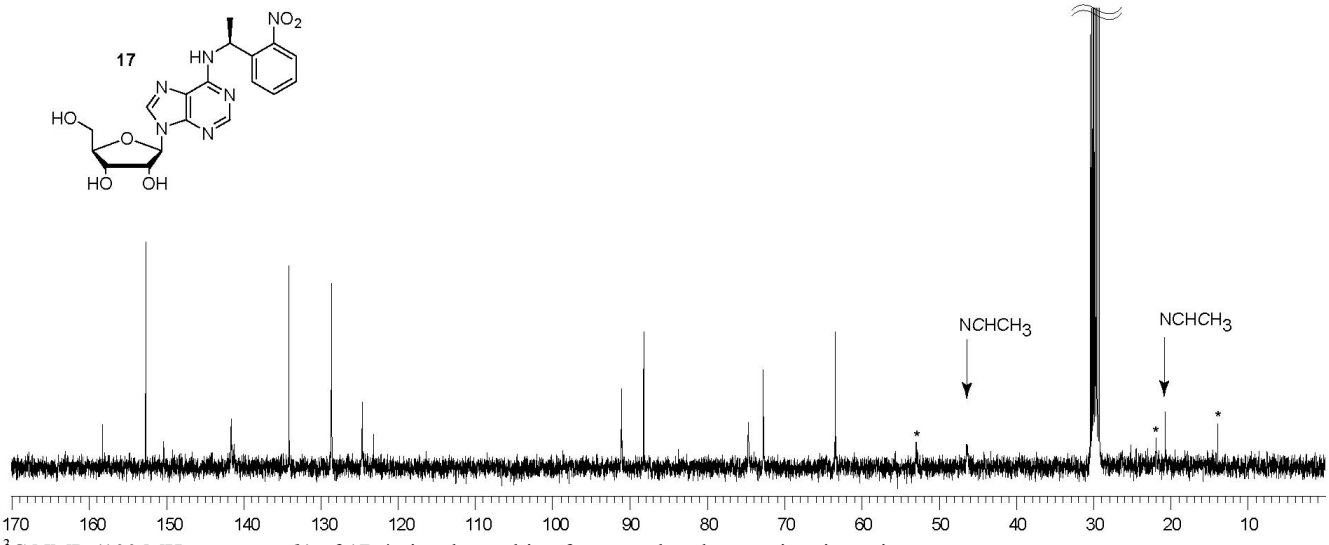
^{1}H NMR (500 MHz, acetone-d_6): δ 1.80 (d, J = 6.9 Hz, 3H, NCHC<u>H</u>$_3$); 3.66, 3.77 (2m, 2H, H$_2$-C(5′)); 4.14 (m, 1H, H-C(4′)); 4.35 (m, 1H, H-C(3′)); 4.41, 4.71 (2br., 2 OH); 4.88 (m, 1H, H-C(2′)); 5.63 (br., 1H. OH); 5.90 (m, 2H, H-C(1′), NC<u>H</u>CH$_3$); 7.45, 7.65 (2m, 2H, H-C(ar)); 7.72 (br.d, J = 7.5 Hz, 1H, NH); 7.90 (m, H-C(ar)); 8.06 (br.s, 1H, H-C(2)); 8.21 (s, 1H, H-C(8)) ppm.

^{13}C NMR (125 MHz, acetone-d_6): δ 20.7 (NCH<u>C</u>H$_3$); 46.4 (N<u>C</u>HCH$_3$); 63.43 (C(5′)); 72.72 (C(3′)); 74.66 (C(2′)); 88.20 (C(4′)); 91.08 (C(1′)); 123.16 (C(5)); 124.62, 128.61, 134.11 (C(ar)); 141.17, 141.59 (C(4), C(ar)); 150.32 (C(8)); 152.65 (C(2)); 154.71 (C(ar)); 158.23 (C(6)) ppm.

ESI-HRMS: m/z calcd. for $C_{18}H_{20}N_6O_6$ $[M+H]^+$ 417.1523, found 417.1524 (Δm +0.0001, error +0.1 ppm).



^{1}H NMR (500 MHz, acetone-d_6) of **17**.



^{13}C NMR (100 MHz, acetone-d_6) of **17**. * signals resulting from tetrabutylammonium impurity.

5′-*O*-(4,4′-Dimethoxytrityl)-N^6-[(*S*)-1-(2-nitrophenyl)ethyl]adenosine (18)

Compound **17** (125 mg, 0.30 mmol; containing <10% tetrabutylammonium salt from the desilylation step) was coevaporated from pyridine (2 × 5 mL) and dissolved in pyridine (2 mL). 4,4′-Dimethoxytrityl chloride (125 mg, 0.37 mmol) was added and the solution was stirred at room temperature for 4 h. Methanol (100 µL) was added and the solvents were removed under vacuum. The residue was dissolved in dichloromethane, extracted with 5% aqueous citric acid, water and saturated aqueous sodium bicarbonate solution. The organic layer was dried over Na_2SO_4 and the solvent was removed under vacuum. The residual yellow foam was chromatographed with 0–3% methanol in dichloromethane.

