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Effect of N3 Modifications on the Affinity of Spin Label ç for Abasic Sites in Duplex DNA

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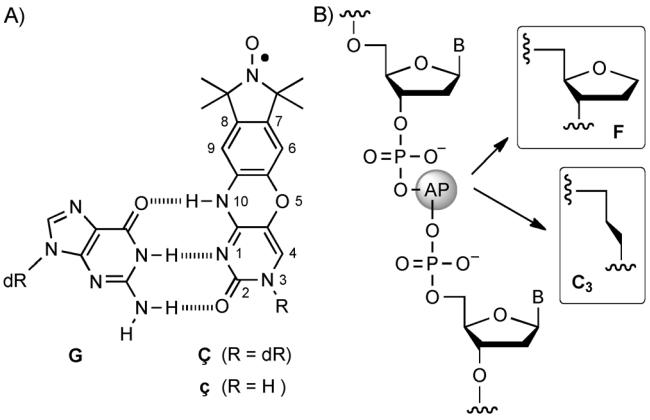
Noncovalent site-directed spin labeling (NC-SDSL) of abasic sites in duplex DNAs with the spin label **ç**, a cytosine analogue, is a promising approach for spin-labeling nucleic acids for EPR spectroscopy. In an attempt to increase the affinity of **ç** for abasic sites, several N3 derivatives were prepared, and their binding affinities were determined by EPR spectroscopy. Most of the N3 substituents had a detrimental effect on binding. The triazole-linked polyethylene-glycol derivative (**12a**) showed a 15-fold decrease in affinity, whereas the binding affinities of ethyl azido (**8b**) and hydroxyl (**8c**) derivatives were five- to sixfold lower. The spin-labeled nucleoside **Ç** showed only a twofold decrease, thus binding better than **8c**, even though it contains the larger 2′-deoxyribose substituent at N3 instead of a 2-hydroxyethyl group. N3 derivatives that contained the basic ethyl amino (**9**) or ethyl guanidino (**10**) substituents had both higher binding affinity and solubility, attributed to their cationic charge at neutral pH. Compounds **9** and **10** are promising candidates for NC-SDSL of nucleic acids, for distance measurements by pulsed EPR spectroscopy.

Introduction

Electron paramagnetic resonance (EPR) spectroscopy is a valuable technique for obtaining information about the structures and conformational dynamics of nucleic acids under biologically relevant conditions.[1] Structural information can be obtained from direct measurement of the distances between paramagnetic centers. Continuous wave (CW) EPR is useful for measuring short to intermediate distances (5–20 Å),[2] while pulsed electron–electron double resonance (PELDOR, also known as double electron–electron resonance (DEER)) can be used to measure long-range distances (20–80 Å).[1a,d,3] EPR also detects spin label motion; this can be used to extract information on the dynamics and local structure of specific sites.[1b,e,f,4]

The study of nucleic acids by EPR spectroscopy requires incorporation of reporter groups that contain unpaired electrons (spin labels). Persistent aminoxyl radicals (nitroxides) are usually the spin labels of choice. To be informative, the spin labels need to be linked to specific sites by using site-directed spin labeling (SDSL).[1b,e,g,5] SDSL of nucleic acids is usually accomplished by one of two methods: incorporation of spin labels during the oligonucleotide synthesis, or by post-synthesis spin labeling, where the label is attached through modification of a uniquely reactive functional group.[6] Both approaches are based on attachment of the spin label by covalent bonds.

We have previously reported a noncovalent SDSL (NC-SDSL) strategy for nucleic acids by utilizing ligand–receptor interactions. In this approach, the rigid spin label **ç**, an analogue of cytosine (C), binds to an abasic site in duplex DNA through hydrogen bonding to a guanine (G) on the complementary strand (Scheme 1 A) and π-stacking interactions with the flanking nucleotides.[7] CW-EPR spectroscopy revealed that **ç** was specifically and fully bound to the abasic site at −30 °C.[7] This NC-SDSL approach is promising for distance measurement in nucleic acids by PELDOR, as this is performed with frozen solutions. Sample preparation is straight-forward as DNA that contains abasic sites can be readily prepared by automated solid-phase synthesis with commercially available phosphoramidite; spin-labeled samples for EPR studies are prepared simply by mixing DNA and the spin label prior to EPR measurements.



Scheme 1. A) Base pairing between guanine **G** and the spin-labeled nucleoside **Ç** (or nucleobase **ç**). B) Structures of the abasic sites used in this study. AP = apurinic or apyrimidinic site. B = nucleobase.

Recently, we have shown that NC-SDSL with **ç** is highly sequence dependent.[8] For some flanking sequences, incomplete binding was observed at −30 °C. At temperatures lower than

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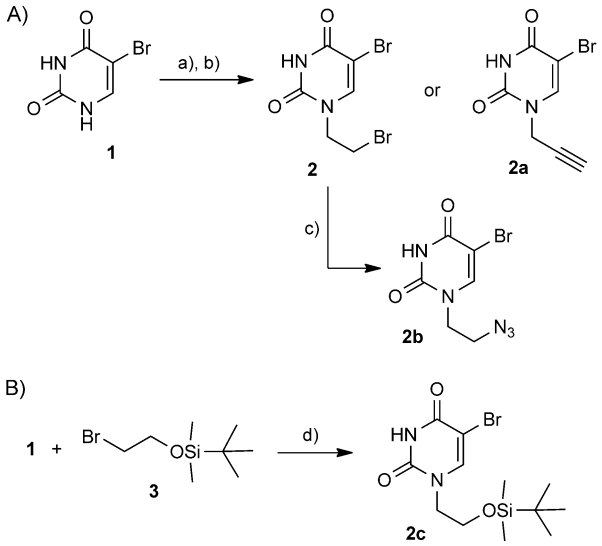
−30 °C, the spin label has limited solubility in aqueous solution, even with 30% ethylene glycol (generally used as a cryoprotectant in pulsed-EPR studies) and a small amount of DMSO. In an attempt to find spin labels that have better solubility and higher affinity for abasic sites, we prepared several N3 derivatives of the spin label **ç**. We show that both the ethyl amino and ethyl guanidine derivatives of **ç** have higher binding affinity for abasic sites in duplex DNA and higher solubility in aqueous solutions than **ç**.

