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A Carbazole-Derived Nitroxide That Is an Analogue of Cytidine: A Rigid Spin Label for DNA and RNA

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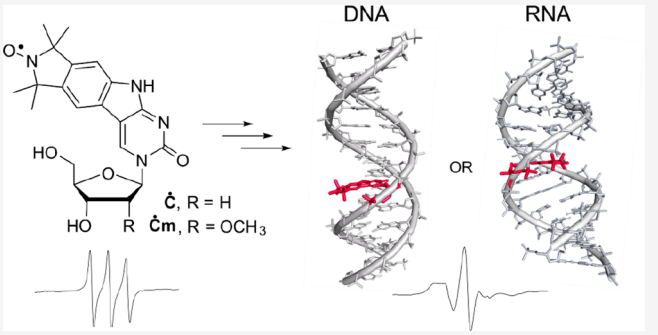
ABSTRACT: A variety of semirigid and rigid spin labels comprise a valuable arsenal for measurements of biomolecular structures and dynamics by electron paramagnetic resonance (EPR) spectroscopy. Here, we report the synthesis and characterization of rigid spin labels **Ċ** and **Ċm** for DNA and RNA, respectively, that are carbazole-derived nitroxides and analogues of cytidine. **Ċ** and **Ċm** were converted to their phosphoramidites and used for their incorporation into oligonucleotides by solid-phase synthesis. Analysis of **Ċ** and **Ċm** by single-crystal X-ray crystallography verified their identity and showed little deviation from planarity of the nucleobase. Analysis of the continuous-wave (CW) EPR spectra of the spin-labeled DNA and RNA duplexes confirmed their incorporation into the nucleic acids and the line-shape was characteristic of rigid spin labels. Circular dichroism (CD) and thermal denaturation studies of the **Ċ**-labeled DNAs and **Ċm**-labeled RNAs indicated that the labels are nonperturbing of duplex structure.



INTRODUCTION

Nucleic acids are the basis of life. Deoxyribonucleic acid (DNA) contains the genetic code and ribonucleic acid (RNA) plays major roles in sustainability of the cell, for example, by transmitting genetic information, regulating gene expression, and catalyzing chemical reactions.[1−4] To gain a deeper understanding into the properties and processes of nucleic acids, it is essential to study their structure and dynamics. Several techniques have been used for this purpose. X-ray crystallography[5,6] and nuclear magnetic resonance (NMR) spectroscopy[7,8] give information about the structure and dynamics of nucleic acids at atomic resolution. In recent years, cryo electron microscopy (EM) has also emerged as a useful technique to gain atomistic structures of biomolecules and their complexes at high resolution.[9−12] Furthermore, Förster resonance energy transfer (FRET) has been used to study tertiary structure and dynamics of nucleic acids[13−16] and can even be used to investigate single molecules.[17,18]

Electron paramagnetic resonance (EPR) spectroscopy is also a very useful method to study structure and dynamics of nucleic acids.[19−22] Using continuous-wave (CW) EPR spectroscopy, the dynamics can be studied by line-shape analysis of the EPR spectra,[23] and distances between two paramagnetic-centers can be measured within a range of 5−25 Å.[24] Pulsed dipolar spectroscopies, such as pulsed electron−electron double resonance (PELDOR), relaxation induced dipolar modulation enhancement (RIDME), single-frequency technique for refocusing dipolar couplings (SIFTER), and double quantum coherence (DQC), have been used to measure distances between 15 and 160 Å.[25−27] With pulsed EPR methods, information can also be obtained about relative orientations between two rigid spin centers.[28]

Most biomolecules, including nucleic acids, are diamagnetic; thus, spin labels need to be incorporated for EPR studies.[29,30] The most commonly used spin labels are aminoxyl radicals, usually called nitroxides. Nitroxides are highly persistent radicals, due to the delocalization of the radical between the nitrogen and the oxygen and because of the shielding effect of the alkyl groups flanking the radical center.[31,32] Spin labels can be incorporated at specific sites in nucleic acids, either covalently or noncovalently by a method called site-directed spin labeling (SDSL).[29,30,33,34] For covalent labeling, the spin labels can be incorporated into the nucleic acids postsynthetically or by using spin-labeled phosphoramidites as building blocks in the chemical synthesis of nucleic acids. The phosphoramidite method enables incorporation of involute labels at specific sites of nucleic acids.[30,35]

Spin labels that are covalently attached through single-bond tethers can move independently of the labeled biomolecule. Such flexibility leads to a large distance distribution.[36]

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Conformationally unambiguous spin labels[37] are semirigid spin labels that give more accurate distance measurements. They contain single-bond tethers, but the bonds lie on the axis of the nitroxide. Thus, the rotation around the single bonds does not cause a displacement of the nitroxide relative to the biomolecule. CW-EPR spectra of conformationally unambiguous benzimidazole-derived spin labels, incorporated into duplex DNA, show restricted mobility[38] and PELDOR distance measurements have shown a strong angular dependence.[39,40] Furthermore, *in-cell* PELDOR experiments of duplex RNA, doubly labeled with such semirigid spin labels, identified small distance changes that indicated compaction of RNA duplexes inside the cell.[41]

Rigid spin labels are even less mobile. In fact, rigid labels can be immobilized in helical regions of nucleic acids where they have no motion independent of the nucleic acid. As a result, they give more accurate distance measurements. Furthermore, the relative orientation of two rigid spin labels can be determined by dipolar EPR spectroscopy.[28,42] With rigid spin labels, room-temperature PELDOR measurements have even become possible,[43] but PELDOR studies are usually carried out at cryogenic temperatures. Rigid spin labels also give more precise information about the dynamics of biomolecules.[44] The nucleoside nitroxide analogues **Ç**[35] and **Çm**[45] (Figure 1) are rigid spin labels for DNA and RNA, respectively, that have been used to gain valuable information on structure and dynamics of nucleic acids. They have been used to obtain information on internal motions of DNA[46] and conformational changes and dynamics of both DNA and RNA.[47−49]

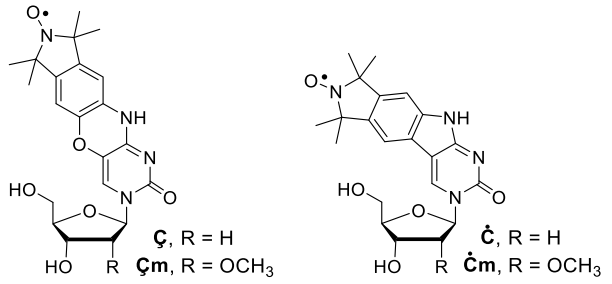


Figure 1. Structures of the rigid nitroxides **Ç**, **Çm**, **Ċ**, and **Ċm**.

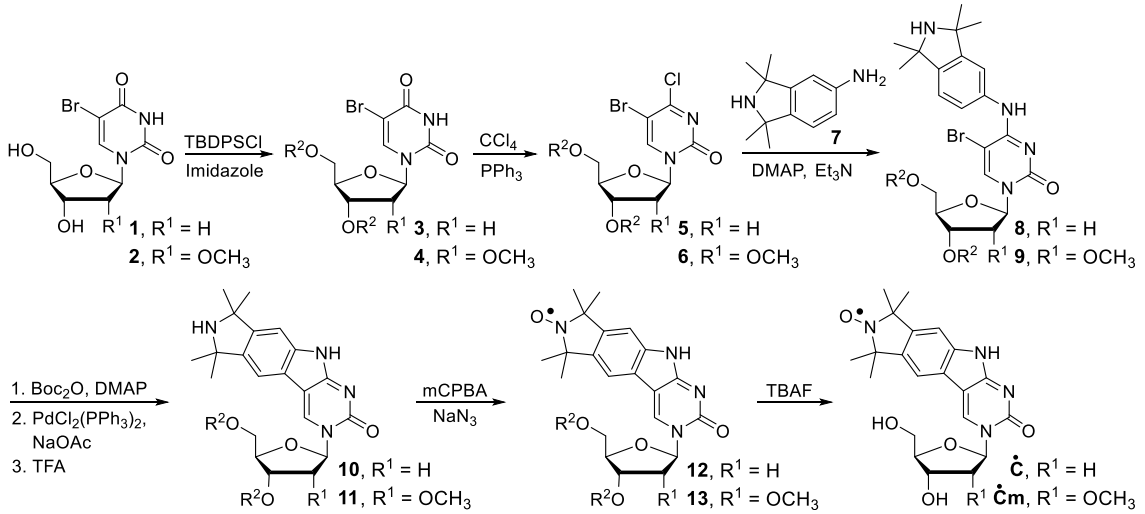
Although **Ç** and **Çm** have shown to be very valuable spin labels due to their rigidity, they seem to have some flexibility in the middle of the conjugated ring system, where the cytidine and the benzene are connected by an oxygen and a nitrogen atom, as indicated by the X-ray structure of **ç**, the nucleobase of **Ç**. Although the crystal structure of **Ç**-labeled DNA showed that **Ç** has a planar geometry inside DNA,[50] the crystal structure of **ç** has a 20° bend over the oxazine linkage. The observed nonplanar geometry of **ç** could be an indication that there is some degree of flexibility at the oxazine linkage that could affect the use of **Ç** and **Çm** as rigid spin labels. Such flexibility would not be surprising since these labels are formally antiaromatic according to the Hückel rule, as they contain 16 π-electrons.

Here we describe the synthesis and characterization of the rigid nitroxide **Ċ** for DNA and **Ċm** for RNA (Figure 1). **Ċ** and **Ċm** are derivatives of **Ç** and **Çm** where the oxygen at the oxazine linkage has been removed, yielding in a carbazole derived nitroxide that is aromatic, which should be more rigid. Spin labels **Ċ** and **Ċm** were incorporated into oligonucleotides by solid-phase synthesis with high spin-labeling efficiency. When paired with guanine, **Ċ** was well-tolerated in B-form DNA duplexes and **Ċm** in A-form RNA duplexes, as judged by thermal denaturation studies and circular dichroism (CD).

RESULTS AND DISCUSSION

Synthesis of Ċ and Ċm. The synthesis of **Ċ** and **Ċm** began by TBDPS protection of the 3′- and 5′-hydroxyl groups of nucleosides **1** and **2**, respectively, followed by chlorination of the fourth position of the nucleobase (Scheme 1). Nucleosides **5** and **6** were subsequentially coupled with tetramethyl isoindoline derivative **7**[51] to yield **8** and **9**, respectively. The secondary amines of **8** and **9** were protected by *tert*-butoxycarbonyl (boc) to avoid poisoning of the palladium catalyst,[52,53] which was used in the next step. An intramolecular Pd-catalyzed C−C cross-coupling reaction[54] fol-

Scheme 1. Synthesis of Nitroxides Ċ and Ċm[a]



[a]Yields were as follows: **3** (95%), **4** (80%), **5** (88%), **6** (77%), **8** (66%), **9** (63%), **10** (47%, 3 steps), **11** (59%, 3 steps), **12** (65%), **13** (76%), **Ċ** (84%), and **Ċm** (67%). R^2 stands for TBDPS.

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lowed by boc-deprotection, yielded carbazole derivatives **10** and **11**. Oxidation with *m*-CPBA only proceeded in good yield when NaN_3 was included in the reaction, which presumably provides transitory protection from side-reactions by a reversible addition to the nucleobase.[38] We have not seen any evidence of oxidation of the sec-amine in the five-membered carbazole ring.[55] Subsequent removal of the TBDPS groups gave rigid nitroxides **Ċ** and **Ċm**.

