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Nitroxide-labeled pyrimidines for noncovalent spin-labeling of abasic sites in DNA and RNA duplexes

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Abstract

Noncovalent and site-directed spin labeling gives an easy access to spin-labeled nucleic acids for the study of their structure and dynamics by electron paramagnetic resonance (EPR) spectroscopy. In a search for improved spin labels for noncovalent binding to abasic sites in duplex DNA and RNA, ten pyrimidine-derived spin labels were prepared in good yields and their binding evaluated by continuous wave (CW)-EPR spectroscopy. Most of the spin labels showed lower binding affinity than the previously reported label **ç** towards abasic sites in DNA and RNA. The most promising labels were triazole-linked spin labels and a pyrrolocytosine label. In particular, the N1-ethylamino derivative of a triazole-linked uracil spin label binds fully to both DNA and RNA containing an abasic site. This is the first example of a spin label that binds fully through noncovalent interactions to an abasic site in RNA.

Introduction

Electron paramagnetic resonance (EPR) spectroscopy is increasingly being used to obtain structural insights of nucleic acids.[1-6] Rapid progress in EPR spectroscopy has been driven by advances in both EPR instrumentation and accessibility of spin-labeled oligonucleotides. EPR is more sensitive than nuclear magnetic resonance (NMR), and is not restricted to molecular size.[7] In addition, EPR is a bio-orthogonal technique, i. e. there are no background signals in biological fluids from radicals, and has been used in in-cell EPR measurements of RNA.[8] EPR enables measurement of distances between two spin centers up to 80 Å using pulsed EPR techniques such as pulsed electron-electron double resonance (PELDOR), also known as double electron-electron

resonance (DEER).[2, 9, 10] EPR also gives valuable information about local dynamics (motion) as well as conformational changes of nucleic acids.[5, 11-15] EPR studies of diamagnetic biopolymers require incorporation of spin labels at specific sites, generally stable aminoxyl (nitroxide) radicals, termed site-directed spin labeling (SDSL).[3, 5, 16, 17]

There are three main approaches available for SDSL, namely spin-labeling during oligonucleotide synthesis, post-synthetic labeling and noncovalent labeling.[3, 16, 17] The first two methods rely on covalent attachment of spin labels, which is often time-consuming and non-trivial. The third approach is based on noncovalent van der Waals- and hydrogen bonding-interactions.[18-21] There are several reports in the literature that describe noncovalent targeting of small molecules, including spin labels, to specific sites in nucleic acids. For example, Nakatani and co-workers developed an approach for a programmable assembly of spin probes on one and two dimensional DNA arrays by using nitronyl nitroxides and 2,2,6,6-tetramethylpiperidine-1-oxyl, (TEMPO) conjugated to the G-G mismatch- binding ligand naphthyridine carbamate dimer (NCD).[19, 21-23] Abasic sites in duplex nucleic acids have also been used as binding sites for ligands. For example, Lhomme et. al. used adenine-acridine conjugates as ligands for abasic sites, including spin-labeled derivatives.[24-27] Similarly, Teramae and coworkers have developed a series of fluorescent ligands that bind specifically to abasic sites and have been used for the detection of single nucleotide polymorphisms (SNPs) in DNAs[28-31] and RNAs.[32, 33] Based on the fluorescence signaling of small molecules upon binding to abasic sites in duplex DNA, an aptasensor for adenosine[34-36] and riboflavin[37] have been developed by the same group, where a nucleotide opposite to the abasic site acts as a receptor for these molecules.

We have previously utilized abasic sites for SDSL using the spin label **ç** (Figure 1A),[20] which is an analogue of the pyrimidine nucleobase cytosine (C) and structurally similar to the rigid spin-labeled nucleoside **Ç**.[38] The spin label **ç** contains a nitroxide-bearing isoindoline fused onto a cytosine base through an oxazine ring, making the nitroxide a part of the nucleobase (Figure 1A). The **ç** spin label binds specifically to an abasic site in a duplex DNA at low temperatures, forming a base-pair with a guanine (G) as an orphan base on the opposite strand.[20] This label was used for determination of distances as well as relative orientations between two spin labels in duplex DNAs, the latter made possible its rigid structure, and enabled the study of DNA-bending by the Lac-repressor protein using PELDOR.[39] This noncovalent labeling approach simply requires mixing the spin label **ç** and the DNA duplex containing an abasic site.

In spite of the advantages of noncovalent spin-labeling using **ç**, its binding is sequence dependent and several flanking sequences show incomplete binding.[40] In addition, **ç** has limited solubility in aqueous solutions.[41] To circumvent these problems, derivatives of **ç** containing N3-ethylamino and N3-ethylguanidino groups were prepared and their binding to abasic sites in duplex DNAs evaluated.[41] Both derivatives showed an improved binding affinity and solubility in aqueous solutions. However, syntheses of **ç** and its derivatives are rather lengthy and low-yielding. Herein, we report the syntheses of a series of pyrimidine-based spin labels for noncovalent binding that were prepared in an attempt to discover readily prepared, high-affinity spin labels that have good solubility in water. Most of these spin labels were prepared by short synthetic routes using bio-orthogonal Sonogashira- and click-reactions. A subset of these compounds showed good binding affinity and specificity towards abasic sites and particularly noteworthy was near complete noncovalent labeling of RNA for two of the derivatives.