Results and Discussion

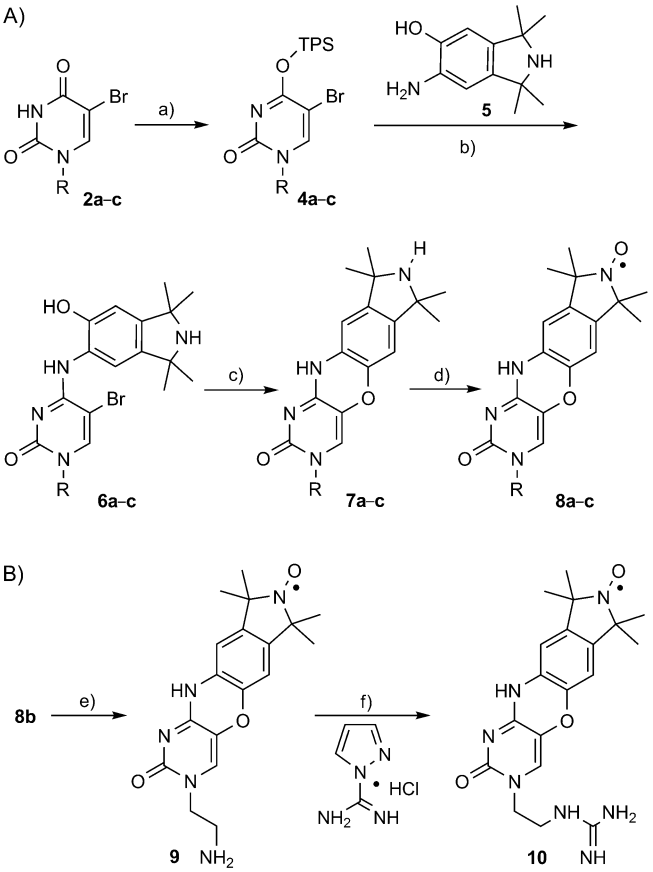
The binding of **ç** to an abasic site in duplex DNA is strongly governed by hydrogen bonding to an orphan base on the complementary strand[7] and stacking interactions with the base pairs flanking the abasic site.[8] Having identified the structural components of the abasic site that are important for noncovalent binding to **ç**, we turned our attention to increasing the binding affinity through structural modifications of **ç**. A logical site for modification is N3 because of its position relative to the structural boundaries of the abasic site pocket and because N3 derivatives can be readily synthesized. We have previously installed nonpolar alkyl substituents at N3 of **ç**, and we have shown that increased alkyl chain length decreases binding affinity and solubility.[8] In this work we examined other structural variations, including aromatics and various polar functional groups. Of particular interest was the incorporation of basic functional groups, such as amino and guanidine groups, that are protonated under physiological conditions and were expected to increase the binding affinity of the spin label to the negatively charged DNA. Alkyne and azide derivatives of **ç** were also prepared for conjugation to various ligands by using the Cu^{I}-catalyzed Huisgen–Meldal–Sharpless [3+2] cycloaddition reaction.[9]

Syntheses of spin label derivatives

The syntheses of the spin label derivatives began with regioselective alkylation of 5-bromouracil at the N1 position by one of two methods, depending on the alkyl halide (Scheme 2). For preparation of compounds **2** and **2a** (Scheme 2A), the alkylation was performed by a one-pot, two-step reaction: silylation with 1,1,1,3,3,3-hexamethyldisilazane (HMDS), followed by treatment with the corresponding alkyl halide in the presence of a catalytic amount of iodine to yield the N1-modified 5-bromouracil (Scheme 2A).[7–8] However, this method could not be used for the preparation of **2c**, presumably because of the lower reactivity of the hydroxyl-protected 2-bromoethanol **3**. Instead, **2c** was synthesized by reacting 5-bromouracil with **3** in the presence of K_2CO_3 in DMSO (Scheme 2B).[8] The aliphatic bromide of **2** was replaced with an azide by treatment with NaN_3 in DMSO to yield **2b**.[10] The N1 derivatives of 5-bromouracil (**2a**–**c**) were treated with 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl), in the presence of triethylamine and a catalytic amount of 4-dimethylaminopyridine (DMAP), to yield the O^4-sulphonyl-activated compounds **4a**–**c** (Scheme 3). Compounds **4a**–**c** were reacted with amino phenol **5**[11] to give con-



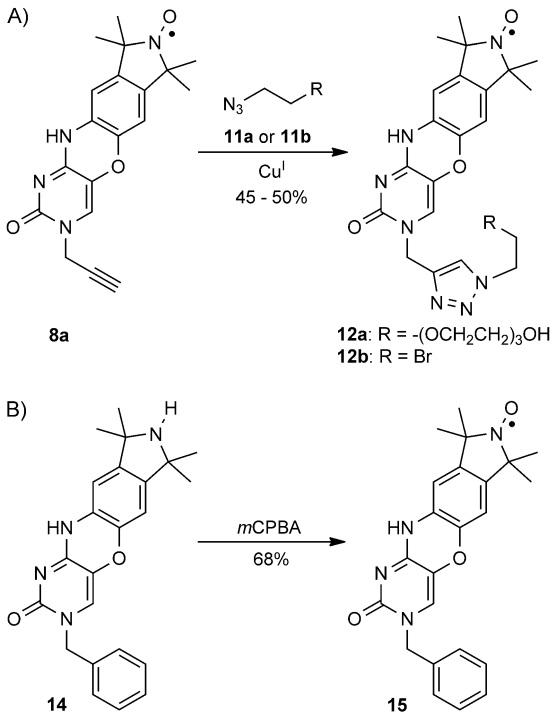
Scheme 2. Regioselective N1 modification of 5-bromouracil. a) HMDS, TMS·Cl; b) $BrCH_2CH_2Br$ or $BrCH_2C{\equiv}CH$, I_2, 40–75%; c) NaN_3, 80%; d) K_2CO_3, 30%.



