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Nitroxide-Labeled Oligonucleotides | *Very Important Paper* |

Benzoyl-Protected Hydroxylamines for Improved Chemical Synthesis of Oligonucleotides Containing Nitroxide Spin Labels

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Abstract: Oligonucleotides containing nitroxide spin labels, used in biophysical studies of nucleic acids, are frequently prepared by chemical synthesis. However, during the synthesis of spin-labeled oligonucleotides, the nitroxides are partially reduced to the corresponding amines. Here we report that a benzoylated hydroxylamine can be used as a protected form of the nitroxide to avoid this reduction. The benzoyl group is stable through the oligonucleotide synthesis and is readily removed under standard oligonucleotide deprotection conditions, yielding a hydroxylamine that is oxidized in situ to the nitroxide. This method was used to incorporate the rigid spin labels **Ç** and **Çm** into DNA and RNA oligonucleotides, respectively, including a doubly labeled 36-nucleotide long DNAzyme. Enzymatic digestion of the spin-labeled oligonucleotides and subsequent HPLC analysis showed that the nitroxides were intact. This protecting group strategy facilitates the high-yielding synthesis of spin-labeled DNA and RNA oligonucleotides using the phosphoramidite method.

Introduction

Nucleic acids are essential for life as they contain the cellular blueprint in living organisms and are active participants in the cellular machinery, for example in regulation of gene expression.[1] Therefore, it is of great interest to determine their structure and dynamics in order to gain insights into their function. Several different techniques are used for such studies. X-ray crystallography can provide three-dimensional structures and precise arrangements of atoms in space, but growing a highly-diffractive single crystal can be a laborious and time-consuming task.[2] Moreover, the crystals of the biomolecules might not represent their biologically active conformation.[3] Nuclear magnetic resonance (NMR) spectroscopy provides high-resolution structural information under biologically relevant conditions as well as information on dynamics.[4] However, NMR has inherently low sensitivity and, therefore, a relatively large amount of sample is required and the measurements can be time-consuming.[5] Förster resonance energy transfer (FRET) is a technique that measures the distances between two or more chromophores.[6] FRET has been used extensively to study tertiary structures of nucleic acids and has enabled studies of single molecules.[7]

Electron paramagnetic resonance (EPR) spectroscopy is another method to study the structure and dynamics of nucleic acids. For structural studies, continuous wave (CW) EPR can be used to measure distances up to 25 Å.[8] Pulsed dipolar spectroscopy, such as pulsed electron-electron double resonance (PELDOR), also called double electron-electron resonance (DEER), relaxation induced dipolar modulation enhancement (RIDME), single frequency technique for refocusing dipolar couplings (SIFTER) and double quantum coherence (DQC) can be used to measure distances between 15–160 Å.[9] Information about dynamics of nucleic acids can be obtained directly from line-broadening of CW-EPR spectra[10] and from orientation studies using pulsed EPR.[11] In EPR spectroscopy, transitions between spin states of unpaired electrons in a magnetic field are measured. Since nucleic acids and most other biomolecules are diamagnetic, a paramagnetic center needs to be introduced. Paramagnetic metal ions have been used as spin labels for EPR[12] but more often organic radicals, such as nitroxides, are employed for spin labeling.[13]

Incorporation of spin labels at specific sites in nucleic acids is called site-directed spin-labeling (SDSL) and is performed either through covalent or noncovalent binding.[14] Covalent spin-labeling is either carried out post-synthetically or by using spin-labeled phosphoramidites as building blocks in chemical synthesis of nucleic acids.[14a,14b,14d] Post-synthetic labeling requires oligonucleotides that have reactive groups at specific sites for modification with the spin label after the oligonucleotide synthesis.[14a,14b,14d] An advantage of post-synthetic labeling is that the label is not exposed to the reagents used for oligonucleotide synthesis, but drawbacks include non-specific reactions with other functional groups present in nucleic acids and incomplete spin labeling. Also, the spin labels that are incorporated post-synthetically contain flexible tethers, that render distance measurements less accurate and relative orientation of labels cannot be determined.[15]

With the phosphoramidite method, it is possible to incorporate intricate labels with unique structural features, such as rigid labels, into nucleic acids.[16] Drawbacks of the phosphoramidite approach include the synthetic effort required to prepare spin-

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labeled phosphoramidites and exposure of the nitroxides to reagents used in the solid-phase synthesis, resulting in a partial[17] or even complete[18] reduction of the nitroxide to its corresponding amine. For oligonucleotides shorter than ca. 15-nucleotide (nt) long, the desired spin-labeled product can be readily separated from the reduced material by denaturing polyacrylamide gel electrophoresis (DPAGE). For longer oligonucleotides, on the other hand, the separation is usually a tedious and nontrivial task that often results in a mixture of spin-labeled and reduced material. Protecting the nitroxide with a group that is stable through the oligonucleotide synthesis would eliminate this drawback of spin-labeling nucleic acids by the phosphoramidite method.

Nitroxides have been protected as *O*-methyl hydroxylamines under reaction conditions that would otherwise reduce the nitroxide.[19] The methyl group can be removed by treatment with *meta*-chloroperbenzoic acid (*m*-CPBA), but this reagent can also oxidize nitrogen atoms in the heterocyclic nucleobases present in nucleic acids. The *tert*-butyldimethylsilyl (TBDMS) group has been used to protect the hydroxylamine of 2,2,6,6-tetramethylpiperidyl-1-oxyl (TEMPO) for the synthesis of a nitroxide-nitroxide biradical,[20] however, less than 50 % of the nitroxide was recovered after removal of the TBDMS groups. Photolabile protecting groups have been used to protect TEMPO during oligonucleotide synthesis, of both DNA and RNA; irradiation with light gave high yields of spin-labeled oligonucleotides.[21] Photoprotection of nitroxides is a useful method for photocaging, allowing a controlled release of the protecting group in functional nucleic acids.[22] However, additional synthetic effort is required to prepare and incorporate the photolabile protecting group and specialized equipment is needed to irradiate the oligomers with the right wavelength for deprotection. An ideal protecting group for routine preparation of spin-labeled oligonucleotides would be removed by using standard oligonucleotide deprotecting conditions, returning the radical without having to include an additional deprotection step. An acetyl group has been used to protect a TEMPO moiety attached to a deoxyuridine phosphoramidite during incorporation into DNA. After oligomer deprotection using standard conditions,[23] complete removal of the acetyl group required additional incubation with aqueous NaOH (0.5 M), conditions that are not compatible with RNA. During the course of this work, we also became aware of a report describing the incorporation of 2-*N*-*tert*-butylaminoxyl-2′-deoxyadenosine into DNA, utilizing acetylated hydroxylamine,[24] however, the structure of this nitroxide is very different from the nitroxides that are normally used for spin labeling.

