

Structural changes of an abasic site in duplex DNA affect noncovalent binding of the spin label ζ

Sandip A. Shelke and Snorri Th. Sigurdsson*

Department of Chemistry, University of Iceland, Science Institute, Dunhaga 3, 107 Reykjavik, Iceland

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ABSTRACT

The influence of structural changes of an abasic site in duplex DNA on noncovalent and site-directed spin labeling (NC-SDSL) of the spin label ζ were examined with electron paramagnetic resonance (EPR) spectroscopy. The binding affinities of ζ to sixteen different DNA duplexes containing all possible sequences immediately flanking the abasic site were determined and the results showed that the binding of ζ is highly flanking-sequence dependent. In general, a 5'-dG nucleotide favors the binding of the spin label. In particular, 5'-d(G__T) was the best binding sequence whereas 5'-d(C__T) showed the lowest affinity. Changing the structure of the abasic site linker from a tetrahydrofuran analog (F) to the anucleosidic C₃-spacer (C₃) does not appreciably affect the binding of ζ to the abasic site. For efficient binding of ζ , the abasic site needs to be located at least four base pairs away from the duplex end. Introducing a methyl substituent at N3 of ζ did not change the binding affinity, but a decreased binding was observed for both N3-ethyl and -propyl groups. These results will guide the design of abasic site receptors and spin label ligands for NC-SDSL of nucleic acids.

INTRODUCTION

Electron paramagnetic resonance (EPR) spectroscopy, also known as electron spin resonance (ESR) spectroscopy, was invented by Zavoisky in 1945 (1). EPR detects paramagnetic centers and has been used for studying nucleic acids since 1965, when McConnell and coworkers reported the intercalation of a chlorpromazine radical to DNA (2). Since then, EPR has advanced significantly and is now routinely applied for studying dynamics and structural properties of biopolymers (3–9). Since nucleic acids are diamagnetic in their native state, a prerequisite for their EPR studies is incorporation of stable free radicals (spin labels). Aminoxyl radicals, commonly referred to as

nitroxide radicals, are normally utilized for site-specific labeling of nucleic acids. Such a labeling is generally termed site-directed spin labeling (SDSL) (10–13).

SDSL of nucleic acids utilizes any one of three approaches (14,15). The first strategy is spin labeling during chemical synthesis of the oligonucleotide, using a spin-labeled phosphoramidite. The second method is post-synthetic spin labeling, in which a uniquely reactive functional group in the nucleic acid, often incorporated at a specific site during the nucleic acid synthesis, is reacted with a spin-labeling reagent. The third approach is noncovalent and site-directed spin labeling (NC-SDSL), where the spin-label binding to the nucleic acids is facilitated by hydrogen bonding, van der Waals forces and π -stacking.

Advantages of NC-SDSL include the ease of spin-label synthesis and its stability, when compared to a spin-labeled phosphoramidite. Furthermore, the spin label does not get exposed to the chemicals used in the synthesis of oligonucleotides, which can partially reduce the nitroxide (16–18), and no tedious purifications of the spin-labeled product are required. Sample preparation is easy as a solution of the spin label and the nucleic acid is simply prepared prior to EPR studies.

Before automated solid-phase synthesis of oligonucleotides became widespread, there were reports of noncovalent DNA binding to spin-labeled derivatives of intercalators, such as ethidium (19,20), acridine (21), nitrobenzenes (22) and other polyaromatic carcinogens (19). However, the binding of multiple spin labels and lack of site-selectivity hampered the use of these compounds as spin labels. Subsequently, Lhomme and coworkers (23,24) tethered a spin-labeled acridine to an adenine that could bind to an abasic site, thereby directing the spin-labeled intercalator to sites proximal to the abasic site. However, the spin-labeled acridine intercalated in two different orientations and non-specific intercalation was also observed (24).

Recently we described a general NC-SDSL strategy for nucleic acids, in which the spin-labeled ligand ζ (Figure 1A) was bound to an abasic site in duplex DNA (25). The spin label ζ is an analogue of cytosine and, therefore, binds to an abasic site through hydrogen

*To whom correspondence should be addressed. Tel: +354 5 25 8 01; Fax: + 354 5 52 89 11; Email: snorrissi@hi.is

bonding to guanine (G) on the opposite strand and π -stacking interactions with the flanking nucleobases. Although the spin label ζ has an estimated K_d in the low-millimolar range at 20°C, it binds fully and specifically to the abasic site at -30°C. Thus, ζ should be useful for distance measurements using pulsed electron-electron double resonance (PELDOR) as they are performed in frozen solutions. It is worth noting that samples can be directly flash-frozen for distance measurements between two noncovalently labeled sites by PELDOR (unpublished results), which avoids conformational changes that might be associated with sample preparation at -30°C. This NC-SDSL approach has the advantage that nucleic acids that contain abasic sites at any desired position can be readily prepared by employing commercially available phosphoramidites. In other words, the receptor for the spin-label ligand can be placed at a chosen position in the nucleic acid duplex. Additionally,

ζ should give accurate distance measurements and enable the determination of relative orientations between two spin labels (26), like the rigid spin label ζ , which is a nucleoside that has ζ covalently linked to the sugar (18,27).

Given the potential that this NC-SDSL approach has for being a valuable spin-labeling technique for EPR studies of nucleic acids, it is important to investigate factors that may influence the binding of ζ to an abasic site. In this paper we show that the degree of spin-label binding is highly dependent on the flanking sequence and that the binding affinity of the spin label decreases when the abasic site is located close to the end of a duplex. Changes in the sugar-phosphate backbone of the abasic site have a minimal effect on binding while structural modifications of the spin label ζ affect its binding affinity. The findings described herein will guide further development of NC-SDSL of nucleic acids through abasic sites.