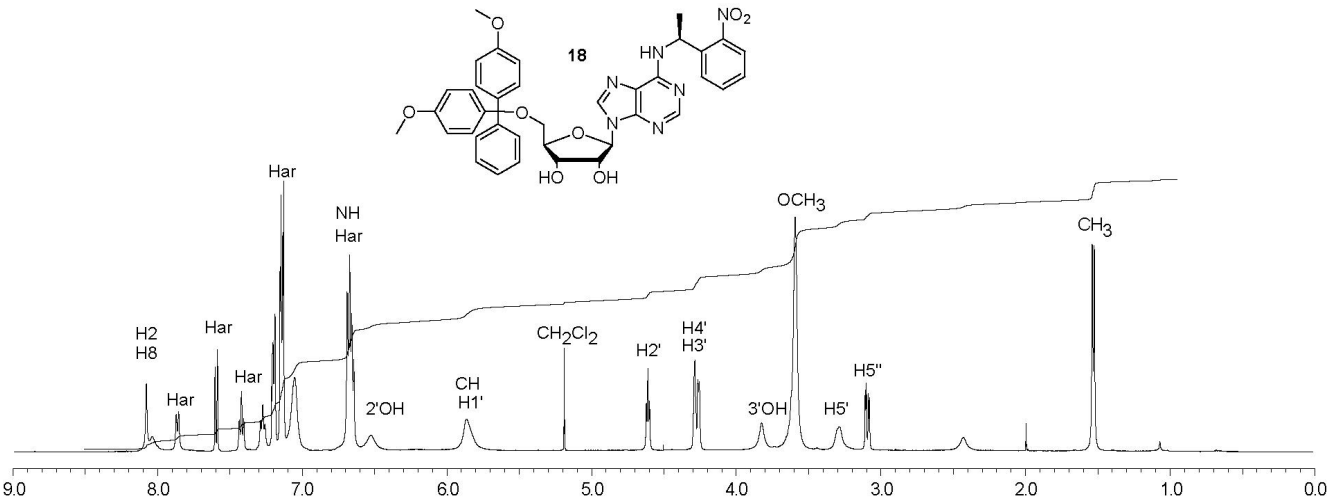
Yield: 142 mg of **18** as a colorless foam (65% over two steps).

TLC (dichloromethane/methanol 93:7): R_f 0.5.

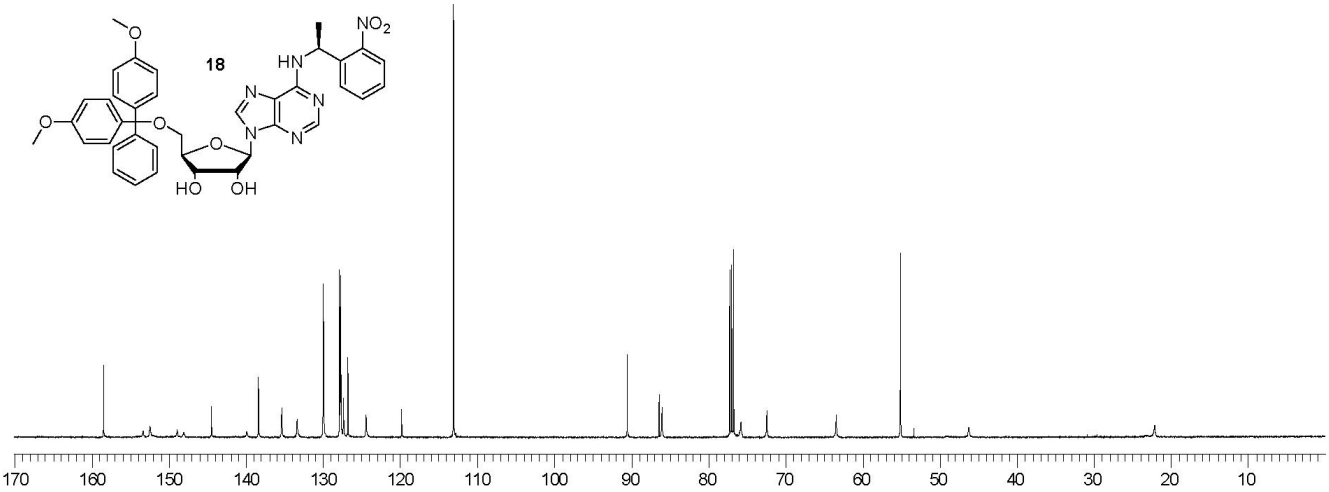
^{1}H NMR (500 MHz, $CDCl_3$): δ 1.72 (d, J = 7.0 Hz, 3H, NCHC$\underline{H}_3$); 3.25 (dd, J = 3.5, 10.4 Hz, 1H, H_1-C(5′)); 3.43 (br., 1H, H^2-C(5′)); 3.79 (s, 6H, 2 OCH_3); 3.96 (br., 1H, 3′-OH); 4.38-4.41 (2m, 2H, H-C(3′), H-C(4′)); 4.72 (t, $J \approx$ 5.4 Hz, 1H, H-C(2′)); 5.95 (br., 2H, H-C(1′), NC$\underline{H}$$CH_3$); 6.60 (br, 1H, 2′-OH); 6.74 (m, 5H, NH, 4 H-C(ar-DMT)); 7.10-7.26 (m, 9H, H-C(ar-DMT)); 7.32, 7.47 (2t, J = 7.7 Hz, 2H, H-C(ar-NPE)); 7.64, 7.90 (2d, J = 7.7 Hz, 2H, H-C(ar-NPE)); 8.10 (br.s, 2H, H-C(2), H-C(8)) ppm.