Crystal Structures of Ċ and Ċm. To investigate the three-dimensional structures of **Ċ** and **Ċm**, particularly with regards to the planarity of the aromatic system, single crystals were grown, and their crystal structures were determined. As expected, the crystal structure of **Ċ** adopts a planar geometry (Figure 2A). **Ċm**, in contrast, is slightly twisted from the plane

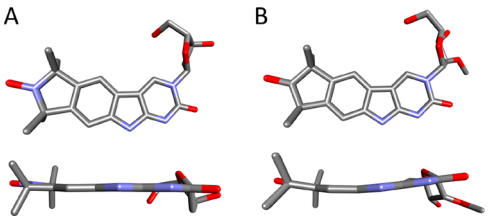


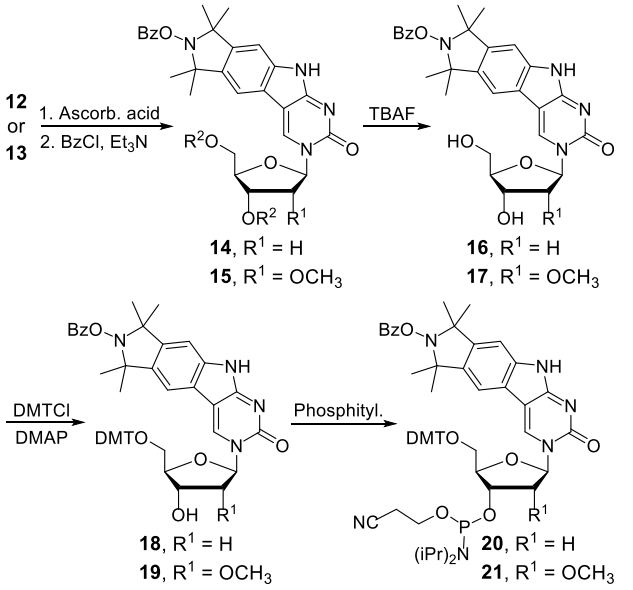
Figure 2. Side and top views of the crystal structures of **Ċ** (A) and **Ċm** (B).

with a 9° overall bend (Figure 2B). The nonplanar geometry is most likely due to the crystal packing. Nonplanar crystal structures of aromatic polycycles, resulting from intermolecular interactions are quite common as the energy required for bending is small.[56,57] The nature of the bend of **Ċm** is different from what was found in the aforementioned **ç**, where the bend is confined to the oxazine linkage, as if it was a hinge. The crystal structure of **Ċm**, however, slightly winds up on itself which induces a bend that is spread over the whole nucleobase. Taking these facts together, namely, that the crystal structure of **Ċ** is planar, that the bend of **Ċm** is smaller than for **ç**, and that **ç** has a bend at one location, indicates that **Ċ** and **Ċm** are more rigid than **Ç** and **Çm**.

Phosphoramidites of Ċ and Ċm. Intricate labels such as **Ċ** and **Ċm** can only be incorporated into nucleic acids through the phosphoramidite approach by solid-phase oligonucleotide synthesis. However, during the synthesis of spin-labeled oligonucleotides, nitroxides are partially reduced to their corresponding EPR-silent amines.[58,59] To circumvent such reduction, we used a recently developed strategy for protecting the nitroxides as benzoyl ethers of the corresponding hydroxylamine.[60] First, nitroxides **12** and **13** were reduced with ascorbic acid to their hydroxyl amines, followed by benzoylation (Scheme 2). The TBDPS groups were subsequently removed to yield nucleosides **16** and **17**, and the 5′-hydroxyl groups were protected as 4,4′-dimethoxytrityl ethers to yield **18** and **19**. Phosphitylation of the 3′-hydroxyl groups yielded phosphoramidites **20** and **21**, used for incorporation of **Ċ** and **Ċm** into DNA and RNA oligonucleotides, respectively, by solid-phase synthesis.

Synthesis and Characterization of Spin-Labeled DNA Oligonucleotides. **Ċ** was incorporated into six DNA oligonucleotides through automated solid-phase synthesis using phosphoramidite **20**. The **Ċ**-labeled oligonucleotides varied in length and position of the spin label (Table S1). The spin-labeled phosphoramidite coupled well during the solid-phase synthesis, as indicated by a strong orange color of the

Scheme 2. Synthesis of Benzoyl-Protected Ċ and Ċm and Their Corresponding Phosphoramidites[a]



[a]Yields were as follows: **14** (51%, 2 steps), **15** (43%, 2 steps), **16** (77%), **17** (74%), **18** (81%), **19** (75%), **20** (91%) and **21** (65%). R^2 stands for TBDPS.

trityl cation that appeared during removal of the DMT group. Moreover, analysis by denaturing polyacrylamide gel electrophoresis (DPAGE) showed no failure bands for the synthesized oligonucleotides (data not shown). After purification of the oligonucleotides by DPAGE, the spin-labeling efficiency was determined (Figure S50) by spin-counting using CW-EPR spectroscopy. As can be seen in Table S1, all oligonucleotides (**I**–**VI**) were quantitatively spin labeled. To further analyze the spin labeling efficiency, oligonucleotides **I**–**VI** were enzymatically digested, and the digests were analyzed by high-performance liquid chromatography (HPLC) (Figure S51). All chromatograms had five peaks, one for each natural nucleoside and one for **Ċ**, the identity of which was confirmed by coinjection of free nucleoside **Ċ**. Quantification of the nucleosides in the digest showed that **Ċ** had the expected ratio compared to the other four nucleosides in all the samples, further confirming quantitative spin labeling of all DNA oligonucleotides.

DNA duplexes **B**–**D**, **F**, and **H** were formed by annealing spin-labeled oligonucleotides **I**–**VI** (Table S1) to their complementary strands (Table 1). The CW-EPR spectra of **Ċ**, the **Ċ**-labeled DNA single-strand **I**, and corresponding DNA duplex **B** are shown in Figure 3. **Ċ** shows three fairly sharp lines (Figure 3A) due to the fast tumbling of the nucleoside in solution. The lines broadened after incorporation of **Ċ** into the oligonucleotide (Figure 3B), consistent with incorporation into an oligonucleotide that has a longer rotational correlative time. Upon annealing to its complementary strand, the CW-EPR spectrum broadened further, showing a splitting of the high- and low-field components (Figure 3C), which is characteristic for duplexes containing rigid spin labels.[35,45]

A molecular model of **Ċ** within a B-form DNA duplex showed a good fit of the spin label in the major groove of the duplex (Figure S49). To analyze experimentally if **Ċ** causes structural perturbation of the DNA duplex, thermal denaturation studies of duplexes **A**–**H** were carried out and their

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Table 1. Sequences of Spin-Labeled DNA and RNA Duplexes and Their Thermal Denaturation Analysis[a]

	sequence	T_M (°C)	ΔT_M
A	5′-d(GACCTCGCATCGTG)-3′ 3′-d(CTGGAGCGTAGCAC)-5′	63.0	
B	5′-d(GACCTCGĊATCGTG)-3′ 3′-d(CTGGAGCGTAGCAC)-5′	60.0	-3.0
C	5′-d(GAĊCTCGCATCGTG)-3′ 3′-d(CTGGAGCGTAGCAC)-5′	60.9	-2.1
D	5′-d(GACCTCGCATCGTG)-3′ 3′-d(CTGGAGCGTAGCAĊ)-5′	62.5	-0.5
E	5′-d(GACCTCGCATCGTGGACCTCGCATCGTGGACCTCGCATCGTG)-3′ 3′-d(CTGGAGCGTAGCACCTGGAGCGTAGCACCTGGAGCGTAGCAC)-5′	83.7	
F	5′-d(GACCTCGĊATCGTGGACCTCGCATCGTGGACCTCGCATCGTG)-3′ 3′-d(CTGGAGCGTAGCACCTGGAGCGTAGCACCTGGAGCGTAGCAC)-5′	83.1	-0.6
G	5′-d(GTCAGTGCGCGCGCGCGATC)-3′ 3′-d(CAGTCACGCGCGCGCGCTAG)-5′	79.2	
H	5′-d(GTĊAGTGCGCGCGCGCGATC)-3′ 3′-d(CAGTCACGCGĊGCGCGCTAG)-5′	77.0	-2.2
I	5′-GACCUCGCAUCGUG-3′ 3′-CUGGAGCGUAGCAC-5′	73.5	-3.2
J	5′-GACCUCGĊmAUCGUG-3′ 3′-CUGGAGCG UAGCAC-5′	70.3	-3.2
K	5′-UGUCAGUCGCGCGCGCGCAUC -3′ 3′- CAGUCAGCGCGCGCGCGUAGU-5′	85.9	
L	5′-UGUĊmAGUCGCGCGCG CGCAUC -3′ 3′- CAG UCAGCGCGCGĊmGCGUAGU-5′	83.4	-2.5
M	5′-d(GACCTCGYATCGTG)-3′ 3′-d(CTGGAGCGTAGCAC)-5′	61.8	-1.2

[a]**Y** stands for nucleoside **25**.

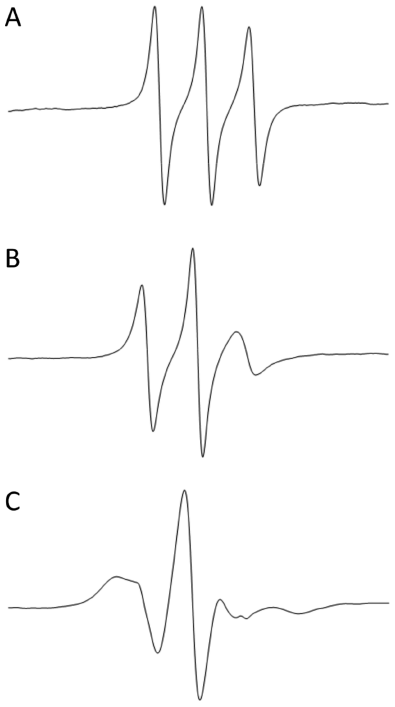

Figure 3. CW-EPR spectra of free nitroxide **Ċ** (A), spin-labeled DNA oligonucleotide **I** (B), and spin-labeled DNA duplex **B** (C).

circular dichroism (CD) spectra were recorded. The thermal denaturation curves showed a cooperative melting-transition (Figure S53) and confirmed duplex formation of the **Ċ**-labeled oligonucleotides. The melting temperatures (T_M's) of the **Ċ**-labeled duplexes were slightly lower than those of the unmodified duplexes (0.5−3.0 °C, Table 1). Comparison of the T_M's of duplex **B** and the same duplex labeled with **Ç** at the same position showed that the T_M of duplex **B** is 1.9 °C lower than that for the **Ç**-labeled duplex.[58] However, in general the decrease of the T_M's compared to the unmodified duplexes is minor and shows that **Ċ** is well-accommodated within DNA duplexes.

The CD spectra of the **Ċ**-labeled and unmodified DNA duplexes (**A**−**H**, Figure S54) all possessed negative and positive molar ellipticities at ca. 250 and 280 nm, respectively, characteristic of right-handed B-DNA (Figure S54). However, the CD spectra were different for the **Ċ**-labeled and unmodified duplexes, namely, there is a decrease in the CD signal between 270 and 280 nm for the modified duplex. A similar result has previously been described by Wypijewska del Nogal et al. for 2CNqA, an analogue of adenosine, in DNA duplexes.[61] The authors ascribe this discrepancy to "differences in molar absorptivity of the adenine that is exchanged with a 2CNqA in the modified duplex",[61] but 2CNqA absorbs strongly between 275 and 290 nm.[62] Nucleoside **Ċ** has an absorption maxima at 279 nm, where the CD spectra differs between the **Ċ**-modified and unmodified duplexes (Figure 4A); thus, the difference in the CD spectra could originate in the strong absorption of **Ċ** at 279 nm.

