Nitroxide-Derived *N*-Oxide Phenazines for Noncovalent Spin-Labeling of DNA

Haraldur Y. Juliusson[a] and Snorri Th. Sigurdsson*[a]

Two *o*-benzoquinone derivatives of isoindoline were synthesized for use as building blocks to incorporate isoindoline nitroxides into different compounds and materials. These *o*-quinones were condensed with a number of *o*-phenylenediamines to form isoindoline-phenazines in high yields. Subsequent oxidation gave phenazine-di-*N*-oxide isoindoline nitroxides that were evaluated for noncovalent and site-directed spin-labeling of duplex DNA and RNA that contained abasic sites. Although only minor binding was observed for RNA, the unsubstituted phenazine-*N,N*-dioxide tetramethyl isoindoline nitroxide showed high binding affinity and selectivity towards abasic sites in duplex DNA that contained cytosine as the orphan base.

Introduction

The field of radical chemistry has advanced considerably since the isolation and characterization of Fremy's salt (**1**; Figure 1), an inorganic compound containing a nitroxide, described in 1845.[1] Over the years, the diversity of organic nitroxide radicals, a.k.a. aminoxyl radicals, has steadily increased. Of note is the synthesis of 2,2,6,6-tetramethyl-4-piperidone-1-oxyl (4-oxo-TEMPO; **2**; Figure 1), described by Lebedev et al. in 1960.[2] The subsequent discovery of Rozantsev and Neiman that nitroxides can be involved in various organic reactions without affecting the aminoxyl group,[3] opened the door for the synthesis of numerous structurally diverse radicals.[4] Stable nitroxide radicals have been used in a variety of applications, such as antioxidants,[5] free-radical scavengers,[6] stabilizers in the materials industry,[7] catalytic oxidizers[8] and site-directed spin labels.[9]

Isoindoline-based nitroxides (**3**; Figure 1) have been the subject of much attention because the fusion of the nitroxide-containing ring to the aromatic ring provides rigidity and renders them resistant to ring opening reactions, which is a significant decomposition pathway for pyrrolidine and piperidine nitroxides.[10] Isoindoline-nitroxides also have narrower electron paramagnetic resonance (EPR) line widths,[11] and the aromatic ring, which can undergo a variety of substitution reactions, can be used to build more complex structures for a wide range of applications.

Nitroxides have been used extensively as spin labels to study the structure and dynamics of biomolecules,[9] in particular nucleic acids and proteins. The term site-directed spin-labeling (SDSL) refers to the process of incorporation of a spin label at specific sites on diamagnetic biopolymers. For nucleic acid spin-labeling, there are three main approaches, namely the phosphoramidite approach, post-synthetic labeling and non-covalent labeling. The first two methods rely on covalent attachment of the spin labels that usually require time-consuming and nontrivial synthetic transformations. On the other hand, noncovalent spin-labeling uses binding through van der Waals interactions, hydrogen bonding and π-stacking. There are reports of spin-labeled intercalators and groove binders that bind to nucleic acids noncovalently, but most of them lack the sequence specificity that is usually required for EPR studies.[12] Noncovalent site-directed spin-labeling of the malachite green (MG) RNA aptamer has recently been reported, which utilized a spin-labeled derivative of tetramethylrosamine (TMR), and is the first example of site-specific spin labeling of a completely unmodified RNA.[13] Abasic sites in duplex nucleic acids have also been used as binding sites for noncovalent binding of small molecules. For example, fluorescent compounds that bind to abasic sites have been used for the detection of single nucleotide polymorphisms (SNPs).[14] An adenine acridine-derived spin-label has been used as an intercalator for noncovalent spin-labeling of a DNA duplex containing an abasic site.[15] The spin label **ç**, a cytosine-based spin label, bound specifically to abasic sites in duplex DNA opposite guanine (G).[16] The semi-flexible G-spin (**Ġ**), a guanine-based spin label, was found to bind to abasic sites in duplex RNA opposite cytosine (C) with high affinity.[17] In spite of these advances, there is still demand for new methods for making

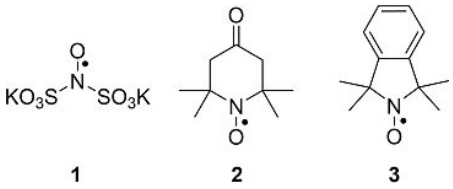

Figure 1. Structures of common aminoxyl radicals: Fremy's salt (**1**), 2,2,6,6-tetramethyl-4-piperidone-1-oxyl (4-oxo-TEMPO) (**2**), and an isoindoline nitroxide (**3**).

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different functionalized nitroxide radicals that might possess improved qualities for noncovalent spin-labeling, and other applications.

Phenazines are an interesting class of compounds as they are redox-active and can therefore accept and donate electrons.[18] They are present in several natural products that are produced by Gram-positive and Gram-negative bacteria or by archaeal *Methanosarcina* species.[19] Phenazines display various interesting biologically relevant functions[20] such as DNA-intercalation,[20–21] inhibition of topoisomerases,[22] production of reactive oxygen species (ROS)[23] and radical scavenging.[24] Oxidized phenazines, phenazine-di-*N*-oxides (**4**; Figure 2), have also been reported to be good intercalators into duplex DNA.[25] The synthesis of substituted phenazines presents a challenge since most of the synthetic methods that have been reported are low yielding and can tolerate a limited number of functional groups.[20] Early approaches of synthesizing phenazines, such as the Wohl-Aue procedure[26] (1901) and the Bamberger-Ham reaction[27] (1911) were performed under harsh conditions, were low yielding and formed a significant number of byproducts. In the mid-1960s, Haddadin and coworkers reported the Beirut reaction[28] which addressed some of the limitations of phenazine substituents met in earlier procedures. The synthesis of phenazines through Pd-catalyzed coupling was reported in late 2000[29] and further improved in 2013 by Laha et al.[30] The condensation of *o*-phenylenediamines with *o*-benzoquinones, generated in situ from the corresponding catechols, has also been an important route that directly gives phenazines in moderate to high yields.[20,31]

Here we describe the synthesis of *o*-benzoquinone derivatives of isoindoline that were condensed with a number of commercially available *o*-phenylenediamines to form isoindoline-phenazine skeletons. Oxidation of the isoindoline-phenazine moieties gave isoindoline-phenazine-*N,N*-dioxide nitroxide radicals, such as **5** (Figure 2), that were evaluated for noncovalent spin-labeling of nucleic acids. Compound **5** showed extensive binding affinity and selectivity towards abasic sites in DNA.