^{13}C NMR (125 MHz, $CDCl_3$): δ 22.15 (NCH$\underline{C}$$H_3$); 46.24 (N$\underline{C}$$HCH_3$); 55.11 ($OCH_3$); 63.44 (C(5′)); 72.43 (C(3′)); 75.81 (C(2′)); 86.04; 86.41 (C(4′)); 90.54 (C(1′)); 113.07 (C(ar)); 119.80 (C(5)); 124.42; 126.76, 127.33, 127.67, 127.73, 127.85, 129.91, 129.97, 133.35 (9 C(ar)); 135.311, 135.38; 138.36 (C(8)); 139.88, 144.46; 148.06 (C(4)); 148.91; 152.44 (C(2)); 153.34 (C(6)); 158.43, 158.47 ppm.

ESI-HRMS: m/z calcd. for $C_{39}H_{38}N_6O_8$ $[M+H]^+$ 719.2829, found 719.2834 (Δm +0.0005, error +0.7 ppm).



^{1}H NMR (500 MHz, $CDCl_3$) of **18**.



^{13}C NMR (125 MHz, $CDCl_3$) of **18**.

5′-*O*-(4,4′-Dimethoxytrityl)-N^6-[(*S*)-1-(2-nitrophenyl)ethyl]-2′-*O*-[[(triisopropylsilyl)oxy]methyl]adenosine (19)

A solution of compound **18** (225 mg, 0.31 mmol) in 1,2-dichloroethane (2 mL) containing diisopropylethylamine (205 µL, 1.24 mmol) was treated with dibutyltin dichloride (104 mg, 0.34 mmol) and stirred at room temperature for 1.5 h. The solution was heated to 70 °C in an oil bath and [(triisopropylsilyl)oxy]methyl chloride (77 mg, 0.34 mmol) was added dropwise. After stirring for 15 min at 70 °C then for 10 min at room temperature, the solution was diluted with dichloromethane and extracted with saturated sodium bicarbonate solution, water and brine. The organic layer was dried over Na_2SO_4, filtered through Celite, and concentrated to give a yellow foam. The 2′- and 3′-*O*-alkylated isomers were separated by chromatography with a gradient of ethylacetate/hexane 3:7–6:4.

