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Chem Soc Rev

TUTORIAL REVIEW

Advances in the synthesis of nitroxide radicals for use in biomolecule spin labelling

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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EPR spectroscopy is an increasingly useful analytical tool to probe biomolecule structure, dynamic behaviour, and interactions. Nitroxide radicals are the most commonly used radical probe in EPR experiments, and many methods have been developed for their synthesis, as well as incorporation into biomolecules using site-directed spin labelling. In this Tutorial Review, we discuss the most practical methods for the synthesis of nitroxides, focusing on the tunability of their structures, the manipulation of their sidechains into spin labelling handles, and their installation into biomolecules.

Introduction

Among various techniques for the structural elucidation of biomolecules, electron paramagnetic resonance (EPR) spectroscopy, which is based on the study of unpaired electron spins in a magnetic field, has become an important tool.[1-3] In order to study biomolecules that are not naturally paramagnetic (e.g. certain metalloproteins or radical enzymes), a persistent radical, termed a 'spin label', must be introduced into a biomolecule through a strategy known as site-directed spin labelling (SDSL). The need for efficient and orthogonal chemistries for SDSL, as well as for radicals with improved stability and spectroscopic properties, has led to the development of a wide variety of spin labels. Commonly used paramagnets include organic-based nitroxide (aminoxyl) or trityl derivatives,[4] or paramagnetic metal ions (e.g. Mn(II), Cu(II), Gd(III)).[1-3] Among these, the nitroxide remains the most widely employed spin label in biomolecular EPR research.[5]

In this Tutorial Review, we survey methods for the preparation of the most important classes of nitroxide and, to illustrate context and applicability, discuss recent case studies of their use in biomolecule spin labelling. Aside from a brief overview of nitroxide properties of relevance to bio-EPR, it is beyond the scope of this Review to offer a comprehensive coverage of applications of EPR in structural biology. For this, the reader is referred to recent excellent treatises. [1-5]

EPR spectroscopy as a tool for investigating biomolecules

EPR spectroscopy is a highly sensitive analytical method which requires relatively small quantities of spin labelled material; as such, it is well-suited to the study of biological systems. As it is also compatible with disordered or inhomogeneous systems of virtually unlimited molecular size,[1] EPR is particularly powerful for the study of biomolecules that are difficult to crystallize, or too large to characterize by other methods such as NMR.

A variety of EPR techniques are available, which afford different types of structural data. Continuous wave (CW) experiments allow the environment and dynamic mobility of the spin labelled molecule to be measured; such data can inform on processes such as the binding of a ligand to a protein. Pulsed EPR methods are also highly informative: a particular focus of the applications described in this Review is double electron-electron resonance (DEER, also known as PELDOR), which measures nanometre-scale distances (typically 15-80 Å) between two unpaired spins in an immobilized system.[1] If the molecular framework carrying the spin labels is geometrically well-defined, information can also be obtained on their relative orientation, which is potentially of great value in the elucidation of tertiary structure.

Notably, the range of distances accessible by DEER coincides with fluorescence-based methods that are widely applied in structural biology, such as Förster resonance energy transfer (FRET),[6] and fluorophore quenching (by proximal spin labels).[7] While fluorescence methods benefit from higher concentration sensitivity than EPR, and can provide distance measurements at room temperature (compared to DEER experiments, which usually require temperatures of around 50 K), they are complicated by the need for two different 'tags', whereas DEER can be performed on pairs of identical labels. In addition, as fluorescent labels are larger and bulkier than most spin labels, and typically use relatively flexible linker groups, the interprobe distance is usually less well-defined. As such, these techniques generally afford less detailed information than a well-designed SDSL/EPR strategy.

The linker selected to attach a spin label to its biomolecule is crucial to the quality of the resulting EPR data. Ideally, the spin label framework and linker should be as conformationally

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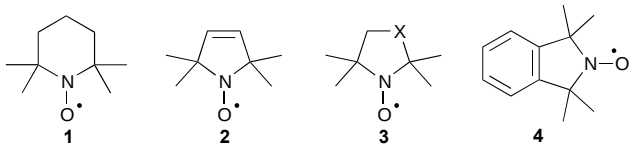

Figure 1. Commonly encountered nitroxides include: Piperidines (**1**), pyrrolines (**2**), pyrrolidines (**3**, X = C), oxazolidines / imidazolidines (**3**, X = O, N), and isoindolines (**4**).

restricted as possible, but equally should not cause extensive structural perturbation of the labelled biomolecule. Conformationally-mobile spin labels report mainly on the motion of the label (and not the biomolecule itself) in CW EPR, while distance distributions derived from DEER experiments widen due to a broader ensemble of conformations, leading to less precise measurements. Achieving rigid but non-perturbing labelling thus remains a prime challenge in SDSL, as well as increasing the range and sensitivity of EPR distance measurements, performing pulsed EPR experiments at ambient temperatures, and conducting EPR *in vivo*.

Nitroxide structure, stability and EPR spectroscopic properties

The popularity of the nitroxide is due in part to its relatively high stability, which derives from the 'steric stabilization' afforded by the substituents of the quaternary carbon atoms that flank the radical (examples of common nitroxide frameworks **1**-**4** are shown in Figure 1). Other attractive features are the highly tunable nature of the heterocyclic scaffold, the ease of introduction of spin labelling 'handles', and the relatively localised nature of the unpaired electron which, being mainly distributed over the N–O bond, intrinsically improves the accuracy of EPR measurements.[1]

Nitroxides are nonetheless susceptible to oxidation to an oxammonium ion, or reduction to a hydroxylamine,[5] the latter being an important factor for use in biological media,[8] which can be reducing environments. The stability of the nitroxide towards these processes depends on a number of factors. For example, the size of the nitroxide-bearing ring greatly influences the susceptibility of the radical to reduction, with six-membered piperidinyl nitroxides such as **1** (Figure 2a) being more prone to reduction than five-membered nitroxides, and unsaturated pyrrolinyl radicals **2** being less stable than saturated pyrrolidinyl radicals **5**.[9, 10] Electron-withdrawing substituents render the radical more reduction-prone, while electron-donating groups have the opposite effect.[11] In the absence of electronic effects, replacing the methyl groups in **1** with spirocyclohexyl groups (**6**, Figure 2b) somewhat increases the stability of the nitroxide towards reduction by ascorbic acid; however, the enhancement is dramatic with tetraethyl substitution (**7**) due to more effective steric shielding by the conformationally mobile ethyl groups.[8, 10] The nitroxide is also influenced by local environment effects such as pH (which can lead to different protonation states of nitroxides such as **3**), or oxygen concentration (due to spin exchange processes).[12]

In pulsed EPR experiments such as DEER, the maximum measurable distance, and the resolution of the distance

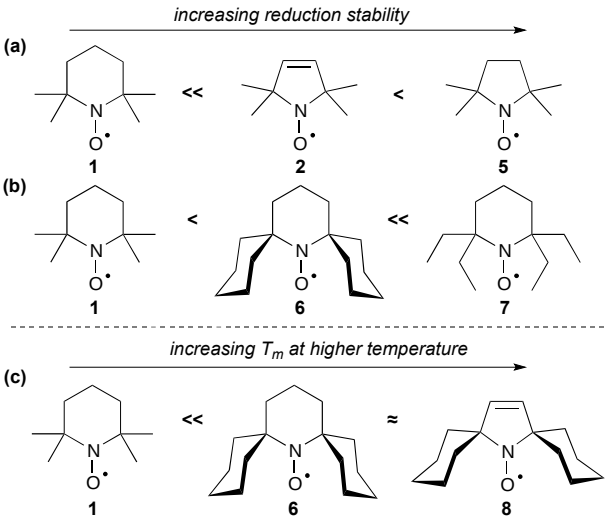

Figure 2. Influence of **(a)** nitroxide ring structure and **(b)** size of the α-substituents on reduction stability; **(c)** influence of ring structure and α-substituents on T_m values.

distributions, is limited by a parameter known as the phase memory time T_m, which should be as high as possible. This is the main reason DEER experiments are typically performed at cryogenic temperatures, and a major challenge for biomolecular applications of EPR spectroscopy is the development of spin labels that exhibit sufficiently long T_m values to enable measurements at room temperature. Spin labels with α-spirocyclohexyl substituents (e.g. **6** or **8**, Figure 2c) significantly extend T_m at temperatures of 60-180 K compared to equivalent tetramethyl substitution,[9, 13-15] as the rotation rate of the methyl group C–C bond contributes to a relaxation mechanism that reduces T_m in this temperature range (which is avoided with spirocyclic substituents). Finally, for biomolecular applications, aqueous solubility is also key.