To investigate this issue further, we synthesized carbazole-analogue **25** (Scheme 3), where the five-membered ring of **Ċ**, containing the nitroxide and the four methyl groups, has been removed. Nucleoside **25** should, therefore, be less perturbing of the DNA duplex structure than **Ċ**. Nucleoside **25** was converted to phosphoramidite **27** (Scheme 3) and incorporated into DNA oligonucleotide **X** (Table S1) through solid-phase synthesis. Thermal denaturation experiments of duplex **M** (Table 1), containing **25**, showed a 1.2 °C decrease of the T_M compared to the unmodified duplex (**A**, Table 1), indicating that nucleoside **25** is nonperturbing of duplex structure. The CD spectrum of DNA duplex **M** (Figure 4B) had a strong resemblance to the CD spectra of the **Ċ**-labeled duplexes, but the decrease in the intensity of the 279 nm peak

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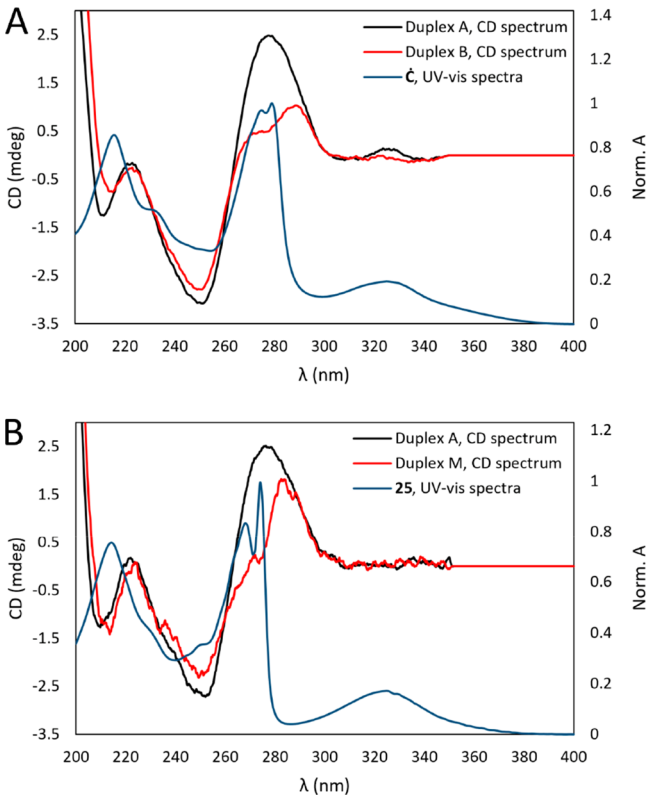
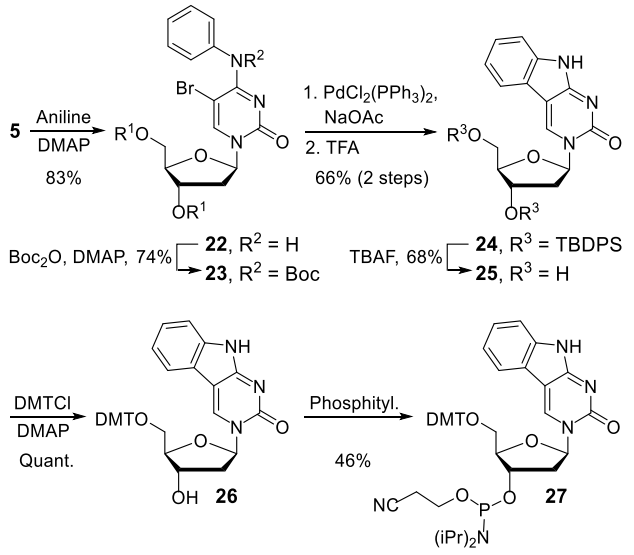


Figure 4. Comparison of CD spectra of DNA duplexes and of absorption spectra of nucleosides. CD spectra of duplex A (black) and B (red) and absorption spectra of nucleoside Ċ (blue) (A). CD spectra of duplex A (black) and M (red) and absorption spectra of nucleoside **25** (blue) (B).

Scheme 3. Synthesis of Nucleoside 25 and Its Corresponding Phosphoramidite[a]



[a]R^1 stands for TBDPS.

was blue-shifted to 274 nm which is the absorption maximum of **25**. It is noteworthy that there are two maxima around 274 nm in the UV spectrum of **25** and that the small dip between the two peaks is mirrored in the CD spectrum. This data is a further indication that the difference in the CD spectra of the Ċ-modified and unmodified DNA duplexes is not due to conformational rearrangement of the duplex itself.

Synthesis and Characterization of Spin-Labeled RNA Oligonucleotides. Phosphoramidite **21** was used to incorporate **Ċm** into one 14- and two 21-nucleotide long RNAs through automated solid-phase synthesis (Table 1). As for the DNA synthesis, the phosphoramidite coupled well during the RNA solid-phase synthesis and spin-counting by CW-EPR spectroscopy confirmed high spin-labeling efficiency (Table S1). Oligonucleotides **VII**–**IX** were enzymatically digested, and the digests were analyzed by HPLC (Figure S52), showing the natural nucleosides and **Ċm**. Quantification of the five peaks gave the expected ratios.

CW-EPR spectra of RNA duplexes **J** and **L** showed the expected line-shape for a rigid spin label in a duplex RNA (Figure S50). A model of an RNA A-form helix, labeled with **Ċm**, showed that the spin label fits well into the major groove of the duplex (Figure S49). Thermal denaturation experiments resulted in sigmoidal melting curves that also confirmed duplex formation of the **Ċm**-labeled oligonucleotides (Figure S53). The melting temperatures of the **Ċm**-labeled duplexes were only ca. 3.2 °C lower (Table 1) than for the corresponding unmodified duplexes, indicating only minor destabilization. The CD spectra for the unmodified and **Ċm**-labeled RNA duplexes showed negative and positive molar ellipticities at ca. 210 and 263 nm, respectively, typical for A-form RNA (Figure S54). Thus, both the thermal denaturation studies and the CD spectra indicate that **Ċm** fits well in A-form RNA duplexes.

CONCLUSION

Rigid spin labels **Ċ** and **Ċm** were synthesized and characterized. Comparison of their single-crystal X-ray structures with the known structure of **ç**,[50] the nucleobase of **Ç** and **Çm**, indicates that **Ċ** and **Ċm** are more rigid than known rigid spin labels **Ç** and **Çm**. However, it remains to be seen if this will result in better performance of **Ċ** and **Ċm** as spin labels compared to **Ç** and **Çm**. Another advantage of **Ċ** and **Ċm**, compared to **Ç** and **Çm**, is that their synthesis is two steps shorter. **Ċ** and **Ċm** were incorporated into DNA and RNA, respectively, with high spin-labeling efficiency. Thermal denaturation experiments and CD spectra indicate that the spin labels are nonperturbing of duplex structures. These new rigid spin labels are promising candidates for future studies of DNA and RNA structures and dynamics by pulsed EPR methods.

EXPERIMENTAL SECTION

General Materials and Methods. All commercially available reagents were purchased from Sigma-Aldrich, Inc. GmbH or Acros Organics and used without further purification, except for diisopropylammonium tetrazolide and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, which were purchased from ChemGenes Corp. All commercial phosphoramidites, controlled pore glass (CPG) solid support, and solutions for oligonucleotide syntheses were also purchased from ChemGenes Corp. Columns for the CPG solid-support were purchased from BioAutomation. 2′-Deoxyuridine and 2′-*O*-methyluridine were purchased from Rasayan Inc. USA. CH_2Cl_2, pyridine, and CH_3CN were dried over CaH and freshly distilled before use. Et_3N, 1,4-dioxane, and DMA where dried over molecular sieves (3 Å). All moisture- and air-sensitive reactions were carried out in oven-dried glassware under an inert atmosphere of argon. Thin-layer chromatography (TLC) was carried out using glass plates precoated with silica gel (0.25 mm, F-25, Silicycle) and compounds were visualized under UV light and by *p*-anisaldehyde

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staining. Column chromatography was carried out using 230−400 mesh silica gel (Silicycle). ^{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 are reported in parts per million (ppm) relative to the partially deuterated NMR solvents $CDCl_3$ (7.26 ppm for ^{1}H NMR and 77.16 ppm for ^{13}C) and DMSO-d_6 (2.50 ppm for ^{1}H NMR and 39.52 ppm for ^{13}C). ^{31}P NMR chemical shifts are reported relative to 85% H_3PO_4 (at 0.0 ppm) as an external standard. All coupling constants were reported 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 carried out on a high-resolution electrospray ionization mass spectrometer (ESI-HRMS) (Bruker, MicrOTOF-Q) in positive ion mode. UV−vis absorption spectra were recorded on a PerkinElmer Lambda 25 UV/Vis Spectrometer.

DNA and RNA solid-phase oligonucleotide syntheses were carried out 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 a 1.5 μmol scale (1000 Å CPG columns). Oxidation was carried out with *tert*-butylhydroperoxide (1.0 M) in toluene. Capping and detritylation were carried out using standard conditions for DNA and RNA syntheses. Molecular weights of the oligonucleotides were determined by MALDI-TOF analysis on a Voyager-DE STR Biospectrometry Workstation. HPLC analyses of enzymatic digests were carried out on an analytical Agilent 1200 HPLC system using a GL Science Inc. C18 4.6 × 150 mm^2 analytical column with UV detection at 260 nm. Solvent gradients for analytical RP-HPLC were run at 1.0 mL/min using the following gradient: solvent A, triethylammonium acetate (TEAA) buffer (50 mM, pH 7.0), solvent B, CH_3CN; 0−4 min isocratic 4% B, 4−30 min linear gradient 4−100% B, 30−34 min isocratic 100% B, 34−36 min linear gradient 100−4% B, and 36−40 min isocratic 4% B. Sample preparation for CD, thermal denaturation, and EPR measurements was as follows: An appropriate quantity of each DNA or RNA stock solution was dried in an Eppendorf Concentrator Plus and dissolved in phosphate buffer (10 mM phosphate, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0). DNA and RNA duplexes were formed by annealing in an MJ Research PTC 200 thermal cycler using the following protocol: 90 °C for 2 min, 60 °C for 5 min, 50 °C for 5 min, 40 °C for 5 min, and 22 °C for 15 min. CW-EPR spectra were recorded on a MiniScope MS200 spectrometer using 100 kHz modulation frequency, 1.0 G modulation amplitude, and 2.0 mW microwave power. The samples were placed in a quartz capillary (BLAUBRAND intraMARK) prior to EPR measurements. CD spectra of the duplexes were recorded on a Jasco J-810 spectropolarimeter and were recorded from 350 to 200 nm at 25 °C.

*5-Bromo-1-((2R,4S,5R)-4-((tert-butyldiphenylsilyl)oxy)-5-(((tert-butyldiphenylsilyl)oxy)methyl)tetrahydrofuran-2-yl)pyrimidine-2,4-(1H,3H)-dione (**3**).* To a solution of compound **1** (10.0 g, 3.3 mmol) in DMF (31 mL) and pyridine (12 mL) were added TBDPSCl (3.3 mL, 13.1 mmol) and imidazole (0.7 g, 9.8 mmol). After stirring at 22 °C for 48 h, aqueous HCl (50 mL, 1 M) was added, the solution extracted with EtOAc (3 × 50 mL) and the combined organic phases washed with aqueous HCl (2 × 50 mL, 1 M) and brine (2 × 50 mL). The organic phase was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (EtOAc/petroleum ether, 10:90 to 20:80) to yield compound **3** (2.4 g, 95% yield) as a white fluffy powder. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.64 (s, 1H), 8.00 (s, 1H), 7.60 (dd, J = 8.0, 1.4 Hz, 2H), 7.54 (td, J = 8.0, 1.3 Hz, 4H), 7.47−7.32 (m, 10H), 7.29−7.24 (m, 4H), 6.43 (dd, J = 9.0, 5.1 Hz, 1H), 4.49 (d, J = 5.3 Hz, 1H), 3.98 (s, 1H), 3.71 (dd, J = 11.7, 2.1 Hz, 1H), 3.28 (dd, J = 11.7, 2.6 Hz, 1H), 2.42 (dd, J = 12.9, 5.7 Hz, 1H), 2.00−1.91 (m, 1H), 1.07 (s, 9H), 0.95 (s, 9H) ppm. $^{13}C\{^{1}H\}$ NMR (101 MHz, $CDCl_3$) δ 158.8, 149.3, 149.3, 139.2, 135.7, 135.6, 135.5, 135.4, 133.1, 133.0, 132.4, 132.1, 130.1, 130.03, 130.0, 127.9, 127.89, 97.0, 88.3, 86.0, 77.2, 74.1, 63.9, 41.9, 27.0, 26.9, 22.7, 19.2, 19.0, 14.1 ppm. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{41}H_{47}BrN_2O_5Si_2Na]^+$ 807.2088. Found 807.2078.