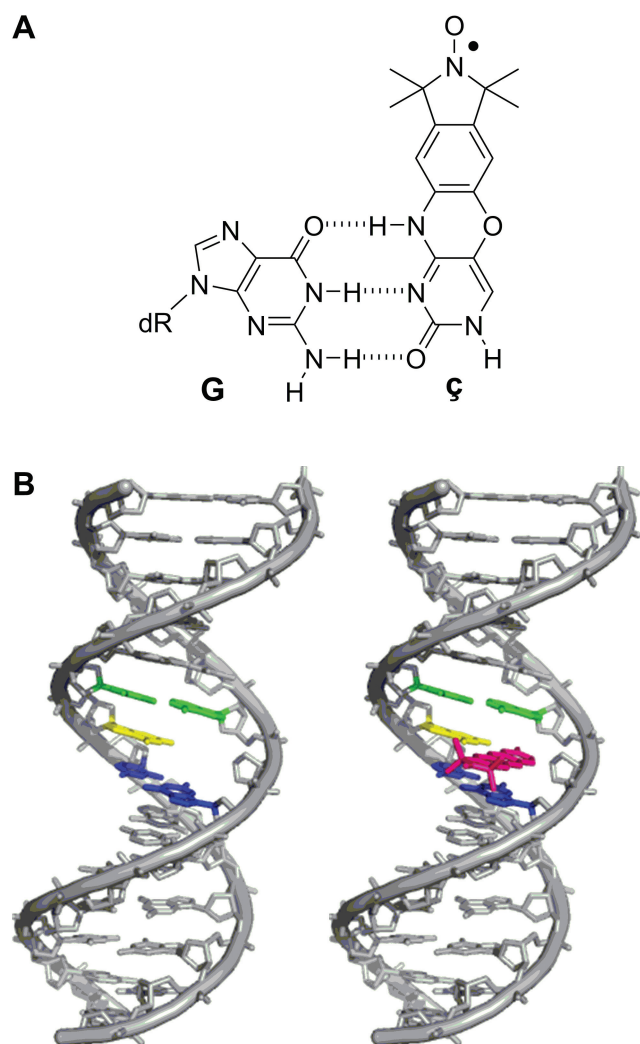


Figure 1. (A). Structure of spin label ζ and its base-pairing scheme with G. (B). A model of a B-DNA duplex that contains an abasic site (left) and the spin label ζ (magenta) bound to the abasic site (right). The green and blue colors identify the flanking base-pairs on the 3'- and 5'-side of the abasic site, respectively, and the orphan base opposite the abasic site is in yellow.

MATERIALS AND METHODS

General

All commercial reagents were purchased from Sigma-Aldrich and used without further purification. Dichloromethane was freshly distilled over calcium hydride prior to use. All air and moisture sensitive reactions were performed in oven-dried reaction flasks, under an argon atmosphere. Thin layer chromatography (TLC) was performed on glass backed TLC with extra hard layer (Kieselgel 60 F₂₅₄, 250 μ m, Silicycle) and compounds were visualized by UV light. Compounds were purified by flash column chromatography using silica gel (230–400 mesh, 60 Å) which was purchased from Silicycle. The petroleum ether used in chromatographic purification of synthetic compounds is of boiling point 60–90°C fraction. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 400 MHz spectrometer and the chemical shifts were reported in parts per million (ppm) relative to the residual proton signal (for ^1H NMR) and the carbon signal (for ^{13}C NMR) of the deuterated solvents used [d_6 -DMSO (2.50 ppm), CDCl_3 (7.26 ppm), d_4 -MeOH (4.84 and 3.31 ppm)] for ^1H NMR; [d_6 -DMSO (39.52 ppm), CDCl_3 (77.0 ppm), d_4 -MeOH (49.05 ppm)] for ^{13}C NMR. All coupling constants were reported in Hertz. Molecular mass of organic compounds were determined by HR-APCI-MS (Bruker, MicroTof-Q). The CW-EPR spectra were recorded on a MiniScope MS200 (Magnettech Germany) spectrometer (100 kHz modulation frequency, 1.0 G modulation amplitude, and 2.0 mW microwave power), using same number of scans. Plots were phase corrected, centered and aligned to the height of the central peak. Temperature was regulated by a temperature controller M01 from Magnettech with error $\pm 0.5^\circ\text{C}$.

DNA synthesis and purification

Unmodified oligonucleotides were purchased from Eurofins MWG Operon, Germany. All commercial

phosphoramidites, CPG columns and solutions for DNA synthesis were purchased from ChemGenes Corporation. Acetonitrile and pyridine were freshly distilled over calcium hydride before use. C₃ phosphoramidite was prepared according to a previously reported procedure (28,29). Oligonucleotides containing an abasic site were synthesized on an automated ASM800 DNA synthesizer (Biosset) using a trityl-off protocol and phosphoramidites with standard protecting groups on a 1.0 μmol scale (1000 Å CPG columns). The oligonucleotides were deprotected from solid support by incubation in concentrated aqueous ammonia solution at 55°C for 8 h and purified by 20% denaturing polyacrylamide gel electrophoresis (DPAGE). The oligonucleotides were visualized under UV light and the bands were excised from the gel, crushed and eluted from gel with a Tris buffer (250 mM NaCl, 10 mM Tris, 1 mM Na₂EDTA, pH 7.5). The DNA elution solutions were filtered through 0.45 μm polyethersulfone membrane (disposable filter device from Whatman) and desalted using Sep-Pak cartridge (Waters Corporation) according to manufacturer's instructions. After removing the solvent in SpeedVac, the DNAs were dissolved in deionized and sterilized water (200 μl). Concentrations of oligonucleotides were calculated from Beer's law based on measurements of absorbance at 260 nm. Extinction coefficients were determined by using the UV WinLab oligonucleotide calculator (V2.85.04, PerkinElmer).

Preparation of EPR samples

A DNA oligomer containing an abasic site and its complementary strand were mixed together (1 : 1.2) along with a stock solution of spin label (in ethanol), and evaporated in SpeedVac. The resulting residue was dissolved in phosphate buffer (10 μl; 10 mM NaHPO₄, 100 mM NaCl, 0.1 mM Na₂EDTA, pH 7.0), annealed (annealing protocol: 90°C for 2 min, 60°C for 5 min, 50°C for 5 min, 40°C for 5 min, 22°C for 15 min) and dried in SpeedVac. The resulting material was dissolved in an aqueous 30% ethylene glycol solution containing 2% DMSO (10 μl) and placed in a 50 μl quartz capillary (BLAUBRAND intraMARK) for the EPR measurements.