Results and Discussion

The goal of this study was to prepare labels that were easier to synthesize, have higher affinity for abasic sites and have higher solubility in aqueous solutions than **ç**. In addition, we wanted to prepare spin labels that would show less orientational effects in PELDOR,[42] which would simplify EPR measurements and data analysis. Flexible spin labels do not show orientational effects, but usually yield less accurate distances because of the movement of the label independent of the nucleic acid to which they are attached. However, labels that only allow rotation of bonds that lie on the same axis as the N-O bond,[43-45] which have been termed conformationally unambiguous,[44] would retain flexibility without compromising the accuracy of distance measurements.

We chose pyrimidines as scaffolds for the spin labels, primarily because of their ease of modification (Figure 1B). Pyrimidine nucleobases in nucleosides have been modified with spin labels at either position four[46-50] or five.[51-56] Four- and five-modified nucleobases preserve Watson-Crick base-pairing properties and direct the spin label into the spacious major groove, where it is unlikely to cause structural perturbations. The pyrimidine chemistry gives either U or C which could pair with A or G on the opposite strand, respectively; **ç** can only bind to G. Additionally, four- and five-modified pyrimidines are readily prepared, the latter through palladium-catalyzed cross-coupling reactions of readily available 5-halogenated pyrimidines.

Amino-, acetylene- and pyrrolo-linked pyrimidine-derived spin labels

The exocyclic amino group of cytosine can be readily modified using the convertible nucleoside approach[57] and the spin label TEMPO has been incorporated into the four-position of pyrimidines in both DNA[46, 50] and RNA using this method.[58] Thus, the 4-amino TEMPO-modified C (**1,** Figure 1B) was a logical target in this series. Its synthesis began by regioselective methylation of uracil (**11**) 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 (Scheme 1). The N1-methyl uracil[59] was treated with 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl) to yield the *O*^{4}-TPS-modified compound, which on incubation with 4-amino TEMPO afforded spin label **1**.

Spin labels have also been attached to the five-position of pyrimidines using a relatively short and semi-rigid acetylene-linker.[51, 53, 54, 60-62] Although rotation around the single bonds flanking the acetylene is possible, it should only cause minimal change in position of the N-O-group relative to the nucleobase, due to a slight pucker in the nitroxide-bearing six-membered ring. Thus, 5-alkyne-linked labels (**2-5**) would allow accurate distance determination by pulsed EPR spectroscopy that should not require taking relative orientation dependence into account.[39, 42] The acetylene-linked spin-label derivatives of cytosine (**2** and **3** Scheme 1B) and uracil (**4** and **5**, Scheme 1C) were prepared in a single step by a palladium-catalyzed Sonogashira cross-coupling reaction between the nitroxide (**15**)[61] and either a pyrimidine base (**13** and **16**) or its N1-methyl analogue (**14** and **17**).

It has been shown that 5-alkynyl-substituted pyrimidines undergo cyclization with heteroatoms in position four upon heating in the presence of base to yield furanouracils or pyrrolocytosines.[63] We hypothesized that the fused ring system of the pyrrolocytosine might have a better ability to stack into the abasic site than the pyrimidines and thereby have a higher affinity. Benzoyl protected N1-methyl-5-iodo cytosine (**18**) was prepared by silylation with HMDS, followed by alkylation with methyl iodide in the presence of a catalytic amount of iodine and benzoylation of amino group in the presence of a base (Supporting Information). A subsequent one-pot, two-step reaction involving a palladium-catalyzed Sonogashira cross-coupling with nitroxide **15**, followed by cyclization at an elevated temperature, yielded the spin-labeled pyrrolocytosine **6** (Scheme 1D).

To test the binding affinity of the spin labels shown in Scheme 1 to abasic sites, each of the spin labels was incubated with a 14-mer duplex DNA containing an abasic site. The uracil nitroxides (**4**

and **5**) were mixed with DNAs containing with adenine (A) as the orphan base opposite to the abasic site and the cytosine labels (**1**, **2**, **3** and **6**) with DNA containing guanine (G) (Figure 2). EPR spectra were recorded in a phosphate buffer (pH 7) containing 30% ethylene glycol (a cryoprotectant that is generally used in pulsed EPR studies)[2] and 2% DMSO, at temperature intervals between 0 °C and −30 °C. The EPR spectra of spin labels without DNA at −30 °C showed relatively narrow lines due to the fast motion of the nitroxides in solution (Figure 2, left), while spectral broadening was observed in the presence of a DNA duplex containing an abasic site (Figure 2, right). Broadening of the EPR spectra indicates slower motion,[64] consistent with spin-label binding to the DNA.