*5-Bromo-1-((2R,3R,4R,5R)-4-((tert-butyldiphenylsilyl)oxy)-5-(((tert-butyldiphenylsilyl)oxy)methyl)-3-methoxytetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione (**4**).* To a solution of compound **2** (3.0 g, 8.9 mmol) in DMF (84 mL) and pyridine (33 mL) were added TBDPSCl (9.2 mL, 35.6 mmol) and imidazole (1.8 g, 26.7 mmol). After stirring at 22 °C for 48 h, aqueous HCl (200 mL, 1 M) was added, and the solution was extracted with EtOAc (3 × 100 mL). The combined organic phases were washed with aqueous HCl (2 × 200 mL, 1 M) and brine (2 × 200 mL). The organic phase was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (EtOAc/petroleum ether, 10:90 to 20:80) to yield compound **4** (5.8 g, 80% yield) as a white fluffy powder. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.63 (s, 1H), 7.83 (s, 1H), 7.69 (d, J = 6.7 Hz, 2H), 7.57 (d, J = 6.7 Hz, 4H), 7.52 (d, J = 8.0 Hz, 2H), 7.45−7.27 (m, 12H), 6.22 (d, J = 6.2 Hz, 1H), 4.33 (dd, J = 4.6, 3.0 Hz, 1H), 4.03 (d, J = 2.2 Hz, 1H), 3.79 (dd, J = 11.9, 1.8 Hz, 1H), 3.60 (dd, J = 6.1, 4.9 Hz, 1H), 3.33 (dd, J = 11.9, 2.6 Hz, 1H), 3.23 (s, 3H), 1.08 (s, 9H), 1.03 (s, 9H) ppm. $^{13}C\{^{1}H\}$ NMR (101 MHz, $CDCl_3$): δ 251.9, 158.6, 149.4, 138.8, 135.9, 135.7, 135.6, 135.4, 133.2, 132.9, 132.5, 132.1, 130.1, 130.05, 130.0, 127.9, 127.85, 127.7, 97.5, 86.3, 85.8, 83.6, 71.03, 63.6, 58.4, 31.9, 27.1, 26.9, 26.5, 22.7, 19.3, 19.3, 14.1 ppm. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{42}H_{49}BrN_2O_6Si_2Na]^+$ 837.2194. Found 837.2163.

*5-Bromo-1-((2R,4S,5R)-4-((tert-butyldiphenylsilyl)oxy)-5-(((tert-butyldiphenylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-4-chloropyrimidin-2(1H)-one (**5**).* To a solution of compound **3** (1.94 g, 2.47 mmol) in CH_2Cl_2 (15 mL) and CCl_4 (15 mL) was added PPh_3 (1.62 g, 6.17 mmol). The reaction mixture was refluxed at 65 °C for 2 h, cooled to 22 °C, and concentrated *in vacuo*, and the residue was purified by flash column chromatography using a gradient elution (EtOAc/petroleum ether, 10:90 to 20:80) to yield compound **5** (1.75 g, 88% yield) as a pale-yellow fluffy powder. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.42 (s, 1H), 7.62−7.24 (m, 23H), 6.34 (dd, J = 8.1, 5.4 Hz, 1H), 4.47−4.40 (m, 1H), 4.06 (q, J = 2.3 Hz, 1H), 3.71 (dd, J = 11.8, 2.3 Hz, 1H), 3.23 (dd, J = 11.8, 2.9 Hz, 1H), 2.88 (ddd, J = 13.4, 5.5, 1.3 Hz, 1H), 2.00 (ddd, J = 13.5, 8.2, 5.5 Hz, 1H), 1.09 (s, 9H), 0.95 (s, 9H) ppm. $^{13}C\{^{1}H\}$ NMR (101 MHz, $CDCl_3$): δ 262.3, 250.6, 165.4, 151.8, 144.4, 135.7, 135.6, 135.5, 135.5, 135.4, 133.2, 132.8, 132.4, 132.1, 130.1, 130.05, 130.0, 127.9, 127.89, 127.87, 127.6, 97.1, 89.5, 89.1, 74.5, 63.9, 43.0, 27.0, 26.9, 26.86, 26.7, 19.2, 19.0, 14.1 ppm. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{41}H_{46}BrClN_2O_4Si_2Na]^+$ 825.1745. Found 825.1738.

*5-Bromo-1-((2R,3R,4R,5R)-4-((tert-butyldiphenylsilyl)oxy)-5-(((tert-butyldiphenylsilyl)oxy)methyl)-3-methoxytetrahydrofuran-2-yl)-4-chloropyrimidin-2(1H)-one (**6**).* To a solution of compound **4** (1.9 g, 2.3 mmol) in CH_2Cl_2 (14 mL) and CCl_4 (14 mL) was added PPh_3 (1.5 g, 5.7 mmol). The reaction mixture was refluxed at 65 °C for 2 h, cooled to 22 °C, and concentrated *in vacuo*, and the residue was purified by flash column chromatography using a gradient elution (EtOAc/petroleum ether, 10:90 to 25:75) to yield compound **6** (1.5 g, 77% yield) as a pale-yellow fluffy powder. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (s, 1H), 7.68 (s, 2H), 7.58 (d, J = 11.4 Hz, 6H), 7.40 (d, J = 31.9 Hz, 11H), 7.29−7.24 (m, 3H), 6.02 (s, 1H), 4.20 (s, 1H), 4.15 (s, 1H), 4.03 (d, J = 13.8 Hz, 1H), 3.61 (d, J = 15.1 Hz, 1H), 3.36 (s, 1H), 3.29 (s, 3H), 1.06 (s, 18H) ppm. $^{13}C\{^{1}H\}$ NMR (101 MHz, $CDCl_3$): δ 165.7, 151.5, 143.8, 135.9, 135.8, 135.7, 135.6, 135.56, 135.5, 135.4, 133.0, 132.9, 132.8, 132.5, 132.1, 130.1, 130.03, 130.0, 128.0, 127.9, 127.85, 127.8, 127.7, 100.0, 97.4, 88.7, 86.2, 85.9, 84.7, 83.9, 83.6, 70.3, 62.9, 58.1, 35.4, 31.9, 31.9, 29.7, 29.4, 29.0, 27.3, 27.1, 26.9, 26.86, 26.5, 22.7, 19.5, 19.3, 19.29, 14.1. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{42}H_{48}BrClN_2O_5Si_2Na]^+$ 855.1819. Found 855.1851.

*5-Bromo-1-((2R,4S,5R)-4-((tert-butyldiphenylsilyl)oxy)-5-(((tert-butyldiphenylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-4-((1,1,3,3-tetramethylisoindolin-5-yl)amino)pyrimidin-2(1H)-one (**8**).* To a solution of nucleoside **5** (4.5 g, 5.6 mmol) in CH_2Cl_2 (147 mL) were added compound **7**[S1] (1.2 g, 6.2 mmol), DMAP (0.07 g, 0.7 mmol), and Et_3N (1 mL, 7.2 mmol), and the resulting solution was stirred at 22 °C for 14 h. H_2O (100 mL) was added, and the organic phase was

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extracted with CH_2Cl_2 (2 × 100 mL). The combined organic phases were washed with saturated aqueous $NaHCO_3$ (50 mL) and brine (100 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution ($MeOH/CH_2Cl_2$, 0:100 to 10:90) to yield compound **8** (3.6 g, 66% yield) as a light yellow foam. ^1H NMR (400 MHz, $CDCl_3$): δ 8.09 (s, 1H), 7.68−7.63 (m, 1H), 7.63−7.58 (m, 2H), 7.54 (s, 4H), 7.34 (d, J = 7.5 Hz, 11H), 7.25−7.21 (m, 1H), 7.11 (d, J = 8.2 Hz, 1H), 6.49−6.43 (m, 1H), 4.46 (s, 1H), 4.03 (s, 1H), 3.71 (d, J = 13.8 Hz, 1H), 3.28 (d, J = 14.5 Hz, 1H), 2.68 (d, J = 19.6 Hz, 1H), 1.97−1.85 (m, 1H), 1.57 (s, 12H), 1.07 (s, 9H), 0.94 (s, 9H) ppm. $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 242.9, 157.5, 154.2, 141.0, 136.9, 135.9, 135.8, 135.7, 135.6, 133.5, 133.2, 132.9, 132.4, 130.1, 130.0, 128.0, 127.95, 122.2, 122.1, 115.0, 88.6, 88.3, 87.6, 74.4, 64.1, 42.9, 31.3, 31.26, 27.2, 27.15, 27.1, 27.0, 19.4, 19.1 ppm. HRMS (ESI) m/z: $(M + H)^+$ Calcd for $[C_{53}H_{64}BrN_4O_4Si_2]^+$ 957.3637. Found 957.3615.

*4-5-Bromo-1-((2R,3R,4R,5R)-4-((tert-butyldiphenylsilyl)oxy)-5-(((tert-butyldiphenylsilyl)oxy)methyl)-3-methoxytetrahydrofuran-2-yl)-4-((1,1,3,3-tetramethylisoindolin-5-yl)amino)pyrimidin-2(1H)-one (**9**).* To a solution of compound **6** (4.4 g, 5.2 mmol) in CH_2Cl_2 (137 mL) were added compound 7[S1] (1.2 g, 6.2 mmol), DMAP (0.04 g, 0.5 mmol), and Et_3N (0.9 mL, 6.5 mmol), and the resulting solution was stirred at 22 °C for 12 h. H_2O (100 mL) was added, and the mixture was extracted with CH_2Cl_2 (2 × 200 mL). The combined organic phases were washed with saturated aqueous $NaHCO_3$ (50 mL) and brine (100 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution ($MeOH/CH_2Cl_2$, 0:100 to 2:90) to yield compound **9** (3.3 g, 63% yield) as a light yellow foam. ^1H NMR (400 MHz, $CDCl_3$): δ 7.83 (s, 1H), 7.73−7.54 (m, 9H), 7.48−7.29 (m, 11H), 7.31−7.20 (m, 2H), 7.09 (d, J = 8.2 Hz, 1H), 6.11 (d, J = 3.1 Hz, 1H), 4.23 (dd, J = 6.5, 4.7 Hz, 1H), 4.17 (ddd, J = 6.5, 3.1, 1.9 Hz, 1H), 4.02 (dd, J = 11.8, 1.9 Hz, 1H), 3.62 (dd, J = 11.8, 3.2 Hz, 1H), 3.37 (dd, J = 4.8, 3.1 Hz, 1H), 3.29 (s, 3H), 1.58 (d, J = 6.6 Hz, 12H), 1.06 (d, J = 1.2 Hz, 18H) ppm. $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 262.4, 251.5, 157.4, 153.9, 147.9, 140.6, 137.0, 136.0, 135.8, 135.7, 135.6, 133.4, 133.3, 133.2, 132.8, 130.1, 130.05, 130.0, 129.98, 128.03, 128.0, 127.9, 127.7, 122.1, 122.0, 114.9, 88.6, 88.2, 84.1, 83.8, 70.6, 64.6, 63.2, 58.0, 53.5, 31.1, 31.05, 27.4, 27.0, 19.6, 19.5 ppm. HRMS (ESI) m/z: $(M + H)^+$ Calcd for $[C_{54}H_{66}BrN_4O_5Si_2]^+$ 987.3743. Found 987.3721.