Here we describe a protecting group strategy for chemical synthesis of nitroxide-labeled DNA and RNA that is based on protection of the corresponding hydroxylamine with a benzoyl group, which is compatible with the conditions of solid-phase synthesis of oligonucleotides. The benzoyl group is quantitatively removed under standard conditions used for oligonucleotide deprotection, yielding a hydroxylamine that oxidizes in situ to the corresponding nitroxide radical.[21a,25] This method was used to synthesize fully spin-labeled DNA and RNA oligonucleotides in high yields.

Results and Discussion

The main incentive for carrying out this work was to enable incorporation of rigid spin labels into long sequences (> 15 nt) by solid-phase synthesis, but such spin labels cannot be incorporated by post-synthetic labeling. Specifically, we were interested in incorporating the spin labels **Ç**[16] and **Çm**[26] (Figure 1) into DNA and RNA oligonucleotides, respectively. These spin labels are valuable probes of both structure and dynamics of nucleic acids.[16,26] An advantage of using these labels for developing a general nitroxide protecting group strategy is that reduction of the nitroxides yields the fluorescent amines **Çf**[16] and **Çmf**[27] (Figure 1), allowing for easy detection.

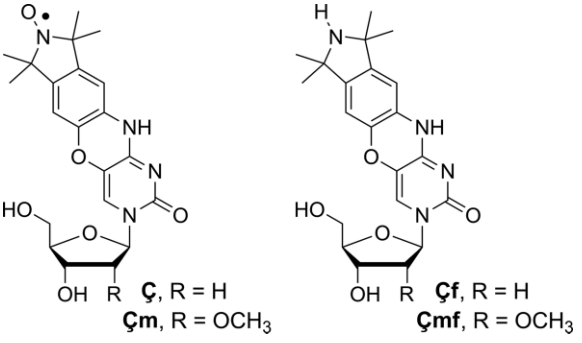


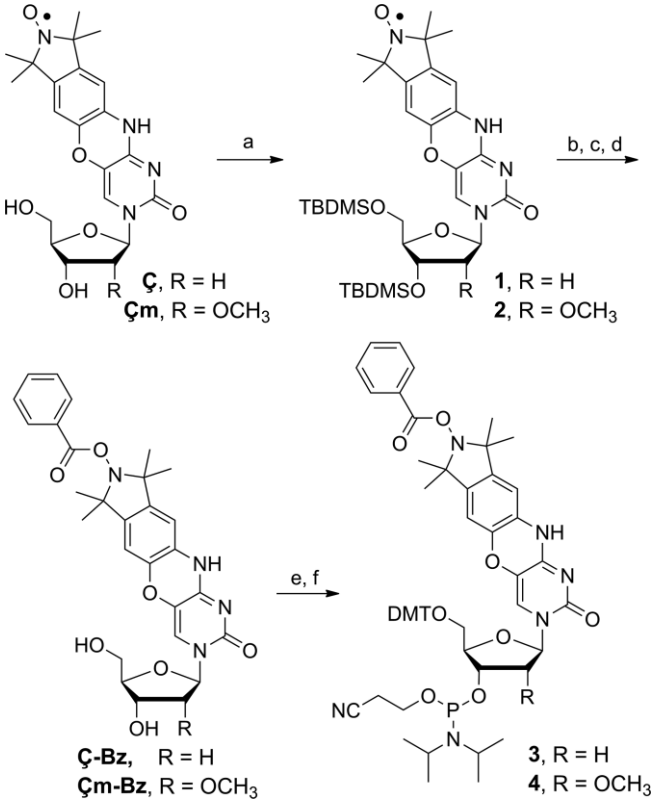
Figure 1. The rigid spin labels **Ç**[16] and **Çm**[26] and their corresponding amines **Çf**[16] and **Çmf**.[27]

The synthesis of **Ç-Bz** and **Çm-Bz** (Scheme 1) began by protecting the 5′- and 3′-hydroxyl groups of **Ç** and **Çm** with TBDMS. The resulting nitroxide radicals **1** and **2** were reduced with ascorbic acid to yield the corresponding hydroxylamines that were subsequently benzoylated, followed by removal of the TBDMS groups to give **Ç-Bz** and **Çm-Bz**. The benzoyl protecting group was shown to be stable under all reaction conditions used for oligonucleotide synthesis for more than five days, except when exposed to either 5-ethylthio tetrazole or 5-benzylthio tetrazole, present in the activation solutions, where slight removal of the benzoyl group was observed after 24 h (data not shown).

The benzoyl protecting group was readily removed within one and two hours under deprotecting conditions for RNA ($MeNH_2/NH_3$ in H_2O/EtOH) and DNA (satd. aq. NH_3), respectively. The 5′-hydroxyl groups of **Ç-Bz** and **Çm-Bz** (Scheme 1) were protected as 4,4′-dimethoxytrityl (DMT) ethers and subsequently phosphitylated to give phosphoramidites **3** and **4**, respectively, in good yields.

Phosphoramidite **4** was used to synthesize an 8-nt long, spin-labeled RNA oligomer (**I**, Table 1). This oligomer was chosen because short oligonucleotides that contain a reduced spin label can be separated by DPAGE from oligomers containing the nitroxide, thus allowing direct visualization of both products. An RNA of the same sequence was also synthesized with a phosphoramidite of the unprotected **Çm**[26] (**II**, Table 1) for comparison.