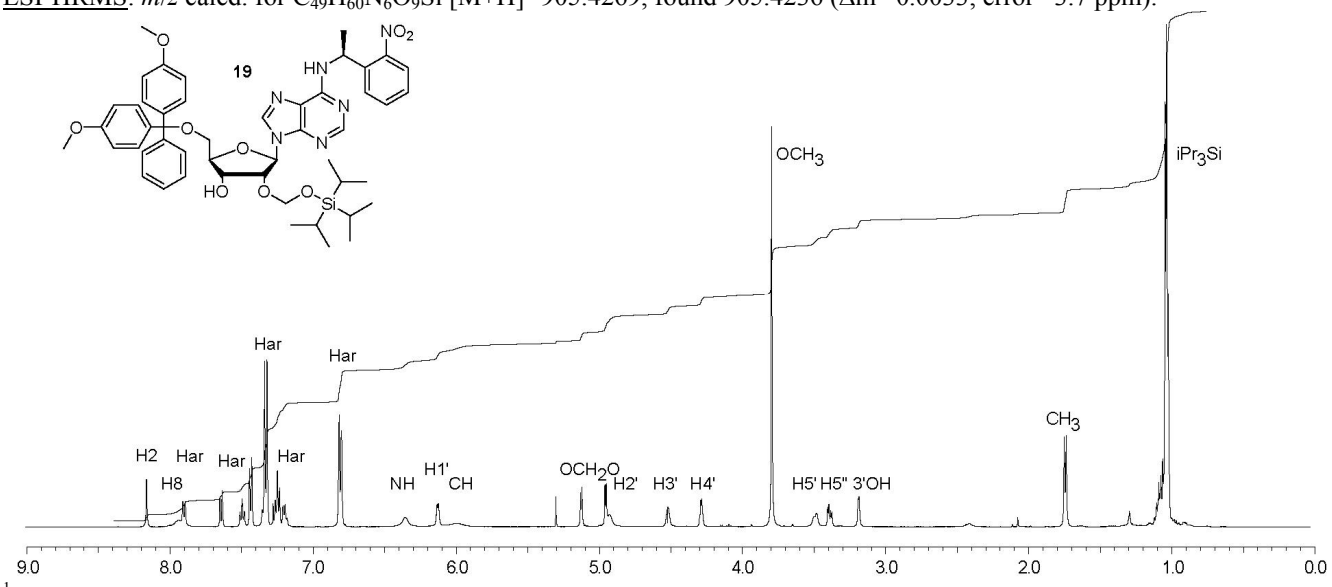
Yield: 100 mg of **19** as a colorless foam (35%) and 125 mg of 3′-*O*-alkylated isomer as a colorless foalm (44%).

TLC (ethylacetate/hexane 6:4): 2′-*O*-TOM isomer R_f 0.6, 3′-*O*-TOM isomer R_f 0.5.

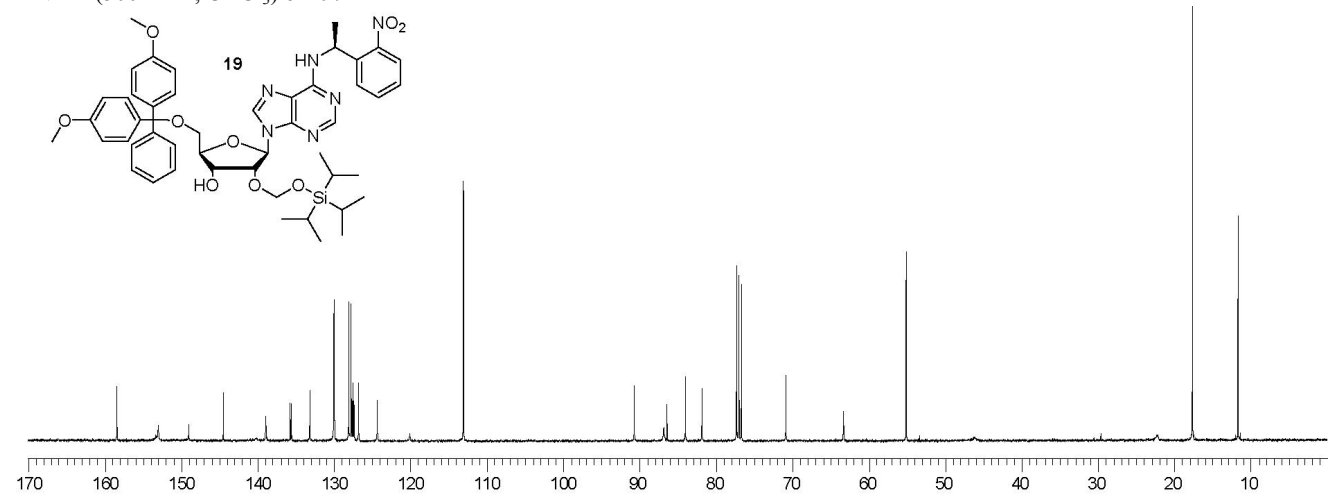
^{1}H NMR (500 MHz, $CDCl_3$): δ 0.94-1.09, (m, 21H, iPr_3Si); 1.72 (d, J = 6.6 Hz, 3H, NCHC$\underline{H}_3$); 3.16 (d, J = 2.7 Hz, 1H, HO-C(3′)); 3.37 (dd, J = 4.4, 10.5 Hz, 1H, H^1-C(5′)); 3.47 (m, 1H, H^2-C(5′)); 3.78 (s, 6H, 2 OCH_3); 4.27 (m, 1H, H-C(4′)); 4.50 (m, 1H, H-C(3′)); 4.85 (m, 1H, H-C(2′)); 4.94, 5.11 (2d, J = 4.7 Hz, 2H, OCH_2O); 5.90 (br., 1H, NC$\underline{H}CH_3$); 6.11 (d, J = 5.0 Hz, 1H, H-C(1′)); 6.34 (br, 1H, NH); 6.80 (m, 4H, H-C(ar-DMT)); 7.17-7.32 (m, 9H, 8 H-C(ar-DMT), 1 H-C(ar-NPE)); 7.42 (d, J = 7.2 Hz, 1H, H-C(ar-DMT)); 7.48 (t, J = 7.7 Hz, 1H, H-C(ar-NPE)); 7.63 (d, J = 7.7 Hz, 1H, H-C(ar-NPE)); 7.90 (m, 2H, H-C(2), H-C(ar-NPE)); 8.15 (s, 1H, H-C(8)) ppm.