*3-((2R,4S,5R)-4-((tert-Butyldiphenylsilyl)oxy)-5-(((tert-butyldiphenylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-6,6,8,8-tetramethyl-6,7,8,10-tetrahydropyrimido[4,5-b]pyrrolo[3,4-f]indol-2(3H)-one (**10**).* To a solution of compound **8** (1150 mg, 1.20 mmol) in 1,4-dioxane (10 mL) were added Boc_2O (2.62 g, 12.02 mmol) and DMAP (15 mg, 0.12 mmol), and the resulting solution was stirred at 60 °C for 12 h. Saturated aqueous $NaHCO_3$ (50 mL) was added, and the mixture was extracted with EtOAc (3 × 20 mL). The combined organic phases were washed with brine (50 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (EtOAc/petroleum ether, 10:90 to 25:75), which yielded a mixture of two compounds that were carried to the next step without further purification by dissolving them (130 mg, 0.11 mmol) in DMA (4.5 mL) and adding anhydrous NaOAc (37 mg, 0.45 mmol) and $PdCl_2(PPh_3)_2$ (16 mg, 0.02 mmol). The resulting solution was stirred at 150 °C for 12 h, cooled to 22 °C, filtered through Celite, and concentrated *in vacuo*. H_2O (20 mL) was added and the mixture extracted with EtOAc (5 × 20 mL). The combined organic phases were washed with brine (3 × 50 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 (20 mL), and TFA (10 mL) was added at 0 °C. The reaction was stirred at 22 °C for 3 h. Saturated aqueous $NaHCO_3$ was added to the solution until the effervescence, by release of CO_2 gas, had stopped. The two phases were separated and the aqueous phase was extracted with EtOAc (4 × 20 mL). The combined organic phases were washed with brine (50 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution ($MeOH/CH_2Cl_2$, 0:100 to 10:90) to yield compound **10** (46 mg, 47%) as a brownish foam. ^1H NMR (400 MHz, $CDCl_3$): δ 8.77 (s, 1H), 7.53 (s, 4H), 7.45 (d, J = 7.3 Hz, 2H), 7.40 (d, J = 7.2 Hz, 2H), 7.25 (s, 9H), 7.16 (q, J = 7.6 Hz, 5H), 6.78 (s, 1H), 6.60 (s, 1H), 4.47 (s, 1H), 4.00 (s, 1H), 3.80 (s, 1H), 3.36 (s, 1H), 3.17 (s, 1H), 2.70 (s, 1H), 1.84 (s, 6H), 1.56 (d, J = 76.4 Hz, 6H), 0.96 (s, 9H), 0.84 (s, 9H) ppm. $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 163.0, 161.7, 154.6, 143.1, 141.4, 137.3, 135.7, 135.7, 135.5, 135.4, 135.3, 135.1, 133.3, 133.1, 132.99, 132.3, 132.2, 130.3, 130.2, 130.0, 129.9, 128.2, 128.1, 127.9, 127.8, 121.2, 112.6, 105.7, 105.2, 88.7, 88.1, 87.9, 72.9, 70.6, 69.5, 67.7, 67.2, 66.7, 66.7, 66.4, 64.1, 63.7, 53.5, 50.5, 43.5, 42.3, 42.0, 30.0, 29.8, 29.6, 29.5, 27.1, 27.0, 26.9, 25.0, 21.4, 19.3, 19.3, 19.1, 19.0, 18.8, 16.7, 15.2, 15.1 ppm. HRMS (ESI) m/z: $(M + H)^+$ Calcd for $[C_{53}H_{63}N_4O_4Si_2]^+$ 875.4382. Found 875.4381.

*3-((2R,3R,4R,5R)-4-((tert-Butyldiphenylsilyl)oxy)-5-(((tert-butyldiphenylsilyl)oxy)methyl)-3-methoxytetrahydrofuran-2-yl)-6,6,8,8-tetramethyl-6,7,8,10-tetrahydropyrimido[4,5-b]pyrrolo[3,4-f]indol-2(3H)-one (**11**).* To a solution of compound **9** (5.02 g, 5.01 mmol) in 1,4-dioxane (42 mL) were added Boc_2O (4.38 g, 40.1 mmol) and DMAP (0.062 g, 0.50 mmol), and the resulting solution was stirred at 60 °C for 12 h. Saturated aqueous $NaHCO_3$ (50 mL) was added, and the mixture was extracted with EtOAc (3 × 30 mL). The combined organic phases were washed with brine (50 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (EtOAc/petroleum ether, 10:90 to 25:75), which yielded a mixture of two compounds that were carried to the next step without further purification by dissolving them (133 mg, 0.11 mmol) in DMA (4.5 mL) and adding anhydrous NaOAc (37 mg, 0.45 mmol) and $PdCl_2(PPh_3)_2$ (16 mg, 0.023 mmol). The resulting solution was stirred at 150 °C for 12 h, cooled to 22 °C, filtered through Celite, and concentrated *in vacuo*. H_2O (25 mL) was added, and the mixture extracted with EtOAc (3 × 20 mL). The combined organic phases were washed with brine (3 × 50 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 (18 mL), and TFA (9 mL) was added at 0 °C. The reaction was stirred at 22 °C for 3 h. Saturated aqueous $NaHCO_3$ was added to the solution until the effervescence, by release of CO_2 gas, had stopped. The two phases were separated, and the aqueous phase was extracted with EtOAc (4 × 40 mL). The combined organic phases were washed with brine (100 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution ($MeOH/CH_2Cl_2$, 2:98 to 10:90) to yield compound **11** (60 mg, 59%) as a brownish foam. ^1H NMR (400 MHz, $CDCl_3$): δ 8.58 (s, 1H), 7.69 (ddd, J = 8.0, 2.7, 1.4 Hz, 4H), 7.63−7.54 (m, 4H), 7.48−7.26 (m, 12H), 7.22 (t, J = 7.3 Hz, 2H), 4.38 (t, J = 4.4 Hz, 1H), 4.19 (d, J = 4.7 Hz, 1H), 4.00 (d, J = 10.6 Hz, 1H), 3.66−3.60 (m, 1H), 3.46 (s, 1H), 3.27 (s, 3H), 1.65 (d, J = 23.4 Hz, 12H), 1.04 (d, J = 9.2 Hz, 18H) ppm. $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 258.3, 161.9, 154.6, 141.6, 136.1, 135.8, 135.5, 135.2, 133.4, 133.2, 133.1, 132.4, 130.5, 130.1, 130.0, 128.4, 128.3, 127.9, 127.7, 121.2, 112.9, 105.7, 100.1, 88.3, 85.4, 84.9, 71.3, 70.7, 66.0, 63.9, 58.4, 29.9, 29.7, 29.4, 27.3, 27.2, 27.1, 19.6, 19.5, 15.4 ppm. HRMS (ESI) m/z: $(M + H)^+$ Calcd for $[C_{54}H_{65}N_4O_5Si_2]^+$ 905.4488. Found 905.4445.

*TBDPDS-Protected Nitroxide **12**.* To a solution of **10** (683 mg, 0.78 mmol) in CH_2Cl_2 (38 mL) was added NaN_3 (203 mg, 3.12 mmol), and the reaction was stirred at 22 °C for 1 h. The solution was cooled to 0 °C, and *m*CPBA (359 mg, 2.08 mmol) was added. The reaction stirred at 22 °C for 4 h. Saturated aqueous $NaHCO_3$ (150 mL) was added, and the mixture extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phases were washed with brine (150 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution ($MeOH/CH_2Cl_2$; 0:100 to 10:90) to yield **12** (449 mg, 65%) as a light yellow solid. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{53}H_{61}N_4O_5Si_2Na]^+$ 912.4073. Found 912.4058.

*TBDPS-Protected Nitroxide **13**.* To a solution of **11** (500 mg, 0.74 mmol) in CH_2Cl_2 (36 mL) was added NaN_3 (193 mg, 2.97 mmol), and the reaction was stirred at 22 °C for 1 h. The solution was cooled to 0 °C, and *m*CPBA (367 mg, 2.12 mmol) was added. The reaction stirred at 22 °C for 3 h. Saturated aqueous $NaHCO_3$ (50 mL) was

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added, and the mixture extracted with CH_2Cl_2 (3 × 30 mL). The combined organic phases were washed with brine (100 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (MeOH/CH_2Cl_2; 0:100 to 5:95), to yield **13** (390 mg, 76%) as a light yellow solid. HRMS (ESI) *m/z*: $(M + Na)^+$ Calcd for $[C_{54}H_{63}N_4O_6Si_2Na]^+$ 942.4178. Found 942.4174.

Nitroxide **Ċ**. To a solution of **12** (200 mg, 0.225 mmol) in THF (12 mL) was added TBAF (674 μL, 0.67 mmol, 1.0 M in THF), and the reaction was stirred at 22 °C for 18 h. Dowex-50 (445 mg) and $CaCO_3$ (148 mg, 1.48 mmol) were added, and the mixture was stirred for 1 h, filtered through a plug of Dowex-50, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (MeOH/CH_2Cl_2; 2:98−20:80) to yield **C** (78 mg, 84%) as a light yellow solid. HRMS (ESI) *m/z*: $(M + Na)^+$ Calcd for $[C_{21}H_{25}N_4O_5Na]^+$ 436.1717. Found 436.1719.

Nitroxide **Cm**. To a solution of **13** (178 mg, 0.19 mmol) in THF (10 mL) was added TBAF (674 μL, 0.67 mmol, 1.0 M in THF), and the reaction stirred at 22 °C for 18 h. Dowex-50 (500 mg) and $CaCO_3$ (150 mg, 1.50 mmol) were added, and the mixture was stirred for 1 h, filtered through a plug of Dowex-50, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (MeOH/CH_2Cl_2; 0:100−10:90) to yield **Cm** (58 mg, 67%) as a light yellow solid. HRMS (ESI) *m/z*: $(M + Na)^+$ Calcd for $[C_{22}H_{27}N_4O_6Na]^+$ 466.1823. Found 466.1815.

*3-((2R,4S,5R)-4-((tert-Butyldiphenylsilyl)oxy)-5-(((tert-butyldiphenylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-6,6,8,8-tetramethyl-2-oxo-2,6,8,10-tetrahydropyrimido[4,5-b]pyrrolo[3,4-f]indol-7(3H)-yl Benzoate (**14**)*. To a solution of **12** (450 mg, 0.506 mmol) in 1,4-dioxane (15 mL) was added L-ascorbic acid (445 mg, 2.53 mmol) in H_2O (6 mL). The reaction mixture was stirred at 40 °C for 4 h, after which CH_2Cl_2 (20 mL) and H_2O (20 mL) were added, and the solution was stirred vigorously for 2 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 (588 μL, 5.06 mmol) and Et_3N (1.4 mL, 10.1 mmol) in CH_2Cl_2 (20 mL). The solution was stirred at 22 °C for 3 h. Saturated aqueous $NaHCO_3$ (100 mL) was added, and the phases were separated. The aqueous phase was extracted with CH_2Cl_2 (3 × 50 mL), and the combined organic phases were washed with brine (100 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (MeOH/CH_2Cl_2; 0:100 to 10:90) to yield **14** (259 mg, 51%) as a light yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.76 (d, *J* = 24.1 Hz, 1H), 8.10−8.00 (m, 2H), 7.62−7.12 (m, 25H), 6.90 (s, 1H), 6.73 (s, 1H), 4.48 (d, *J* = 5.4 Hz, 1H), 4.07 (s, 1H), 3.83 (d, *J* = 12.9 Hz, 1H), 3.26 (d, *J* = 9.5 Hz, 1H), 2.77 (dd, *J* = 13.0, 5.3 Hz, 1H), 1.97 (dt, *J* = 13.5, 7.3 Hz, 1H), 1.58−1.07 (m, 12H), 1.01 (s, 9H), 0.83 (s, 9H) ppm. ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$): δ 259.0, 171.1, 166.6, 162.5, 162.3, 161.2, 155.1, 155.05, 145.0, 144.2, 141.0, 140.8, 139.0, 138.3, 136.1, 136.0, 135.8, 135.5, 135.3, 135.1, 133.7, 133.5, 133.45, 133.3, 133.2, 132.6, 131.3, 130.5, 130.45, 130.4, 130.34, 130.3, 130.0, 129.8, 128.9, 128.6, 128.4, 128.3, 128.2, 128.2, 120.4, 120.1, 113.5, 106.4, 106.2, 106.1, 101.8, 89.0, 88.9, 88.5, 88.4, 74.9, 69.2, 68.7, 68.0, 67.4, 66.9, 64.5, 63.4, 43.8, 29.5, 29.2, 27.3, 27.2, 26.2, 19.63, 19.6, 19.4 ppm. HRMS (ESI) *m/z*: $(M + Na)^+$ Calcd for $[C_{60}H_{66}N_4O_6Si_2Na]^+$ 1017.4413. Found 1017.4420.