The phosphoramidite containing the protected spin label coupled well during the solid-phase synthesis as indicated by a strong orange color of the trityl cation that appears during re-



Scheme 1. Synthesis of **Ç-Bz** and **Çm-Bz** and their corresponding phosphoramidites. **a**. Imidazole, *tert*-butyldimethylsilyl chloride (TBDMSCl), DMF, pyridine. **b**. *L*-Ascorbic acid, 1,4-dioxane, H_2O. **c**. Benzoyl chloride (BzCl), Et_3N. **d**. *tert*-Butyl ammonium fluoride (TBAF), THF. **e**. 4,4′-Dimethoxytrityl chloride (DMTCl), 4-dimethylaminopyridine (DMAP), pyridine. **f**. 2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, diisopropylammonium tetrazolide (DIPAT), CH_2Cl_2.

Table 1. Spin-labeled DNA and RNA oligonucleotides synthesized by solid-phase synthesis. Oligonucleotides **II*** and **VI*** were synthesized with the phosphoramidite of unprotected nitroxide spin-labels **Çm**[26] and **Ç**,[16] respectively. PHO is a phosphate.

No.	Sequence
I	5′-UGCAU**Çm**UU-3′
II*	5′-UGCAU**Çm**UU-3′
III	5′-AGA-UGC-GCG-**Çm**GC-GCG-ACU-GAC-3′
IV	5′-PHO-d(TGAGGTAGTAGGTTGTATA**Ç**T)-3′
V*	5′-PHO-d(TGAGGTAGTAGGTTGTATA**Ç**T)-3′
VI	5′-d(TGTAA**Ç**GCACTACCAGCGGCTGGAAATCT**Ç**TCTCGT)-3′

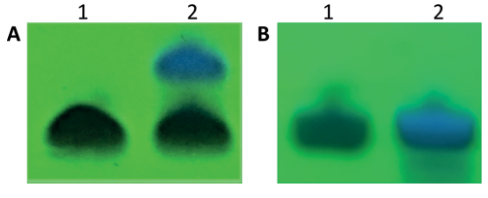


Figure 2. Analysis of spin-labeled oligonucleotides by DPAGE. **A**. Crude 8-mer RNA oligonucleotides **I** (lane 1) and **II** (lane 2) (5′-UGCAU**Çm**UU-3′), synthesized using **4** and the phosphoramidite of unprotected **Çm**,[26] respectively. **B**. Crude 21-mer DNA oligonucleotides **IV** (lane 1) and **V** (lane 2) [5′-d(PHO-TGAGGTAGTAGGTTGTATA**Ç**T)-3′], synthesized using **3** and the phosphoramidite of unprotected **Ç**,[16] respectively. PHO is a phosphate.

moval of the DMT group. Figure 2**A** shows a denaturing polyacrylamide gel of crude RNA **I** (lane 1) and RNA **II** (lane 2). No fluorescent band was detected for RNA **I** (Figure 2**A**, lane 1), synthesized with **Çm-Bz**. In contrast, RNA **II** (Figure 2**A**, lane 2), synthesized with the unprotected **Çm**, contained a strong fluorescent by-product, which indicated a partial reduction of the nitroxide to the corresponding **Çmf**.

For further analysis, crude RNAs **I** and **II** were digested with snake venom phosphodiesterase, nuclease P1 and calf spleen alkaline phosphatase, and the digest was analyzed by HPLC (Figure 3).[16] The HPLC chromatogram for RNA **I** (Figure 3**A**) contained five peaks, one for each natural nucleoside and a strongly retained nucleoside that was shown by co-injection to be **Çm**, while RNA **II** (Figure 3**B**) showed a peak for both **Çm** and **Çmf** along with the natural unmodified nucleosides.

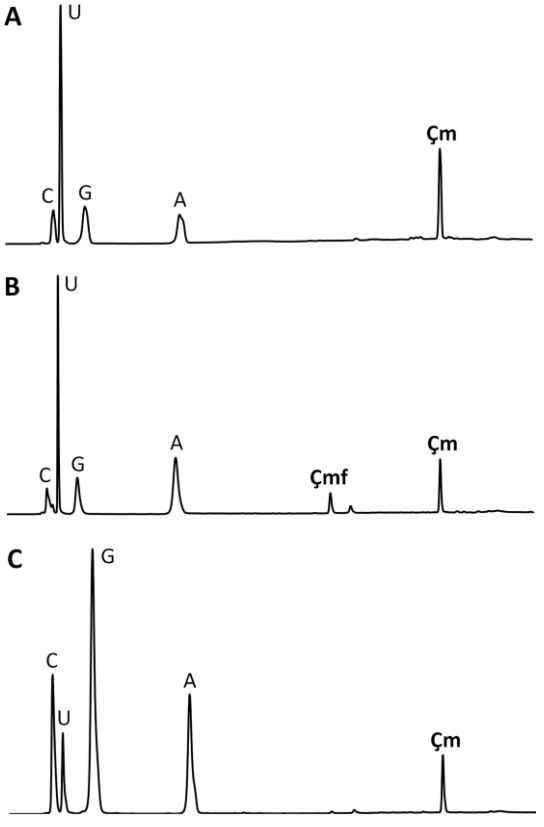


Figure 3. HPLC chromatograms of RNA oligonucleotides after enzymatic digestion with snake venom phosphodiesterase, nuclease P1, and alkaline phosphatase. **A**. Crude RNA **I** (5′-UGCAU**Çm**UU-3′) synthesized using **4**. **B**. Crude RNA **II** (5′-UGCAU**Çm**UU-3′) synthesized using the phosphoramidite of unprotected **Çm**.[26] **C**. RNA **III** (5′-AGAUGCGCG**Çm**GCGCGACUGAC-3′) synthesized using **4**.

Phosphoramidite **4** was also used to synthesize a 21-nt long RNA (**III**, Table 1), which was enzymatically digested and analyzed by HPLC (Figure 3**C**). In this case, a small peak (< 5 %) can be seen for **Çmf**, along with **Çm** and the natural nucleosides. These results show that the benzoyl protecting group on **Çm** was stable during the oligonucleotide synthesis and was completely removed during the deprotection, giving high yields of spin-labeled RNA.