Results and Discussion

The main incentive of this study was to prepare an isoindoline skeleton that would enable easy synthesis of structurally diverse nitroxide radicals, in particular by condensation with *o*-phenylenediamines to create isoindoline-phenazine structures. We designed *o*-benzoquinone derivatives of both tetramethyl (**6**) and tetraethyl (**7**) isoindoline, as tetraethyl isoindoline nitroxides are more resistant to reduction,[32] for condensation with *o*-phenylenediamines.

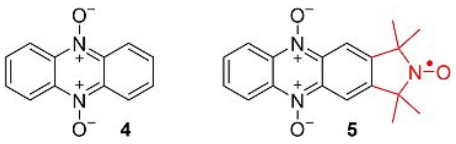


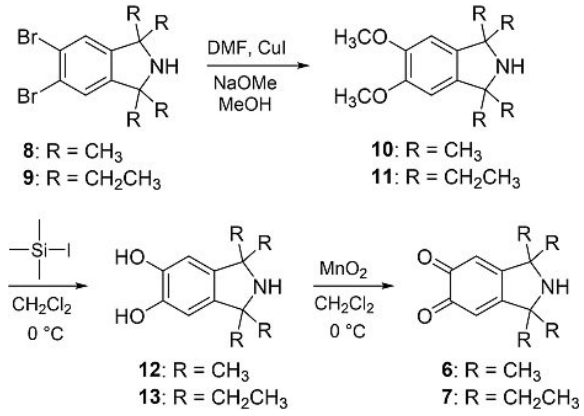
Figure 2. Structures of phenazine-di-*N*-oxide (**4**) and an isoindoline nitroxide-derived phenazine-di-*N*-oxide (**5**).

Synthesis of *o*-quinone isoindolines 6 and 7

The synthesis of **6** and **7** (Scheme 1) started with substitution of the bromines on tetramethylisoindoline **8**[33] and tetraethylisoindoline **9**[34] with methoxide to yield compounds **10**[35] and **11**, respectively. Initial attempts to prepare *o*-quinone derivatives **6** and **7** from **10** and **11**, respectively, by AgO and nitric acid-assisted oxidation[36] gave **6** and **7** only in very low yields. Therefore, dihydroxyl derivatives **12** and **13** were first prepared by demethylation of **10** and **11**, respectively. Reactions of **10** and **11** with boron tribromide or iodotrimethylsilane, commonly used for demethylation of methyl ethers,[37] under a variety of conditions led to complex mixtures of products, which at best resulted in very low yields of **12** and **13**. However, demethylation with iodotrimethylsilane, followed by a water-free work-up and precipitation gave **12** and **13** in excellent yields. *O*-quinones **6** and **7** were subsequently obtained in excellent yields by oxidation of **12** and **13** with MnO_2 in the presence of air.[38]

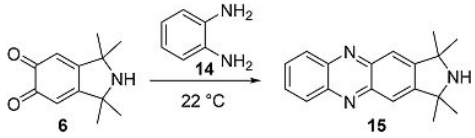
Condensation of 6 and 7 with o-phenylenediamines to give phenazines

Several different conditions were evaluated for condensation of **6** with *o*-phenylenediamine **14** (Table 1) to obtain **15**. Most of the solvents that were evaluated resulted in moderate to excellent yields. Acetic acid (Table 1, entry 3) and EtOH (70% in H_2O) (Table 1, entry 5) proved to be the best conditions. Using catalytic amount of a base led only to rapid decomposition of the diketones (Table 1, entries 6–8). In contrast, acetic acid was very effective as a catalyst. The optimal conditions proved to be 70% EtOH (aq.) with catalytic amount of acetic acid (Table 1, entry 11).



Scheme 1. Synthesis of *o*-quinone isoindolines **6** and **7**. Yields: **10** (95%), **11** (62%), **12** (91%), **13** (77%), **6** (88%), **7** (93%).

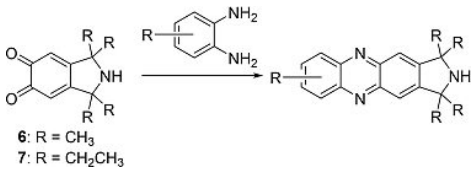
Table 1. Evaluation of reaction conditions for the synthesis of phenazine **15** from *o*-quinone **6**.



	Solvent	Catalyst	*t* [min]	Yield [%]
1	EtOH	–	40	90
2	CH_2Cl_2	–	60	70
3	AcOH	–	<1	95
4	CH_3CN	–	50	83
5	70% EtOH	–	30	94
6	70% EtOH	KOH	<5	0
7	EtOH	pyridine	10	<1
8	EtOH	Et_3N	10	<5
9	CH_3CN	AcOH	10	95
10	CH_2Cl_2	AcOH	10	95
11	70% EtOH	AcOH	<1	99
12	EtOH	AcOH	5	95

A number of commercially available *o*-phenylenediamines with different functional groups were condensed with *o*-benzoquinones **6** and **7** to both investigate the scope of the condensation reaction and to create a small library of compounds for noncovalent spin-labeling (Table 2). All the selected *o*-phenylenediamines condensed readily with the diketones to give the desired products in near quantitative yields (>93%; Table 2, entries 1–6), except for **24** (Table 2, entry 7) which did not give the desired product using any of the reaction conditions listed in Table 1. We postulate that the low solubility of **24** was the cause; low rate of condensation led to decomposition of *o*-quinones **6** and **7**, both of which had limited stability in solution at room temperature (complete decomposition in 1–2 h). Compound **24** was soluble in DMSO but did not yield any condensed products upon incubation with **6** (in the presence or absence of a catalytic amount of acetic acid).