Preparation of the nitroxide functionality

Nitroxides are most commonly introduced by oxidation of the corresponding secondary amine. This is achieved by reaction with *m*-CPBA (e.g. **9**→**10,** Scheme 1a), or with H_2O_2 and catalytic Na_2WO_4 (e.g. **11**→**12**).[5, 13] The former is significantly more rapid, especially for hydrophobic amines, as it can be performed in less polar solvents in which the substrates are more soluble. The oxidations likely proceed *via* initial conversion of the amine to a hydroxylamine **13**, and then to an oxammonium salt **14**. This either disproportionates (with **13**) to give the nitroxide, or oxidizes H_2O_2 to O_2, also forming **12**.[5] Nitroxides can further be prepared from hydroxylamines by treatment with mild oxidants such as MnO_2 or Cu(II) salts in the presence of O_2 (e.g. **15**→**16**, Scheme 1b),[14] and under non-acidic conditions these can undergo spontaneous oxidation by atmospheric oxygen. These milder methods have primarily been used in synthetic routes where hydroxylamine intermediates are isolated: for example, addition of ethynylmagnesium bromide to nitrone **17** generated a hydroxylamine, which was then oxidized with

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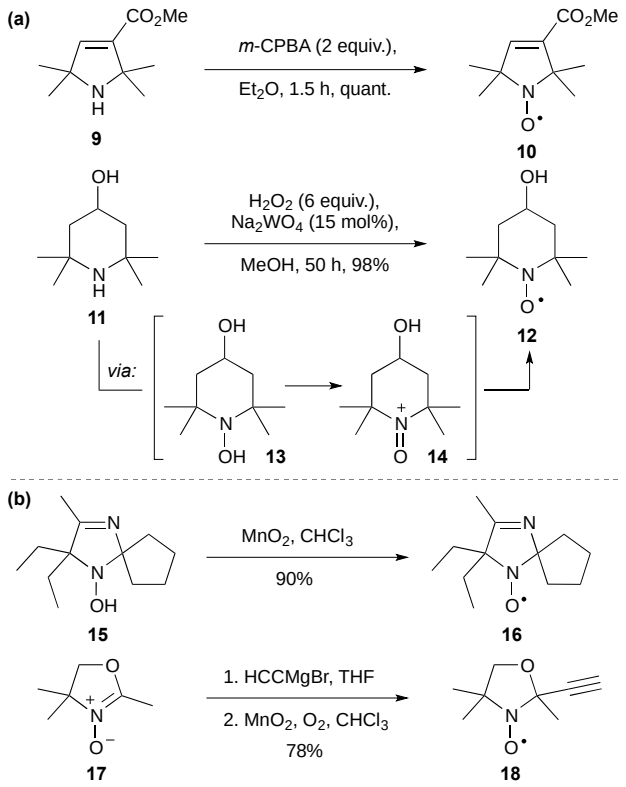


Scheme 1. Common methods to prepare nitroxides by **(a)** oxidation of secondary amines; **(b)** oxidation of hydroxylamines.

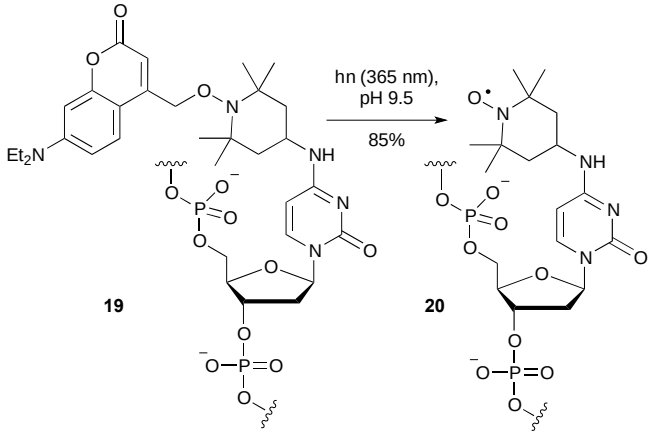
MnO_2 and oxygen to give nitroxide **18**.[16]

As nitroxides are susceptible to degradation under oxidizing or acidic conditions (as used in solid-phase oligonucleotide synthesis), protecting group strategies have also been developed.[17] Particularly attractive in terms of potential biological applications are photolabile protecting groups such as the cytidine derivative **19** (*ortho*-nitrobenzyl groups were also used).[18] Irradiation of **19** with UV light under air revealed the spin labelled DNA **20**.

Variation of the nitroxide flanking substituents

A number of strategies have been developed to tune the structural elements of the nitroxide framework, and hence its properties. This includes variation of the ring structure, the nature of the α-substituents, and the functional groups used to attach the spin label to its target biomolecule. In this section, variation of the substituents flanking the nitroxide is discussed, as in most approaches to nitroxides these are modified at an early stage in the synthesis.

Most syntheses of common monocyclic nitroxides begin with the commercially available tetramethylpiperidone **21** (Scheme 3a). Variation of the α-substituents is most conveniently achieved through a ketone exchange process: for example, **21**, or its *N*-methyl derivative **22**, are converted to the spirocyclohexyl derivative **23** through NH_4Cl-catalyzed exchange of acetone with cyclohexanone.[19] This protocol was reported to give higher yields using tertiary amine **22**; however



Scheme 2. Photolabile protection of nitroxide spin labels as *O*-alkyl hydroxylamines.

we have found that superior results can be achieved from **21** simply by extending the reaction time.[13] Whilst such ketone exchange methods have been demonstrated only using cyclic ketones, popular tetraethyl-substituted piperidines can nevertheless be accessed by reaction of piperidone **22** with thiopyranone **24**, which gives spirocyclic intermediate **25**. Reduction of the C–S bonds with Raney-Ni (which incidentally also reduces the ketone) gives tetraethylpiperidine **26**.[10, 19] A less common approach from acetonin (**27**, Scheme 3b) has been used to access hydroxylated spirocycle **28**, albeit in moderate yield, which led to a nitroxide with high aqueous solubility.[20]

The tetrasubstituted piperidine ring structure has also been assembled *via* a double Horner-Wadsworth-Emmons strategy from bisphosphonate **29** (Scheme 3c). A double aza-Michael addition of ammonia to the intermediate dienone **30** (formed as a mixture of double bond regioisomers), gave the corresponding tetraethylpiperidone.[21] This route has afforded both tetraethyl and bis(spirocyclohexyl) substituted piperidine nitroxides, as well as non-symmetric variants.

Isoindoline-based nitroxides are the subject of much attention due to the enhanced rigidity of their benzannulated frameworks, which in equivalent environments can lead to narrower distance distributions compared to aliphatic skeletons. Adjustment of the flanking substituents of these derivatives is achieved in the course of their synthesis from *N*-benzylphthalimide (**31**, Scheme 3d) via a challenging fourfold Grignard addition. This proceeds best when hemiaminal **32** is isolated (from a single addition), and then resubmitted to excess Grignard reagent, giving tetrasubstituted isoindoline **33** in respectable yield.[13, 22] Subsequent functional group manipulations and *N*-oxidation are carried out to afford the isoindoline spin label.