In an analogous manner to the RNA synthesis, phosphoramidite **3** and a phosphoramidite of **Ç** that does not contain

the benzoyl protecting group were used to synthesize two 21-mer DNA oligonucleotides of the same sequence, (**IV** and **V**, Table 1). Here the spin-label was placed close to the 3′-end, which increases the exposure of the label to the chemicals used in each cycle of the oligonucleotide synthesis. Figure 2**B** shows DPAGE analysis of crude DNA **IV** (Figure 2**B**, lane 1) and crude DNA **V** (Figure 2**B**, lane 2). No fluorescent band was detected for DNA **IV** while DNA **V** showed a strong fluorescent band that overlapped with the band of the spin-labeled oligonucleotide.

These crude DNA samples were digested and analyzed by HPLC (Figure 4). While the digest of oligomer **IV** (Figure 4**A**) showed the natural nucleosides and **Ç**, the digest of oligomer **V** contained a very small peak for **Ç** along with some strongly retained impurities (Figure 4**B**). The quality of the synthesis of spin-labeled DNA was also reflected in the yields of purified material obtained from a 1 µmol synthesis, that gave 180 and 11 nmols of oligomers **VI** and **V**, respectively. Moreover, the small amount of oligo **V** obtained after repeated purifications gave a ca. 50:50 mixture of oligonucleotides containing **Ç** and **Çf**. To demonstrate the use of this method for the synthesis of a longer oligonucleotide, a 36-nt DNAzyme (**VI**, Table 1) containing **Ç** at two positions (6 and 31) was prepared. No fluorescent bands were detected upon purification of the oligonucleotide by DPAGE. Enzymatic digestion followed by HPLC analysis showed four peaks for the natural nucleosides and a peak for **Ç** (Figure 4**C**) in the ratios expected for a fully spin-labeled oligonucleotide.

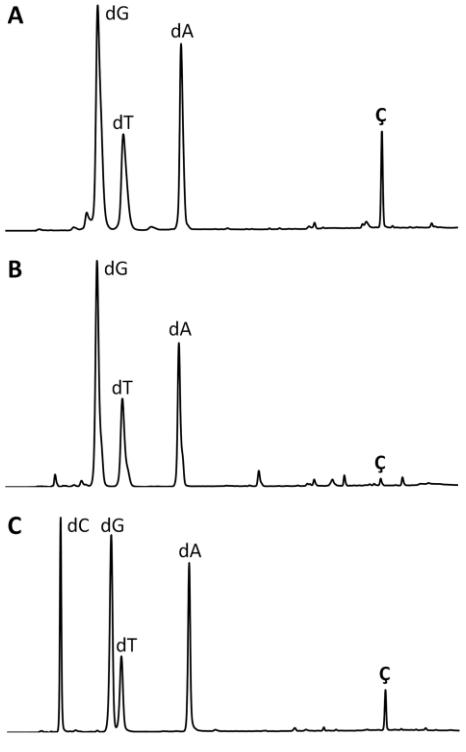


Figure 4. HPLC chromatograms of DNA oligonucleotides after enzymatic digestion with snake venom phosphodiesterase, nuclease P1, and alkaline phosphatase. **A**. Crude DNA **IV** [5′-d(PHO-TGAGGTAGTAGGTTGTATA**Ç**T)-3′] synthesized using **3**. **B**. Crude DNA **V** [5′-d(PHO-TGAGGTAGTAGGTTGTATA**Ç**T)-3′] synthesized using the phosphoramidite of unprotected **Ç**.[16] **C**. DNAzyme **VI** [5′-d(TGTAA**Ç**GCACTACCAGCGGCTGGAAATCT**Ç**TCTCGT)-3′] synthesized using **3**. PHO is a phosphate.

Conclusions

The nitroxide functional groups of **Ç** and **Çm** were protected by benzoylation of the corresponding hydroxylamines. The resulting **Ç-Bz** and **Çm-Bz** were converted into phosphoramidites and used for oligonucleotide synthesis of DNA and RNA of various lengths. The benzoyl protecting group was stable through the chemical synthesis of the oligonucleotides and was readily removed by standard oligonucleotide deprotection conditions to give nitroxide-labeled oligonucleotides; enzymatic digestion and HPLC analysis were used to quantify the spin label in the samples. These results show that this protecting group strategy for nitroxides can be used as a general method to prepare spin-labeled nucleic acids using the phosphoramidite approach.

Experimental Section

General Materials and Methods. All commercially available reagents were purchased from Sigma-Aldrich, Inc. or Acros Organics and used without further purification. 2′-Deoxyuridine and 2′-*O*-methyluridine were purchased from Rasayan Inc. USA. CH_2Cl_2, pyridine, and CH_3CN were dried with calcium hydride and freshly distilled before use. All moisture- and air-sensitive reactions were carried out in oven-dried glassware under an inert atmosphere of Ar. Thin-layer chromatography (TLC) was performed using glass plates pre-coated with silica gel (0.25 mm, F-25, Silicycle) and compounds were visualized under UV light and by *p*-anisaldehyde staining. Column chromatography was performed using 230–400 mesh silica gel (Silicycle). For acid-sensitive compounds the silica gel was basified by passing 3 % Et_3N in CH_2Cl_2 through the column before use. ^{1}H-, ^{13}C- and ^{31}P-NMR spectra were recorded with a Bruker Avance 400 MHz spectrometer. Commercial grade $CDCl_3$ was passed over basic alumina shortly before dissolving tritylated nucleosides for NMR analysis. Chemical shifts (δ) are reported in parts per million (ppm) relative to the partially deuterated NMR solvent $CDCl_3$ (7.26 ppm for ^{1}H NMR and 77.16 ppm for ^{13}C). ^{31}P-NMR chemical shifts are reported relative to 85 % H_3PO_4 as an external standard. All coupling constants were measured in Hertz (Hz). Nitroxide radicals show broadening and loss of NMR signals due to their paramagnetic nature and therefore, those NMR spectra are not shown. Mass spectrometric analyses of all organic compounds were performed on an ESI-HRMS (Bruker, MicrOTOF-Q) in a positive ion mode.