Scheme 3. Syntheses of N3 derivatives of **ç** containing polar functional group side-chains. **a**: R = $CH_2C{\equiv}CH$; **b**: R = $CH_2CH_2N_3$; **2c**, **4c**, **6c**: R = CH_2CH_2O·TBDMS; **7c**, **8c**: R = CH_2CH_2OH). a) TPS-Cl, DMAP, Et_3N, 30–70%; b) Et_3N, 50–70%; c) CsF, 50%; d) *m*CPBA, 50–65%; e) PPh_3, 80%; f) DIPEA, 50%.

jugates **6a**–**c** and subsequently phenoxazine derivatives **7a**–**c** upon cyclization with cesium fluoride. For **6c**, cesium fluoride treatment also removed the TBDMS group to yield the desired hydroxyl compound **7c**. Oxidation of the aliphatic amines of **7a**–**c** to nitroxides with *meta*-chloroperbenzoic acid (*m*CPBA) afforded spin labels **8a**–**c**.

The azide group of nitroxide **8b** was reduced by using Staudinger conditions[12] to give spin label **9**, which was subsequently guanidinylated to yield spin label derivative **10**.[13] Spin label **8a** (containing a terminal acetylene) was prepared with the intention of conjugating various ligands to spin label **ç** through a Cu^{I}-catalyzed 1,3-dipolar cycloaddition reaction. To this end, an azide containing a poly(ethylene glycol) chain (**11a**) and 1-azido-2-bromoethane (**11b**) were conjugated to **8a** to yield triazole-containing spin labels **12a** and **12b**, respectively (Scheme 4A).



Scheme 4. A) Modification of spin label **8a** by a Cu^{I}-catalyzed Huisgen–Meldal–Sharpless [3+2] cycloaddition. B) Synthesis of an N3-benzyl derivative of spin label **ç**.

Binding affinity of ç derivatives monitored by CW-EPR spectroscopy

To quantify the effect of N3 substituents on the binding affinity of **ç**, the derivatives were individually incubated with a 14-mer duplex DNA that contained an abasic site (**F**, Scheme 1B and Figure 1), and the EPR spectra were recorded in a phosphate buffer (pH 7) containing 30% ethylene glycol and 2% DMSO. The spectra were recorded at several temperatures between 0 and −30 °C. At 0 °C, about 30 to 60% binding was observed, whereas the labels were mostly bound at −30 °C (data not shown). The EPR spectra recorded at −10 °C were chosen for determination of the dissociation constants (see the Supporting Information).[7] The data show that there was considerable variation in binding affinity within the family of N3 derivatives (Figure 1). The propargyl derivative **8a** had a similar affinity to that of **ç**, while the affinity of propyl derivative **13** was nearly three times weaker. The higher binding affinity of **8a** compared with that of **13** might be attributable to the smaller size and linear geometry of the terminal acetylene (thus, more easily accommodated in the abasic pocket than the sp^3-hybrid-

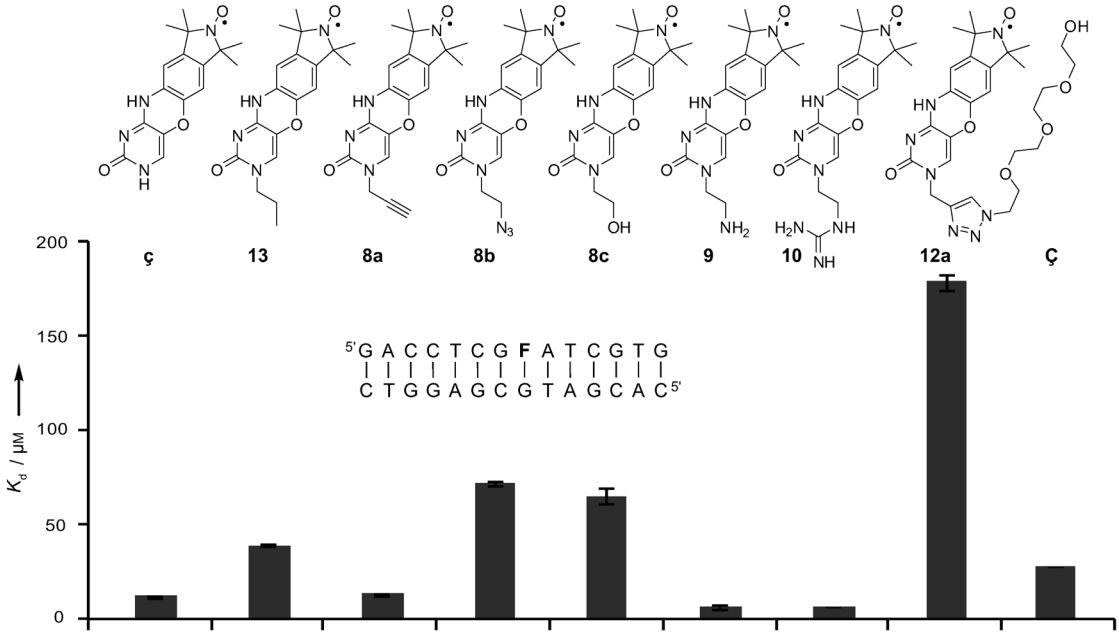


Figure 1. Structures of N3 derivatives of the spin label **ç** (top). Dissociation constants (bottom) upon binding to a DNA duplex containing the abasic site **F** as determined from EPR data collected at −10 °C. The structure of **Ç** is shown in Scheme 1.

ized carbon chain of the propyl group of **13**). It was somewhat surprising that derivatives **8b** and **8c** had about two times lower affinity than the propyl derivative **13**. They have similar sizes, but **8b** and **8c** have polar groups at the ends of their chains; these could interact with the phosphate backbone or the solvent.