^{13}C NMR (125 MHz, $CDCl_3$): δ 11.70 (Si($\underline{C}$H(CH$_3$)$_2$)$_3$); 17.66 (Si(CH($\underline{C}$H$_3$)$_2$)$_3$); 22.36 (NCH$\underline{C}$H$_3$); 46.12 (N$\underline{C}$HCH$_3$); 55.13 (OCH_3); 63.32 (C(5′)); 70.86 (C(3′)); 81.85 (C(2′)); 84.01 (C(4′)); 86.43; 86.85 (C(1′)); 90.1 (OCH_2O); 113.06 (C(ar)); 120.09 (C(5)); 124.34; 126.79, 127.39, 127.56, 127.76, 128.06, 129.96, 130.05, 133.16 (9 C(ar)); 135.57, 135.74; 138.93 (C(8)); 144.45; 149.01 (C(4)); 153.05 (C(2)); 153.31 (C(6)); 158.40, 158.43 ppm.

ESI-HRMS: m/z calcd. for $C_{49}H_{60}N_6O_9Si$ $[M+H]^+$ 905.4269, found 905.4236 (Δm –0.0033, error –3.7 ppm).



^{1}H NMR (500 MHz, $CDCl_3$) of **19**.



^{13}C NMR (125 MHz, $CDCl_3$) of **19**.

5′-*O*-(4,4′-Dimethoxytrityl)-N^6-[(*S*)-1-(2-nitrophenyl)ethyl]-2′-*O*-[[(triisopropylsilyl)oxy]methyl]adenosine 3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (20)

A solution of compound **19** (80 mg, 88 µmol) in dry dichloromethane (1 mL) containing ethyldimethylamine (96 µL, 0.9 mmol) was treated with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (23 mg, 97 µmol). After stirring for 1.5 h at room temperature, the sample was quenched with methanol (100 µL), diluted with dichloromethane, extracted with semi-saturated sodium bicarbonate solution, dried over Na_2SO_4, and evaporated. The crude product was chromatographed with ethyl acetate/hexane 1:4–3:2 containing 2% triethylamine.

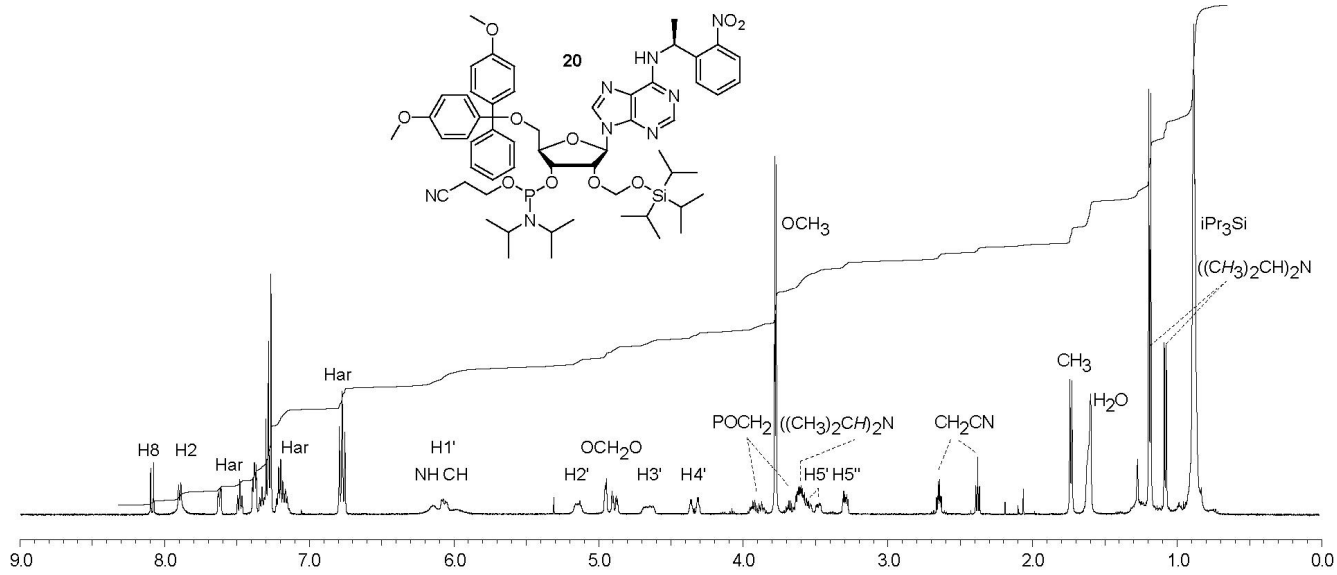
Yield: 82 mg of **20** as a colorless foam (1:1 mixture of diastereoisomers, 84 %).