TBDMS-Ç (1). To a solution of **Ç**[16] (130 mg, 0.30 mmol) in DMF (3 mL) and pyridine (3 mL) were added imidazole (62 mg, 0.91 mmol) and TBDMSCl (137 mg, 0.91 mmol) and the resulting solution was stirred at 22 °C for 24 h. H_2O (50 mL) was added and the organic phase was extracted with EtOAc (3 × 10 mL). The combined organic phases were dried with Na_2SO_4 and concentrated in vacuo. The residue was purified by flash column chromatography using gradient elution ($MeOH/CH_2Cl_2$, 0:100 to 10:90), to yield **1** (160 mg, 95 %) as a yellow solid. HRMS (ESI): *m/z* calcd. for $C_{33}H_{53}N_4O_6Si_2 + Na^+$: 680.3401 $[M + Na]^+$, found 680.3396.

TBDMS-Ç-Bz (5). To a solution of **1** (53 mg, 0.08 mmol) in 1,4-dioxane (3.5 mL) was added *L*-ascorbic acid (72 mg, 0.41 mmol) in H_2O (1 mL) and the reaction mixture stirred at 40 °C for 30 min. H_2O (3.5 mL) and CH_2Cl_2 (5 mL) were added and the mixture was stirred for 5 min. The organic phase was separated and used directly in the next step by passing it through a plug of Na_2SO_4 under an inert atmosphere of Ar, into a solution of BzCl (46 µL, 0.41 mmol) and Et_3N (23 µL, 0.16 mmol) in CH_2Cl_2 (2 mL). The reaction was

stirred at 22 °C for 30 min, the solvent removed in vacuo and the residue purified by flash column chromatography using gradient elution ($MeOH/CH_2Cl_2$, 0:100 to 10:90), to yield **5** (55 mg, 95 %) as a yellow solid. ^1H NMR (400 MHz, $CDCl_3$): δ = 8.10–8.03 (m, 2H), 7.53 (d, J = 7.4 Hz, 1H), 7.40–7.44 (m, 3H), 7.19 (s, 1H), 6.34 (s, 1H), 6.22 (t, J = 6.1 Hz, 1H), 4.40–4.24 (m, 1H), 3.84 (dt, J = 8.6, 2.9 Hz, 2H), 3.76–3.66 (m, 1H), 2.36–2.22 (m, 1H), 2.26–2.32 (m, 1H), 2.06–1.90 (m, 1H), 1.42 (d, J = 10.1 Hz, 12H), 0.90 (s, 9H), 0.81 (s, 9H), 0.09 (d, J = 8.8 Hz, 6H), 0.00 ppm (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 166.13, 160.85, 142.58, 140.14, 139.53, 133.08, 129.62, 128.54, 127.76, 121.42, 112.27, 108.11, 100.88, 87.59, 85.80, 71.24, 68.60, 67.27, 66.52, 62.49, 41.78, 26.06, 25.71, 18.49, 17.95, –4.55 –4.85, –5.46 ppm; HRMS (ESI): m/z calcd. for $C_{40}H_{58}N_4O_7Si_2 + Na^+$: 785.3742 $[M + Na]^+$, found 785.3736.

Ç-Bz. To a solution of **5** (118 mg, 0.16 mmol) in THF (3 mL) was added TBAF (0.5 mL, 0.50 mmol, 1.0 M in THF) at 0 °C and the reaction mixture was stirred at 0 °C for 8 h. The solvent was removed in vacuo and the residue purified by flash column chromatography using gradient elution ($MeOH/CH_2Cl_2$, 0:100 to 25:75), to yield **Ç-Bz** (67 mg, 81 %) as a yellow solid. ^1H NMR (400 MHz, $CDCl_3$): δ = 8.09 (d, J = 7.3 Hz, 2H), 7.70–7.62 (m, 2H), 7.55 (t, J = 7.7 Hz, 2H), 6.66 (s, 2H), 6.23 (t, J = 6.4 Hz, 1H), 4.39 (dt, J = 6.6, 3.5 Hz, 1H), 3.94 (m, J = 3.1 Hz, 1H), 3.81–3.73 (m, 2H), 2.34 (ddd, J = 13.5, 6.0, 3.7 Hz, 1H), 2.17 (dt, J = 13.4, 6.5 Hz, 1H), 1.47 ppm (s, 12H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.9, 146.8, 143.8, 143.0, 137.4, 133.1, 132.5, 112.7, 91.5, 90.1, 74.6, 72.4, 65.2, 62.1, 62.1, 44.3, 31.7, 27.4, 23.3, 16.5 ppm; HRMS (ESI): m/z calcd. for $C_{28}H_{30}N_4O_7 + Na^+$: 557.2012 $[M + Na]^+$, found 557.2007.

Ç-Bz-DMT (6). Ç-Bz (16 mg, 0.03 mmol), DMTCl (21 mg, 0.06 mmol) and DMAP (0.8 mg, 0.01 mmol) were added to a round-bottomed flask and dried in vacuo for 16 h. Pyridine (2 mL) was added and the solution stirred for 16 h, after which MeOH (0.50 mL) was added and the solvent removed in vacuo. The residue was purified by flash column chromatography using gradient elution ($MeOH/CH_2Cl_2$/Et_3N, 0:99:1 to 95:4:1), to yield **6** (20 mg, 95 %) as a yellow solid. ^1H NMR (400 MHz, $CDCl_3$): δ = 8.18–8.09 (m, 3H), 7.64–7.56 (m, 2H), 7.49 (d, J = 7.8 Hz, 4H), 7.40 (d, J = 8.2 Hz, 4H), 7.31 (t, J = 7.6 Hz, 2H), 7.20 (t, J = 7.3 Hz, 1H), 6.86 (dd, J = 8.7, 3.8 Hz, 4H), 6.52–6.46 (m, 1H), 6.43 (s, 1H), 6.25 (s, 1H), 4.70–4.56 (m, 1H), 4.15 (dd, J = 8.8, 5.2 Hz, 1H), 3.75 (d, J = 6.6 Hz, 6H), 3.41 (ddd, J = 26.5, 10.7, 3.2 Hz, 2H), 2.71 (d, J = 12.8 Hz, 1H), 2.37–2.21 (m, 1H), 1.48 ppm (d, J = 12.2 Hz, 12H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 166.21, 159.56, 154.79, 154.60, 148.34, 144.57, 142.44, 139.75, 139.32, 136.66, 133.17, 130.10, 130.05, 129.60, 129.38, 128.60, 128.15, 128.14, 128.05, 127.97, 126.91, 126.30, 122.05, 113.27, 108.58, 106.64, 86.88, 86.33, 86.26, 71.33, 68.65, 68.54, 63.40, 60.42, 55.21, 41.79, 39.06, 29.37, 28.87, 25.44, 21.07, 14.22 ppm; HRMS (ESI): m/z calcd. for $C_{49}H_{48}N_4O_9 + Na^+$: 859.3319 $[M + Na]^+$, found 859.3313.