The highest binding affinity was observed for the amino (**9**) and the guanidino (**10**) spin label derivatives: about twofold higher than **ç**. The enhanced binding affinities of **9** and **10** were most likely the result of ionic interactions between the negatively charged phosphate backbone and the positively charged amino and guanidine groups, both of which are protonated at pH 7. In an attempt to gain an insight into how the sugar-phosphate backbone at the abasic site could accommodate the spin-label side chains, a molecular model of spin label **9** in a 1:1 complex with a 14-mer DNA duplex containing an abasic site was generated (Figure 2A). In the model, the spin label is stacked in the abasic pocket, where it forms three hydrogen bonds to the guanine on the complementary strand, while the abasic sugar is flipped outwards. The tethered ammonium cation was oriented towards the sugar-phosphate backbone where it formed two hydrogen bonds with non-bridging oxygen atoms of two phosphodiesters.

A few of the derivatives contain rings at the N3 position. The poly(ethylene glycol) chain of the triazole derivative **12a** was installed to increase the solubility of the spin label in aqueous solutions. However, the binding affinity of **12a** was about 15-fold lower than that of **ç**. This dramatically lower binding of **12a** could be due to the hydrophilicity of the poly(ethylene glycol) chain, which increases the affinity of the label for the polar solvent over the hydrophobic abasic site or the triazole ring. Neither **12b** nor benzyl derivative **15** (prepared in one step from an intermediate (**14**) in the synthesis of **ç**,[7] Scheme 4B) was sufficiently soluble to enable determination of their binding affinities. We also evaluated the spin-labeled nucleoside **Ç**,[11] which contains a 2-deoxyribose at the N3 position (Scheme 1). Interestingly, **Ç** bound with only twofold lower affinity than **ç** in spite of having a rather bulky group at the N3 position. A molecular model of **Ç** noncovalently bound to an abasic site in duplex DNA shows that the abasic linker is oriented outwards, which enables the sugar ring of **Ç** to be nicely accommodated at the abasic site (Figure 2B). Furthermore, the 3′- and 5′-hydroxyl groups of the sugar form hydrogen bonds with the non-bridging oxygen atoms of the phosphodiesters.

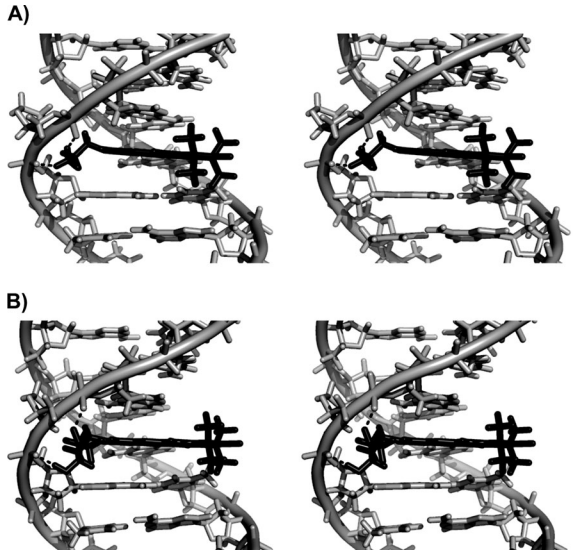


Figure 2. Stereo views of molecular models of A) spin label **9** and B) spin-labeled nucleoside **Ç** (black) bound noncovalently to duplex DNA (gray) that contains an abasic site. Hydrogen bonds between the ammonium group of **9** or the hydroxyls on the 2′-deoxyribose of **Ç** with non-bridging oxygen atoms of the phosphate backbone are shown as dotted lines. During the energy minimization, the sugar-phosphate backbone around the abasic site and the spin label side chains were allowed to move, while the rest of the structure was constrained.

Effect of abasic site linker: C_3 versus F

We have previously shown that the affinities of N3-alkyl derivatives was similar for an abasic site containing the tetrahydrofuran analogue **F** and for one containing the propane-1,3-diol-derived $\mathbf{C_3}$ linker, an acyclic analogue of 2′-deoxyribofuranose (Scheme 1B).[8] Because of the large variation in the structures of the side chains in **8**–**13**, we decided to evaluate their binding to a DNA duplex containing $\mathbf{C_3}$. Only a minor variation in binding affinity of the spin labels was observed for the two abasic-site linkers and most of the derivatives bound with slightly better affinity for **F** than for $\mathbf{C_3}$ (data not shown). The larger size and lower flexibility of **F** clearly does not have a detrimental effect on binding when compared with $\mathbf{C_3}$. This is presumably due to the fact that the tetrahydrofuran ring can rotate outwards to provide access for the N3 side chains.

Conclusion

Several N3 derivatives of spin label **ç** were synthesized, and their binding affinities for an abasic site in duplex DNA were quantified by EPR spectroscopy. All but two of the derivatives had lower affinity for the abasic site than did **ç**. However, with the exception of poly(ethylene glycol) derivative **12a**, the variation in binding affinities was less than fivefold. The size, shape, and polarity of the side chains influence the binding affinity. For example, the N3-ethyl alcohol derivative **8c** had more than twofold lower affinity than **Ç**; this could be due to a better fit of the 2′-deoxyribose in the abasic pocket, as indicated by molecular modeling. The amino (**9**) and guanidino (**10**) derivatives showed enhanced binding and increased solubility, presumably because of their positive charge at neutral pH. The apparent high binding affinities of derivatives **9** and **10** makes them promising candidates for distance measurements by pulsed EPR. Studies into this will be reported in due course.