TLC (ethyl acetate/hexanes 6:4): R_f 0.7.

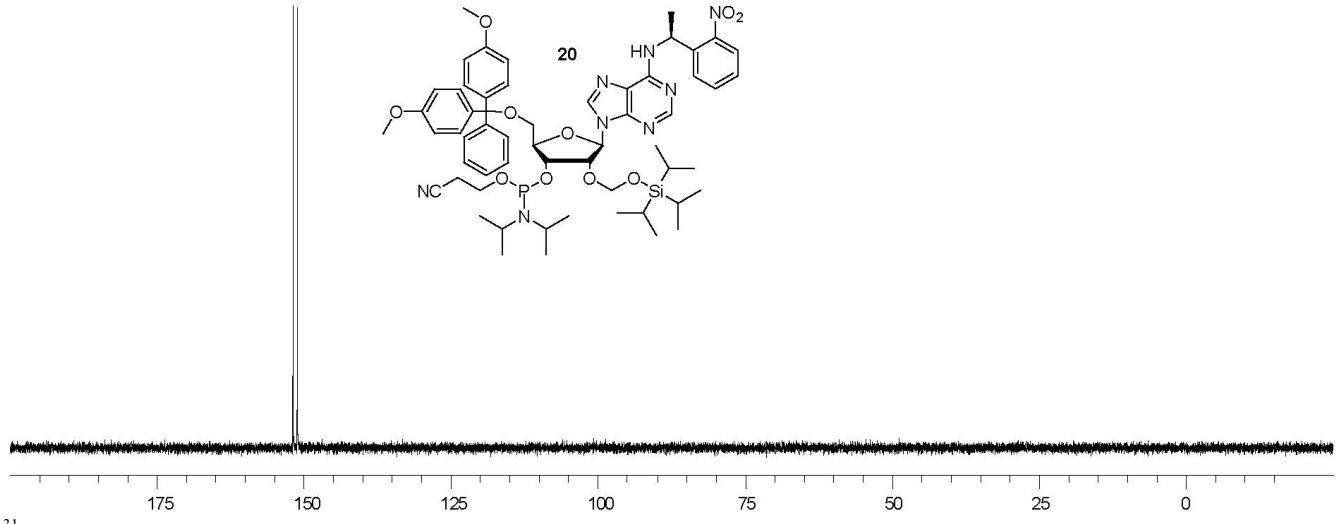
^{1}H NMR (500 MHz, $CDCl_3$): δ 0.82-0.92 (m, 42H, iPr_3Si); 1.07, 1.17 (2d, J = 6.8 Hz, 24 H, $((C\underline{H}_3)_2CH)_2N$); 1.72 (d, J = 6.8 Hz, 6H, $NCHC\underline{H}_3$); 2.37 (m, 2H, CH_2CN); 2.64 (m, 2H, CH_2CN); 3.28 (m, 1H, H^1-C(5′)); 3.45-3.70 (m, 8H, H^2-C(5′), $POCH_2$, $((CH_3)_2C\underline{H})_2N$); 3.76, 3.77 (2s, 12H, OCH_3); 3.84-3.95 (m, 2H, $POCH_2$); 4.31, 4.36 (2m, 2H, H-C(4′)); 4.61-4.68 (m, 2H, H-C(3′)); 4.86-4.94 (m, 4H, OCH_2O); 5.11-5.16 (m, 2H, H-C(2′)); 5.98-6.18 (m, 6H, H-C(1′), $NC\underline{H}CH_3$, NH); 6.77 (m, 8H, H-C(ar-DMT)); 7.15-7.39 (m, 20H, 9 H-C(ar-DMT), 1 H-C(ar-NPE)); 7.48, 7.62 (2m, 4H, H-C(ar-NPE)); 7.89, 7.91 (2s, 2H, H-C(2)); 8.08, 8.10 (2s, 2H, H-C(8)) ppm.

^{31}P NMR (202 MHz, acetone-d_6): δ 151.09, 151.82 ppm.