Ç-Bz phosphoramidite (3). A solution of compound **6** (21 mg, 0.02 mmol) in CH_2Cl_2 (1 mL) was treated with diisopropyl ammonium tetrazolide (5 mg, 0.03 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphane (11 µL, 0.03 mmol). The resulting solution was stirred at 22 °C for 2 h. CH_2Cl_2 (50 mL) was added and the solution washed with satd. aq. $NaHCO_3$ (3 × 50 mL) and brine (50 mL), dried with Na_2SO_4 and concentrated in vacuo. The residue was dissolved in a minimum amount of CH_2Cl_2 (few drops), followed by slow addition of *n*-hexane (40–50 mL) at 22 °C. The solvent was decanted from the precipitate and discarded. This procedure was repeated four times to give **3** (20 mg, 80 %) as a yellowish solid. ^1H NMR (400 MHz, $CDCl_3$): δ = 8.06–8.04 (m, 2H), 7.53 (s, 1H), 7.46–7.34 (m, 6H), 7.32–7.24 (m, 6H), 7.16 (dd, J = 7.4, 4.0 Hz, 1H), 6.79 (dq, J = 6.3, 2.2 Hz, 4H), 6.30–6.19 (m, 1H), 6.17 (d, J = 8.2 Hz, 1H), 4.58 (d, J = 14.0 Hz, 1H), 4.08 (t, J = 3.8 Hz, 1H), 3.69 (dd, J = 5.3, 2.1 Hz, 6H), 3.61–3.25 (m, 6H), 2.52 (q, J = 10.7, 8.4 Hz, 2H), 2.38 (t, J = 6.4 Hz, 1H), 2.22 (d, J = 11.4 Hz, 1H), 1.41 (t, J = 11.0 Hz, 12H), 1.13–0.97 (m, 12H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 158.61, 144.36, 139.29, 133.10, 130.18, 130.13, 130.10, 129.62, 129.47, 128.56, 128.26, 127.96, 126.99, 113.28, 113.25, 108.46, 86.88, 85.76, 77.22, 68.66, 68.59, 62.60, 58.36, 58.16, 55.24, 55.21, 43.38, 43.26, 43.20, 40.45, 31.59, 25.49, 24.65, 24.58, 24.51, 22.66, 20.25, 20.18, 14.12 ppm; ^{31}P NMR (162 MHz, $CDCl_3$): δ = 149.25, 148.63 ppm.

TBDMS-Çm (2). To a solution of **Çm**[26] (290 mg, 0.63 mmol) in DMF (6 mL) and pyridine (1.5 mL) were added TBDMSCl (286 mg, 1.89 mmol) and imidazole (129 mg, 1.89 mmol). The resulting solution was stirred at 22 °C for 16 h. H_2O (50 mL) and EtOAc (20 mL) were added, the organic phase separated and washed with satd. aq. $NaHCO_3$ (5 × 50 mL). The organic phase was dried with Na_2SO_4, concentrated in vacuo and purified by flash column chromatography using gradient elution ($MeOH/CH_2Cl_2$; 0:100 to 10:90), yielding **2** (299 mg, 81 %) as a yellow solid. HRMS (ESI): m/z calcd. for $C_{34}H_{55}N_4O_7Si_2 + Na^+$: 710.3501 $[M + Na]^+$, found 710.3500.

TBDMS-Çm-Bz (7). To a solution of **2** (235 mg, 0.34 mmol) in 1,4-dioxane (30 mL) was added *L*-ascorbic acid (301 mg, 1.71 mmol) in H_2O (5 mL). The reaction mixture was stirred at 40 °C for 1 h, after which CH_2Cl_2 (30 mL) and H_2O (30 mL) were added and the solution was stirred vigorously for 2 min. The organic phase was separated and filtered through a short pad of Na_2SO_4, in a dropping funnel under an inert atmosphere of Ar, into a solution of BzCl (198 µL, 0.34 mmol), Et_3N (953 µL, 6.83 mmol) in CH_2Cl_2 (10 mL). The solution was stirred at 22 °C for 2 h, washed with satd. aq. $NaHCO_3$ (3 × 100 mL), the organic phase dried with Na_2SO_4, concentrated in vacuo and the residue purified by flash column chromatography using gradient elution ($MeOH/CH_2Cl_2$; 0:100 to 10:90), to yield **7** (269 mg, quant.) as a yellow solid. ^1H NMR (400 MHz, $CDCl_3$): δ = 8.14 (s, 2H), 7.81 (s, 1H), 7.59 (t, J = 7.5 Hz, 1H), 7.49 (s, 1H), 7.51–7.44 (m, 2H), 6.41 (s, 1H), 5.88 (s, 1H), 4.22 (dt, J = 8.7, 4.3 Hz, 1H), 4.14–4.02 (m, 2H), 3.81 (dd, J = 11.9, 1.8 Hz, 1H), 3.68 (d, J = 4.6 Hz, 1H), 3.64 (s, 3H), 1.49 (s, 12H), 1.02 (s, 9H), 0.90 (s, 9H), 0.22 (dd, J = 7.5, 2.4 Hz, 6H), 0.09 ppm (dd, J = 5.9, 2.4 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 171.21, 166.14, 160.84, 155.20, 153.56, 142.62, 140.04, 139.53, 133.19, 133.15, 130.29, 130.16, 129.63, 129.44, 129.03, 128.58, 128.29, 127.69, 126.27, 122.06, 111.93, 108.14, 88.08, 87.47, 84.14, 83.11, 68.71, 68.63, 68.61, 68.52, 68.03, 62.97, 61.64, 60.51, 57.98, 28.93, 26.37, 26.34, 25.82, 25.76, 25.49, 18.78, 18.73, 18.15, 18.10, –4.45, –4.50, –4.83, –5.11, –5.20, –5.29, –5.34 ppm; HRMS (ESI): m/z calcd. for $C_{41}H_{60}N_4O_8Si_2 + Na^+$: 815.3842 $[M + Na]^+$, found 815.3853.