Determination of dissociation constants

Dissociation constants were determined as previously described (25). The EPR spectrum of the spin label (A) and the EPR spectrum of the spin label in the presence of duplex DNA that contains an abasic site (B) were normalized with respect to their double-integrated (30) area values in a MATLAB-based program to get the normalized spectra (A1 and B1). Subsequently, the A1 spectrum was manually and fractionally subtracted from B1, yielding a subtracted spectrum. Plotting the EPR spectrum of spin label ζ in an abasic DNA at -30°C (ζ fully bound) and the subtracted spectrum on the same graph, the fraction value α was adjusted to get the visual

best fit. To calculate the dissociation constant (K_d), the α was inserted into the following equation:

$$K_d = \frac{[Y - (1 - \alpha) * X][\alpha * X]}{(1 - \alpha) * X}$$

where X and Y are the initial concentrations of spin labels and DNA duplex that contains an abasic site, respectively.

Synthetic procedures

Compound 2a. A suspension of 5-bromouracil (3.5 g, 18.32 mmol) in 1,2-dichloroethane (1,2-DCE) (20 ml) was treated subsequently with HMDS (4.8 ml, 22.48 mmol) and trimethylsilyl chloride (TMS-Cl) (0.23 ml, 1.81 mmol). The reaction mixture was stirred under reflux until it became clear, cooled to 60°C and the solvent was removed *in vacuo* to yield a colorless oil. The residue was dissolved in 1,2-DCE (20 ml) and treated with iodomethane (1.71 ml, 27.46 mmol) and a catalytic amount of I₂ (0.03 g, 0.11 mmol) at 25°C. The reaction mixture was refluxed for 12 h and cooled to 25°C prior to addition of H₂O (15 ml). The precipitate was filtered off, washed with EtOAc (5 ml) and recrystallized from EtOAc to give 2a as a white solid (3.2 g, 80% yield). ¹H NMR (DMSO-*d*₆): δ 11.71 (s, 1H, NH), 8.19 (s, 1H, CH), 3.24 (s, 3H, CH₃). ¹³C NMR (DMSO-*d*₆): δ 159.80, 150.64, 146.18, 94.00, 35.43. HR-APCI-MS: *m/z* 226.9413 [M + Na]⁺ calculated for C₅H₅BrN₂O₂ 203.9534.

Compounds 2b and 2c. To a solution of 5-bromouracil (1 g, 5.23 mmol) and K₂CO₃ (0.72 g, 5.20 mmol) in DMSO (10 ml) was added a solution of alkyl halide (3.66 mmol) in DMSO (5 ml) and resulting reaction mixture stirred for 2 h at 25°C. The reaction mixture was diluted with H₂O (50 ml) in an ice-bath and stirred for 5 min. The precipitate was filtered and purified by flash column chromatography (3% MeOH in CH₂Cl₂) to yield the N3-alkylated 5-bromouracil derivatives as a white solid. 2b: yield 35%. ¹H NMR (DMSO-*d*₆): δ 11.70 (s, 1H, NH), 8.23 (s, 1H, CH), 3.70 (q, *J* = 7.1 Hz, 2H, CH₂), 1.16 (t, *J* = 7.1 Hz, 3H, CH₃). ¹³C NMR (DMSO-*d*₆): δ 159.68, 150.10, 145.14, 94.50, 43.21, 14.09. HR-APCI-MS: *m/z* 240.9575 [M + Na]⁺ calculated for C₆H₇BrN₂O₂ 217.9691. 2c: yield 30%. ¹H NMR (DMSO-*d*₆): δ 11.71 (s, 1H, NH), 8.23 (s, 1H, CH), 3.63 (t, 2H, *J* = 8, 4 Hz, CH₂), 1.59 (dd, *J* = 14.6, 7.3 Hz, 2H), 0.84 (t, *J* = 7.4 Hz, 3H). ¹³C NMR (DMSO-*d*₆): δ 159.64, 150.30, 145.39, 94.41, 49.33, 40.14, 21.65, 10.55. HR-APCI-MS: *m/z* 254.9710 [M + Na]⁺ calculated for C₇H₉BrN₂O₂ 231.9847.

Compounds 3a-c. A suspension of compounds 2a-c (3.5 mmol) in CH₂Cl₂ (25 ml) was treated with TPS-Cl (2.12 g, 7.0 mmol) and DMAP (42.7 mg, 0.35 mmol) at 0°C. Et₃N (1.9 ml, 14 mmol) was added dropwise at 0°C and the reaction mixture was stirred for 6 h, diluted with CH₂Cl₂ (20 ml) and washed sequentially with H₂O (2 × 20 ml), saturated aqueous solution of NaHCO₃ (15 ml) and brine (15 ml). The organic layer was dried over anhydrous Na₂SO₄ and the solvent was removed *in vacuo*. The residue was purified by flash column

chromatography (10% EtOAc in petroleum ether) to yield compounds 3a–c as white solids. 3a: yield 64%. ^1H NMR (CDCl_3): δ 7.76 (s, 1H, CH), 7.20 (s, 2H, 2Ar-CH), 4.39–4.19 (m, 1H, CH), 3.46 (s, 3H, CH_3), 2.99–2.83 (m, 1H), 1.29 (d, $J = 6.8$ Hz, 12H, 4 CH_3), 1.25 (d, $J = 6.9$ Hz, 6H, CH_3). ^{13}C NMR (CDCl_3): δ 163.06, 154.90, 151.68, 150.07, 130.41, 124.27, 86.29, 38.67, 34.42, 29.80, 24.65, 23.59. HR-APCI-MS: m/z 493.0737 $[\text{M} + \text{Na}]^+$ calculated for $\text{C}_{20}\text{H}_{27}\text{BrN}_2\text{O}_4\text{S}$ 470.0875. 3b: yield 44%. ^1H NMR (CDCl_3): δ 7.74 (s, 1H, CH), 7.20 (s, 2H, 2Ar-CH), 4.38–4.21 (m, 2H, 2CH), 3.85 (q, $J = 7.2$ Hz, 2H, CH_2), 2.90 (dd, $J = 13.8, 6.9$ Hz, 1H, CH), 1.34 (t, $J = 7.2$ Hz, 3H, CH_3), 1.30 (d, $J = 6.8$ Hz, 12H, 4 CH_3), 1.25 (d, $J = 6.9$ Hz, 6H, 2 CH_3). ^{13}C NMR (CDCl_3): δ 162.83, 154.88, 153.03, 151.67, 149.06, 130.44, 124.27, 86.39, 46.66, 34.42, 29.80, 24.66, 23.59, 14.32. HR-APCI-MS: m/z 507.0904 $[\text{M} + \text{Na}]^+$ calculated for $\text{C}_{21}\text{H}_{29}\text{BrN}_2\text{O}_4\text{S}$ 484.1031. 3c: yield 48%. ^1H NMR (CDCl_3): δ 7.72 (s, 1H, CH), 7.20 (s, 2H, 2Ar-CH), 4.38–4.19 (m, 2H, 2CH), 3.80–3.68 (m, 2H, CH_2), 2.90 (dt, $J = 13.8, 6.9$ Hz, 1H, CH), 1.75 (dd, $J = 14.9, 7.4$ Hz, 2H, CH_2), 1.29 (d, $J = 6.7$ Hz, 12H, 4 CH_3), 1.25 (d, $J = 6.9$ Hz, 6H, 2 CH_3), 0.93 (t, $J = 7.4$ Hz, 3H, CH_3). ^{13}C NMR (CDCl_3): δ 162.79, 154.88, 153.12, 151.68, 149.51, 130.41, 124.27, 86.14, 53.17, 34.41, 29.79, 24.66, 23.58, 22.18, 11.05. HR-APCI-MS: m/z 521.1047 $[\text{M} + \text{Na}]^+$ calculated for $\text{C}_{22}\text{H}_{31}\text{BrN}_2\text{O}_4\text{S}$ 498.1188.