Five-membered ring monocyclic nitroxides with different α-substituents are generally prepared by ring contraction of the corresponding tetrasubstituted piperidones (see below), or in the course of ring synthesis, as shown in the following section for cyclic *N*,*N*- or *N*,*O*-acetal nitroxides. Nucleophilic addition processes can also be used, such as discussed above in the preparation of **18** (Scheme 1b).[16]

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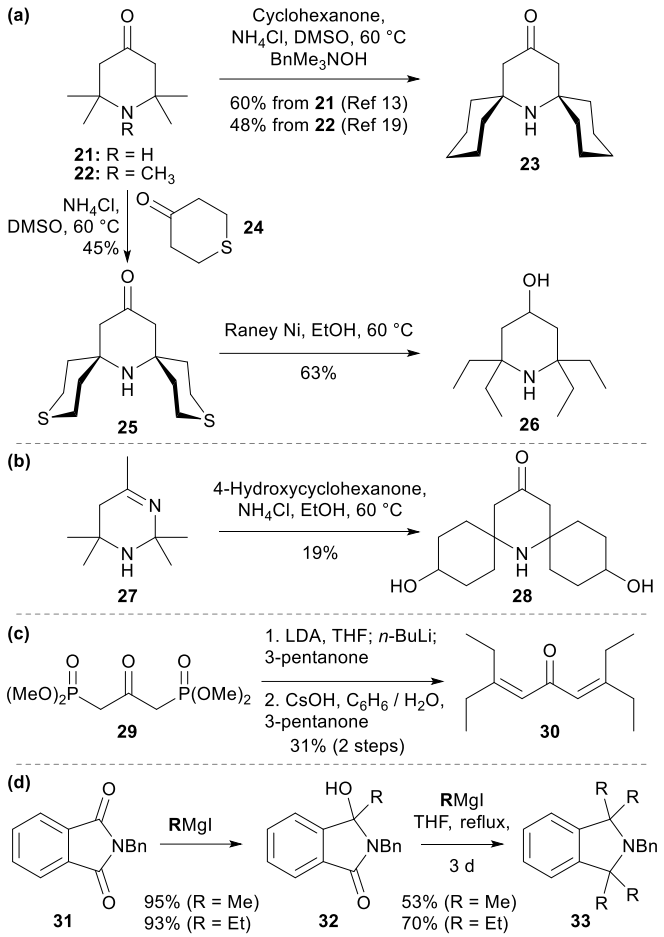
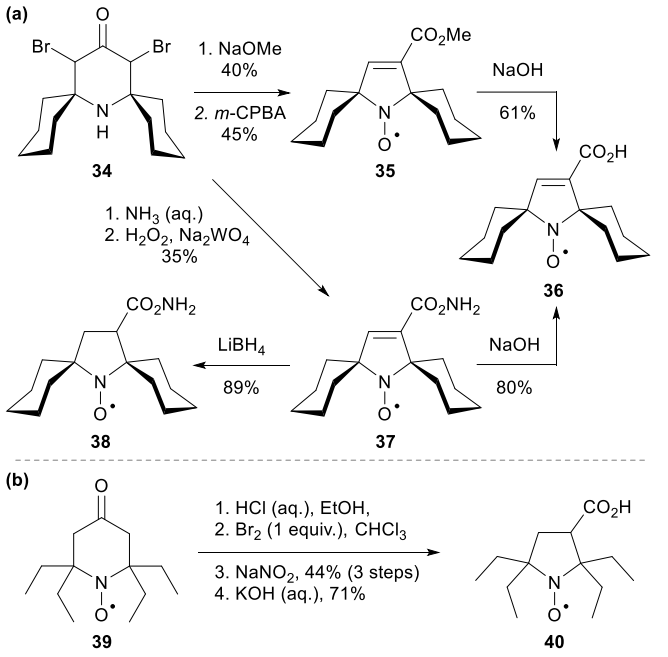


Scheme 3. Strategies to adjust the α-substituents of nitroxide precursors by **(a)**, **(b)** ketone exchange, **(c)** olefination/Michael addition and **(d)** Grignard addition strategies.



Scheme 4. Favorskii rearrangement routes to prepare pyrroline and pyrrolidine nitroxides from piperidones by **(a)** dibromination and **(b)** monobromination.

Variation of the nitroxide ring size and structure

Structural variation of the nitroxide-containing ring can be accomplished in a number of ways. As discussed above, five-membered nitroxides are particularly attractive due to improved rigidity and reduction stability compared to six-membered rings; the most versatile route to access these motifs involves Favorskii ring-contraction of the corresponding piperidone, a tactic which has been applied to rings with methyl, ethyl, and spirocyclohexyl α-substituents. Thus, the unsaturated pyrroline is accessed *via* α,α'-dibromination of the piperidone (e.g. **34**, Scheme 4a), with Favorskii rearrangement / dehydrohalogenation giving the unsaturated product **35** (due to the sensitivity of the nitroxide to bromine, N-oxidation is usually carried out after the rearrangement). Depending on the reaction conditions, or if a subsequent hydrolysis is carried out, this route can afford the ester, amide, or carboxylic acid derivatives **35**-**37**,[9, 13, 23] which can be converted into a range of functionalities for SDSL. Saturated pyrrolidine nitroxides are obtained by borohydride reduction of the α,β-unsaturated carboxylated pyrrolines (e.g. **37**→**38**),[9] or by monobromination / Favorskii rearrangement (**39**→**40**, Scheme 4b); in the example shown, the nitroxide functionality was temporarily protected as a hydroxylamine by treatment with HCl.[10]

Isoindoline-based nitroxides are usually synthesized from *N*-benzylphthalimide (see Scheme 3d); typically, the aromatic ring of **33** is functionalized after this process to incorporate spin labelling handles (see below). However, some (lengthier) routes to isoindolines have been reported that have the potential to introduce substituents on the arene in the course of ring synthesis, such as the synthesis of isoindoline **41** (Scheme 5a) from pyrroline **42** by Suzuki coupling / Horner-Wadsworth-Emmons olefination / 6π-electrocyclization.[24] An alternative approach to functionalized isoindolines involves direct bromination of the benzene ring (**43**→**44**), which enables the introduction of a variety of substituents including water-solubilizing carboxylic acid **45**, which could be introduced by lithiation / quench with CO_2, or cyanation (**46**) followed by nitrile hydrolysis.[25]

Cyclic *N,N*- and *N,O*-acetal nitroxides benefit from concise, modular preparation sequences. For example, oxazolidine nitroxide **47** (Scheme 5b) was assembled by condensation of aminoalcohol **48** with ketone **49**; *N*-oxidation of the resulting oxazolidine followed by phthalimide deprotection gave **47**.[26] Similarly, condensation of cyclopentanone with hydroxylamine **50** in the presence of ammonium acetate afforded an intermediate hydroxylamine, oxidation of which gave imidazoline nitroxide **51**.[14] *N*-Methylation and reduction of the iminium ion gave saturated imidazolidine **52**. Despite these convenient sequences and substituent flexibility, this class of nitroxide is yet to see extensive use in biomolecule SDSL. Further examples of ring structure variation are discussed later in the context of amino acid and nucleoside analogues.