Çm-Bz. To a solution of **7** (271 mg, 0.34 mmol) in THF (18 mL) was added TBAF (1.2 mL, 1.20 mmol, 1.0 M in THF) and the reaction stirred at 22 °C for 18 h. The reaction was concentrated in vacuo and the residue purified by flash column chromatography using gradient elution ($MeOH/CH_2Cl_2$; 0:100–10:90), to yield **Çm-Bz** (139 mg, 72 %) as a yellow solid. ^1H NMR (400 MHz, $CDCl_3$): δ = 8.11 (td, J = 7.3, 6.6, 1.4 Hz, 2H), 7.59 (td, J = 7.4, 3.6 Hz, 1H), 7.57–7.51 (m, 1H), 7.45 (dt, J = 18.4, 7.7 Hz, 2H), 6.45 (d, J = 18.7 Hz, 1H), 5.72–5.67 (m, 1H), 4.95 (s, 1H), 4.38 (t, J = 4.8 Hz, 1H), 4.31 (dt, J = 9.1, 4.8 Hz, 1H), 4.00 (d, J = 6.5 Hz, 2H), 3.89 (q, J = 5.4 Hz, 1H), 3.62 (d, J = 3.9 Hz, 3H), 1.51–1.24 ppm (m, 12H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.91, 166.35, 160.98, 154.87, 153.61, 142.50, 142.19, 141.16, 140.16, 139.44, 133.30, 130.12, 129.62, 129.23, 128.62, 128.36, 128.03, 127.93, 125.91, 125.39, 123.08, 111.54, 108.77, 100.90, 89.88, 84.49, 82.98, 77.39, 77.28, 77.08, 76.76, 68.61, 68.56, 68.11, 67.31, 67.26, 66.55, 63.66, 62.97, 60.69, 58.53, 53.46, 29.26, 28.77, 25.41, 24.99 ppm; HRMS (ESI): m/z calcd. for $C_{29}H_{32}N_4O_8 + Na^+$: 587.2112 $[M + Na]^+$, found 587.2096.

Çm-Bz-DMT (8). Toluene (3 × 5 mL) was evaporated from **Çm-Bz** (139 mg, 0.25 mmol), followed by sequential addition of pyridine (4 mL), DMTCl (834 mg, 2.46 mmol) and DMAP (6 mg, 0.05 mmol). The solution was stirred for 14 h, MeOH (400 µL) was added and the solvent removed in vacuo. The residue was purified by flash column chromatography using gradient elution ($MeOH/CH_2Cl_2/Et_3N$; 0:99:1 to 1:98:1), to yield **8** (87 mg, 41 %) as a yellow solid. ^1H NMR (400 MHz, $CDCl_3$): δ = 8.13 (d, J = 7.2 Hz), 7.72 (s, 1H), 7.60 (t, J = 7.7 Hz, 1H), 7.51–7.50 (m, 2H), 7.49–7.48 (m, 2H), 7.43–7.40 (m, 4H), 7.33–7.31 (m, 2H), 7.22–7.18 (m, 1H), 6.88 (s, 1H), 6.88–6.85 (m, 4H), 6.11 (s, 1H), 5.87 (s, 1H), 4.55–4.52 (m, 1H), 4.02 (d, J = 8.6 Hz, 1H), 3.93 (bs, 1H), 3.75 (s, 6H), 3.73 (s, 3H), 3.60–3.49 (m, 2H), 1.51 ppm (d, J = 25.2 Hz, 12H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 166.15, 158.57, 155.18, 153.79, 142.30, 139.88, 139.26, 135.57, 133.13, 130.09, 129.62, 129.47, 128.57, 128.23, 128.01, 127.84, 126.92, 125.98, 121.80, 113.36, 113.31, 111.78, 108.71, 87.87, 86.82, 83.77, 82.91, 68.70, 68.61, 68.19, 60.84, 58.56, 55.21, 45.82, 29.72, 25.47 ppm; HRMS (ESI): m/z calcd. for $C_{50}H_{50}N_4O_{10}$ + Na^+: 889.3419 $[M + Na]^+$, found 889.3395.