Compounds 5a–c. A solution of compounds 3a–c (0.50 mmol) in CH_2Cl_2 (10 ml) was treated sequentially with amino phenol 4 (27) (0.124 g, 0.6 mmol) followed by Et_3N (0.14 ml, 1.0 mmol). The resulting reaction mixture was stirred at 25°C for 48 h in the dark, the solvent removed *in vacuo* and the oily residue was triturated with CH_3CN (2 ml). The precipitated product was filtered and dried *in vacuo* and used for the next step without further purification. 5a: yield 80%. ^1H NMR (95:5 CDCl_3 : CD_3OD): δ 8.47 (s, 1H, CH), 7.96 (s, 1H, NH), 7.62 (s, 1H, Ar-CH), 6.58 (s, 1H, Ar-CH), 3.37 (s, 3H, CH_3), 1.69 (s, 6H, 2 CH_3), 1.61 (s, 5H, 2 CH_3). ^{13}C NMR (95:5 CDCl_3 : CD_3OD): δ 148.04, 145.98, 129.67, 113.31, 106.54, 96.60, 68.61, 67.80, 37.90, 29.59, 28.43. HR-APCI-MS: m/z 393.0896 $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{17}\text{H}_{21}\text{BrN}_4\text{O}_2$ 392.0848. 5b: yield 75%. ^1H NMR (95:5 CDCl_3 : CD_3OD): δ 7.97 (s, 1H, CH), 7.56 (s, 1H, Ar-CH), 6.59 (s, 1H, Ar-CH), 3.80 (dd, $J = 14.0, 6.9$ Hz, 2H, CH_2), 1.46 (s, 6H, 2 CH_3), 1.40 (s, 6H, 2 CH_3), 1.29 (t, $J = 7.0$ Hz, 3H, CH_3). ^{13}C NMR (95:5 CDCl_3 : CD_3OD): δ 157.16, 155.43, 147.69, 144.73, 138.56, 126.13, 114.44, 108.66, 88.72, 64.15, 63.78, 45.84, 30.77, 30.64, 29.69, 14.41. HR-APCI-MS: m/z 407.1052 $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{18}\text{H}_{23}\text{BrN}_4\text{O}_2$ 406.1004. 5c: yield 78%. ^1H NMR (95:5 CDCl_3 : CD_3OD): δ 8.06 (s, 1H, CH), 7.54 (s, 1H, Ar-CH), 6.61 (s, 1H, Ar-CH), 3.75–3.68 (m, 2H, CH_2), 1.71 (dd, $J = 14.7, 7.3$ Hz, 2H, CH_2), 1.54 (s, 6H, 2 CH_3), 1.47 (s, 6H, 2 CH_3), 0.91 (t, $J = 7.4$ Hz, 3H, CH_3). ^{13}C NMR (95:5 CDCl_3 : CD_3OD): δ 157.13, 155.58, 147.90, 145.22, 126.57, 114.30, 108.42, 100.06, 88.51, 52.37, 30.26, 30.14, 22.36, 10.90. HR-APCI-MS: m/z 421.1199 $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{18}\text{H}_{23}\text{BrN}_4\text{O}_2$ 420.1161.

Compounds 6a–c. A solution of compounds 5a–c (0.2 mmol) in EtOH (5 ml) was treated with CsF (0.303 g, 2.0 mmol) and stirred at 85°C for 3 days. The solvent was evaporated and the crude product was purified by flash column chromatography [4% MeOH in CH_2Cl_2 , containing 1% NH_3 (aq)] to give cyclized compounds 6a–c as yellowish solids. 6a: yield 40%. ^1H NMR (CDCl_3): δ 7.42 (s, 1H, CH), 6.74 (s, 1H, Ar-CH), 6.44 (s, 1H, Ar-CH), 3.33 (s, 3H, CH_3), 1.43 (s, 6H, 2 CH_3), 1.39 (s, 6H, 2 CH_3). ^{13}C NMR (CDCl_3): δ 154.82, 154.73, 144.87, 144.42, 142.18, 128.02, 126.30, 125.90, 111.84, 108.16, 62.89, 62.77, 37.40, 31.95, 31.87. HR-APCI-MS: m/z 313.1658 $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{17}\text{H}_{20}\text{N}_4\text{O}_2$ 312.1586. 6b: yield 45%. ^1H NMR (CDCl_3): δ 7.54 (s, 1H, CH), 6.76 (s, 1H, Ar-CH), 6.42 (s, 1H, Ar-CH), 3.74 (q, $J = 7.2$ Hz, 2H, CH_2), 1.43 (s, 6H, 2 CH_3), 1.39 (s, 6H, 2 CH_3), 1.31 (t, $J = 7.2$ Hz, 3H, CH_3). ^{13}C NMR (CDCl_3): δ 154.61, 154.08, 144.53, 144.22, 142.00, 127.94, 126.13, 124.63, 111.79, 107.85, 62.74, 62.62, 44.82, 31.78, 31.70, 14.24. HR-APCI-MS: m/z 327.1791 $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{18}\text{H}_{22}\text{N}_4\text{O}_2$ 326.1743. 6c: yield 44%. ^1H NMR (CDCl_3): δ 7.49 (s, 1H, CH), 6.73 (s, 1H, Ar-CH), 6.43 (s, 1H, Ar-CH), 3.69–3.62 (m, 2H, CH_2), 1.74 (dd, $J = 14.5, 7.3$ Hz, 2H, CH_2), 1.42 (s, 6H, 2 CH_3), 1.38 (s, 6H, 2 CH_3), 0.97 (t, $J = 7.4$ Hz, 3H, CH_3). ^{13}C NMR (CDCl_3): δ 154.50, 154.21, 144.60, 144.26, 142.02, 127.73, 126.20, 125.10, 111.83, 107.87, 62.71, 62.59, 51.33, 31.78, 31.72, 22.20, 11.02. HR-APCI-MS: m/z 341.1973 $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{19}\text{H}_{24}\text{N}_4\text{O}_2$ 340.1899.