Introduction of single-point labelling functionalities

The above chemistries provide a number of options for the synthesis of nitroxide-containing rings adorned not only with

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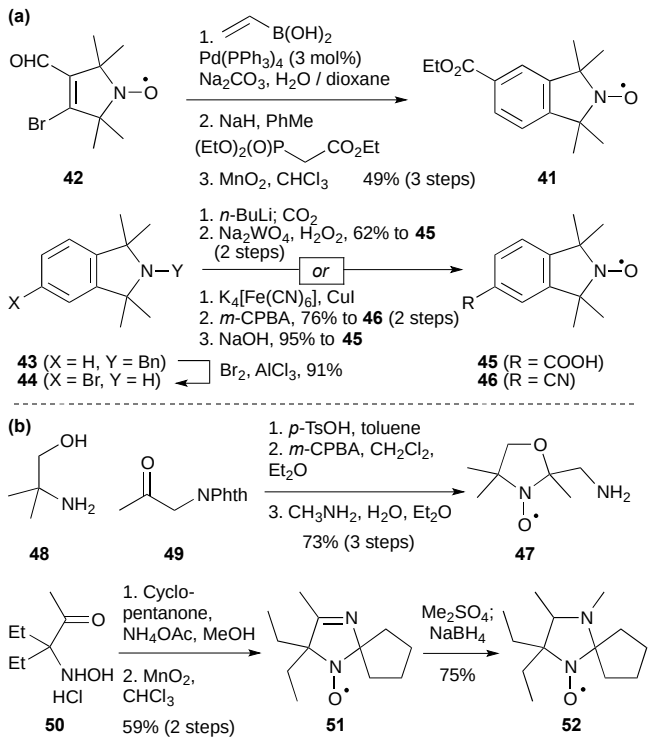
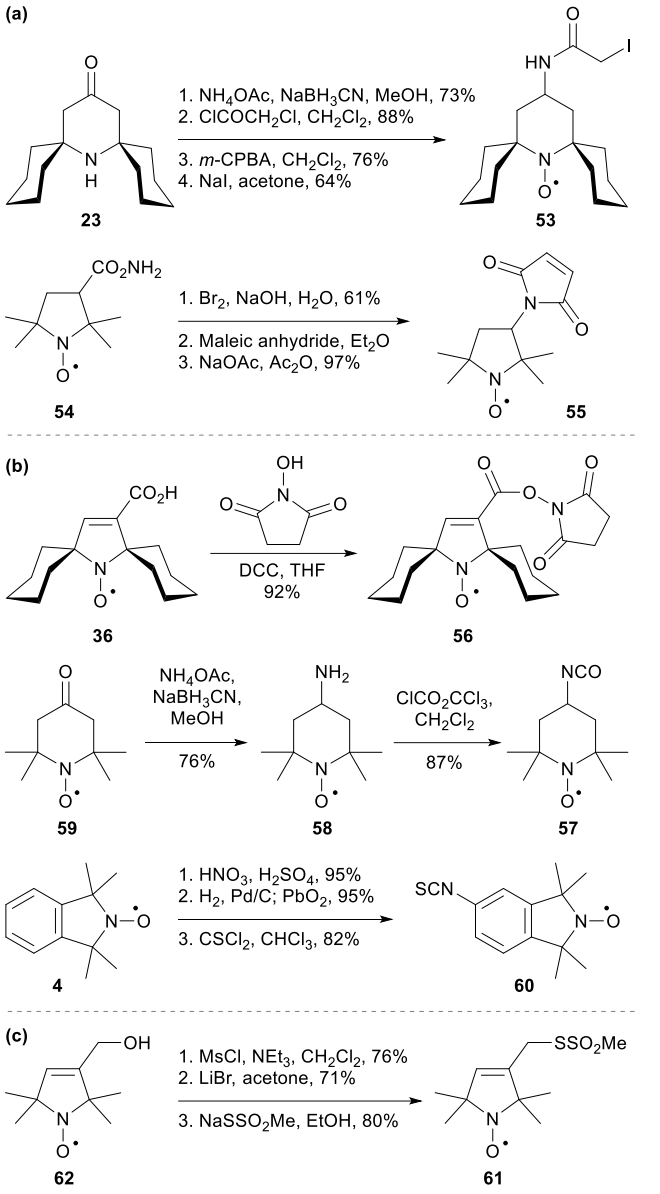


Scheme 5. (a) Routes toward functionalized isoindoline nitroxides; **(b)** synthesis of oxazolidine, imidazoline and imidazolidine based nitroxides.

tuneable α-substituents that influence the properties of the radical, but also with residual functional groups that are ideal for the introduction of SDSL handles – i.e. conversion into functionalities that enable attachment to biomolecules. In this section, the installation of a single SDSL site on the spin label scaffold is discussed from the perspective of different SDSL strategies, and their associated functional groups.

Alkylation and acylation are popular methods for the spin labelling of nucleophilic amino acid sidechains of proteins (usually cysteines), using electrophilic nitroxide derivatives. For example, α-iodoacetamides such as **53** (Scheme 6a) are readily available from the corresponding ketone (**23**) by reductive amination, acylation with chloroacetyl chloride, oxidation to the nitroxide, and iodination.[15] Maleimides have also been used as electrophiles for bioconjugation to cysteines: Hofmann rearrangement of amide **54**, and two-step condensation of the amine with maleic anhydride (*via* the intermediate amide-acid) gives commercially available **55**.

The activated *N*-hydroxysuccinimide ester **56** (Scheme 6b) is a popular acylating agent for the spin labelling of lysine sidechains, and is prepared in high yield from acid **36** by DCC-mediated esterification.[9, 27] Isocyanates have been used extensively as acylating spin labels for nucleotides; isocyanate **57** was prepared from commercial amine **58** (accessed by reductive amination of ketone **59**) *via* reaction with diphosgene.[28] Isothiocyanates based on the isoindoline scaffold have been reported to be more hydrolytically stable acylating agents compared to isocyanates; nitroxide **60** was prepared from the corresponding aniline (derived from isoindoline **4** by nitration, hydrogenation and oxidation of the



Scheme 6. Routes towards a selection of spin labels used in **(a)** alkylation, **(b)** acylation and **(c)** disulfide formation SDSL strategies.

resulting hydroxylamine) by treatment with thiophosgene.[29]

Disulfides enable cysteine-specific labelling – for which the popular pyrroline label methanethiosulfonate **61** ('MTSSL', Scheme 6c) can be prepared from alcohol **62**[13] in three steps, including a nucleophilic displacement of bromide by methanethiosulfonate.[30] A bis(spirocyclohexyl) version of this spin label has been prepared *via* a similar route.[9]

Cu-catalysed click chemistry is well-established as an orthogonal and efficient strategy for bioconjugation, and a range of azide spin labels have been used for this purpose. One example is azide **63** (Scheme 7a) which is easily prepared from piperidone **21** in four steps.[13] Aromatic azides are also known: isoindoline azide **64** was generated from aniline **65** through a diazo transfer reaction with triflyl azide, followed by oxidation to the nitroxide.[13, 31] It is also possible to spin label biomolecules with alkynylated nitroxides through click or