*3-((2R,3R,4R,5R)-4-((tert-Butyldiphenylsilyl)oxy)-5-(((tert-butyldiphenylsilyl)oxy)methyl)-3-methoxytetrahydrofuran-2-yl)-6,6,8,8-tetramethyl-2-oxo-2,6,8,10-tetrahydropyrimido[4,5-b]pyrrolo[3,4-f]indol-7(3H)-yl Benzoate (**15**)*. To a solution of **13** (500 mg, 0.54 mmol) in 1,4-dioxane (16 mL) was added L-ascorbic acid (478 mg, 2.72 mmol) in H_2O (6 mL). The reaction mixture was stirred at 40 °C for 4 h, after which CH_2Cl_2 (20 mL) and H_2O (20 mL) were added, and the solution was stirred vigorously for 2 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 (631 μL, 5.43 mmol) and Et_3N (1.5 mL, 10.9 mmol) in CH_2Cl_2 (20 mL). The solution was stirred at 22 °C for 3 h. Saturated aqueous $NaHCO_3$ (100 mL) was added, and the phases were separated. The aqueous phase was extracted with CH_2Cl_2 (3 × 50 mL), and the combined organic phases were washed with brine (100 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (MeOH/CH_2Cl_2; 1:99 to 4:96), to yield **15** (239 mg, 43%) as a light yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.55 (d, *J* = 18.4 Hz, 1H), 8.20−8.15 (m, 2H), 7.81−7.29 (m, 23H), 7.25 (t, *J* = 7.1 Hz, 1H), 6.82 (s, 1H), 4.41−4.27 (m, 2H), 3.73−3.66 (m, 1H), 3.62−3.52 (m, 1H), 3.40 (d, *J* = 5.0 Hz, 3H), 1.63 (s, 6H), 1.46−1.26 (m, 6H), 1.10 (d, *J* = 13.9 Hz, 18H) ppm. ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$): δ 170.9, 166.3, 162.1, 161.9, 160.9, 154.6, 154.5, 145.0, 144.1, 140.7, 138.8, 138.0, 135.9, 135.7, 135.5, 135.2, 134.7, 133.3, 133.2, 133.1, 133.0, 132.4, 130.8, 130.2, 130.1, 130.0, 129.9, 129.7, 129.5, 128.6, 128.3, 128.2, 128.1, 127.8, 127.78, 127.6, 119.8, 119.5, 113.3, 106.2, 106.1, 101.0, 88.5, 84.8, 84.5, 71.0, 68.9, 68.4, 67.7, 67.1, 66.6, 63.8, 63.0, 58.3, 29.2, 28.9, 27.1, 27.0, 26.9, 25.8, 19.5, 19.45, 19.4 ppm. HRMS (ESI) *m/z*: $(M + Na)^+$ Calcd for $[C_{61}H_{68}N_4O_7Si_2Na]^+$ 1047.4519. Found 1047.4509.

*3-((2R,4S,5R)-4-Hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-6,6,8,8-tetramethyl-2-oxo-2,6,8,10-tetrahydropyrimido[4,5-b]pyrrolo[3,4-f]indol-7(3H)-yl Benzoate (**16**)*. To a solution of **14** (332 mg, 0.33 mmol) in THF (18 mL) was added TBAF (1 mL, 1.00 mmol, 1.0 M in THF), and the reaction was stirred at 22 °C for 14 h. Saturated aqueous $NaHCO_3$ (50 mL) was added, and the mixture was extracted with EtOAc (3 × 50 mL). The combined organic phases were washed with brine (50 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (MeOH/CH_2Cl_2; 2:98−10:90), to yield **16** (134 mg, 77%) as a light yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.05 (d, *J* = 26.1 Hz, 1H), 8.00 (d, *J* = 7.2 Hz, 2H), 7.51 (s, 1H), 7.45 (s, 1H), 7.38 (s, 2H), 6.99 (d, *J* = 24.0 Hz, 1H), 6.26 (s, 1H), 4.35 (d, *J* = 5.3 Hz, 1H), 3.96−3.86 (m, 2H), 3.76 (d, *J* = 11.9 Hz, 1H), 2.50 (d, *J* = 13.4 Hz, 1H), 2.15 (s, 1H), 1.45 (s, 12H) ppm. ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$): δ 166.9, 161.1, 155.6, 143.5, 139.9, 138.1, 136.0, 133.4, 129.4, 128.8, 128.6, 120.4, 113.4, 105.6, 105.0, 87.7, 87.5, 69.9, 69.3, 68.8, 68.5, 60.8, 49.6, 49.3, 49.1, 48.9, 48.7, 48.5, 48.3, 48.1, 41.7, 28.6, 25.8, 25.6 ppm. HRMS (ESI) *m/z*: $(M + Na)^+$ Calcd for $[C_{28}H_{30}N_4O_6Na]^+$ 541.5058. Found 541.2055.

*3-((2R,3R,4R,5R)-4-Hydroxy-5-(hydroxymethyl)-3-methoxytetrahydrofuran-2-yl)-6,6,8,8-tetramethyl-2-oxo-2,6,8,10-tetrahydropyrimido[4,5-b]pyrrolo[3,4-f]indol-7(3H)-yl Benzoate (**17**)*. To a solution of **15** (464 mg, 0.45 mmol) in THF (24 mL) was added TBAF (1.4 mL, 1.40 mmol, 1.0 M in THF), and the reaction was stirred at 22 °C for 13 h. Saturated aqueous $NaHCO_3$ (100 mL) was added, and the mixture was extracted with EtOAc (3 × 70 mL). The combined organic phases were washed with brine (70 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (MeOH/CH_2Cl_2; 2:98−10:90), to yield **17** (184 mg, 74%) as a light yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.26 (d, *J* = 42.3 Hz, 1H), 8.04−7.97 (m, 2H), 7.55−7.48 (m, 1H), 7.44−7.36 (m, 3H), 6.96 (d, *J* = 31.9 Hz, 1H), 6.00 (s, 1H), 4.19 (dd, *J* = 8.6, 4.9 Hz, 1H), 4.02 (dd, *J* = 20.5, 10.7 Hz, 2H), 3.84−3.71 (m, 2H), 3.60 (s, 3H), 1.44 (d, *J* = 10.7 Hz, 12H) ppm. ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$): δ ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.9, 161.0, 155.2, 143.7, 139.9, 138.2, 136.6, 136.3, 133.4, 129.5, 128.8, 128.6, 120.5, 113.3, 105.5, 105.0, 89.4, 84.1, 68.8, 68.5, 66.9, 59.2, 58.5, 49.6, 49.4, 49.2, 49.0, 48.8, 48.6, 48.3, 48.1, 28.6, 25.8, 14.8 ppm. HRMS (ESI) *m/z*: $(M + Na)^+$ Calcd for $[C_{29}H_{32}N_4O_7Na]^+$ 571.2163. Found 571.2136.

*3-((2R,4S,5R)-5-((Bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-hydroxytetrahydrofuran-2-yl)-6,6,8,8-tetramethyl-2-oxo-2,6,8,10-tetrahydropyrimido[4,5-b]pyrrolo[3,4-f]indol-7(3H)-yl Benzoate (**18**)*. Toluene (3 × 5 mL) was evaporated from **16** (95 mg, 0.18 mmol), followed by sequential addition of pyridine (4 mL), DMTCl (93 mg, 0.27 mmol) and DMAP (3 mg, 0.02 mmol). The solution was stirred for 12 h, MeOH (400 μL) was added and the solvent removed *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (MeOH/CH_2Cl_2/Et_3N; 0:99:1 to 5:94:1), to yield **18** (122 mg, 81%) as a light yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.22 (s, 1H), 8.16−8.02 (m, 2H),

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7.49 (ddt, J = 47.4, 32.1, 8.7 Hz, 10H), 7.23−7.05 (m, 3H), 6.75 (dd, J = 8.6, 3.2 Hz, 5H), 6.60 (d, J = 17.9 Hz, 1H), 4.61 (d, J = 24.3 Hz, 1H), 4.32 (s, 1H), 3.67 (s, 7H), 3.24 (s, 1H), 3.01 (d, J = 12.0 Hz, 1H), 2.47 (s, 1H), 1.99 (s, 1H), 1.44 (s, 6H), 1.26−0.94 (m, 6H) ppm. $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 250.2, 166.3, 162.3, 158.7, 155.6, 143.9, 140.5, 138.1, 136.0, 135.8, 135.5, 133.2, 130.0, 129.7, 129.5, 128.7, 128.2, 128.0, 127.3, 120.1, 113.54, 113.5, 113.46, 106.1, 105.8, 100.1, 88.2, 87.1, 87.0, 72.2, 70.7, 68.9, 68.3, 55.3, 43.1, 29.1, 28.9, 25.7 ppm. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{49}H_{48}N_4O_8Na]^+$ 843.3364. Found 843.3346.

*3-((2R,3R,4R,5R)-5-((Bis(4-methoxyphenyl)(phenyl)methoxy)-methyl)-4-hydroxy-3-methoxytetrahydrofuran-2-yl)-6,6,8,8-tetramethyl-2-oxo-2,6,8,10-tetrahydropyrimido[4,5-b]pyrrolo[3,4-f]-indol-7(3H)-yl Benzoate (**19**).* Toluene (3 × 5 mL) was evaporated from **17** (128 mg, 0.23 mmol), followed by sequential addition of pyridine (5 mL), DMTCl (239 mg, 0.64 mmol), and DMAP (3 mg, 0.02 mmol). The solution was stirred for 10 h. MeOH (0.5 mL) was added, and the solvent was removed *in vacuo*. The residue was purified by flash column chromatography using a gradient elution ($MeOH/CH_2Cl_2/Et_3N$; 0:99:1 to 5:94:1), to yield **19** (148 mg, 75%) as a light yellow solid. ^1H NMR (400 MHz, $CDCl_3$): δ 9.34 (s, 1H), 8.09 (d, J = 7.6 Hz, 2H), 7.64−7.39 (m, 10H), 7.33−7.12 (m, 3H), 6.87−6.71 (m, 4H), 6.40 (d, J = 8.9 Hz, 1H), 6.28 (s, 1H), 4.65 (d, J = 12.5 Hz, 1H), 4.25−4.09 (m, 2H), 3.87 (s, 3H), 3.88−3.83 (m, 1H), 3.70 (s, 6H), 3.33 (dd, J = 11.3, 2.5 Hz, 1H), 2.76 (s, 1H), 2.05 (s, 1H), 1.64−1.44 (m, 6H), 1.23−0.73 (m, 6H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$) δ 249.0, 166.2, 162.4, 158.7, 155.1, 143.9, 140.5, 137.9, 136.1, 135.4, 133.1, 129.8, 129.6, 129.5, 128.6, 128.2, 127.9, 127.3, 119.9, 114.0, 113.5, 113.4, 105.9, 105.7, 89.7, 86.8, 84.0, 83.7, 68.6, 68.2, 68.0, 65.9, 62.5, 59.1, 55.2, 46.0, 31.6, 29.1, 28.7, 25.8, 22.7, 15.3, 14.2 ppm. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{50}H_{50}N_4O_9Na]^+$ 873.3470. Found 873.3442.

*3-((2R,4S,5R)-5-((Bis(4-methoxyphenyl)(phenyl)methoxy)-methyl)-4-(((2-cyanoethoxy)(diisopropylamino)phosphaneyl)oxy)-tetrahydrofuran-2-yl)-6,6,8,8-tetramethyl-2-oxo-2,6,8,10-tetrahydropyrimido[4,5-b]pyrrolo[3,4-f]indol-7(3H)-yl Benzoate (**20**).* A solution of **18** (596 mg, 0.77 mmol) in CH_2Cl_2 (25 mL) was treated with diisopropyl ammonium tetrazolide (370 mg, 2.18 mmol) and 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphane (692 μL, 2.18 mmol). The reaction was stirred at 22 °C for 6 h. CH_2Cl_2 (30 mL) was added, and the solution was washed with saturated aqueous $NaHCO_3$ (3 × 30 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was dissolved in Et_2O (15 mL) and a few drops of CH_2Cl_2, followed by slow addition of *n*-hexane (80 mL), and the mixture was centrifuged for 10 min. The solvent was decanted from the precipitate and discarded. This procedure was repeated five times to yield **20** (673 mg, 91%) as a light yellow solid. ^1H NMR (400 MHz, $CDCl_3$): δ 9.22 (d, J = 22.6 Hz, 2H), 8.10 (dt, J = 8.5, 1.5 Hz, 4H), 7.64−7.56 (m, 2H), 7.56−7.32 (m, 18H), 7.25−7.14 (m, 6H), 6.84−6.72 (m, 8H), 6.65 (d, J = 6.8 Hz, 1H), 6.62 (t, J = 6.0 Hz, 1H), 6.51 (d, J = 17.8 Hz, 2H), 4.67 (s, 2H), 4.30 (d, J = 9.4 Hz, 2H), 3.82−3.75 (m, 2H), 3.70 (d, J = 2.0 Hz, 12H), 3.62−3.49 (m, 6H), 3.24 (d, J = 10.1 Hz, 2H), 2.59 (t, J = 6.3 Hz, 2H), 2.50 (s, 2H), 2.41−2.33 (m, 2H), 1.55 (d, J = 19.1 Hz, 12H), 1.18−1.10 (m, 18H), 1.03 (d, J = 6.8 Hz, 6H) ppm. $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 166.0, 162.1, 158.5, 154.9, 143.6, 140.2, 137.8, 135.5, 135.1, 132.9, 129.7, 129.4, 129.3, 128.4, 127.9, 127.1, 119.9, 117.3, 117.2, 113.2, 113.15, 105.6, 105.4, 86.7, 86.0, 85.8, 68.6, 68.0, 58.3, 58.2, 58.1, 58.0, 55.03, 55.0, 43.2, 43.1, 43.0, 42.98, 41.4, 28.6, 25.7, 25.6, 24.5, 24.4, 24.36, 24.3, 20.2, 20.16, 20.0, 19.9 ppm. ^{31}P NMR (162 MHz,$CDCl_3$): δ 149.0, 148.5 ppm. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{58}H_{65}N_6O_9PNa]^+$ 1043.4443. Found 1043.4412.