Experimental Section

General: Compounds **3**,[14] **5**,[11] **11a**,[15] **11b**,[16] **13**,[8] **14**[7] and **Ç**[11] were prepared according to previously reported procedures. All reactions were carried out in oven-dried reaction flasks under an

argon atmosphere. Reactions were monitored by thin layer chromatography (TLC), performed on glass-backed TLC plates with an extra hard layer (Kieselgel 60 F_{254}, 250 μm; Silicycle, Quebec, Canada), and compounds were visualized with UV light. Dichloromethane, pyridine, and acetonitrile were freshly distilled over calcium hydride prior to use. All commercial reagents were purchased from Sigma–Aldrich and used without further purification. Flash column chromatography was carried out with silica gel (230–400 mesh, 60 Å) as the stationary phase (Silicycle). The petroleum ether used for chromatography was from distillation between 60 and 90 °C. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer, and coupling constants are reported in Hertz. The chemical shifts are reported in ppm relative to the residual proton signal (for ^{1}H NMR) and the carbon signal (for ^{13}C NMR) of the deuterated solvents: [D_6]DMSO (2.50 ppm), $CDCl_3$ (7.26 ppm), [D_4]MeOH (4.84 and 3.31 ppm) for ^{1}H NMR; [D_6]DMSO (39.52 ppm), $CDCl_3$ (77.0 ppm), [D_4]MeOH (49.05 ppm) for ^{13}C NMR. Molecular masses of organic compounds were determined by HR-ESI-MS (MicroTof-Q, Bruker). The CW-EPR spectra were recorded on a MiniScope MS200 spectrometer (modulation frequency100 kHz, modulation amplitude1.0 G, microwave power 2.0 mW; Magnettech, Berlin, Germany). Temperature was regulated by an M01 temperature controller (error ±0.5 °C; Magnettech).

DNA synthesis and purification: Deoxyoligonucleotides (either unmodified DNA or containing an **F** or $\mathbf{C_3}$ abasic site) were prepared by phosphoramidite chemistry on an automated ASM800 DNA synthesizer (Biosset, Novosibirsk, Russia) by using a trityl-off protocol and phosphoramidites with standard protecting groups on a 1.0 μmol scale (1000 Å CPG columns). All commercial phosphoramidites, CPG columns and chemical reagents for DNA synthesis were purchased from ChemGenes Corporation (Wilmington, MA). The $\mathbf{C_3}$ phosphoramidite was prepared according to a previously reported procedure.[17] After deprotection of oligonucleotides and cleavage from the solid support by incubation in concentrated aqueous ammonia solution at 55 °C for 8 h, oligomers were purified by denaturing polyacrylamide (20%) gel electrophoresis (DPAGE). The oligonucleotides were visualized under UV light, and the bands were excised from the gel, crushed and eluted from gel in Tris buffer (Tris (10 mM, pH 7.5), NaCl (250 mM), Na_2EDTA (1 mM)). The DNA elution solutions were filtered through 0.45 μm polyethersulfone membrane (Whatman) and desalted in a Sep-Pak cartridge (Waters Corporation). Solvent was removed in a SpeedVac (Thermo Fisher), and DNA was dissolved in deionized and sterilized water (200 μL). UV absorbance at 260 nm was used to calculate the concentrations of oligonucleotides according to Beer's law. Extinction coefficients were determined by using the UV WinLab oligonucleotide calculator (V2.85.04; PerkinElmer).

Molecular modeling: Molecular modeling was carried out with B-form DNA duplex generated in Spartan 10 (Wavefunction, Irvine, CA) with default parameters. The abasic site was generated by deleting the corresponding cytosine (C) base and a hydrogen atom at the anomeric carbon of the 2′-deoxysugar. The spin label **ç** was manually docked into the abasic pocket so that it formed three hydrogen bonds with the G on the opposing strand. The energy minimization to obtain equilibrium geometry at ground state was performed by using molecular mechanics (MMFF). The sugar-phosphate backbone around the abasic site and the spin-label side chains were allowed to move while the rest of the helix was constrained. The energy-minimized models were exported as PDB files and visualized in PyMOL (DeLano Scientific LLC).

Compounds 2 and 2a: HMDS (16.45 mL, 78.53 mmol) and trimethylsilyl chloride (TMS-Cl, 1.56 mL, 13.08 mmol) were added to a suspension of 5-bromouracil (5 g, 26.18 mmol) in 1,2-dichloroethane (1,2-DCE, 25 mL). The reaction mixture was refluxed for 5 h, after which it became clear; it was cooled to 60 °C, and the solvent was removed in vacuo to yield a colorless oil. The residue was dissolved in DMF (25 mL) and treated with an alkyl halide (dibromoethane (9.022 mL, 104.7 mmol) for **2,** propargyl bromide (4.51 mL, 52.35 mmol) for **2a**)) and a catalytic amount of I_2 (0.067 g, 0.26 mmol) at 25 °C. The reaction mixtures were refluxed for 12 h, cooled to 25 °C, diluted with H_2O (25 mL), and extracted with CH_2Cl_2. The combined organic layers were washed with a saturated aqueous solution of $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude mixtures were purified by flash column chromatography (MeOH (3%) in CH_2Cl_2) to yield white solids. **2:** Yield 40%; ^{1}H NMR ([D_6]DMSO): δ = 11.85 (s, 1H; NH), 8.24 (s, 1H; CH), 4.07 (t, J = 6.4 Hz, 2H; CH_2), 3.71 (t, J = 6.4 Hz, 2H; CH_2); ^{13}C NMR ([D_6]DMSO): δ = 159.57, 150.19, 145.33, 94.54, 48.89, 40.15, 30.30; HR-ESI-MS: *m/z* 318.8688 [*M*+Na]$^+$, calcd for $C_6H_6Br_2N_2O_2$ 295.8696. **2a:** Yield 76%. ^{1}H NMR ([D_6]DMSO): δ = 8.26 (s, 1H; CH), 4.50 (d, J = 2.5 Hz, 2H; CH_2), 3.44 (t, J = 2.4 Hz, 1H; CH); ^{13}C NMR ([D_6]DMSO): δ = 159.47, 149.71, 144.13, 95.28, 78.18, 76.10, 37.11; HR-ESI-MS: *m/z* 228.9780 [*M*+H]$^+$, calcd for $C_7H_5BrN_2O_2$ 227.9534.