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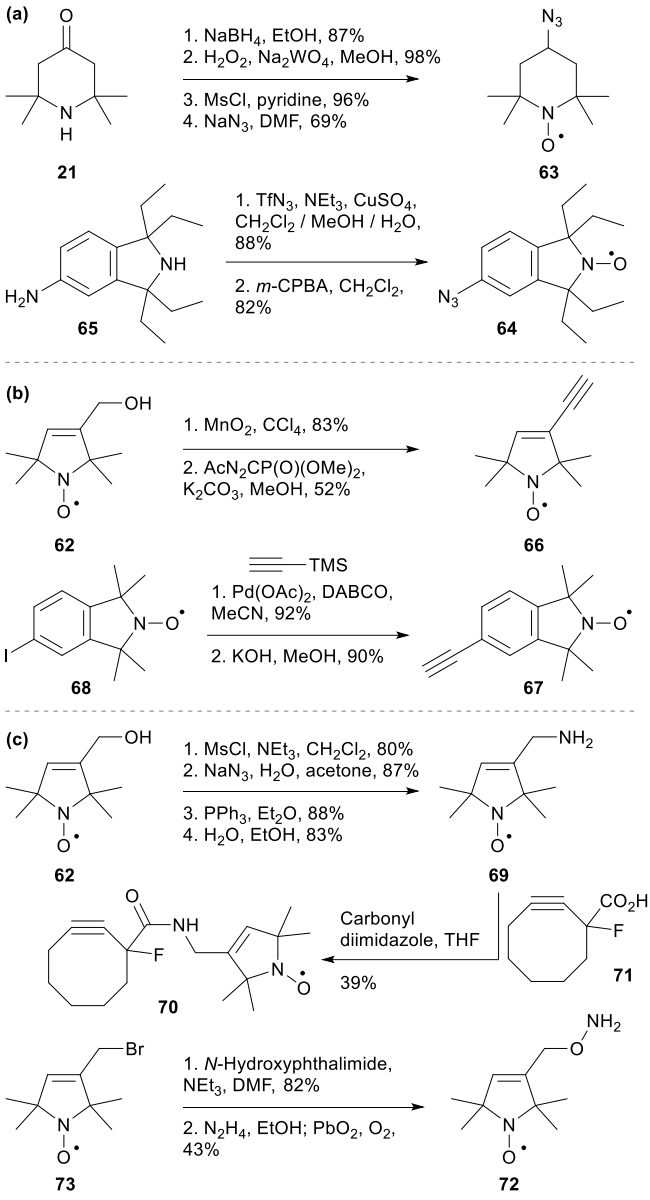
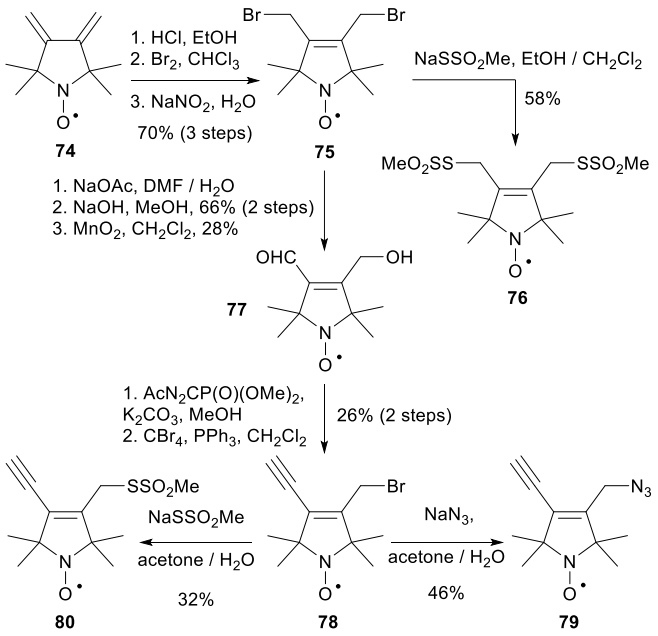


Scheme 7. Synthesis of spin labels containing **(a)** azides and **(b)** alkynes, suitable for SDSL *via* click chemistry/Sonogashira coupling, and **(c)** bioorthogonal SDSL spin labels.

Sonogashira cross-coupling SDSL strategies. Amongst the most well-established is enyne **66** (Scheme 7b; also known as 'TPA'), which is prepared from alcohol **62** by oxidation to the aldehyde and subsequent alkynylation by the Ohira-Bestmann protocol.[32] The equivalent isoindoline alkyne **67** has been synthesized from iodide **68** by Cu-free Sonogashira cross-coupling with trimethylsilylacetylene.[33] Finally, bioorthogonal strategies are highly attractive from the perspective of achieving intracellular SDSL. Aside from the biostability of the nitroxide, additional factors (such as selectivity, efficiency, reaction rate, and toxicity of reagents / side products) are important for the choice of these labelling chemistries. Several spin labels have been designed for this purpose; for example, amine **69** (Scheme 7c), prepared from alcohol **62** by straightforward manipulations, was converted into cyclooctyne spin label **70** through amide coupling with **71**.[34]



Scheme 8. Preparation of bifunctional spin labels from diene **74**.

Another biocompatible spin label is the *O*-alkyl hydroxylamine **72**, which has been used to form oxime ethers with *p*-acetylphenylalanine residues in proteins.[35] This spin label was prepared from bromide **73** by substitution with *N*-hydroxyphthalimide, followed by phthalimide cleavage. Reoxidation with PbO_2 is required since hydrazine reduces the nitroxide.

Introduction of two-point labelling functionalities

The incorporation of a spin label into a biomolecule through two points of attachment can increase the conformational rigidity of the spin labelled system compared to attachmentthrough one linker group. Although only a few applications of this strategy in biomolecule spin labelling have been described to date, a number of interesting nitroxides bearing two labelling groups have been developed; we expect this tactic to become more widely used, albeit it also relies on the presence of two suitably proximal anchoring points on the biomolecule.

The diene nitroxide **74** (Scheme 8, prepared in 6 steps from **10**, Scheme 1) is a common intermediate in the syntheses of several bifunctional pyrroline spin labels.[36] **74** was converted to dibromide **75** by reaction with bromine, after temporary protection of the nitroxide as a hydroxylamine. As well as serving as a double alkylating spin label in its own right,[37] dibromide **75** can be transformed into other spin labels: for example, treatment of **75** with nucleophiles such as sodium methanethiosulfonate leads to bis-MTS **76**.[36] Alternatively, a two-step hydrolysis followed by mono-oxidation gave aldehyde **77**; subsequent alkynylation and various manipulations gave bromide **78**, azide **79** or MTS **80**.[32]

Maleimide sidechains equipped with leaving groups enable dual attachment of spin labels. A dibromomaleimide nitroxide

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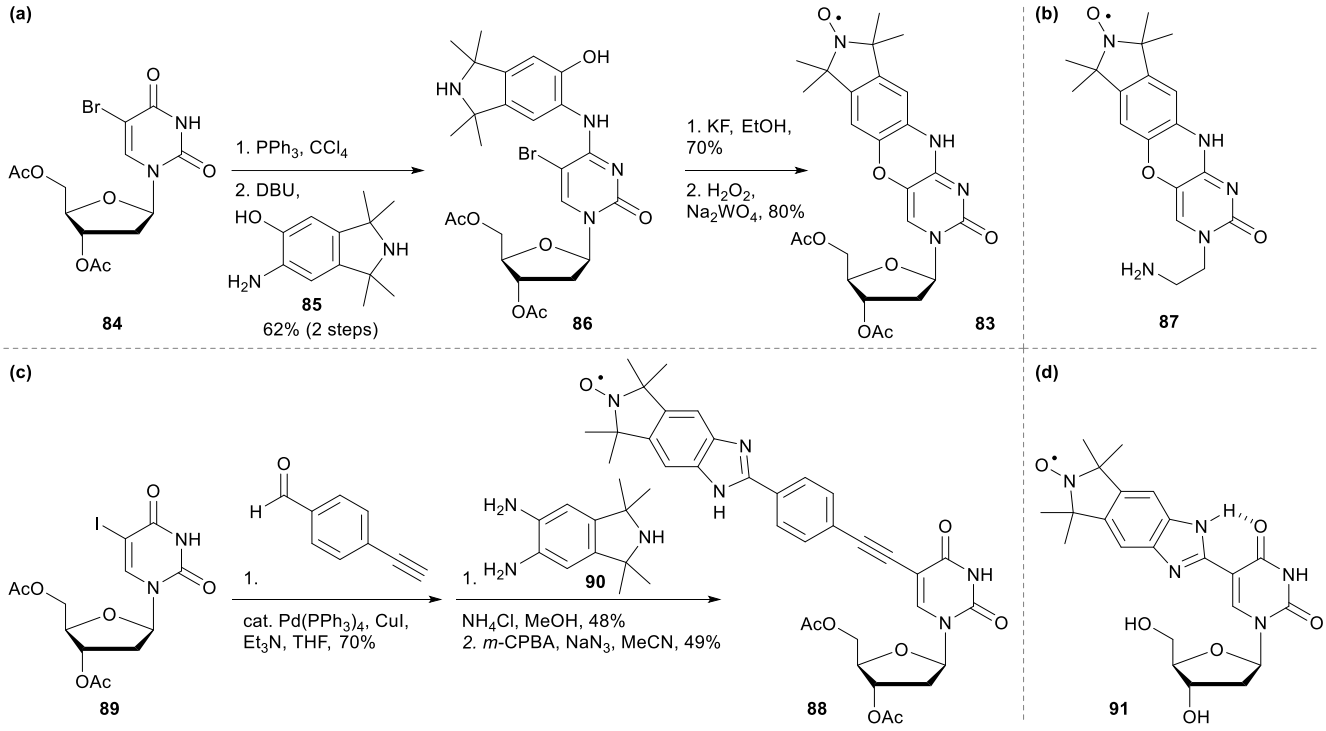
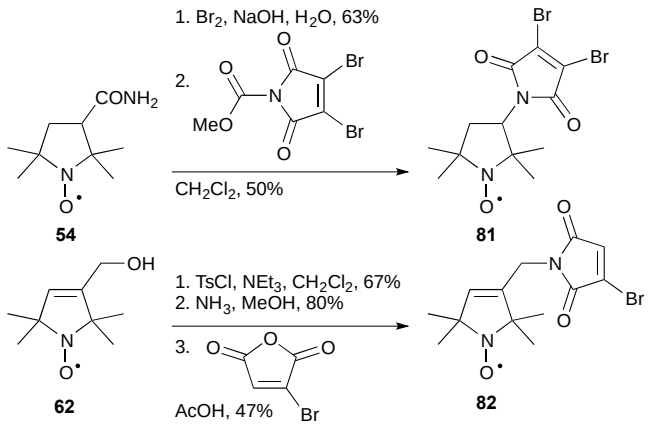