All the spin labels showed some binding affinity towards abasic sites, except **1**, which does not seem to bind at all. This was somewhat unexpected since nucleosides containing this modification have shown only a minor destabilization of a duplex DNA (3 °C)[50] and RNA (6 °C).[58] On the other hand, the acetylene-linked spin labels showed moderate binding affinity (Ca. 30-40%). The acetylene-based cytosine derivatives (**2** and **3**) bind more efficiently than the uracil derivatives (**4** and **5**), presumably due to the increased number of hydrogen bonds. The pyrrolocytosine spin label (**6**) showed better binding than the acetylene-linked spin labels (Ca. 60%), possibly due to better stacking of the fused ring-system with the nucleobases flanking the abasic site.

To determine if the binding of the labels in Scheme 1 was specific to the abasic site, spin labels were individually incubated with an unmodified 14-mer DNA duplex, which showed no detectable binding (Supporting Information). Comparison of the EPR spectra of the acetylene-linked spin labels (**2**-**5**) and pyrrolocytosine **6** revealed that the spectra of the latter were slightly broader. The slower mobility of the nitroxide in **6,** once bound to DNA, than in **2-5** is most likely due to placement of the nitroxide closer to the side of the major groove, which could restrict its motion through steric interactions. In addition, spin label **6** contains only one rotatable single bond between the base and the label.

Triazole-linked spin labels

We have previously used the copper-catalyzed Huisgen–Meldal–Sharpless [3+2] cycloaddition[65, 66] ("click chemistry")[67] for post-synthetic spin labeling of DNA.[56] Specifically, an oligonucleotide containing an alkyne was reacted with an azido-isoindoline nitroxide and used for detection of DNA structural lesions, such as abasic sites and mismatches.[56] Due to the ease of synthesis, we also

prepared a few pyrimidine-based spin labels using click-chemistry, although they might not be optimal for distance measurements due to movement of the nitroxide upon rotation of the single bonds connecting the rings.

Syntheses of triazole-linked spin labels **7** and **8** (Scheme 2) started with a Sonogashira reaction between trimethylsilyl acetylene and 5-iodo-1-methyl uracil[41] to yield compound **19** (Scheme 2A), which afforded 5-ethynyl-uracil derivative **20** by treatment with NH_3. Compound **20** was incubated with azide functionalized nitroxide **21**[56] in the presence of Cu(I) to afford spin label **7** in good yield. The synthesis of the triazole-linked cytosine spin label derivative began by treatment of intermediate **19** with TPS-Cl in the presence of triethylamine and a catalytic amount of 4-dimethylaminopyridine (DMAP). Reaction of the resulting O4-sulphonyl-activated compound **22** with NH_3 resulted into displacement of the O4-sulphonyl leaving group and removal of trimethylsilyl group in one step to afford 5-ethynyl-cytosine derivative **23**. The Huisgen–Meldal–Sharpless [3+2] cycloaddition between **23** and azide **21** yielded spin label **8**.

The temperature dependent binding of spin labels **7** and **8** to a 14-mer DNA duplex containing an abasic site was determined by EPR spectroscopy (Figure 3A, right). A substantially higher binding to the DNA was observed for both triazole labels than for spin labels **1**-**6**, in particular at −30 °C, where **7** was almost fully bound. However, asymmetrical lines and broadening of the EPR spectra of spin label **7,** both in the presence and absence of DNA, indicates some aggregation of the spin label, which complicates analysis of the data.

Surprisingly, the triazole-linked cytosine derivative **8** showed lower binding affinity (ca. 70%) than **7**, although it can form three hydrogen bonds with G. Molecular modeling of the cytosine spin label **8** showed that there is a ca. 25° twist between the triazole ring and the pyrimidine ring due to formation of a hydrogen bond between one of the nitrogens of the triazole with the exocyclic amino group of the cytosine. The resulting non-planar structure might affect stacking interactions of the label at the abasic site and thereby reduce its binding affinity.

Ethylamino derivatives of triazole-linked spin labels

Low solubility of **7** in aqueous solutions prompted us to prepare more polar analogues. We chose to replace the N1-methyl groups in **7** and **8** with ethylamino groups, which had shown favorable effects on binding affinity of the spin label **ç**.[41] The syntheses of N1-ethylamino spin labels **9** and **10** (Scheme 3) is very similar to the syntheses of their corresponding methyl derivatives (Scheme

2). Alkylation of 5-iodo-uracil (**16**) with N-benzoyloxycarbonyl-protected 2-bromoethylamine in the presence of potassium carbonate in DMSO afforded **24** in moderate yield (Scheme 3A). A Sonogashira reaction, followed by removal of trimethylsilyl group yielded **26**, with which a Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction with azide **21** gave nitroxide **27**. Deprotection of the amino group was achieved by hydrogenation, which also reduced the nitroxide group to a hydroxyl amine, which upon oxidation by treatment with copper acetate in the presence of oxygen afforded spin label **9** (Scheme 3A). The synthesis of the N1-ethylamino spin label derivative **10** was achieved by a similar synthetic strategy (Scheme 3B).