Compound 2b: NaN_3 (0.6 g, 10 mmol) was added to a solution of **2** (1.6 g, 5.37 mmol) in DMSO (20 mL), and the resulting mixture was stirred for 24 h at 25 °C. The reaction mixture was diluted with water (60 mL) in an ice bath and extracted with CH_2Cl_2 (3×20 mL). The combined organic layers were washed with water and brine and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo to afford **2b** as a white solid, which was used without further purification. Yield 80%; ^{1}H NMR ([D_6]DMSO): δ = 11.83 (s, 1H; NH), 8.22 (s, 1H; CH), 3.87 (t, J = 7.6 Hz, 2H; CH_2), 3.60 (t, J = 5.6 Hz, 2H; CH_2); ^{13}C NMR ([D_6]DMSO): δ = 159.57, 150.28, 145.30, 94.70, 48.82, 46.93, 41.85; HR-ESI-MS: *m/z* 281.9597 [*M*+Na]$^+$, calcd for $C_6H_6BrN_5O_2$ 258.9705.

Compound 2c: K_2CO_3 (0.5 g, 3.61 mmol) and **3** (0.44 g, 1.83 mmol) were added to a solution of 5-bromouracil (0.5 g, 2.61 mmol) in DMSO (10 mL). After the resulting mixture had been stirred for 2 h at 25 °C, the reaction mixture was cooled to 10 °C, diluted with H_2O (50 mL) and extracted with CH_2Cl_2. The combined organic layers were washed with saturated $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, and concentrated. The crude product was purified by flash column chromatography (MeOH (2%) in CH_2Cl_2) to yield **2c** as a white solid. Yield 30%; ^{1}H NMR ($CDCl_3$): δ = 8.57 (s, 1H; NH), 7.65 (s, 1H; CH), 3.88 (m, 2H; CH_2), 3.81 (m, 2H; CH_2), 0.88 (s, 9H; $3CH_3$), 0.03 (s, 6H; $2CH_3$); ^{13}C NMR ($CDCl_3$): δ = 159.45, 150.05, 146.04, 95.28, 60.98, 51.24, 25.95, 18.30, −4.40; HR-ESI-MS: *m/z* 371.0397 [*M*+Na]$^+$, calcd for $C_{12}H_{21}BrN_2O_3Si$ 348.0505.

Compounds 4a–c: TPS-Cl (2.12 g, 7.0 mmol), DMAP (42.7 mg, 0.35 mmol), and Et_3N (1.9 mL, 14 mmol) were added to solutions of **2a–c** (3.5 mmol) in CH_2Cl_2 (25 mL) at 0 °C. The resulting reaction mixtures were stirred for 6 h at 0 °C, diluted with CH_2Cl_2 (20 mL), and washed sequentially with H_2O (2×20 mL), saturated $NaHCO_3$ (15 mL), and brine (15 mL). The organic layers were dried over anhydrous Na_2SO_4 and concentrated. The residues were purified by flash column chromatography (EtOAc (10%) in petroleum ether) to yield compounds **4a–c** as white solids. **4a:** Yield 40%; ^{1}H NMR ($CDCl_3$): δ = 8.16 (s, 1H; CH), 7.20 (s, 2H; 2Ar-CH), 4.59 (d, J = 2.6 Hz, 2H; CH_2), 4.25–4.32 (m, 2H; 2CH), 2.88–2.92 (m, 1H; CH), 2.65 (t, J = 2.6 Hz, 1H; CH), 1.29 (d, J = 6.7 Hz, 12H; $4CH_3$), 1.25 (d, J = 6.9 Hz, 6H; $2CH_3$); ^{13}C NMR ($CDCl_3$): δ = 163.21, 155.01, 152.60, 151.72, 147.69, 130.27, 124.29, 87.02, 78.43, 74.95, 39.45, 34.42, 29.79, 24.64, 23.57; HR-ESI-MS: *m/z* 517.0767 [*M*+Na]$^+$, calcd for

$C_{22}H_{27}BrN_2O_4S$ 494.0875. **4b:** Yield 33%; ^1H NMR ($CDCl_3$): $\delta = 7.80$ (s, 1H; CH), 7.21 (s, 2H; 2Ar-CH), 4.24–4.31 (m, 2H; 2CH), 3.88 (dd, $J =$ 6.2, 4.3 Hz, 2H; CH_2), 3.70 (dd, $J = 6.2$, 4.3 Hz, 2H; CH_2), 2.87–2.92 (m, 1H; CH), 1.29 (d, $J = 6.8$ Hz, 12H; $4CH_3$), 1.25 (d, $J = 6.9$ Hz, 6H; $2CH_3$); ^{13}C NMR ($CDCl_3$): $\delta = 163.33$, 155.01, 152.95, 151.69, 150.42, 130.22, 124.27, 86.51, 50.72, 48.75, 34.39, 29.79, 24.62, 23.54; HR-ESI-MS: *m/z* 548.0914 $[M+Na]^+$, calcd for $C_{21}H_{28}BrN_5O_4S$ 525.1045. **4c:** Yield 68%; ^1H NMR ($CDCl_3$): $\delta = 7.82$ (s, 1H; CH), 7.20 (s, 2H; 2Ar-CH), 4.29–4.32 (m, 2H; 2CH), 3.91 (m, 2H; CH_2), 3.79 (m, 2H; CH_2), 2.88–2.92 (m, 1H; CH), 1.29 (d, $J = 6.7$ Hz, 12H; $4CH_2$), 1.25 (d, $J = 6.9$ Hz, 6H; $2CH_3$), 0.82 (s, 9H; $3CH_3$), −0.09 (s, 6H; $2CH_3$); ^{13}C NMR ($CDCl_3$): $\delta = 154.83$, 153.10, 151.67, 151.50, 130.43, 124.22, 100.12, 85.37, 59.99, 53.06, 34.42, 29.78, 25.85, 24.66, 23.60, 18.17, −5.57; HR-ESI-MS: *m/z* 637.1737 $[M+Na]^+$, calcd for $C_{27}H_{43}BrN_2O_5SSi$ 614.1845.