Scheme 10. Synthesis of nitroxide nucleobase analogues: **(a)** cytidine mimic **83** (Ç); **(b)** non-covalent cytosine mimic **87**; **(c)** conformationally unambiguous benzimidazole **88**; **(d)** conformationally restricted benzimidazole **91**.

81 (Scheme 9) has been prepared from amide **54** *via* Hofmann rearrangement to an intermediate amine, then reaction with *N*-methoxycarbonyldibromomaleimide.[38] Monobrominated maleimide **82** has been accessed from alcohol **62** by tosylation, substitution with ammonia, and condensation with bromomaleic anhydride.[39] These electrophiles could be used to label two spatially proximal cysteines;[38] label reactivity can also be tuned by use of phenol or thiophenol leaving groups.

Synthesis of nitroxide nucleoside and amino acid analogues

Appropriately functionalized isoindolines can be converted into spin label analogues of the nucleobases found in DNA and RNA. Arguably the most significant example of this strategy is the cytidine analogue **83** (Scheme 10; known as Ç, or 'C-spin'), which has been shown by EPR studies to adopt highly rigid conformations on guanosine base pairing. This is prepared in a relatively straightforward manner by formal condensation of 5-bromouridine **84** and aminophenol isoindoline **85** on activation by triphenylphosphine, followed by S_NAr cyclization of the intermediate bromide **86**.[40] Oxidation to the nitroxide affords **83**, and this nucleoside was incorporated into nucleic acids using solid-supported phosphoramidite chemistry under conditions modified to prevent nitroxide degradation. The free nucleobase analogue **87**, prepared *via* a similar route from 5-bromouracil, has been used for non-covalent spin labelling of nucleic acids by occupation of an abasic site opposite a complementary guanosine.[41] A related guanine mimic has also been reported (see Examples section, below).[42]



Scheme 9. Synthesis of nitroxides carrying dibrominated and monobrominated maleimide groups for dual-point attachment to two cysteines.

Benzimidazole-linked isoindoline nitroxides have also been developed. One example is **88**, which was prepared from 5-iodouridine **89** and triamine **90** by a Sonogashira coupling / oxidative condensation sequence.[43] This spin label has free rotation around the acetylene axis, but as this is co-axial with the nitroxide, it nonetheless provides a highly defined spin label environment. The related uridine benzimidazole nitroxide **91** is rigidified by an internal hydrogen bond, rendering it similarly restricted to Ç **83**.

Various amino acid mimics are available. One of the more recent advances in this area is the phenylglycine mimic 'TOPP' (**92**, Scheme 11).[44] Again due to the arene rotation axis that also contains the nitroxide, quite narrow distance distributions were obtained in DEER experiments. A drawback is its rather lengthy 11 step synthesis from hydroxyphenylglycine **93** (albeit

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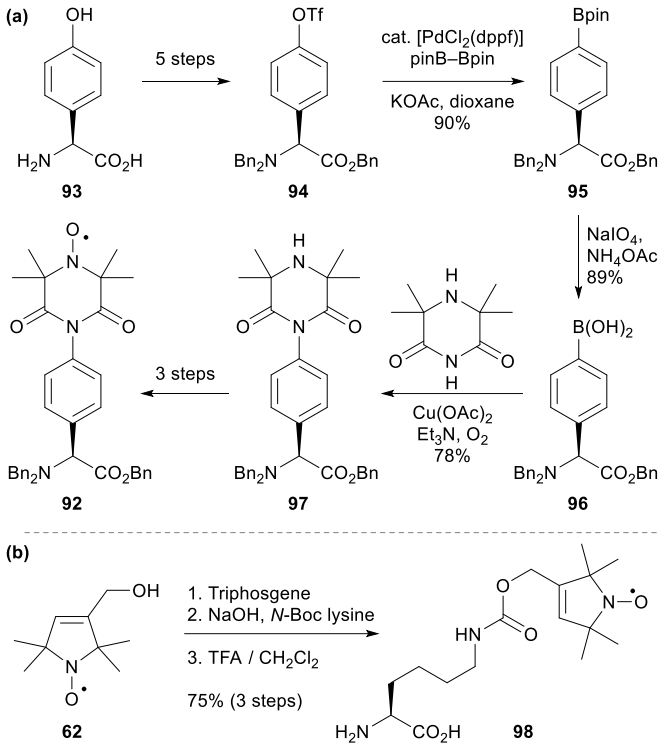


Scheme 11. Amino acid mimics **(a)** phenylglycine mimic **92** (TOPP), and **(b)** glutamine mimic **98**, for biosynthetic incorporation into proteins.

proceeding in 17% overall yield); key features of this route are the Miyaura borylation (**94**→**95**) and Chan-Lam coupling (**96**→**97**) to install the piperazine dione, itself a relatively unusual spin label scaffold. More recently, the glutamine analogue **98**, synthesized in three steps from nitroxide **62**, has been impressively incorporated into proteins biosynthetically using amber stop codon technology, albeit requiring quite flexible linker groups.[45]

Recent examples of biomolecule spin labelling

In this final section, recent applications of biomolecule spin labelling are described which reflect the state of the art in the use of EPR spectroscopy to characterize biomolecule interactions and dynamics. Due to the limitations of space, we here focus on selected examples that illustrate the use of each of the various nitroxide spin labels described above; for fuller discussions, the reader is referred to recent reviews.[1-5]

The methanethiosulfonate (MTS) label **61** (Scheme 6c) remains arguably the most general and commonly used method for protein labelling, as being cysteine-specific it can be readily installed using site-specific mutagenesis. A recent elegant example that illustrates its use is the spin labelling of six different cysteine mutants of the copper-binding protein azurin (Figure 3).[46] DEER was used to measure nitroxide–Cu(II) ion distance distributions, which afforded a position estimate for the metal ion. Whilst the overall distance distribution calculated for the Cu(II) ion was relatively small, it was noted that its location as predicted from EPR was of the order of 2 Å displaced from that determined by X-ray crystallography. This