Spin labels 7a–c. To a solution of compounds 6a–c (0.033 mmol) in CH_2Cl_2 (10 ml) was added a solution of *m*CPBA (8.6 mg, 0.05 mmol) in CH_2Cl_2 (2 ml) at 0°C. The resulting reaction mixture was stirred for 6 h at 0°C and concentrated *in vacuo*. The residue was purified by column chromatography (2% MeOH in CH_2Cl_2) to afford spin labels 7a–c as pale yellow solids. 7a: yield 60%. ^1H NMR (CDCl_3): δ 9.17 (s, 1H), 8.61 (bs, 1H), 3.23 (s, 1H). HR-APCI-MS: m/z 328.1534 $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{17}\text{H}_{19}\text{N}_4\text{O}_3$ 327.1457. 7b: yield 58%. ^1H NMR (CDCl_3): δ 6.70 (bs, 1H), 3.90 (bs, 2H, CH_2), 1.40 (bs, 6H). HR-APCI-MS: m/z 342.1686 $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{18}\text{H}_{21}\text{N}_4\text{O}_3$ 341.1614. 7c: yield 65%. ^1H NMR (CDCl_3): δ 6.55 (s, 1H), 3.75 (s, 4H), 1.91–1.82 (m, 2H), 1.26 (s, 7H), 0.92–0.74 (m, 10H). HR-APCI-MS: m/z 356.1859 $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{19}\text{H}_{23}\text{N}_4\text{O}_3$ 355.1843. Note: due to the paramagnetic nature of nitroxides the NMR spectra of these compounds shows significant broadening of the signals for which reason some peaks, particularly of nuclei close to the radical, are not seen in the spectra.

RESULTS AND DISCUSSION

Effect of flanking sequences on spin label binding

Abasic sites in nucleic acids have been used as receptors for binding small organic ligands (31–36). A major factor that governs the binding of ligands to abasic sites is hydrogen bonding with the orphan base on the complementary strand, opposite to the abasic site (33–36).

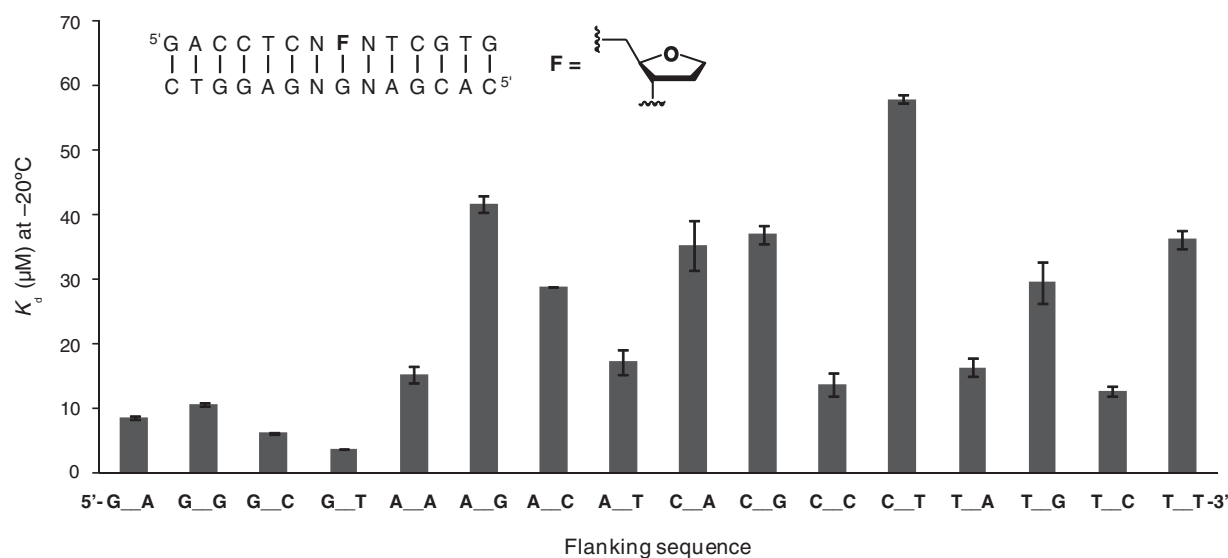


Figure 2. A plot of the dissociation constants of ̑-binding to duplex DNA containing an abasic site as a function of flanking sequence (150 μ M duplex DNA, 100 μ M ̑, phosphate buffer, pH 7.0, 30% ethylene glycol, 2% DMSO). Insert shows the DNA construct used for this study, where N represents the nucleobases A, G, C and T.

We have previously shown that spin label ̑ binds most efficiently when paired to a guanine (G), with which it forms three hydrogen bonds; when paired with adenine (A), cytosine (C) or thymine (T) the binding affinity was 4-to 6-fold lower (25). In addition to hydrogen bonding, it has been shown that stacking interactions with the flanking nucleobases also contribute to binding (33,35,37). However, the influence of the base-pairs flanking the abasic site on the binding affinity of ̑ remains unanswered.