Compounds 6a–c: Amino phenol **5** (0.130 g, 0.63 mmol) and Et_3N (0.14 mL, 1.0 mmol) were added to solutions of **4a–c** (0.57 mmol) in CH_2Cl_2 (10 mL). The resulting reaction mixtures were stirred at 25 °C for 48 h in the dark, solvent was removed in vacuo, and the residues were purified by flash column chromatography (MeOH (2%) in CH_2Cl_2 containing NH_3 (1%)) to yield **6a–c** as pale yellow solids. **6a:** Yield 70%; ^1H NMR ([D$_6$]DMSO): $\delta = 8.27$ (s, 1H; CH), 7.72 (s, 1H; Ar-CH), 6.66 (s, 1H; Ar-CH), 6.58 (s, 1H; Ar-CH), 4.53 (d, $J = 2.4$ Hz, 2H; CH_2), 3.4 (t, $J = 2.4$ Hz, 1H; CH), 1.34 (s, 12H; $4CH_3$); ^{13}C NMR ([D$_6$]DMSO): $\delta = 153.27$, 145.51, 116.25, 107.80, 99.51, 87.22, 78.82, 75.75, 38.12, 32.04, 31.78; HR-ESI-MS: *m/z* 417.0930 $[M+H]^+$, calcd for $C_{19}H_{21}BrN_4O_2$ 416.0848. **6b:** Yield 55%; ^1H NMR ($CDCl_3$/CD_3OD 95:5): $\delta = 8.03$ (s, 1H; CH), 7.57 (s, 1H; Ar-CH), 6.57 (s, 1H; Ar-CH), 3.87–3.81 (m, 2H; CH_2), 3.70–3.63 (m, 2H; CH_2), 1.44 (s, 6H; $2CH_3$), 1.37 (s, 6H; $2CH_3$); ^{13}C NMR ($CDCl_3$/CD_3OD 95:5): $\delta =$ 157.34, 155.32, 147.47, 145.59, 125.76, 114.47, 108.37, 88.93, 63.67, 63.32, 30.97, 30.82, 29.68; HR-ESI-MS: *m/z* 448.1066 $[M+H]^+$, calcd for $C_{18}H_{22}BrN_7O_2$ 447.1018. **6c:** Yield 52%; ^1H NMR ($CDCl_3$): $\delta = 7.70$ (s, 1H; CH), 6.99 (s, 1H; Ar-CH), 6.82 (s, 1H; Ar-CH), 3.95 (d, $J =$ 4.1 Hz, 2H; CH_2), 3.85 (d, $J = 3.5$ Hz, 2H; CH_2) 1.43 (s, 12H; $4CH_3$), 0.88 (s, 9H; $3CH_3$), 0.01 (s, 6H; $2CH_3$); ^{13}C NMR ($CDCl_3$): $\delta = 149.66$, 149.46, 141.58, 116.07, 114.09, 62.79, 62.55, 60.85, 52.34, 32.22, 32.12, 31.89, 25.94, 18.24, −5.43; HR-ESI-MS: *m/z* 537.1878 $[M+H]^+$, calcd for $C_{24}H_{37}BrN_4O_3Si$: 536.1818.

Compounds 7a–c: CsF (0.56 g, 3.72 mmol) was added to solutions of **6a–c** (0.37 mmol) in EtOH (10 mL), and the resulting reaction mixtures were stirred at 85 °C for three days. Solvent was evaporated, and the crude products were purified by flash column chromatography (MeOH (5%) in CH_2Cl_2 containing NH_3 (1%)) to give cyclized compounds **7a–c** as pale yellow solids. **7a:** Yield 50%; ^1H NMR (95:5 $CDCl_3$:[D$_6$]DMSO): $\delta = 7.08$ (s, 1H; CH), 6.69 (s, 1H; Ar-CH), 6.44 (s, 1H; Ar-CH), 4.50 (s, 2H; CH_2), 2.48 (s, 1H; CH), 1.38 (s, 6H; $2CH_3$), 1.37 (s, 6H; $2CH_3$); ^{13}C NMR (95:5 $CDCl_3$:[D$_6$]DMSO): $\delta = 154.44$, 144.79, 143.94, 142.33, 128.68, 126.03, 123.58, 110.33, 108.61, 75.73, 63.00, 38.17, 31.46, 31.39; HR-ESI-MS: *m/z* 337.1605 $[M+H]^+$, calcd for $C_{19}H_{20}N_4O_2$ 336.1586. **7b:** Yield 50%; ^1H NMR ($CDCl_3$): $\delta = 7.54$ (s, 1H; CH), 6.83 (s, 1H; Ar-CH), 6.45 (s, 1H; Ar-CH), 3.79–3.81 (m, 2H; CH_2), 3.66–3.69 (m, 2H; CH_2), 1.45 (s, 6H; $2CH_3$), 1.42 (s, 6H; $2CH_3$) ^{13}C NMR ($CDCl_3$): $\delta = 155.29$, 154.53, 142.11, 127.98, 126.32, 125.73, 111.59, 108.23, 49.82, 49.73, 31.72, 29.83; HR-ESI-MS: *m/z* 368.1836 $[M+H]^+$, calcd for $C_{18}H_{21}N_7O_2$ 367.1757. **7c:** Yield 25%; ^1H NMR ([D$_6$]DMSO): $\delta = 7.27$ (s, 1H; CH), 6.62 (s, 1H; Ar-CH), 6.52 (s, 1H; Ar-CH), 3.64 (brs, 2H; CH_2), 3.56 (brs, 2H; CH_2), 1.27 (s, 12H; $4CH_3$); ^{13}C NMR ([D$_6$]DMSO): $\delta = 153.48$, 144.20, 143.51, 141.66, 125.94, 109.41, 108.24, 69.77, 61.92, 61.87, 58.64, 51.04, 40.14, 31.89, 31.72; HR-ESI-MS: *m/z* 343.1765 $[M+H]^+$, calcd for $C_{18}H_{22}N_4O_3$: 342.1652.