ESI-HRMS: m/z calcd. for $C_{58}H_{77}N_8O_{10}PSi$ $[M+H]^+$ 1105.5348, found 1105.5399 (Δm +0.0051, error +4.6 ppm).



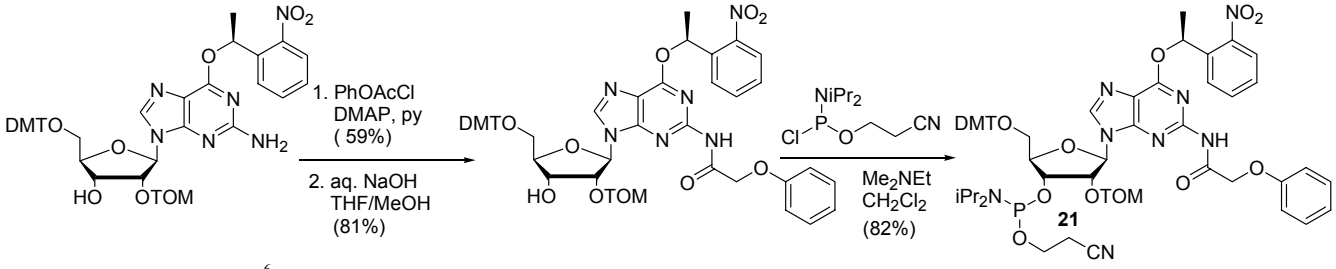
^{1}H NMR (500 MHz, $CDCl_3$) of **20**.



^{31}P NMR (202 MHz, $CDCl_3$) of **20**.

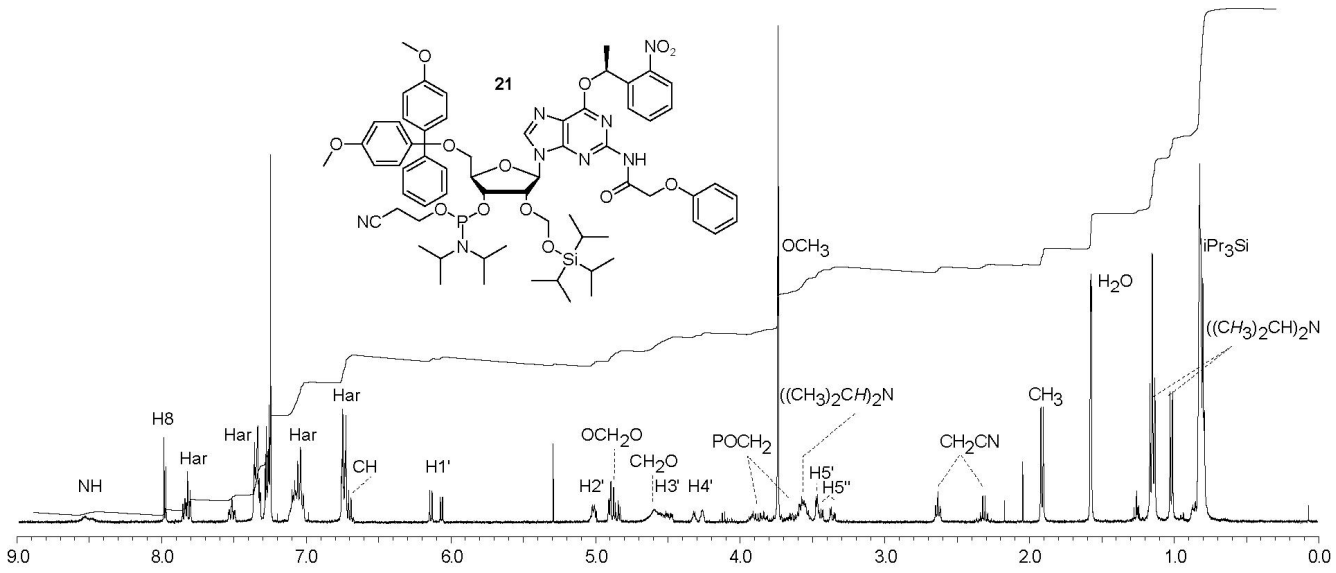
Synthesis of (*S*)-NPE-caged guanosine nucleoside phosphoramidite

The synthesis of 5′-*O*-(4,4′-dimethoxytrityl)-O^6-[(*S*)-1-(2-nitrophenyl)ethyl]-N^2-phenoxyacetyl-2′-*O*-[[(triisopropylsily)oxy]methyl]guanosine 3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) was performed as described for the analogous N^2-(4-isopropylphenoxy)acetyl derivative[12] by using phenoxyacetyl chloride instead of (4-isopropyl)phenoxyacetyl chloride. ^{1}H NMR, ^{31}P NMR, and ESI-HRMS data for the phenoxyacetyl-protected NPE-guanosine phosphoramidite **21** are given below.