To interrogate the flanking sequence dependence of ̑-binding, a series of 16 14-mer oligonucleotide duplexes, containing all possible combinations of base pairs immediately flanking the abasic site, were prepared and incubated with ̑. The EPR spectra were recorded in an aqueous solution containing 30% ethylene glycol and 2% DMSO. Ethylene glycol is frequently used as a cryoprotectant for pulsed EPR experiments (38,39) and DMSO was added to avoid precipitation of ̑ at low temperatures. Upon visual inspection of the EPR spectra, a high degree of variation was observed for different flanking sequences (Figure S1). To quantify the extent of binding of the spin label, dissociation constants (K_d) were determined at -20°C (Table S1) and subsequently plotted as a function of the flanking sequence (Figure 2). Binding of the spin label is strongly flanking-sequence dependent, varying as much as 15-fold. It is worth noting that the identity of the nucleotide present at the 5'-side of the abasic site has a large effect, which is in consistent with the reported data for the binding of alloxazine to an abasic site (33). The spin label binds most efficiently when the abasic site is flanked by a 5'-dG; the sequence 5'-d(G__T) showed the highest affinity. In contrast, the binding is considerably worse when the abasic site is flanked by 5'-dC, with the sequence 5'-d(C__T) binding most poorly of all flanking sequences. It is also noteworthy that in

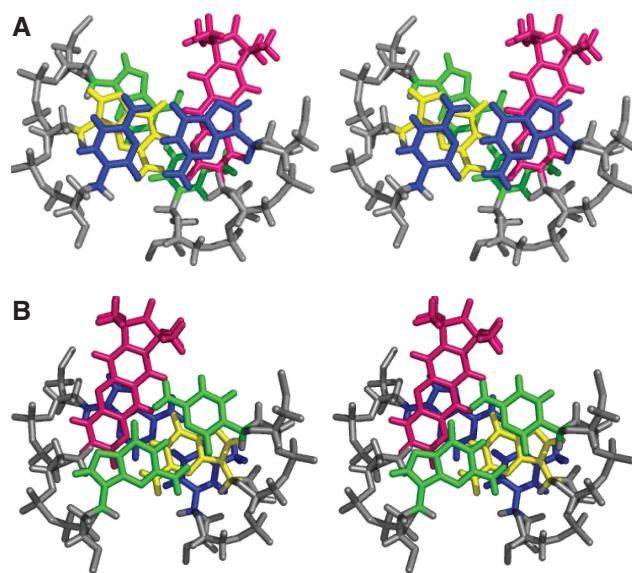


Figure 3. Overhead stereo views of a model of a B-DNA duplex containing ̑ (magenta) bound to an abasic site at the flanking sequences 5'-d(G__T) (A) and 3'-d(G__A) (B). The base pairs on the 3'- and 5'-side of the abasic site are shown in green and blue, respectively.

general, a 3'-dG disfavored the spin label binding relative to the other bases, irregardless of what the flanking base on the 5'-side is.

It is perhaps not surprising that there is relatively larger variation in K_d as the identity of the 5'-flanking base is changed, because it makes a large contribution to stacking. Figure 3 shows how the right twist of B-DNA results in a much larger stacking area between the 5'-base and ̑ than with the 3'-base. It is noteworthy that a 5'-G contributes more to binding than an A, both of which are purines. This could be due to the higher polarity of G and

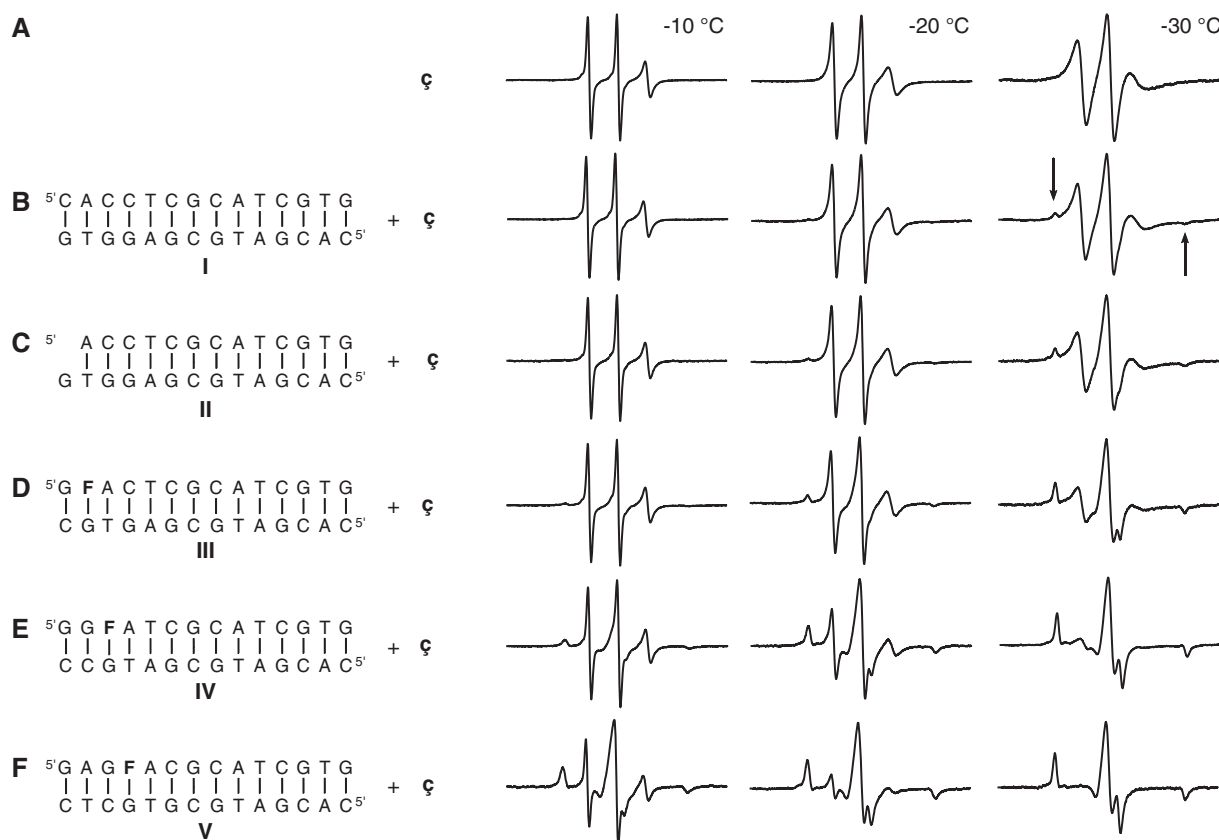


Figure 4. Binding of ϕ to an unmodified B-DNA duplex and to DNAs containing abasic sites at different locations relative to the duplex end. (A). EPR spectra of spin label ϕ . (B). EPR spectra of ϕ (100 μ M) in the presence of an unmodified DNA duplex (150 μ M). (C–F). EPR spectra of spin label ϕ in presence of DNA duplexes I–V containing an abasic site (F). Arrows indicate peaks associated with a slow-moving component of the spectrum that reflects mobility of ϕ bound to DNA.