The binding of spin labels **9** and **10** to an abasic site in a DNA duplex were evaluated at different temperatures and revealed that both **9** and **10** had increased solubility and higher binding affinity than their N1-methyl analogues (Figure 3B). Spin label **9** showed significant binding even at 0 °C and at −30 °C it was fully bound. The binding efficiency of spin label **9** and its EPR spectrum is very similar to the N3-ethylamino derivative of **ç**.[41] The increased binding affinity of these labels is accredited to the amino group, which would be protonated at pH 7 and could form salt bridges with the negatively charged phosphate backbone.[41]

The specificity of the triazole-linked spin labels towards abasic sites was investigated by incubating each label with an unmodified DNA duplex of the same sequence. The spin labels were highly specific to an abasic site; the N1-methyl derivatives **7** and **8** did not show binding to the unmodified DNA duplex, whereas, the N1-ethylamino analogues showed only ca. 10% non-specific binding at −30 °C (Supporting Information). To determine the specificity of binding at the abasic sites as a function of the identity of the orphan base, the spin labels were incubated with a DNA duplex containing either A, C, G or T opposite the abasic site. EPR spectra that were recorded at −30 °C showed that cytosine derivatives **8** and **10** were not selective, whereas spin labels **7** and **9** showed higher affinity towards A, T and G (Supporting Information).

Binding of spin labels to abasic sites in RNA

We have previously[40, 41, 68] and in this paper demonstrated noncovalent binding of spin labels to abasic sites in duplex DNA. However, noncovalent spin-labeling of RNA has yet to be demonstrated. For example, only ca. 30% of the spin label **ç** was found to bind to an RNA duplex containing an abasic site (Figure 4A). Spin labels **1-10** were incubated with an RNA duplex that contained an abasic site in a phosphate buffer (pH 7) and EPR spectra were recorded at different

temperatures. Most of the spin labels do not bind to abasic sites in RNA (data not shown), with the exception of the triazole-linked spin labels derivatives. N1-methyl derivatives **7** and **8** showed moderate to low binding affinity (ca. 60% and 20%), while the N1-ethylamino analogues **9** and **10** were almost fully bound (100% and 90%, respectively). This is the first example of complete spin labeling of RNA using noncovalent labels. However, substantial binding of **9** and **10** to an unmodified 14-mer RNA duplex was also observed, ca. 50% and 90%, respectively (Figure 4B). However, comparison of the spectra of spin label **9** in the presence of RNA with and without the abasic site reveals signs of aggregation with the unmodified RNA, as manifested in the irregular baseline that could result from strong dipolar coupling between radicals.[69] The absence of such signs in the presence of the RNA with the abasic site might indicate that the label is indeed bound at the abasic site, but the extent of binding will need further experimental determination.

Conclusions

The syntheses and evaluation of ten pyrimidine-derived nitroxide labels for noncovalent and site-directed spin labeling of nucleic acids are reported in this paper. Most of the nitroxides were synthesized by relatively short synthetic routes in good yields. Amino-modified spin label **1** does not bind at all to an abasic site in duplex DNA, whereas acetylene-linked spin labels showed moderate binding affinities. The pyrrolocytosine spin-label **6** showed higher binding affinity, presumably due to better stacking with the flanking bases, and less mobility than the acetylene-linked labels. Of the triazole-linked derivatives, N1-ethylamino analogues of spin label **9** and **10** showed both increased binding affinity and solubility in aqueous solutions. Of the spin labels reported here, only **9** and **10** showed significant binding to an RNA duplex containing an abasic site. In fact, **9** is the first example of quantitative spin-labeling of abasic sites in duplex RNA. However, **9** and **10** showed considerable nonspecific binding to an unmodified RNA and, therefore, the specificity of binding requires further investigation. Although some of the labels reported here show promise for use as noncovalent labels in distance measurements with pulsed EPR, most of the labels show moderate to low binding affinity. The use of structural scaffolds other than pyrimidines in a quest for improved spin labels is currently under investigation and the results will be reported in due course.

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Experimental details, synthetic procedures and characterization of synthetic compounds.