Table 2. Condensation of **6** and **7** with different derivatives of *o*-diamines.



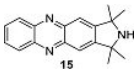
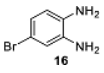
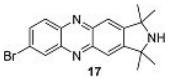
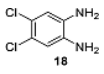
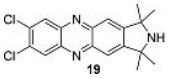
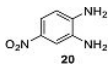
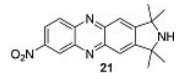
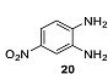
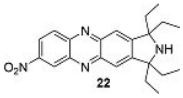
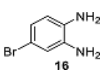
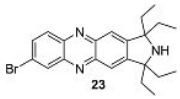
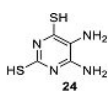
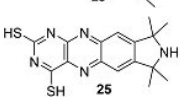
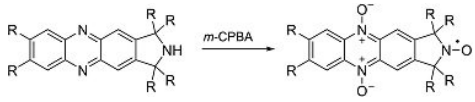
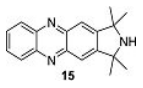
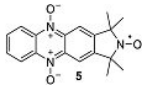
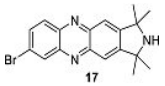
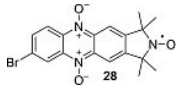
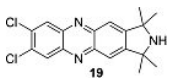
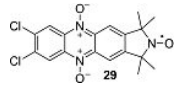
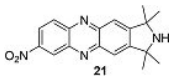
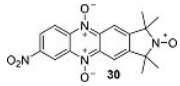
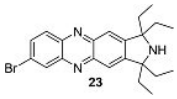
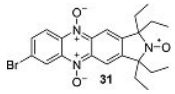
Diamine	*o*-quinone	Product	Yield [%]
	**6**		99
	**6**		95
	**6**		95
	**6**		93
	**7**		90
	**7**		96
	**6**		0

Table 3. Oxidation of isoindoline-phenazines to the corresponding isoindoline-phenazine di-N-oxide nitroxide radicals.



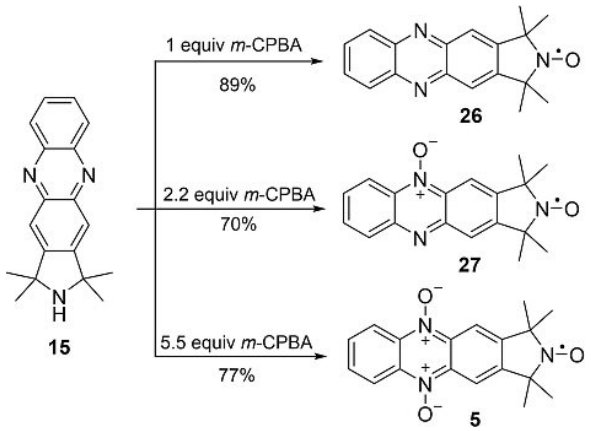
Starting compound	Product	Yield [%]
		77
		72
		70
		78
		75

Phenazine-*N*-oxide derived nitroxides

While oxidizing the phenazines with *m*-CPBA, it became apparent that the level of oxidation could be controlled by the number of equivalents of the oxidizing agent used in the reaction. When **15** was treated with one equivalent of *m*-CPBA, only nitroxide **26** was obtained (Scheme 2). The mono-*N*-oxide aminoxyl radical **27** was obtained with 2.2 eq. of the oxidizing agent and 5.5 equiv. gave the di-*N*-oxide radical **5** (Scheme 2), all in very good to excellent yields.

For evaluating the isoindoline nitroxide-derived phenazines as noncovalent spin labels for duplex DNA and RNA containing abasic sites, the di-*N*-oxides were prepared, since **26** and **27** had limited or no solubility in water. Oxidation of compounds **15**, **17**, **19**, **21** and **23** gave the desired phenazine di-*N*-oxide radicals **5** and **28**–**31** in very good yields (Table 3).

Since NMR spectra of the paramagnetic phenazine di-*N*-oxide nitroxides give limited information due to peak-broadening and even loss of NMR signals, UV-vis spectroscopy was used for spectroscopic characterization of the di-*N*-oxides (Figure S30), in addition to HRMS. Absorption peaks at 250 and



Scheme 2. Reaction of phenazine **15** with different amounts of oxidizing agent.

370 nm for the phenazine derivatives were red-shifted to 290 and 490 nm upon oxidation to the di-*N*-oxide radicals. The phenazine di-*N*-oxide skeleton rendered the radicals water soluble to some extent, with the exception of the tetraethyl derivative **31**, which made it possible to evaluate almost all as spin labels.