*3-((2R,3R,4R,5R)-5-((Bis(4-methoxyphenyl)(phenyl)methoxy)-methyl)-4-(((2-cyanoethoxy)(diisopropylamino)phosphaneyl)oxy)-3-methoxytetrahydrofuran-2-yl)-6,6,8,8-tetramethyl-2-oxo-2,6,8,10-tetrahydropyrimido[4,5-b]pyrrolo[3,4-f]indol-7(3H)-yl Benzoate (**21**).* A solution of **19** (322 mg, 0.38 mmol) in CH_2Cl_2 (13 mL) was treated with diisopropyl ammonium tetrazolide (193 mg, 1.14 mmol) and 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphane (361 μL, 1.14 mmol). The reaction was stirred at 22 °C for 12 h. CH_2Cl_2 (25 mL) was added, and the solution was washed with saturated aqueous $NaHCO_3$ (3 × 25 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was dissolved in Et_2O (10 mL) and a few drops of CH_2Cl_2, followed by slow addition of *n*-hexane (60 mL), and the mixture was centrifuged for 10 min. The solvent was decanted from the precipitate and discarded. This procedure was repeated six times to yield **21** (258 mg, 65%) as a light yellow solid. ^1H NMR (400 MHz, $CDCl_3$): δ 9.44 (s, 1H), 9.35 (s, 1H), 8.09 (dd, J = 7.4, 2.0 Hz, 4H), 7.62−7.34 (m, 20H), 7.24−7.11 (m, 6H), 6.77 (t, J = 9.6 Hz, 8H), 6.38−6.19 (m, 4H), 4.64 (d, J = 37.0 Hz, 2H), 4.38 (dd, J = 13.9, 8.2 Hz, 2H), 4.24 (s, 2H), 3.91 (dd, J = 22.3, 10.9 Hz, 2H), 3.81 (s, 3H), 3.79 (s, 3H), 3.74−3.66 (m, 12H), 3.56 (ddp, J = 10.6, 7.2, 3.3 Hz, 4H), 3.24 (d, J = 11.2 Hz, 2H), 2.54 (s, 2H), 2.32−2.10 (m, 4H), 1.61−1.45 (m, 12H), 1.16−1.08 (m, 18H), 1.00 (d, J = 6.8 Hz, 6H) ppm. $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$): δ 262.5, 249.6, 166.2, 162.5, 162.48, 158.7, 155.2, 155.1, 143.7, 140.6, 137.7, 136.1, 135.4, 135.3, 133.1, 130.4, 129.8, 129.6, 129.5, 128.5, 128.14, 128.1, 127.3, 120.0, 119.97, 117.6, 117.5, 114.1, 113.8, 113.4, 113.36, 113.3, 106.0, 105.8, 105.7, 90.8, 86.8, 86.7, 84.2, 82.9, 82.5, 82.3, 82.27, 69.5, 69.4, 68.8, 68.2, 59.1, 58.8, 58.6, 58.4, 58.3, 58.1, 55.2, 55.2, 43.3, 43.30, 43.2, 43.18, 29.1, 28.7, 25.8, 25.5, 24.7, 24.67, 24.6, 24.5, 20.4, 20.3, 20.0 ppm. ^{31}P NMR (162 MHz,$CDCl_3$): δ 150.3, 149.7 ppm. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{59}H_{67}N_6O_{10}PNa]^+$ 1073.4548. Found 1073.4549.

*5-Bromo-1-((2R,4S,5R)-4-((tert-butyldiphenylsilyl)oxy)-5-(((tert-butyldiphenylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-4-(phenylamino)pyrimidin-2(1H)-one (**22**).* To a solution of nucleoside **5** (8.4 g, 10.5 mmol) in CH_2Cl_2 (250 mL) were added aniline (1.2 g, 16.2 mmol), DMAP (0.13 g, 1.1 mmol), and Et_3N (1.7 mL, 23.2 mmol), and the resulting solution was stirred at 22 °C for 12 h. Saturated aqueous $NaHCO_3$ (200 mL) was added, and the organic phase extracted with CH_2Cl_2 (2 × 100 mL). The combined organic phases were washed with saturated aqueous $NaHCO_3$ (50 mL) and brine (100 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (EtOAc/petroleum ether, 10:90 to 20:80) to yield compound **22** (7.5 g, 83% yield) as a white foam. ^1H NMR (400 MHz, $CDCl_3$): δ 8.08 (s, 1H), 7.71 (d, J = 7.8 Hz, 2H), 7.61 (d, J = 8.0 Hz, 2H), 7.55 (d, J = 6.2 Hz, 4H), 7.49−7.25 (m, 18H), 7.15 (t, J = 7.4 Hz, 1H), 6.47 (dd, J = 8.3, 5.4 Hz, 1H), 4.48 (d, J = 5.6 Hz, 1H), 4.05−4.01 (m, 1H), 3.72 (dd, J = 11.6, 2.3 Hz, 1H), 3.28 (dd, J = 11.6, 2.9 Hz, 1H), 2.68 (ddd, J = 13.4, 5.4, 1.6 Hz, 1H), 1.97−1.89 (m, 1H), 1.08 (s, 9H), 0.95 (s, 9H) ppm. $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$) δ 157.1, 140.8, 137.3, 135.7, 135.7, 135.6, 135.6, 135.4, 133.3, 133.1, 132.8, 132.3, 130.0, 129.9, 129.91, 129.1, 127.9, 127.88, 127.8, 125.0, 121.5, 88.4, 88.36, 87.4, 74.3, 64.0, 42.7, 35.5, 27.0, 26.92, 26.9, 23.9, 20.8, 19.2, 19.0, 18.98 ppm. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{47}H_{52}BrN_3O_4Si_2Na]^+$ 882.2563. Found 882.2564.

*tert-Butyl(5-bromo-1-((2R,4S,5R)-4-((tert-butyldiphenylsilyl)oxy)-5-(((tert-butyldiphenylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)(phenyl)carbamate (**23**).* To a solution of compound **22** (7.0 g, 8.2 mmol) in 1,4-dioxane (67 mL) were added Boc_2O (5.3 g, 24.5 mmol) and DMAP (99 mg, 0.8 mmol), and the resulting solution was stirred at 60 °C for 12 h. Saturated aqueous $NaHCO_3$ (100 mL) was added, and the mixture was extracted with EtOAc (3 × 50 mL). The combined organic phases were washed with brine (100 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution (EtOAc/petroleum ether, 10:90 to 25:75) to yield compound **23** (5.8 g, 74% yield) as a white foam. ^1H NMR (400 MHz, $CDCl_3$): δ 8.44 (s, 1H), 7.62−7.51 (m, 6H), 7.48−7.32 (m, 14H), 7.29−7.25 (m, 5H), 6.39 (dd, J = 8.2, 5.4 Hz, 1H), 4.44 (dd, J = 5.5, 1.5 Hz, 1H), 4.08−4.05 (m, 1H), 3.71 (dd, J = 11.8, 2.4 Hz, 1H), 3.26 (dd, J = 11.7, 3.2 Hz, 1H), 2.83 (ddd, J = 13.3, 5.4, 1.4 Hz, 1H), 2.03−1.93 (m, 1H), 1.49, (s, 9H), 1.08 (s, 9H), 0.95 (s, 9H) ppm. $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$) δ 165.4, 154.2, 151.7, 144.8, 139.4, 135.7, 135.6, 135.5, 135.49, 133.2, 132.9, 132.5, 132.2, 130.1, 130.0, 128.9, 128.1, 127.9, 127.86, 127.4, 127.2, 97.1, 89.1, 88.7, 83.0, 74.4, 63.8, 42.9, 35.4, 28.1, 26.9, 26.88, 26.5,

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26.3, 22.7, 19.2, 19.0, 14.1 ppm. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{52}H_{60}BrN_3O_6Si_2Na]^+$ 982.3089. Found 982.3090.

*3-((2R,4S,5R)-4-((tert-Butyldiphenylsilyl)oxy)-5-(((tert-butyldiphenylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-3,9-dihydro-2H-pyrimido[4,5-b]indol-2-one (**24**).* To a solution of compound **23** (110 mg, 0.11 mmol) in DMA (4.6 mL) were added anhydrous NaOAc (38 mg, 0.46 mmol) and $PdCl_2(PPh_3)_2$ (16 mg, 0.023 mmol). The resulting solution was stirred at 150 °C for 12 h, cooled to 22 °C, filtered through Celite, and concentrated *in vacuo*. H_2O (20 mL) was added, and the mixture was extracted with EtOAc (5 × 20 mL). The combined organic phases were washed with brine (3 × 50 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 (20 mL), TFA (10 mL) was added at 0 °C, and the reaction was stirred at 22 °C for 3 h. Saturated aqueous $NaHCO_3$ was added to the solution until the effervescence, by release of CO_2 gas, had stopped. The two phases were separated and the aqueous phase was extracted with EtOAc (4 × 20 mL). The combined organic phases were washed with brine (50 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution ($MeOH/CH_2Cl_2$, 0:100 to 10:90) to yield compound **24** (59 mg, 66%) as a light brown foam. ^1H NMR (400 MHz, $CDCl_3$) δ 8.76 (s, 1H), 7.73−7.69 (m, 1H), 7.67−7.58 (m, 6H), 7.49−7.45 (m, 2H), 7.45−7.42 (m, 1H), 7.41−7.28 (m, 10H), 7.28−7.21 (m, 3H), 6.98−6.92 (m, 2H), 6.75 (dd, J = 8.3, 5.4 Hz, 1H), 4.56 (d, J = 4.1 Hz, 1H), 4.15 (s, 1H), 3.87 (dd, J = 11.6, 2.3 Hz, 1H), 3.35 (dd, J = 11.6, 2.9 Hz, 1H), 2.89 (dd, J = 12.8, 6.0 Hz, 1H), 2.13−2.04 (m, 1H), 1.11 (s, 9H), 0.94 (s, 9H) ppm. $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$) δ 162.0, 154.9, 140.7, 135.8, 135.7, 135.5, 135.3, 135.2, 133.4, 133.1, 133.0, 132.4, 130.1, 130.0, 129.99, 129.97, 128.0, 127.9, 127.86, 127.2, 121.6, 120.1, 119.8, 112.7, 105.8, 88.6, 88.2, 74.4, 64.2, 53.4, 43.4, 27.0, 26.9, 19.1 ppm. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{47}H_{51}N_3O_4Si_2Na]^+$ 800.3310. Found 800.3336.

*3-((2R,4S,5R)-4-Hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-3,9-dihydro-2H-pyrimido[4,5-b]indol-2-one (**25**).* To a solution of **24** (1220 mg, 1.57 mmol) in THF (85 mL) was added TBAF (4.7 mL, 4.7 mmol, 1.0 M in THF), and the reaction was stirred at 22 °C for 4 h. Dowex-50 (2830 mg) and $CaCO_3$ (942 mg, 9.41 mmol) were added, and the mixture was stirred for 1 h, filtered through a plug of Dowex-50, and concentrated *in vacuo*. The residue was purified by flash column chromatography using a gradient elution ($MeOH/CH_2Cl_2$; 0:100−10:90), to yield **25** (320 mg, 68%) as a white solid. ^1H NMR (400 MHz, DMSO-d_6) δ 11.64 (s, 1H), 9.14 (s, 1H), 7.79 (d, J = 7.6 Hz, 1H), 7.36−7.24 (m, 2H), 7.17 (t, J = 7.4, 1H), 6.27 (t, J = 6.2 Hz, 1H), 5.34−5.21 (m, 2H), 4.34−4.26 (m, 1H), 3.94−3.89 (m, 1H), 3.81−3.73 (m, 1H), 3.72−3.64 (m, 1H), 2.41−2.31 (m, 1H), 2.16−2.06 (m, 1H) ppm. $^{13}C\{^1H\}$ NMR (101 MHz, DMSO-d_6) δ 162.0, 154.9, 140.5, 137.2, 127.0, 121.7, 121.1, 120.4, 111.8, 104.1, 88.2, 87.2, 70.0, 61.2, 41.8, 40.6, 40.4, 40.2, 40.0, 39.8, 39.6, 39.3 ppm. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{15}H_{15}N_3O_4Na]^+$ 324.0955. Found 324.0952.