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List of Figures

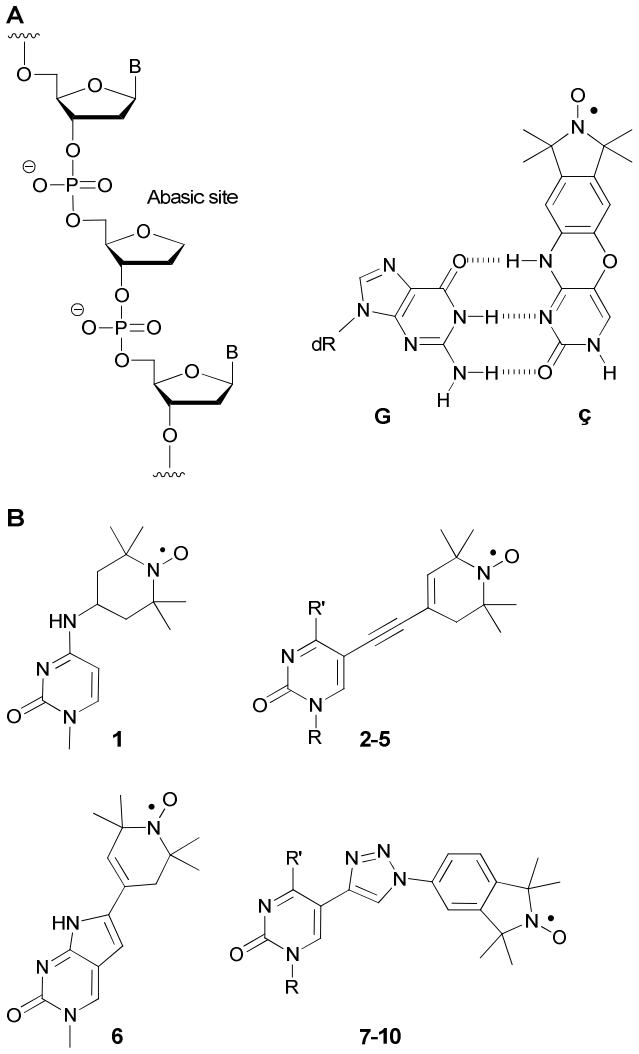
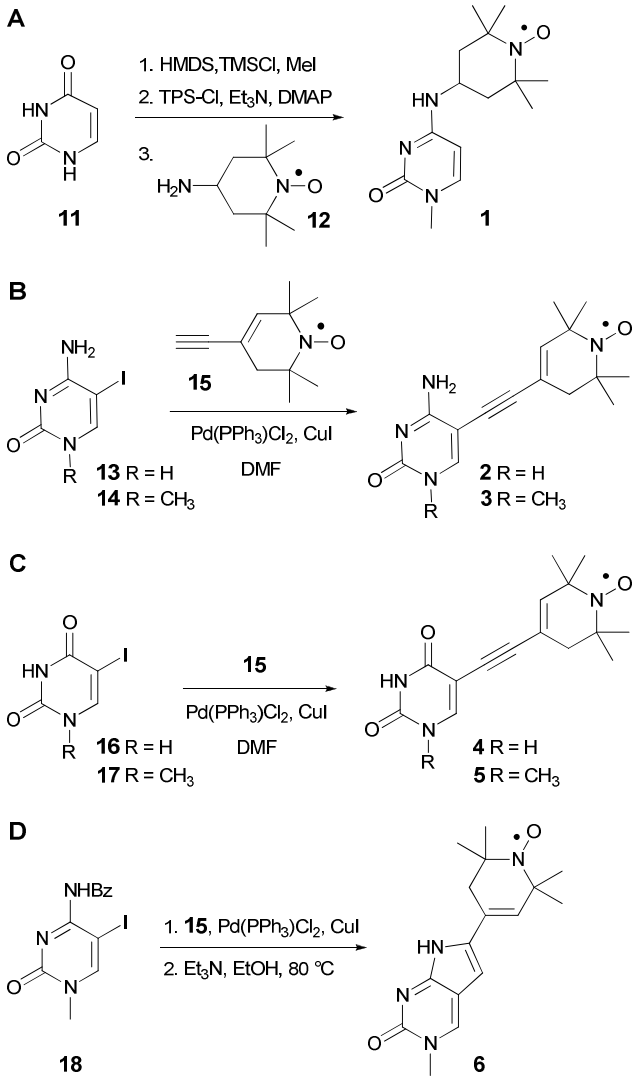


Figure 1 A. Left. Structure of an abasic site. **Right.** Structure and base pairing scheme of the spin label **ç** with guanine (G). dR = deoxyribose. **B.** Structures of pyrimidine-derived spin labels prepared in this study. R = -H, $-CH_3$ or $-CH_2CH_2NH_2$ and R' = -OH, $-NH_2$.



Scheme 1: Synthesis of 4-amino- (**A**), acetylene- (**B** and **C**), and pyrrolo- (**D**) linked pyrimidine spin labels. Bz = benzoyl.