Spin-labeling of abasic sites in duplex oligonucleotides

Binding of **5**, **28**, **29** and **30** to abasic sites in DNA duplexes was evaluated by EPR spectroscopy.[16] Each spin label was incubated with a 14-mer duplex DNA containing an abasic site with either G, T, A or C as the orphan base at −30 °C (Figure 3). The EPR spectrum of each spin label in the absence of DNA (Figure 3, top row) showed comparatively narrow lines, consistent with fast tumbling of the radical in solution. In contrast, an EPR spectrum of a spin label bound to a DNA duplex is wider due to a longer rotational correlation time,[16] as observed for spin label **5**, which showed high binding affinity and specificity

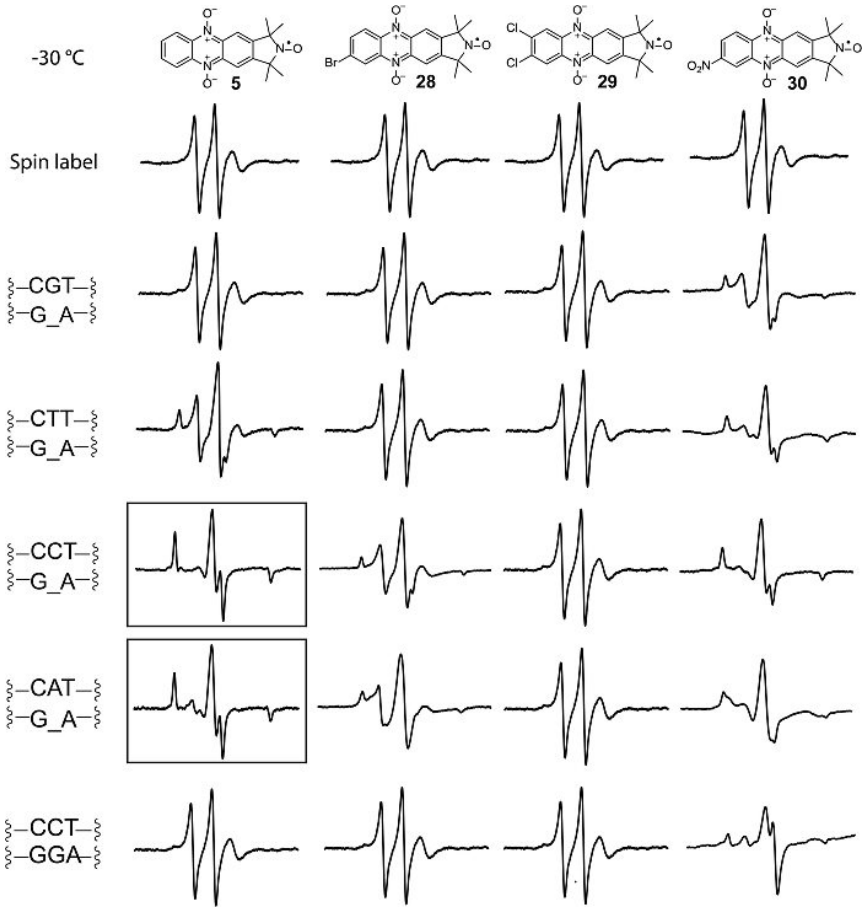


Figure 3. Noncovalent spin labeling of abasic sites in DNA duplexes evaluated by EPR spectroscopy at −30 °C. EPR spectra of the labels without DNA (top row) and in the presence of unmodified DNA (bottom row) are shown for comparison. The central four rows show the spin labels in the presence of DNA duplexes containing an abasic site (denoted by "_") opposite the orphan bases G, T, A and C. Only a part of the DNA construct is shown to the left; the complete sequence is 5′-d(GACCTCG_ATCGTG)-3′·5′-d(CACGATXCGAGGTC)-3′, where × represents the orphan base. EPR spectra of the spin-labels (100 μM) in the presence of DNA duplexes (200 μM) were recorded in phosphate buffer (10 mM $NaHPO_4$, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0) containing 30% ethylene glycol and 2% DMSO at −30 °C.

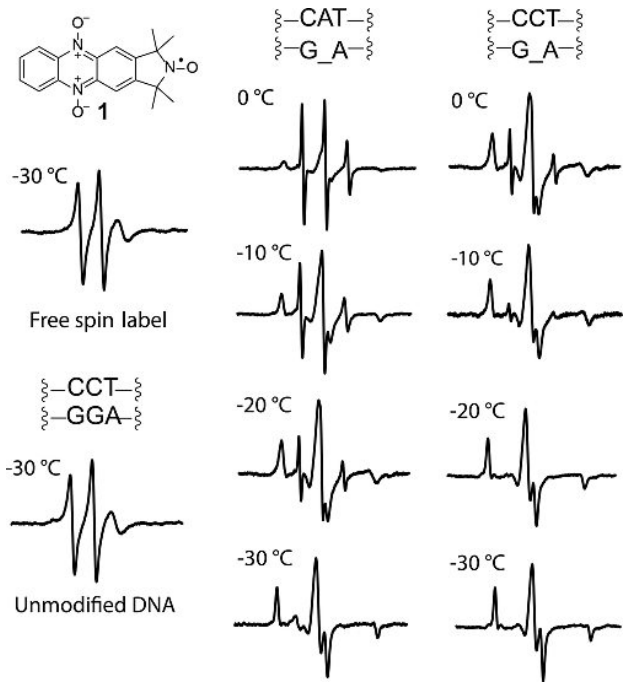


Figure 4. Evaluation of temperature-dependent noncovalent spin-labeling of spin label **5** to abasic sites in DNA duplexes opposite A (center) and C (right), by EPR spectroscopy. The EPR spectra of **5** alone and in the presence of an unmodified DNA at −30 °C are shown to the left. The EPR measurements were performed under the same conditions as listed in figure legend to Figure 3.

towards abasic sites opposite C (Figure 3, spectrum in upper box). Nitroxide **5** also showed nearly full binding (ca. 90%) opposite A (Figure 3, spectrum in lower box), but a moderate to low binding opposite T, represented by the mixture of slow- and fast-moving components in its EPR spectrum. Very little binding of **5** was observed to abasic sites opposite G (<4%) or to an unmodified duplex (<2%; see also Figure S31). Spin label **28** and **29** showed limited binding to abasic sites, whereas **30** showed extensive binding to abasic sites opposite all the nucleobases (C, A, G and T). However, substantial binding of **30** was also observed to the unmodified duplex to an extent of about 85% (Figure 3, bottom row to the right). The extensive nonspecific binding of **30** is most likely due to a large dipole moment, because of the nitro group, which facilitates intercalation.[39]