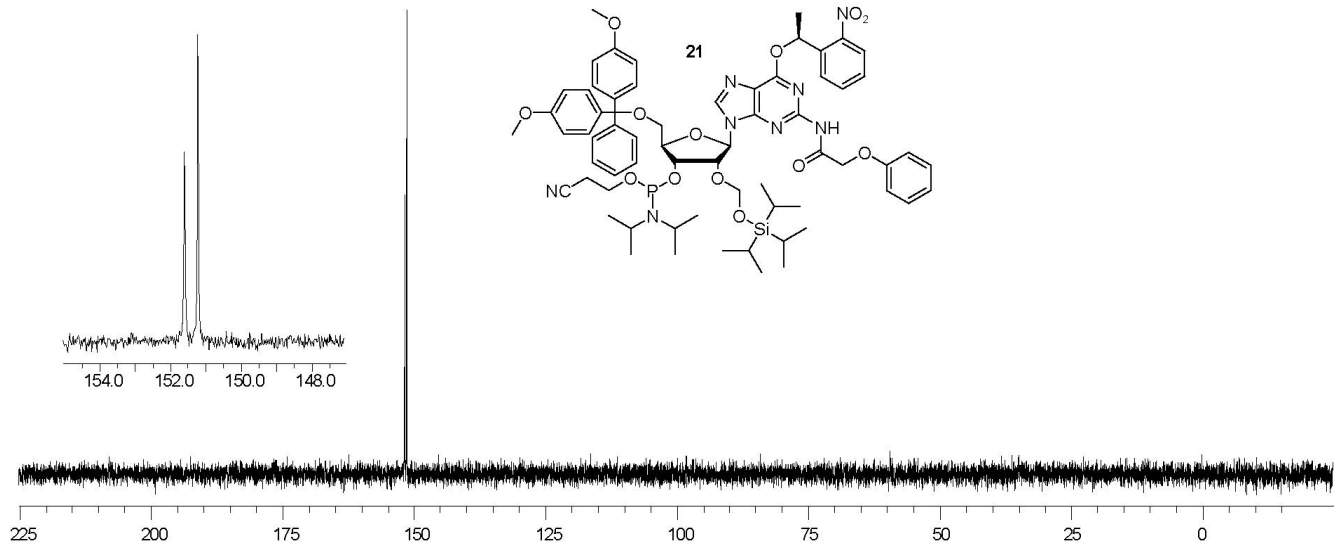


Scheme S3. Synthesis of O^6-(*S*)-NPE-guanosine phosphoramidite **21**.

Data of **21**: TLC (ethyl acetate/hexanes 3:7): R_f 0.6.
^{1}H NMR (400 MHz, $CDCl_3$): δ 0.75-0.87 (m, 42H, iPr_3Si); 1.02 (d, J = 6.8 Hz, 6 H, $((C\underline{H}_3)_2CH)_2N$); 1.15 (m, 18H, $((C\underline{H}_3)_2CH)_2N$); 1.91 (d, J = 6.4 Hz, 6H, $NCHC\underline{H}_3$); 2.31 (m, 2H, CH_2CN); 2.63 (m, 2H, CH_2CN); 3.34-3.70 (m, 10H, H_2-C(5′), $POCH_2$, $((CH_3)_2C\underline{H})_2N$); 3.74, 3.75 (2s, 12H, OCH_3); 3.77-3.95 (m, 2H, $POCH_2$); 4.26, 4.32 (2m, 2H, H-C(4′)); 4.47-4.66 (m, 6H, H-C(3′), $COCH_2O$); 4.83-4.92 (m, 4H, OCH_2O); 5.01 (m, 2H, H-C(2′)); 6.07, 6.14 (2d, J = 6.4, 6.8 Hz, 2H, H-C(1′)); 6.68 (m, 10H, $NC\underline{H}CH_3$, H-C(ar-DMT)); 7.03-7.11 (m, 16H, H-C(ar)); 7.24-7.38 (m, 16H, H-C(ar)); 7.52 (m, 2H, H-C(ar)); 7.81-7.83 (m, 4H, H-C(ar)); 7.98, 8.00 (2s, 2H, H-C(8)); 8.48, 8.55 (2br.s., 2H, NH) ppm.
^{31}P NMR (162 MHz, $CDCl_3$): δ 151.20, 151.57 ppm.
ESI-HRMS: *m*/*z* calcd. for $C_{66}H_{83}N_8O_{13}PSi$ $[M+H]^+$ 1255.5665, found 1255.5668 (Δm +0.0003, error +0.2 ppm).



^{1}H NMR (400 MHz, $CDCl_3$) of **21**.



^{31}P NMR (162 MHz, $CDCl_3$) of **21**.

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