Spin labels 8a–c, 15: Solutions of **7a–c** and **14** (0.054 mmol) in CH_2Cl_2 (10 mL) were treated with a solution of *m*CPBA (11.25 mg, 0.065 mmol) in CH_2Cl_2 (2 mL) at 0 °C. The reaction mixtures were stirred for 6 h at 0 °C, and solvent was removed in vacuo. The residues were purified by preparative TLC (MeOH (8%) in CH_2Cl_2) to afford **8a–c** as pale yellow solids. **8a:** Yield 65%; ^1H NMR ($CDCl_3$): $\delta = 7.05$ (s, 1H; CH), 4.72 (brs, 2H; CH_2), 2.55 (s, 1H; CH), 1.29 (s, 1H); HR-ESI-MS: *m/z* 352.1521 $[M+H]^+$, calcd for $C_{19}H_{19}N_4O_3$ 351.1457. **8b:** Yield 60%; ^1H NMR ($CDCl_3$/CD_3OD 95:5): $\delta = 8.09$ (brs, 1H; CH), 6.69 (brs, 1H; CH), 3.90 (brs, 2H; CH_2), 3.72 (brs, 2H; CH_2), 1.26 (brs, 4H); HR-ESI-MS: *m/z* 405.1568 $[M+Na]^+$, calcd for $C_{18}H_{20}N_7O_3$ 382.1628. **8c:** Yield 50%; ^1H NMR ($CDCl_3$/CD_3OD 95:5): $\delta = 8.23$ (brs, 1H), 8.10 (s, 1H; CH), 1.26 (s, 1H); HR-ESI-MS: *m/z* 380.1481 $[M + Na]^+$, calcd for $C_{18}H_{21}N_4O_4$ 357.1563. **15:** Yield 68%; ^1H NMR ($CDCl_3$): $\delta = 7.34$–7.42 (m, 4H; Ar-CH), 4. 96 (brs, 1H; CH), 1.26 (brs, 2H); HR-ESI-MS: *m/z* 426.1641 $[M+Na]^+$, calcd for $C_{23}H_{23}N_4O_3$ 403.1770. Note: Because of the paramagnetic nature of nitroxides, the NMR spectra of spin labels show significant broadening of the signals; thus some peaks (particularly of nuclei close to the radical) are not seen in the spectra.

Spin label 9: PPh_3 (41 mg, 0.156 mmol) was added to a solution of **8b** (40 mg, 0.104 mmol) in dry THF (2 mL), and the reaction mixture was stirred for 30 min at 25 °C prior to addition of H_2O (2.1 μL, 0.11 mmol). The resulting reaction mixture was stirred for 12 h at 25 °C and concentrated in vacuo. The residue was purified by preparative TLC (MeOH (10%) in CH_2Cl_2) to yield **9** as a pale yellow solid. Yield 80%; ^1H NMR ($CDCl_3$/CD_3OD 95:5): $\delta = 8.01$ (brs, 1H; CH), 4.61 (brs, 1H; CH), 1.18 (brs, 4H; CH_3); HR-ESI-MS: *m/z* 357.1811 $[M+H]^+$, calcd for $C_{18}H_{22}N_5O_3$ 356.1723.

Spin label 10: A solution of **9** (20 mg, 0.056 mmol) in DMF (2 mL) was treated with 1H-pyrazole-1-carboxamidine hydrochloride (10 mg, 0.067 mmol) and diisopropylethyl amine (15 μL, 0.084 mmol). The resulting reaction mixture was stirred for 48 h at 25 °C, then concentrated, and the residue was purified by preparative TLC (MeOH (20%) in CH_2Cl_2, ammonia (2%)) to yield **10** as a pale yellow solid. Yield 50%; ^1H NMR ($CDCl_3$/CD_3OD/[D$_6$]DMSO 90:8:2): $\delta = 7.91$ (brs, 1H; CH), 7.03 (brs, 1H; CH), 3.78 (brs, 2H; CH_2), 3.34 (brs, 2H; CH_2); HR-ESI-MS: *m/z* 399.2010 $[M+H]^+$, calcd for $C_{19}H_{24}N_7O_3$ 398.1941.

Spin labels 12a, b: A solution of **8a** (10 mg, 0.0284 mmol) in acetone was treated with either **11a**[15] or **11b**[16] (0.034 mmol) in the presence of a catalytic amount of CuI (1 mg, 0.0028 mmol). The resulting reaction mixtures were stirred under reflux for 12 h. After cooling, the reaction mixtures were filtered, and the filtrates were concentrated in vacuo. The residues were purified by preparative TLC (MeOH (10%) in CH_2Cl_2) to afford the spin labels as pale yellow solids. **12a:** Yield 50%; ^1H NMR ($CDCl_3$): $\delta = 8.11$ (s, 1H; CH), 7.93 (brs, 1H; CH), 6.96 (brs, 1H; CH), 5.06 (brs, 2H; CH_2), 4.55 (brs, 2H; CH_2), 3.62–3.89 (m, 16H; $8CH_2$), 1.27 (brs, 2H; CH_3); HR-ESI-MS: *m/z* 593.2568 $[M+Na]^+$, calcd for $C_{27}H_{36}N_7O_7$ 570.2676. **12b:** Yield 45%; ^1H NMR ($CDCl_3$): $\delta = 7.91$ (s, 1H; CH), 7.02 (brs, 1H; CH), 4.97 (brs, 2H; CH_2), 4.71 (brs, 2H; CH_2), 3.72 (brs, 2H; CH_2), 1.20 (brs, 3H; CH_3); HR-ESI-MS: *m/z* 503.1134 $[M+2H]^+$, calcd for $C_{21}H_{23}BrN_7O_3$ 500.1046.

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