Çm-Bz phosphoramidite (4). A solution of **8** (20 mg, 0.02 mmol) in CH_2Cl_2 (1 mL) was treated with diisopropyl ammonium tetrazolide (6 mg, 0.04 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphane (22 µL, 0.07 mmol). The reaction was stirred at 22 °C for 18 h. CH_2Cl_2 (15 mL) was added and the solution washed with satd. aq. $NaHCO_3$ (3 × 30 mL), dried with Na_2SO_4 and concentrated in vacuo. The residue was dissolved in Et_2O (2 mL) followed by slow addition of *n*-hexane (10 mL). The solvent was decanted from the precipitate and discarded. This procedure was repeated six times to yield **4** (18 mg, 71 %) as a yellow solid. ^1H NMR (400 MHz, $CDCl_3$): δ = 8.12 (dd, J = 8.3, 1.4 Hz, 4H), 7.64–7.57 (m, 3H), 7.53–7.44 (m, 10H), 7.44–7.34 (m, 11H), 7.31 (td, J = 7.8, 2.1 Hz, 6H), 7.22 (tt, J = 7.4, 1.9 Hz, 3H), 6.86 (ddt, J = 8.7, 5.9, 3.4 Hz, 10H), 6.14 (d, J = 15.5 Hz, 1H), 6.06 (d, J = 7.6 Hz, 1H), 5.97 (s, 1H), 4.29 (tt, J = 5.0, 2.6 Hz, 1H), 4.25–4.20 (m, 2H), 3.98 (q, J = 7.0, 6.4 Hz, 2H), 3.77–3.73 (m, 13H), 3.65 (dd, J = 4.0, 1.2 Hz, 6H), 2.46–2.38 (m, 2H), 1.55–1.38 (m, 28H), 1.26 (ddt, J = 5.7, 3.6, 2.2 Hz, 15H), 1.22–1.18 (m, 11H), 1.18–1.03 (m, 15H), 0.92–0.84 ppm (m, 8H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 165.92, 158.38, 155.03, 153.66, 142.02, 132.86, 130.10, 129.37, 128.32, 127.69, 126.77, 125.79, 121.65, 117.33, 113.00, 111.78, 108.29, 88.35, 86.49, 77.16, 68.45, 54.98, 43.16, 24.51, 20.10 ppm; ^{31}P NMR (162 MHz, $CDCl_3$): δ = 150.52, 150.08 ppm; HRMS (ESI): m/z calcd. for $C_{50}H_{60}N_4O_{10}$ + Na^+: 1089.4498 $[M + Na]^+$, found 1089.4498.

DNA and RNA Syntheses, Purification and Characterization. All commercial phosphoramidites, CPG columns, and solutions for oligonucleotide syntheses were purchased from ChemGenes Corp., USA. DNA and RNA solid-phase oligonucleotide syntheses were performed on an automated ASM800 DNA/RNA synthesizer (BIOSSET Ltd., Russia) using phosphoramidite chemistry. Unmodified and spin-labeled oligonucleotides were synthesized using a trityl-off protocol and phosphoramidites with standard protecting groups on 1 µmol scale (1000 Å CPG columns). Oxidation was performed with *tert*-butylhydroperoxide in toluene (1.0 M). Capping and detritylation were performed under standard conditions for DNA and RNA oligonucleotide synthesis.

The **Ç**-modified DNAs were synthesized using phosphoramidite **3**. Unmodified phosphoramidites were dissolved in CH_3CN (100 mM) and **3** was dissolved in 1,2-dichloroethane (100 mM). 5-Ethylthiotetrazole (250 mM) was used as a coupling agent and the coupling time was set to 1.5 min for unmodified phosphoramidites and to 5 min for the **Ç**-modified phosphoramidites. The DNAs were deprotected in satd. aqueous NH_3 at 55 °C for 8 h and dried in vacuo.

For the RNA synthesis, 2′*O*-TBDMS protected ribonucleoside phosphoramidites were used and dissolved in CH_3CN (100 mM). The **Çm**-modified RNAs were synthesized using phosphoramidite **4**, dissolved in 1,2-dichloroethane (100 mM). 5-Ethylthiotetrazole (250 mM) was used as a coupling agent for phosphoramidite **4**, 5-benzylthiotetrazole (250 mM) was used as a coupling agent for unmodified phosphoramidites and the coupling time was set to 7 min. The RNAs were deprotected and cleaved from the resin by adding a 1:1 solution (2 mL) of CH_3NH_2 (8 M in EtOH) and NH_3 (satd. in H_2O) and heating at 65 °C for 1 h. The solvent was removed in vacuo and the 2′*O*-TBDMS groups were removed by incubation in $Et_3N{\cdot}3HF$ (300 µL) in DMF (100 µL) at 55 °C for 1.5 h, followed by addition of deionized and sterilized water (100 µL). This mixture was transferred to a 50 mL Falcon tube and *n*-butanol (20 mL) was added and stored at –20 °C for 14 h, centrifuged and the solvent decanted from the RNA pellet.

All oligonucleotides were subsequently purified by 20 % DPAGE and extracted from the gel slices using the "crush and soak method" with Tris buffer (250 mM NaCl, 10 mM Tris, 1 mM Na_2EDTA, pH 7.5). The solutions were filtered through GD/X syringe filters (0.45 µm, 25 mm diameter, Whatman, USA) and were subsequently desalted using Sep-Pak cartridges (Waters, USA), following the instructions provided by the manufacturer. Dried oligonucleotides were dissolved in deionized and sterilized water (200 µL for each oligonucleotide). Concentrations of the oligonucleotides were determined by measuring absorbance at 260 nm using a Perkin Elmer Inc. Lambda 25 UV/Vis spectrometer and calculated by Beer's law. Mass spectrometric analyses of **Ç**- and **Çm**-labeled oligonucleotides were performed on an HRMS (ESI) (Bruker, MicrOTOF-Q) in negative ion mode.

Enzymatic Digestion of DNA and RNA and HPLC Analysis. To the oligonucleotide (4 nmol) in sterile water (8 µL) was added calf intestinal alkaline phosphatase (1 µL, 2 U), snake venom phosphodiesterase I (4 µL, 0.2 U), nuclease P1 from penicillium citrinum (5 µL, 1.5 U) and Tris buffer (2 µL, 500 mM Tris and 100 mM $MgCl_2$). The samples were incubated at 37 °C for 50 h. Enzymatically digested oligonucleotides were run on a Beckman Coulter Gold HPLC system using Beckman Coulter Ultrasphere C18 4.6 × 250 mm analytical column with UV detection at 254 nm. Solvent gradients for analytical RP-HPLC were run at 1.0 mL/min using following gradient program: solvent A, TEAA buffer (50 mM, pH 7.0); solvent B, CH_3CN; 0–4 min isocratic 4 % B, 4–14 min linear gradient 4–20 % B, 14–24 min linear gradient 20–50 % B, 24–29 min linear gradient 50–80 % B, for 29–30 min isocratic 80 % B, 30–35 min linear gradient 80–4 % B and 35–45 min isocratic 4 % B.

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