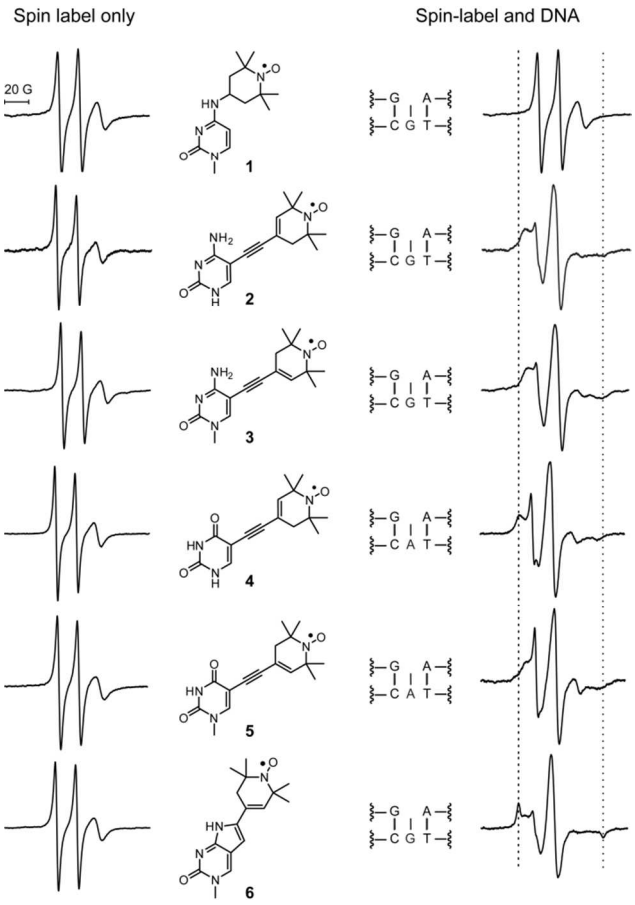
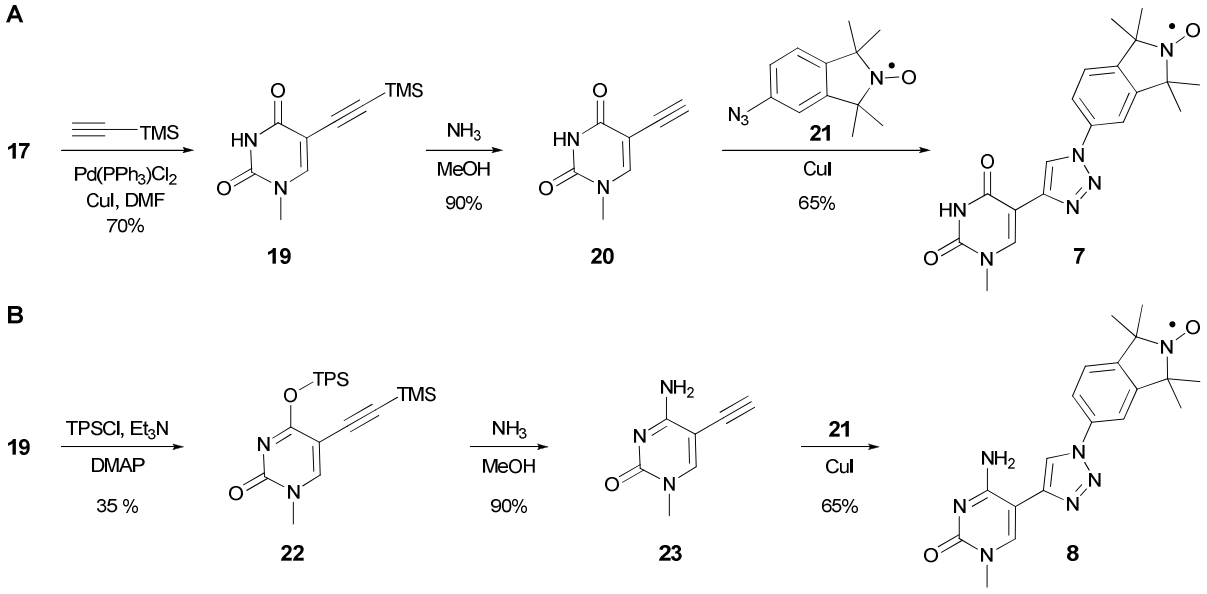


Figure 2: EPR spectra of spin labels (left) and in the presence of an abasic site containing DNA duplex at −30 °C (right). Dotted lines indicate the relative broadening of the EPR spectra of spin labels. For clarity, only a part of the DNA construct is shown. The complete DNA sequence is 5'-d(GAC CTC G__A TCG TG)-3' and its complementary strand 5'-d(CAC GAT **X**CG AGG TC)-3', where __ is an abasic site and **X** is an orphan base opposite the abasic site.



Scheme 2: Syntheses of triazole-linked spin-label derivatives **7** and **8** of uracil (**A**) and cytosine (**B**), respectively.