As spin label **5** exhibited full binding to abasic sites opposite C and almost full binding opposite A, the temperature dependence was investigated to obtain information about the relative affinity of the spin label to the abasic sites in question (Figure 4). Significant binding was already observed for the abasic site opposite C at 0 °C, while close to no binding was seen for A, and almost full binding was observed at −20 °C (> 95%). Little to no binding was observed at +10 °C and no binding was seen at +20 °C (data not shown). The dissociation constant (K_d) for **5** was determined to be 1.4×10^{-6} M at 0 °C by quantifying the amount of bound spin label from a doubly integrated EPR spectrum of **5** in the presence of the duplex containing the abasic site opposite C (see the Supporting Information). Comparison with the binding affinity of the previously reported noncovalent spin labels **Ǵ** ($K_d=6.02\times10^{-6}$ M at 0 °C)[17b] and **ç** ($K_d=1.36\times10^{-4}$ M at 0 °C)[16] to abasic sites in DNA opposite C, shows that spin label **5** possesses higher affinity. The underlying structural reason behind the observed selectivity of **5** towards abasic sites opposite C (and to some extent A) was not obvious when we tried to model the label into an abasic site in duplex DNA. Although the abasic site is necessary for binding, one cannot rule out groove binding in the vicinity of the abasic site, as opposed to directly to the abasic site. Spin label **5** showed very minor binding to 14-mer duplex RNA containing an abasic site opposite G, U, A or C at −30 °C (<3%, Figure S32), demonstrating its selectivity for DNA. In contrast, **Ǵ** shows extensive binding to abasic sites in RNA opposite C, even at +20 °C. The selectivity of **5** for abasic sites in DNA versus RNA may originate in the narrower major groove of RNA which might not be able to accommodate this relatively large label for binding to the abasic site.

Conclusion

We have demonstrated the synthesis of *o*-benzoquinone derivatives for both tetramethyl- and tetraethyl-isoindoline that were readily condensed with a number of commercially available *o*-phenylenediamines in near quantitative yields. Oxidation of the isoindoline-phenazines gave phenazine di-*N*-oxide nitroxide radicals that were evaluated as noncovalent spin labels for DNA and RNA containing abasic sites. Spin label **5** showed high binding affinity and selectivity towards abasic sites opposite C in DNA and is therefore a promising spin label for future EPR studies of DNAs. Moreover, the *o*-benzoquinone isoindoline derivatives **6** and **7** give access to various diversely substituted phenazine structures carrying the isoindoline moiety and thereby facilitate the incorporation of paramagnetic centers into other functional systems.

Experimental Section

General materials and methods. All commercially available reagents were purchased from Sigma-Aldrich or Acros Organics and used without further purification. CH_2Cl_2 was dried over calcium hydride and freshly distilled before use. All moisture- and air-sensitive reactions were carried out in oven-dried glassware under an inert atmosphere of Ar. Thin-layer chromatography (TLC) was performed using glass plates pre-coated with silica gel (0.25 mm, F-25, Silicycle) and compounds were visualized under UV light and by *p*-anisaldehyde staining. Column chromatography was performed using 230–400 mesh silica gel (Silicycle). ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance 400 MHz spectrometer. 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 CD_3OD (3.35, 4.78 ppm for ^{1}H NMR and 49.3 ppm for ^{13}C). Nitroxide radicals show broadening and loss of NMR signals due to their paramagnetic nature and therefore, those NMR spectra are not shown. Mass spectrometric analyses of all organic compounds were performed on an HRMS (ESI) (Bruker, MicrOTOF-Q) in positive ion mode.

DNA and RNA synthesis and purifications. All commercial phosphoramidites, CPG columns, 5-ethylthiotetrazole and acetonitrile for oligomer synthesis were purchased from Chem-Genes Corp. (USA). All other required reagents and solvents were purchased from Sigma-Aldrich. Unmodified oligonucleotides and those containing abasic sites were synthesized on an automated ASM800 DNA synthesizer (Biosset, Russia) by using a trityl-off protocol and commercially available phosphoramidites with standard protecting groups on a 1.0 μmol scale (1000 Å CPG columns). The DNA oligomers were deprotected in satd. NH_3 at 55 °C for 8 h and dried in vacuo. The RNA oligomers were deprotected in a 1:1 solution (2 mL) of CH_3NH_2 (8 M in EtOH) and NH_3 (33% w/w in H_2O) at 65 °C for 45 min. The solvent was removed in vacuo and the TBDMS-protecting groups were removed by incubation in $NEt_3 \cdot 3HF$ (600 μL) for 90 min at 55 °C in DMF (200 μL), followed by addition of water (200 μL). This solution was transferred to a 50 mL Falcon tube, *n*-butanol (20 mL) was added, and the mixture stored at −20 °C for 14 h, then centrifuged at 2.95 g while holding the temperature steady at 4 °C for 2 h. After which the solvent was decanted from the RNA pellet. The oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis. The oligonucleotides were visualized by UV light and the bands were excised from the gel, crushed and extracted from the gel matrix with a Tris buffer (250 mM NaCl, 10 mM Tris, 1 mM Na_2EDTA, pH 7.5). The extracts were filtered through 0.45 μm, 25 mm diameter GD/X syringe filters (Whatman, USA) and desalted using Sep-Pak cartridges (Waters, USA), according to the manufacturer's instructions. After removing the solvent in vacuo, the oligomers were dissolved in deionized and sterilized water (200 μL). Oligonucleotides were quantified using Beer's law and measurements of absorbance at 260 nm, using extinction coefficients determined by using the WinLab oligonucleotide calculator (V2.85.04, PerkinElmer).