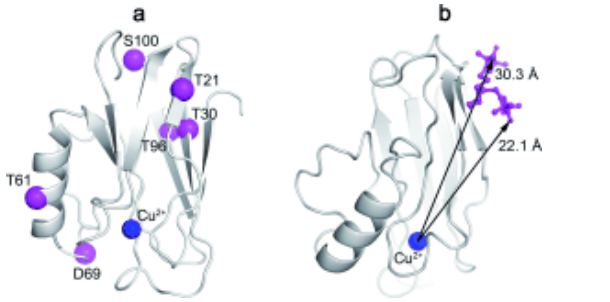
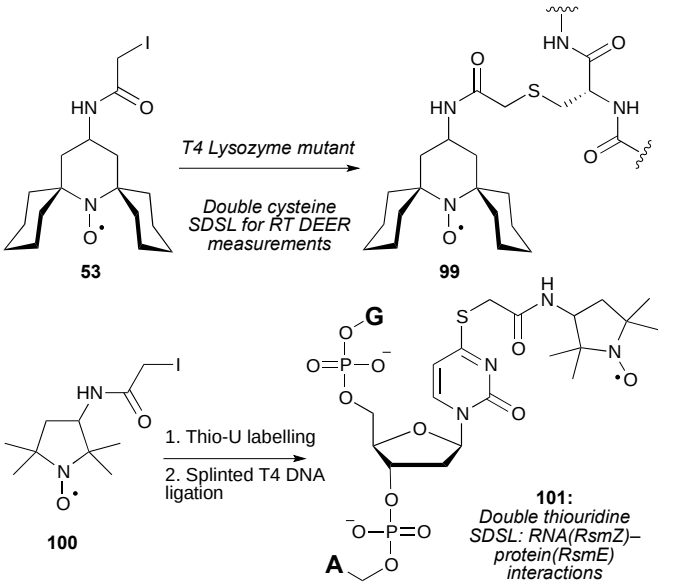


Figure 3. MTS-based multi-measurement DEER location of a copper(II) ion. Figure reproduced with permission from Reference 46.

discrepancy arises from a) the fact that the MTS label populates a number of different conformations; b) the cysteine modification was also found to populate at least two conformations; c) the location of a high degree of the copper ion 'spin' on its cysteine ligand introduces a systematic error. Nonetheless, this is an impressive demonstration of how a 'triangulation' approach can afford high levels of structural information. Examples of two-point attachment using the MTS radicals **75** and **76** (Scheme 8) have also been described: **76** has been used to label proximal cysteines in α-helices and β-sheets, where double-point labelling showing significant conformational advantages over single-point MTS labelling;[47] **75** has been applied to the double alkylation of proximal phosphorothioates in an oligonucleotide.[37]

Iodoacetamides are suited to both protein and nucleic acid spin labelling, as illustrated in the recent report of the first use of a spirocyclohexyl piperidinoxyl iodoacetamide **53** to spin label a double cysteine mutant of the well-studied T4 lysozyme (**99**, Scheme 12).[15] The T_m value for this label is sufficiently long that room temperature DEER measurements proved possible, and indeed this label compared favourably with MTS. The resulting distance distributions were informative, but indicated conformational flexibility in the label, underlining the importance of the linker in defining spin label position.



Scheme 12. Iodoacetamide derivatives in protein and RNA spin labelling.

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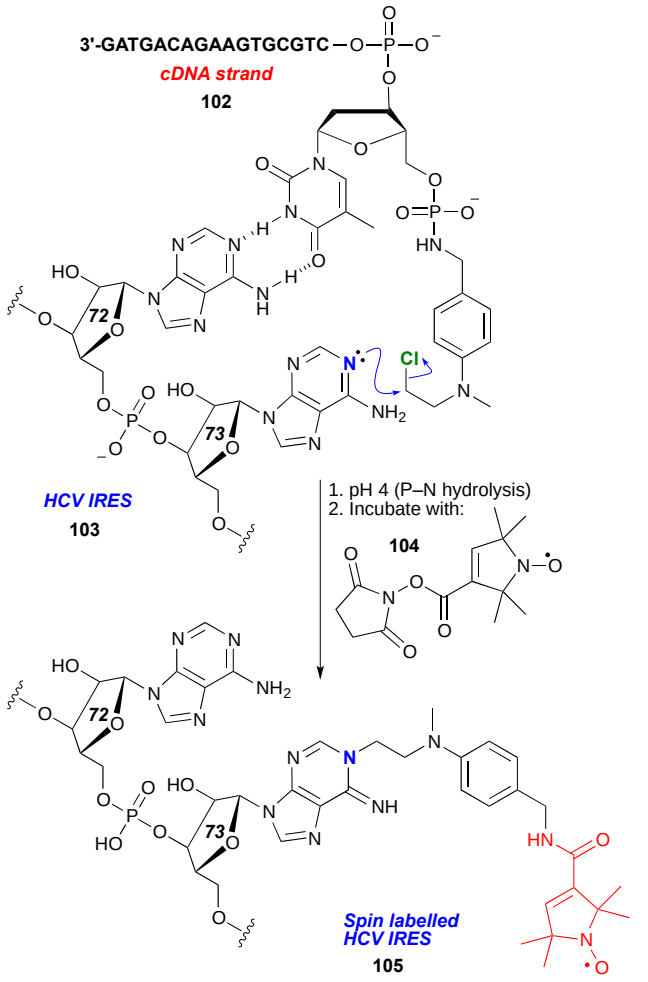
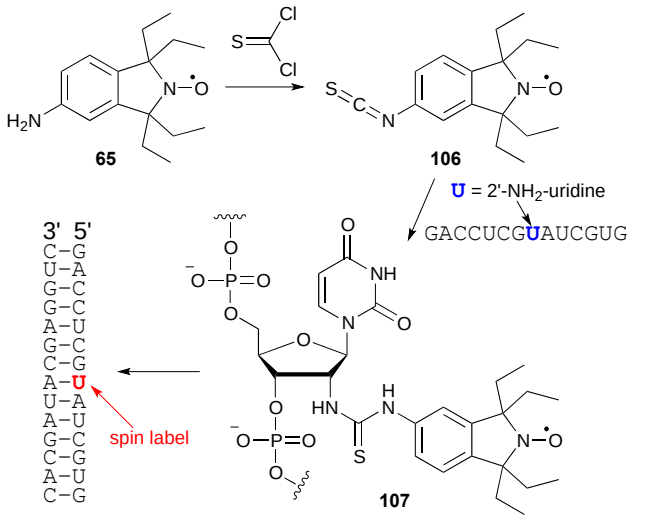


Scheme 13. Templated site-directed modification of long RNA (HCV IRES), and spin labelling with *N*-hydroxysuccinimide nitroxide **104**.

Meanwhile, the tetramethyl pyrrolidinoxyl iodoacetamide **100** has been used to spin label synthetic RNA oligonucleotides at thiouridine.[48] These labelled oligonucleotides were then incorporated into a relatively large double spin labelled non-coding RNA RsmZ (**101**; 72 nucleotides) using splinted T4 DNA ligation (notably, it proved important to carry out spin labelling before the ligation process, which was proposed to hydrolyze unmodified thiouridines). The interactions of the RNA RsmZ with RsmE protein heterodimers, a process that regulates initiation of translation in bacteria, were studied.

The activated ester approach to spin labelling has recently been applied in a number of ambitious contexts. Among the most impressive is modification of the 332 nucleotide internal ribosome entry site (IRES) of HCV RNA using a templated approach (Scheme 13).[49] Hybridization of a complementary DNA (cDNA) strand **102** to the HCV RNA **103** stimulates transfer of a benzylamine SDSL handle by site-selective modification of the HCV RNA at the adjacent adenine base. Hydrolysis of the P–N linkage on the cDNA strand releases the benzylamine nucleophile, which is then alkylated with *N*-hydroxy- succinimide ester **104** to afford the spin-labelled RNA **105**.