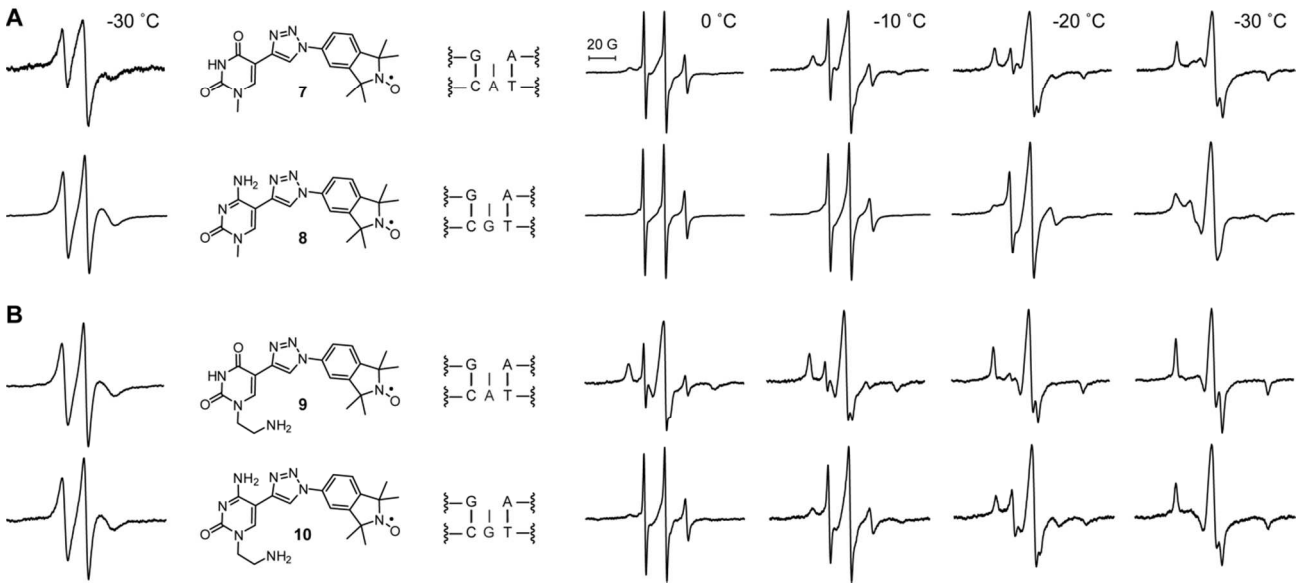
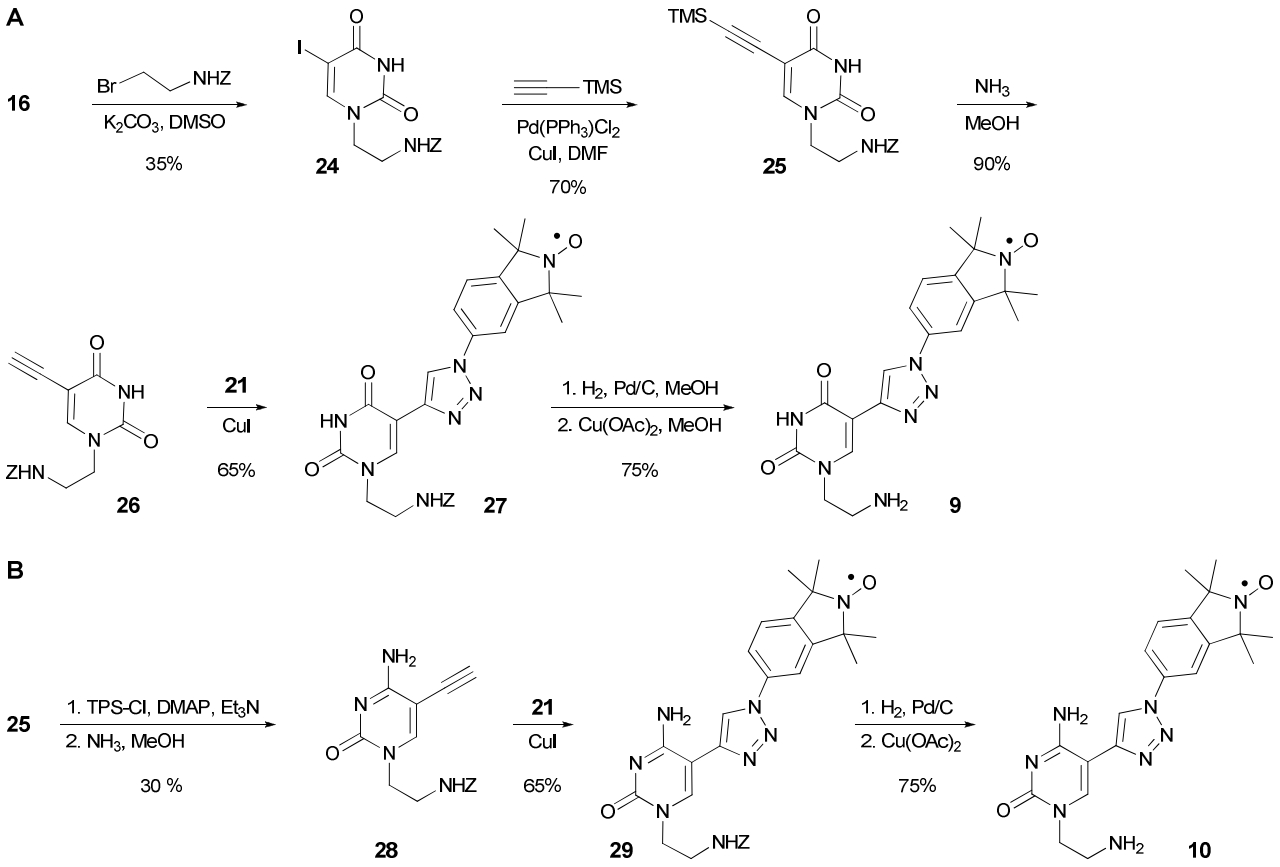