EPR measurements. Solutions for CW-EPR experiments were prepared by mixing aliquots of stock solutions of a single-stranded oligomer containing an abasic site, its complementary strand and the spin label in question (1:1.2:0.5). The solvent was evaporated in vacuo and the resulting residue was dissolved in phosphate buffer (10 μL; 10 mM $NaHPO_4$, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0) and annealed as followed: 90 °C for 2 min, 60 °C for 5 min, 50 °C for 5 min, 22 °C for 15 min and dried using a SpeedVac. The residue was dissolved in an aqueous 30% ethylene glycol solution (10 μL) containing 2% DMSO and placed in a 50 μL quartz capillary (BLAUBRAND intraMARK) (final concentration of nucleic acid duplex 200 μM). The EPR spectra were recorded using 100–200 scans on a MiniScope MS200 (Magnettech Germany) spectrometer (100 kHz modulation frequency, 1.0 G modulation amplitude and 2.0 mW microwave power). A magnettech temperature controller M01 (± 0.5 °C) was used as temperature regulator.

5,6-Dimethoxy-1,1,3,3-tetramethylisoindoline (10). A solution of **8** (350 mg, 1.05 mmol) in DMF (1.5 mL) was added to a stirred solution of NaOMe in MeOH (5 M, 2 mL). Copper iodide (60 mg, 0.3 mmol) was added, and the mixture was heated at 145 °C for 29 h. The reaction mixture was cooled to room temperature, poured onto ice and extracted with Et_2O (3×30 mL). The combined organic phases were washed with H_2O (2×30 mL) and brine (3× 30 mL), dried over Na_2SO_4 and concentrated in vacuo to yield **10** (160 mg, 95%) as a brown-orange solid, which was directly used for the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.62$ (s, 2H), 3.90 (s, 6H), 1.45 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 148.9$, 140.3, 104.5, 62.8, 56.5, 32.0. HRMS (ESI) *m/z* calcd for $C_{14}H_{22}NO_2^+$: 236.1645 $[M+H]^+$; found 236.1632.

1,1,3,3-Tetramethylisoindoline-5,6-diol (12). A solution of **10** (100 mg, 0.425 mmol) in CH_2Cl_2 (5 mL) was treated with iodotrimethylsilane (0.2 mL, 1.40 mmol) at 0 °C and the reaction mixture was stirred at 22 °C for 72 h. MeOH (10 mL) was added and the solution was stirred for 1 h and concentrated in vacuo to yield a black oily residue that subsequently solidified. The black residue was dissolved in a minimum amount of MeOH (1 mL) followed by a slow addition of CH_2Cl_2 (20 mL) to yield a gray precipitate. The solid was filtered and washed with cold CH_2Cl_2 to yield **12** (80 mg, 91%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): $\delta = 6.72$ (s, 2H), 1.67 (s, 12H). ^{13}C NMR (101 MHz, CD_3OD): $\delta = 148.7$, 131.0, 109.3, 77.1, 25.5. HRMS (ESI) *m/z* calcd for $C_{12}H_{18}NO_2^+$: 208.1332 $[M+H]^+$; found 208.1336.

1,1,3,3-Tetramethylisoindoline-5,6-dione (6). A solution of **12** (25 mg, 0.12 mmol) in MeOH (0.5 mL) was added to a stirred solution of MnO_2 (1.0 g) in CH_2Cl_2 (20 mL) and the resulting mixture stirred at 22 °C for 3 h. The reaction mixture was filtered through a Büchner funnel, MnO_2 was recovered, the filtrate was concentrated in vacuo and the residue purified by flash-column chromatography, using a gradient elution (CH_2Cl_2/MeOH 100:0 to 90:10), to yield **6** (22 mg, 88%) as a dark brown solid. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta =$ 6.17 (s, 2H), 1.44 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 178.7$, 167.3, 120.8, 70.6, 60.6, 31.6, 30.6, 29.7, 27.5. HRMS (ESI) *m/z* calcd for $C_{12}H_{16}NO_2^+$: 206.1176 $[M+H]^+$; found 206.1172.

1,1,3,3-Tetraethyl-5,6-dimethoxyisoindoline (11). A solution of **9** (350 mg, 0.90 mmol) in anhydrous DMF (1.5 mL) was added to a stirred solution of NaOMe in MeOH (5 M, 2 mL). Copper iodide (60 mg, 0.3 mmol) was added and the mixture was heated at 145 °C for 29 h. The reaction mixture was cooled to room temperature, poured onto ice and extracted with Et_2O (3×30 mL). The combined organic phases were washed with H_2O (2×30 mL) and brine (3× 30 mL), dried over Na_2SO_4 and concentrated in vacuo to yield **11** (160 mg, 62%) as a brown-orange solid, which was directly used for the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 6.56$ (s, 2H), 3.87 (s, 6H), 1.75–1.57 (m, 8H), 0.88 (t, $J = 7.5$ Hz, 12H).

1,1,3,3-Tetraethylisoindoline-5,6-diol (13). A solution of **11** (160 mg, 0.552 mmol) in CH_2Cl_2 (5 mL) was treated with iodotrimethylsilane (0.26 mL, 1.82 mmol) at 0 °C and the solution was stirred at 22 °C for 72 h. MeOH (10 mL) was added and the solution stirred for 1 h and concentrated in vacuo to yield a black oily residue that subsequently solidified. The black residue was dissolved in minimum amount of MeOH (1 mL) followed by a slow addition of CH_2Cl_2 (20 mL) to yield a gray precipitate. The solid was filtered and washed with cold CH_2Cl_2 to yield **13** (112 mg, 77%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): $\delta = 6.64$ (s, 2H), 2.04 (q, $J =$ 7.4 Hz, 8H), 1.03 (t, $J = 7.4$ Hz, 12H). ^{13}C NMR (101 MHz, CD_3OD): $\delta =$ 147.9, 132.2, 110.7, 76.8, 31.7, 8.7. HRMS (ESI) *m/z* calcd for $C_{16}H_{26}NO_2^+$: 264.1958 $[M+H]^+$; found 264.1948.