C (40), which results in the GC base pair being more polarized (40). This may explain why ϕ , which is a C-analogue, has relatively good binding affinities toward the sequences 5'-d(G__G, G__C, and C__C), compared to flanking sequences containing AT base pairs (41).

While considering the effect of flanking bases on ϕ binding one also needs to consider that the orphan base on the other strand can be in a dynamic equilibrium between intrahelical and extrahelical conformation, depending on the nature of the flanking base pairs (42–44). However, an extrahelical conformation of the orphan base, which collapses the abasic site, is more pronounced for pyrimidines than purines. For example, a pyrimidine orphan base that is flanked by pyrimidines has a high propensity to flip out, whereas it tends to stack when flanked by two purines (43). Thus, the flanking nucleobases not only affect the binding affinity through stacking with ϕ but may also influence the structural integrity of the abasic site.

Dependence of spin-label binding on the location of the abasic site

Since base pairs at the ends of duplexes are more dynamic than those present towards the center (45,46), abasic sites that are placed close to the duplex termini may not have the structural integrity that is required for efficient binding

of ϕ . To investigate the effect of the abasic site location, eight different 14-mer DNA duplexes were prepared such that the abasic site was moved one base pair at a time from the duplex end towards its center (Figure 4). The structural components of the abasic site were retained by keeping the same flanking nucleobases and the same orphan base (G) on the opposite strand.

The EPR spectrum of ϕ in the presence of an unmodified duplex at -30 °C shows small peaks at higher and lower field (arrows in Figure 4B), indicative of a slow-moving component that is consistent with a small amount of non-specific ϕ -binding to DNA I (Figure 4B). DNA II contains a G-overhang and provides a potential binding site for ϕ (Figure 4C); if the G-overhang stacks on top of the duplex (41), it forms a partial abasic site that can provide stacking on one side and base pairing. A similar amount of binding (ca. <5%) of ϕ was observed for DNAs I and II, indicating that the spin label requires a pocket formed by flanking bases on both sides. In fact, at the next site (DNA III), significantly more spin-label binding was observed (Figure 4D). As the abasic site was moved further toward the center of the duplex, the binding of ϕ continued to increase until it became fully bound at the fourth position from the terminus (Figure 4F, -30 °C). A similar binding trend was also observed from the 3'-end (data not shown).

Thus, structural integrity of the abasic site that is required for the efficient binding of ζ is compromised when the abasic site is placed close to a duplex end.

Spin-label binding to an abasic site prepared by a C_3 -spacer (C_3)

We next investigated how spin label binding was affected by changing the backbone of the abasic site from the tetrahydrofuran analogue (**F**) to the more flexible C_3 -spacer (C_3) (28). C_3 (derived from 1, 3-propanediol) is an acyclic analogue of 2'-deoxyribofuranose, which mimics the C-3/C-5 carbon chain of DNA backbone, but lacks the 1' and 2' carbons of the sugar as well as the nucleobase (Figure 5A). 14-mer DNA duplexes containing either **F** or C_3 abasic sites were incubated with ζ at three different temperatures and the EPR spectra compared (Figure 5B and C). The spin label ζ binds with slightly higher affinity to **F** than C_3 at -10°C and -20°C . At -30°C , however,

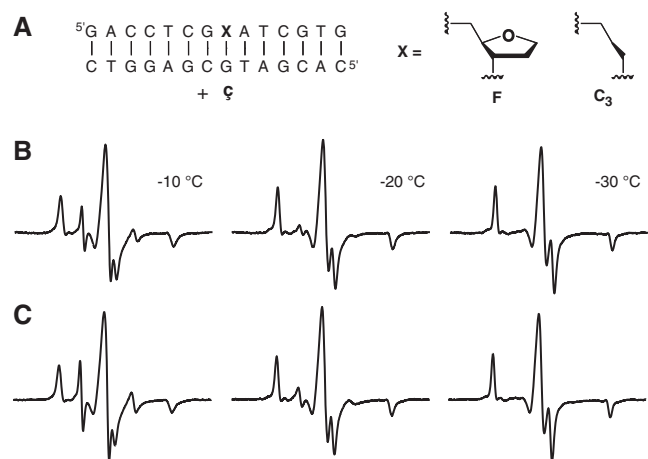


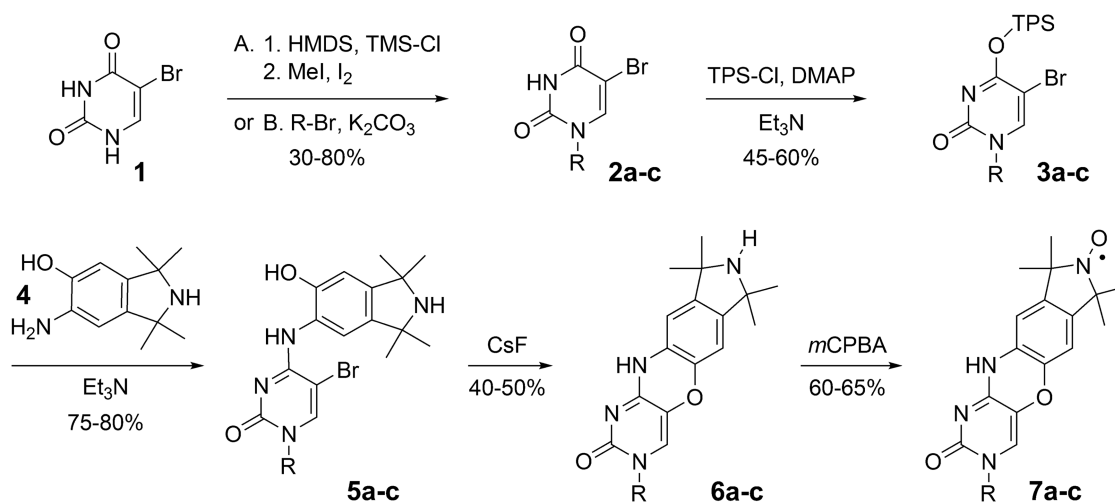
Figure 5. Binding of spin label ζ (200 μM) to DNA duplexes (400 μM) containing the abasic sites **F** or C_3 . (A). The structure of a DNA duplex, the abasic sites **F** and C_3 . (B). EPR spectra of ζ in the presence of a DNA duplex containing the abasic site **F**. (C). EPR spectra of ζ in the presence of duplex DNA that contains the abasic site C_3 .