*3-((2R,4S,5R)-5-((Bis(4-methoxyphenyl)(phenyl)methoxy)-methyl)-4-hydroxytetrahydrofuran-2-yl)-3,9-dihydro-2H-pyrimido-[4,5-b]indol-2-one (**26**).* Toluene (3 × 5 mL) was evaporated from **25** (100 mg, 0.33 mmol), followed by sequential addition of pyridine (7.4 mL), DMTCl (169 mg, 0.50 mmol), and DMAP (4 mg, 0.03 mmol). The solution was stirred for 12 h. MeOH (0.8 mL) was added, and the solvent was removed *in vacuo*. The residue was purified by flash column chromatography using a gradient elution ($MeOH/CH_2Cl_2/Et_3N$; 0:99:1 to 2:97:1) to yield **26** (205 mg, quant.) as a white solid. ^1H NMR (400 MHz, $CDCl_3$) δ 12.83 (s, 1H), 9.05 (s, 1H), 7.65 (d, J = 8.0 Hz, 1H), 7.53 (d, J = 7.7 Hz, 2H), 7.41 (d, J = 8.5 Hz, 4H), 7.30 (t, J = 5.0 Hz, 2H), 7.26−7.19 (m, 2H), 6.89 (t, J = 7.6 Hz, 1H), 6.82 (d, J = 7.5, 4H), 6.72 (t, J = 6.2 Hz, 1H), 6.64 (d, J = 7.8 Hz, 1H), 4.71−4.65 (m, 1H), 4.41−4.36 (m, 1H), 3.72 (s, 3H), 3.71 (s, 3H), 3.63 (dd, J = 10.7, 3.1 Hz, 1H), 3.42 (dd, J = 10.7, 3.9 Hz, 1H), 3.11−3.01 (m, 1H), 2.53−2.41 (m, 1H) ppm. $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$) δ 162.0, 158.7, 155.5, 144.4, 140.5, 135.7, 135.4, 130.0, 129.97, 128.2, 128.1, 127.2, 127.1, 121.7, 120.1, 120.0, 113.4, 112.4, 106.0, 88.0, 87.0, 86.7, 71.7, 68.0, 63.4, 55.2, 53.5, 45.9, 42.8 ppm. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{36}H_{33}N_3O_6Na]^+$ 626.2262. Found 626.2265.

*(2R,3S,5R)-2-((Bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2-oxo-2,9-dihydro-3H-pyrimido[4,5-b]indol-3-yl)tetrahydrofuran-3-yl(2-cyanoethyl) Diisopropylphosphoramidite (**27**).* A solution of **26** (175 mg, 0.29 mmol) in CH_2Cl_2 (10 mL) was treated with diisopropyl ammonium tetrazolide (74 mg, 0.43 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphane (138 μL, 0.43 mmol). The reaction was stirred at 22 °C for 5 h. CH_2Cl_2 (25 mL) was added, and the solution was washed with saturated aqueous $NaHCO_3$ (3 × 25 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was dissolved in Et_2O (8 mL) and a few drops of CH_2Cl_2, followed by slow addition of *n*-hexane (40 mL), and the mixture was centrifuged for 10 min. The solvent was decanted from the precipitate and discarded. This procedure was repeated five times to yield **27** (107 mg, 46%) as a white solid. ^1H NMR (400 MHz, $CDCl_3$) δ 13.51 (s, 2H), 9.03 (s, 1H), 8.98 (s, 1H), 7.67 (dd, J = 8.0, 2.7 Hz, 2H), 7.51 (t, J = 7.2 Hz, 4H), 7.39 (t, J = 7.4, 8H), 7.31−7.21 (m, 8H), 6.87−6.78 (m, 10H), 6.62−6.51 (m, 3H), 6.45 (d, J = 7.8 Hz, 1H), 4.77−4.63 (m, 2H), 4.35−4.28 (m, 2H), 3.89−3.68 (m, 10H), 3.70 (s, 6H), 3.65−3.55 (m, 6H), 3.43−3.34 (m, 2H), 2.95−2.80 (m, 2H), 2.63 (t, J = 6.4 Hz, 2H), 2.54−2.44 (m, 2H), 2.42 (t, J = 6.4 Hz, 2H), 1.17 (d, J = 6.8 Hz, 18H), 1.06 (d, J = 6.8 Hz, 6H) ppm. $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$) δ 162.1, 158.8, 155.0, 144.3, 144.2, 140.7, 140.66, 135.6, 135.57, 135.3, 130.1, 130.07, 128.4, 128.3, 128.1, 127.3, 127.2, 127.1, 127.08, 121.6, 120.1, 120.0, 119.9, 117.5, 117.4, 113.4, 112.5, 112.4, 105.9, 105.89, 87.6, 87.5, 87.0, 86.9, 85.8, 85.7, 85.6, 73.0, 72.9, 71.9, 71.7, 62.9, 62.2, 58.5, 58.4, 58.3, 58.2, 55.23, 55.2, 43.4, 43.3, 43.25, 43.2, 41.8, 41.79, 41.44, 41.4, 31.6, 25.6, 24.7, 24.62, 24.6, 24.55, 24.5, 22.7, 20.4, 20.35, 20.2, 20.17, 14.2 ppm. ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.4, 148.7 ppm. HRMS (ESI) m/z: $(M + Na)^+$ Calcd for $[C_{45}H_{50}N_5O_7PNa]^+$ 826.3340. Found 826.3343.

X-ray Crystallography. X-ray quality single crystals of $\dot{\mathbf{C}}$ and $\dot{\mathbf{C}}$**m** were obtained by dissolving the compounds in 10% $MeOH/CHCl_3$ and layering the solution with Et_2O. The crystals were isolated from the solvent, immersed in cryogenic oil, and mounted on a Bruker D8 VENTURE (Photon100 CMOS detector) diffractometer equipped with a Cryostream open-flow nitrogen cryostat. The data were collected using Mo Kα (λ = 0.71073 Å) radiation. The unit cell determination, data collection, data reduction, structure solution/refinement, and empirical absorption correction (SADABS) were carried out using Apex-III (Bruker AXS: Madison, WI, 2015). The structures were solved by a direct method and refined by full-matrix least-squares on F^2 for all data using SHELXTL version 2017/1[63] and Olex2 software.[64] All nondisordered non-hydrogen atoms were refined anisotropically except the disordered oxygen atom of the methoxy group in $\dot{\mathbf{C}}$**m**, which was refined using free variable (FVAR) instruction. The hydrogen atoms were placed in the calculated positions and refined using a riding model.

DNA Synthesis. Unmodified 2′-deoxy phosphoramidites were dissolved in CH_3CN (0.1 M) and phosphoramidite **20** was dissolved in 1,2-dichloroethane (0.1 M). 5-Ethylthiotetrazole (0.25 M in CH_3CN) was used as a coupling agent for the unmodified phosphoramidites and **20**. The coupling time was 1.5 min for unmodified DNA phosphoramidites, whereas **20** was coupled manually for 10 min. After completion of the DNA synthesis, the DNAs were cleaved from the resin and deprotected in a saturated aqueous NH_3 solution at 55 °C for 8 h, after which the solvent was removed *in vacuo*.

RNA Synthesis. Unmodified 2′O-TBDMS phosphoramidites were dissolved in CH_3CN (0.1 M), and phosphoramidite **21** was dissolved in 1,2-dichloroethane (0.1 M). 5-Benzylthiotetrazole (0.25 M in CH_3CN) was used as a coupling agent for the unmodified RNA phosphoramidites, and 5-ethylthiotetrazole (0.25 M in CH_3CN) was used for **21**. The coupling time was 7 min for the unmodified RNA phosphoramidites, and **21** was coupled manually for 10 min.

The RNAs were deprotected and cleaved from the resin in a 1:1 solution (2 mL) of CH_3NH_2 (8 M in EtOH) and saturated aqueous NH_3 at 65 °C for 1 h. The solvent was removed *in vacuo*, and the 2′O-TBDMS groups were removed by incubation in a solution of $Et_3N\cdot$

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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 solution was transferred to a 50 mL Falcon tube, and *n*-butanol (20 mL) was added. The mixture was stored at −20 °C for 14 h and centrifuged (4000 rpm) at 4 °C for 1 h. The solvent was decanted from the RNA pellet, and the pellet was dried *in vacuo*.

DNA and RNA Purification. All oligonucleotides were 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; 2×(3 mL gel/9 mL buffer)). The solutions were filtered through GD/X syringe filters (0.45 μm, 25 mm diameter, Whatman) and were subsequently desalted using Sep-Pak cartridges (Waters), following the instructions provided by the manufacturer. The dried oligonucleotides were dissolved in deionized and sterilized water (200 μL for each oligonucleotide). Concentrations of the oligonucleotides were determined by measuring UV absorbance at 260 nm and calculation using Beer's law.

CW-EPR Measurements and Spin Counting. Samples of spin-labeled oligonucleotides for EPR measurements were prepared by dissolving single-stranded DNA or RNA (2.0 nmol) in phosphate buffer (10 μL, 10 mM phosphate, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0, oligonucleotide final concentration 200 μM). DNA and RNA duplexes were prepared by dissolving complementary single-stranded oligonucleotides (2.0 nmol of each) in a phosphate buffer (10 μL; 10 mM phosphate, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0, duplex final concentration 200 μM) and annealing (Figures 3 and S50). The amount of spin labels in each oligonucleotide was determined by spin counting. A stock solution of 4-hydroxy-TEMPO (1.0 M) was prepared in phosphate buffer (10 mM phosphate, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0). The stock solution was diluted into samples of different concentrations (0−0.5 mM), and each sample was measured by EPR spectroscopy. The area under the peaks of each spectrum, obtained by double integration, was plotted against its concentration to yield a standard curve, which was used to determine the spin-labeling efficiency with an error margin of 5−10% (Table S1).

Enzymatic Digestion of Oligonucleotides and HPLC Analysis. To an 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 24 h, after which they were analyzed by HPLC chromatography (Figures S51 and S52).

Thermal Denaturing Experiments. To determine if **Ċ** and **Ċm** affected the stability of the DNA and RNA duplexes, respectively, the thermal denaturation curves of unmodified and spin-labeled oligomers were recorded. Both DNA and RNA duplexes were prepared by dissolving complementary single-stranded oligonucleotides (4.0 nmol) in a phosphate buffer (100 μL; 10 mM phosphate, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0), followed by annealing. The samples were diluted to 1.0 mL with the phosphate buffer (pH 7.0), degassed with Ar and heated from 24 to 90 °C (1.0 °C/min). The absorbance at 260 nm was subsequently recorded at 0.2 °C intervals (Table 1 and Figure S53).

CD Measurements. To determine if **Ċ** and **Ċm** labels had any effect on the conformation of the DNA and RNA duplexes, CD spectra of all unmodified duplexes and their spin-labeled counterparts were recorded. DNA and RNA duplexes were prepared by dissolving complementary single-stranded oligonucleotides (2.5 nmol of each) in a phosphate buffer (100 μL; 10 mM phosphate, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0) and annealing. The annealed samples were diluted to 200 μL with the same buffer before CD measurements (Figure S54).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.joc.1c01176.

Crystallographic data for **Ċ** and **Ċm**, extinction coefficients and UV−vis absorption spectra. Models of spin-labeled duplexes, spin-labeled oligonucleotides, and their analysis by MS and EPR spectroscopy, ^{1}H, ^{13}C, and ^{31}P NMR spectra, HPLC analyses of enzymatic digests, thermal denaturing experiments of spin-labeled duplexes, CD spectra of oligonucleotide duplexes and CW-EPR spectra of spin-labeled oligonucleotides (PDF)

Accession Codes

CCDC 2084624 and 2084625 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interest.

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