Figure 3: EPR spectra of triazole-linked N1-methyl (**A**) and N1-ethylamino (**B**) spin labels (left) and in the presence of duplex DNA containing an abasic site (right). For clarity, only a part of the DNA construct is shown. The complete DNA sequence is 5'-d(GAC CTC G__A TCG TG)-3' and its complementary strand 5'-d(CAC GAT **X**CG AGG TC)-3', where __ is an abasic site and **X** is the orphan base opposite the abasic site.



Scheme 3: Synthesis of N1-ethylamino triazole-linked spin labels **9** and **10**. Z = carboxybenzyl.

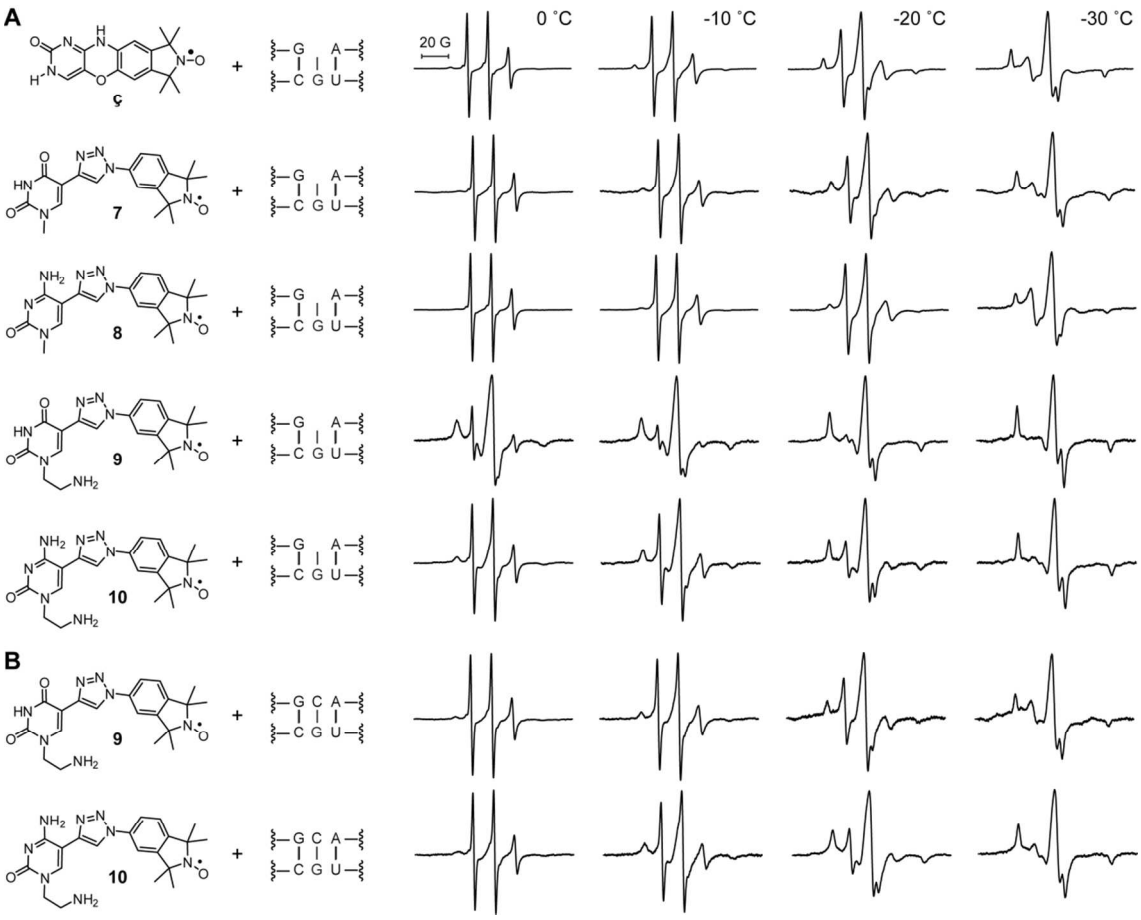


Figure 4: EPR spectra of triazole-linked spin labels in the presence of both an RNA duplex containing an abasic site (**A**) and an unmodified RNA duplex (**B**). For clarity, only a part of the RNA construct is shown. The complete RNA sequences are 5'-GAC CUC G__A UCG UG-3' and 5'-CAC GAU GCG AGG UC-3', where __ is an abasic site.

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Of ten new pyrimidine-derived nitroxide spin labels, an N1-ethylamino triazole-linked uracil derivative binds fully to both DNA and RNA duplexes containing an abasic site, as determined by CW-EPR.