1,1,3,3-Tetraethylisoindoline-5,6-dione (7). A solution of **13** (27 mg, 0.10 mmol) in MeOH (0.5 mL) was added to a stirred solution of MnO_2 (1000 mg) in CH_2Cl_2 (20 mL) and the resulting mixture stirred for 3 h at room temperature. The reaction mixture was filtered through a Büchner funnel, MnO_2 was recovered, the filtrate was concentrated in vacuo and the residue purified by flash-column chromatography (CH_2Cl_2 100%), to yield **7** (25 mg, 93%) as a dark brown solid. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.10$ (s, 2H), 1.65 (qd, $J = 14.1$, 7.3 Hz, 8H), 0.93 (t, $J = 7.4$ Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 178.8$, 166.4, 121.6, 66.5, 32.9, 8.5. HRMS (ESI) *m/z* calcd for $C_{16}H_{24}NO_2^+$: 262.1802 $[M+H]^+$; found 262.1801.

Isoindoline phenazine 15. To a solution of 70% EtOH in H_2O (4 mL) containing 1 drop of AcOH was added **6** (98.4 mg, 0.48 mmol) and **14** (73.5 mg, 0.48 mmol) and the solution was stirred at 22 °C for 30 min. The solvent was removed in vacuo and the residue purified by flash-column chromatography, using a gradient elution (CH_2Cl_2/MeOH, 100:0 to 90:10), to yield **15** (131.8 mg, 99%) as a yellowish

solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.11 (dd, J = 6.7, 3.4 Hz, 2H), 7.85 (s, 2H), 7.69 (dd, J = 6.7, 3.4 Hz, 2H), 1.53 (s, 13H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 154.95, 142.95, 130.03, 129.43, 120.76, 103.38, 62.29, 32.11. HRMS (ESI) *m/z* calcd for $C_{18}H_{20}N_3^+$: 278.1652 $[M+H]^+$; found 278.1633.

Bromo isoindoline phenazine 17. To a solution of 70% EtOH in H_2O (2.5 mL) containing 1 drop of AcOH was added **6** (16.4 mg, 0.079 mmol) and **16** (14.9 mg, 0.079 mmol) and the solution was stirred at 22 °C for 30 min. The solvent was removed in vacuo and the residue purified by flash-column chromatography, using a gradient elution (CH_2Cl_2/MeOH 100:0 to 90:10), to yield **17** (26.7 mg, 95%) as a brownish-yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.41 (d, J = 2.0 Hz, 1H), 8.07 (d, J = 9.2 Hz, 1H), 7.92 (s, 2H), 7.85 (dd, J = 9.2, 2.1 Hz, 1H), 1.67–1.59 (m, 13H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 155.9, 155.5, 143.8, 143.5, 143.2, 141.6, 133.8, 131.5, 130.7, 124.5, 121.0, 120.9, 62.5, 32.1, 29.7. HRMS (ESI) *m/z* calcd for $C_{18}H_{19}BrN_3^+$: 356.0757 $[M+H]^+$; found 356.0757.

Dichloro isoindoline phenazine 19. To a solution of 70% EtOH in H_2O (2.5 mL) containing 1 drop of AcOH was added **6** (12.0 mg, 0.058 mmol) and **18** (10.3 mg, 0.058 mmol) and the solution was stirred at 22 °C for 30 min. The solvent was removed in vacuo and the residue purified by flash-column chromatography, using a gradient elution (CH_2Cl_2/MeOH 100:0 to 90:10), to yield **19** (19.1 mg, 95%) as a brownish yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.35 (s, 2H), 7.91 (s, 2H), 1.65 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 144.0, 141.6, 129.8, 121.1, 117.6, 77.5, 77.2, 76.8, 62.7, 32.1, 29.8. HRMS (ESI) *m/z* calcd for $C_{18}H_{18}Cl_2N_3^+$: 346.0872 $[M+H]^+$; found 346.0862.

Nitro isoindoline phenazine 21. To a solution of 70% EtOH in H_2O (4 mL) containing 1 drop of AcOH was added **6** (24.4 mg, 0.12 mmol) and **20** (18.2 mg, 0.12 mmol) and the solution was stirred at 22 °C for 30 min. The solvent was removed in vacuo and the residue purified by flash-column chromatography, using a gradient elution (CH_2Cl_2/MeOH 100:0 to 90:10), to yield **21** (35.7 mg, 93%) as a bright yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.16 (d, J = 2.2 Hz, 1H), 8.54 (dd, J = 9.5, 2.5 Hz, 1H), 8.36 (d, J = 9.5 Hz, 1H), 7.99 (d, J = 6.1 Hz, 2H), 1.69 (d, J = 2.0 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 147.9, 144.9, 144.3, 141.2, 131.4, 126.6, 123.0, 121.3, 121.2, 62.8, 32.0, 29.8. HRMS (ESI) *m/z* calcd for $C_{18}H_{19}N_4O^{2+}$: 323.1503 $[M+H]^+$; found 323.1501.

Ethylnitro isoindoline phenazine 22. To a solution of 70% EtOH in H_2O (2.5 mL) containing 1 drop of AcOH was added **7** (50 mg, 0.191 mmol) and **20** (29.3 mg, 0.191 mmol) and the solution was stirred at 22 °C for 30 min. The solvent was removed in vacuo and the residue purified by flash-column chromatography, using a gradient elution (CH_2Cl_2/MeOH 100:0 to 95:5), to yield **22** (65.2 mg, 90%) as a brownish yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.17 (d, J = 2.4 Hz, 1H), 8.53 (d, J = 2.5 Hz, 1H), 8.35 (d, J = 9.5 Hz, 1H), 7.93 (d, J = 5.4 Hz, 2H), 1.97–1.79 (m, 8H), 0.98 (td, J = 7.4, 1.5 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 155.9, 154.9, 146.8, 143.8, 143.7, 143.2, 140.0, 130.3, 125.5, 121.8, 120.9, 120.7, 67.2, 67.1, 33.0, 28.7, 7.9, 0.0. HRMS (ESI) *m/z* calcd for $C_{22}H_{27}N_4O_2^+$: 379.2129 $[M+H]^+$; found 379.2126.