ζ is fully bound to the DNA duplexes containing both the abasic site **F** and C_3 . These data indicate that the change in the backbone of the abasic site does not appreciably affect the binding affinity of ζ .

Effect of structural modification of ζ on its binding affinity

To further probe the interactions of ζ with the abasic site, we synthesized several N3-derivatives of the spin label (Scheme 1). Since the abasic site is hydrophobic in nature we introduced alkyl substituents, specifically methyl, ethyl and propyl groups. The alkyl derivatives of ζ were synthesized by a strategy similar to the one used for the preparation of spin label ζ (Scheme 1) (25).

The regioselective alkylation of 5-bromouracil at the N1 position was achieved by either of two methods. For the methyl derivative (7a), alkylation was done by a one-pot, two-step reaction: silylation with 1,1,1,3,3,3-hexamethyldisilazane (HMDS), followed by alkylation with methyl iodide in the presence of a catalytic amount of iodine to afford 2a in good yields. For the ethyl- and propyl-derivatives, this method resulted in poor yields, even after long reaction times, presumably because of the lower reactivities of ethyl and propyl bromides. Instead, the alkylations were performed by treatment with the corresponding bromides in presence of K_2CO_3 in DMSO (47). This procedure afforded the mono- as well as the dialkylated products, which were separated by column chromatography to yield N1-ethyl- (2b) and N1-propyl- (2c) 5-bromouracil in moderate yields. The N1-alkylated compounds 2a–c were reacted with 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl) to get the O^4 -TPS derivatives 3a–c, that were subsequently coupled with amino phenol 4 (27) in the presence of triethylamine to yield conjugates 5a–c. The coupled products were cyclized to phenoxazine derivatives 6a–c by treatment with cesium fluoride, and finally oxidized with meta-chloroperoxybenzoic acid (*m*CPBA) to afford the spin label derivatives 7a–c.



Scheme 1. Synthesis of N3-alkyl derivatives of spin label ζ . Letters next to the compound numbers indicate the identity of **R** (a: **R** = $-\text{CH}_3$; b: **R** = $-\text{CH}_2\text{CH}_3$ and c: **R** = $-\text{CH}_2\text{CH}_2\text{CH}_3$).

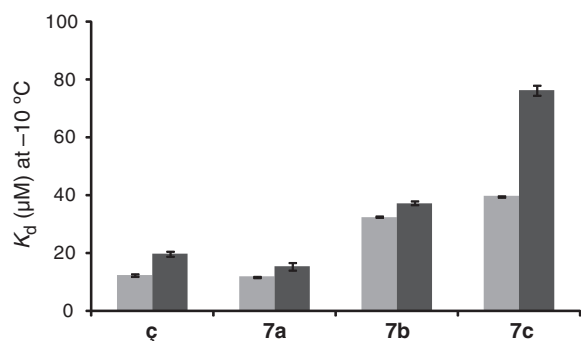


Figure 6. A plot of dissociation constants of spin label ϕ and its alkyl derivatives (200 μM) in presence of a DNA duplex (400 μM) containing the abasic site **F** (light gray) or C_3 (dark gray) at -10°C . Structure of the DNA duplex and the abasic sites are shown in Figure 5A.

Spin labels 7a–c as well as ϕ , were incubated with 14-mer DNA duplexes containing either **F** or C_3 as the abasic site and the EPR spectra recorded at different temperatures. The EPR spectra revealed a variation between the different compounds (Figure S2), indicating that the alkyl derivatives have different binding affinities. The dissociation constants for all the spin labels were subsequently determined at -10°C (Figure 6). For **F**, spin label 7a binds with similar affinity as ϕ ; however, the binding affinity of derivatives 7b and 7c decreased by 2.6- and 3.2-fold, respectively (Table S2). These results indicate that, as the size of the alkyl chain at the N3 position of the spin label increases its binding affinity decreases. The binding affinities of spin labels ϕ , 7a and 7b were similar for DNAs containing either the abasic site **F** (Figure 6, light gray bars) or C_3 (Figure 6, dark gray bars). The abasic site **F** showed slightly better binding to ϕ and 7a–b than C_3 whereas, **F** bound the propyl derivative (7c) with 2-fold higher affinity than C_3 .

CONCLUSIONS

We have shown that the binding of spin label ϕ to an abasic site in duplex DNA is highly flanking-sequence dependent, with up to 15-fold difference in binding affinities between flanking sequences. This variation is on the same order of magnitude as variation in dissociation constants upon changing the base-pairing partner of ϕ (25). The 5'-nucleotide is the main factor in flanking-sequence dependence of spin labeling. In particular a 5'-G favors spin label binding, with the sequence 5'-d(G__T) binding the spin label with the highest efficiency and 5'-d(C__T) the lowest. Furthermore, the abasic site needs to be located at least four base pairs from the duplex end for efficient binding. Changing the structure of the abasic site from the tetrahydrofuran analogue (**F**) to C_3 does not appreciably affect the binding of ϕ . Finally, N3-alkyl derivatives of the spin label were prepared and binding studies showed that the increased carbon chain length at the N3-position of the spin label decreases its binding affinity to both **F** and C_3 . These findings provide detailed insights into a structure–function relationship of ligand binding to abasic

sites, facilitated by straight forward quantification of binding by EPR spectroscopy. The data will guide the design of abasic site receptors and spin label ligands for NC–SDSL of nucleic acids.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2, Supplementary Figures 1 and 2, and NMR spectra of synthetic compounds.

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