Ethylbromo isoindoline phenazine 23. To a solution of 70% EtOH in H_2O (2.5 mL) containing 1 drop of AcOH was added **7** (12.6 mg, 0.048 mmol) and **16** (8.9 mg, 0.048 mmol) and the solution was stirred at 22 °C for 30 min. The solvent was removed in vacuo and the residue purified by flash-column chromatography, using a gradient elution (CH_2Cl_2/MeOH 100:0 to 95:5), to yield **23** (19 mg, 96%) as a brownish-yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.41 (d, J = 2.1 Hz, 1H), 8.07 (d, J = 9.2 Hz, 1H), 7.87 (s, 2H), 7.85 (dd, J = 9.2, 2.1 Hz, 1H), 1.95–1.77 (m, 9H), 0.97 (t, J = 7.4 Hz, 13H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 142.6, 142.4, 142.1, 140.5, 132.7, 130.4, 129.7, 123.2, 120.7, 120.6, 67.0, 33.0, 7.7, 0.0. HRMS (ESI) *m/z* calcd for $C_{22}H_{27}BrN_3^+$: 412.1383 $[M+H]^+$; found 412.1383.

Isoindoline phenazine radical 26. To a solution of **15** (10 mg, 0.036 mmol) in CH_2Cl_2 (2 mL), was added *m*-chloroperoxybenzoic acid (6.2 mg, 0.036 mmol) and the resulting solution stirred at 22 °C for 12 h in a closed reaction vial. The solution was poured onto ice, extracted with CH_2Cl_2 (3×10 mL), dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by preparative TLC (CH_2Cl_2/MeOH 95:5) to yield **26** (9.4 mg, 89% yield) as a yellow solid. HRMS (ESI) *m/z* calcd for $C_{18}H_{18}N_3O^+$: 315.1348 $[M+Na]^+$; found 315.1308.

Dioxidized isoindoline phenazine 27. To a solution of **15** (10 mg, 0.036 mmol) in CH_2Cl_2 (2 mL), was added *m*-chloroperoxybenzoic acid (13.6 mg, 0.079 mmol) and the resulting solution stirred at 22 °C for 12 h in a closed reaction vial. The solution was poured onto ice, extracted with CH_2Cl_2 (3×10 mL), dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by preparative TLC (CH_2Cl_2/MeOH 95:5) to yield **27** (7.8 mg, 70% yield) as an orange-red solid. HRMS (ESI) *m/z* calcd for $C_{18}H_{18}N_3O_2^+$: 331.1297 $[M+Na]^+$; found 331.1286.

Trioxidized isoindoline phenazine 5. To a solution of **15** (10 mg, 0.036 mmol) in CH_2Cl_2 (2 mL), was added *m*-chloroperoxybenzoic acid (34.2 mg, 0.198 mmol) and the resulting solution stirred at 22 °C for 12 h in a closed reaction vial. The solution was poured onto ice, extracted with CH_2Cl_2 (3×10 mL), dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by preparative TLC (CH_2Cl_2/MeOH 95:5) to yield **5** (9 mg, 77% yield) as a bright red solid. HRMS (ESI) *m/z* calcd for $C_{18}H_{18}N_3O_3^+$: 347.1240 $[M+Na]^+$; found 347.1215.

Trioxidized bromo isoindoline phenazine 28. To a solution of **17** (10 mg, 0.028 mmol) in CH_2Cl_2 (2 mL), was added *m*-chloroperoxybenzoic acid (26.6 mg, 0.154 mmol) and the resulting solution stirred at 22 °C for 12 h in a closed reaction vial. The solution was poured onto ice, extracted with CH_2Cl_2 (3×10 mL), dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by preparative TLC (CH_2Cl_2/MeOH 95:5) to yield **28** (8.1 mg, 72% yield) as a deep brown-red solid. HRMS (ESI) *m/z* calcd for $C_{18}H_{17}N_3O_3Br^+$: 425.0345 $[M+Na]^+$; found 425.0360.

Trioxidized dichloro isoindoline phenazine 29. To a solution of **19** (10 mg, 0.029 mmol) in CH_2Cl_2 (2 mL), was added *m*-chloroperoxybenzoic acid (26.6 mg, 0.154 mmol) and the resulting solution stirred at 22 °C for 12 h in a closed reaction vial. The solution was poured onto ice, extracted with CH_2Cl_2 (3×10 mL), dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by preparative TLC (CH_2Cl_2/MeOH 95:5) to yield **29** (7.9 mg, 70% yield) as a brownish solid. HRMS (ESI) *m/z* calcd for $C_{18}H_{17}N_3O_3Cl_2^+$: 393.0641 $[M+H]^+$; found 393.0556.

Trioxidized nitro isoindoline phenazine 30. To a solution of **21** (10 mg, 0.031 mmol) in CH_2Cl_2 (2 mL), was added *m*-chloroperoxybenzoic acid (29.4 mg, 0.171 mmol) and the resulting solution stirred at 22 °C for 12 h in a closed reaction vial. The solution was poured onto ice, extracted with CH_2Cl_2 (3×10 mL), dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by preparative TLC (CH_2Cl_2/MeOH; 95:5) to yield **30** (8.9 mg, 78% yield) as a bright red solid.

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