Isocyanates have seen extensive use in nucleic acid spin



Scheme 14. Isothiocyanate-based spin labelling of RNA with tetraethylisoindoline isothiocyanate nitroxide **106**.

labelling. One much-studied system is the hammerhead ribozyme (HHRz), where two 2'-aminonucleotides in the *S. mansoni* HHRz were modified with TEMPO-isocyanate **57** (see Scheme 6b).[50] DEER experiments on this double spin labelled RNA were used to observe a clear conformational change in the HHRz tertiary structure, induced by increasing magnesium ion concentration. The measured distance distribution was in excellent agreement with that predicted from the X-ray crystal structure of the HHRz, based on the conjecture that the spin labels adopt minor groove-bound conformations. Although isothiocyanates are less common compared to isocyanates, the use of isoindoline isothiocyanate **106**[29] (see also **60** in Scheme 6b) in the highly efficient, rapid (~2-4 h) spin labelling of 14-mer RNA oligonucleotides (**107**, Scheme 14) has been described. These nitroxides adopt conformationally well-defined environments in spin labelled RNA duplexes, with the tetraethyl-flanked isoindoline being completely unaffected by ascorbate over a two hour period.

Compared to macromolecules, the use of spin labelled small molecules is comparatively rare in the study of biological interactions. One recent elegant example,[51] is spin labelling of the natural product archazolid A (**108**, Scheme 15). This was used to study the structure of a Vacuolar-type ATPase (V-ATPase), in combination with modification of essential glutamate residues in the V-ATPase decamer using the DCC-TEMPO derivative **109**. Both CW and DEER experiments were deployed to assess binding of the (modified or unmodified) product to (modified or unmodified) protein. Although broad EPR distance data resulted, these could in part be explained by rearrangement of the initial carboxylate-carbodiimide adduct to give two different nitroxide environments **110** and **111** (as observed in a model with protected glutamic acid **112**). Nevertheless, the use of labelled small molecules was shown to be an interesting concept for bio-EPR applications.

Nucleobase modification can conveniently be achieved post-oligonucleotide synthesis by S_NAr reactions on appropriately modified bases. For example, rigid spin labelled

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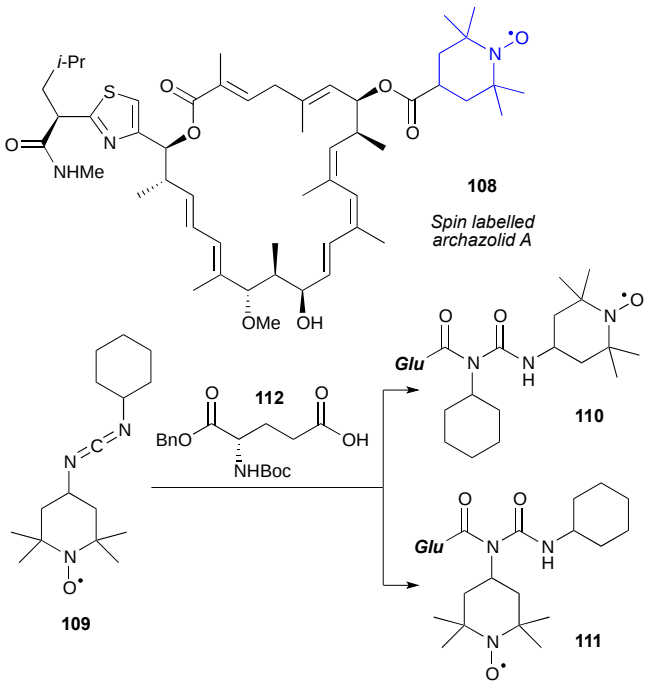


Scheme 15. Spin labelling of the natural product archazolid A, and rearrangement in glutamate labelling with DCC-TEMPO **109**.

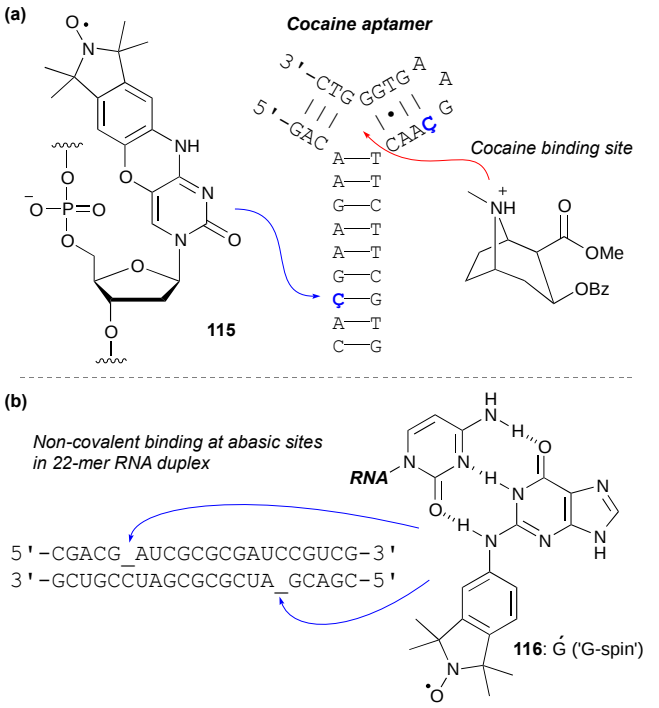
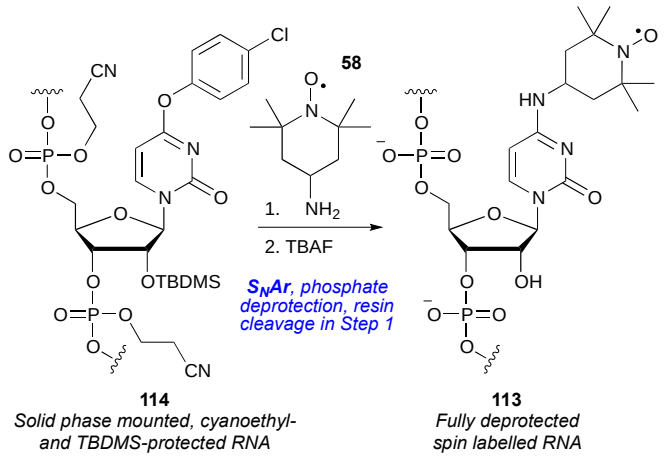


Figure 4. Nucleobase mimics **(a) 115** (C-spin) and **(b) 116** (G-spin).

cytidine **113** can be prepared by reaction of electrophilic O^4-chlorophenyl uridine **114** with amino-TEMPO **58** (Scheme 16).[52] This method affords exceptionally narrow distance distributions in DEER experiments on RNA duplexes containing a double spin labelled strand, by virtue of the rigidifying effect of base pairing coupled with the enforced positioning of the label in the major groove of the duplex.

Nucleobase surrogates have been extensively developed and applied, showing this to be a versatile strategy that affords high quality EPR data. This tactic has been used for both covalent and non-covalent spin labelling, to date mainly demonstrated with the cytosine mimic 'Ç' (**115**, Figure 4a). One recent application[53] is the characterization (by DEER) of the structural change in the three-way junction of cocaine aptamers on binding of cocaine, showing that: a) the aptamer becomes more rigid (evidenced by narrower distance distributions); b) helix elongation takes place (an interspin distance change of just 0.3 nm was detectable); c) the orientation of the helices is affected (as detected by orientation changes of the nitroxides). This principle was recently extended to the guanine mimic **116** (G-spin, Figure 4b, which is readily prepared by S_NAr reaction of 2-bromohypoxanthine with an isoindoline amine nitroxide.[42] **116** was shown to bind to cytosine in abasic sites in a 22-mer RNA duplex, and afforded DEER data.



Scheme 16. S_NAr approach to RNA oligonucleotide spin labelling.

Alkynes offer versatile handles for the SDSL through either Sonogashira coupling, or copper-catalyzed azide-alkyne cycloaddition (CuAAC / click chemistry). The former of these has found many uses in nucleic acid labelling due to the availability of 5-iodouridine (**117**→**118**, Scheme 17). This strategy has been extensively explored for pioneering work on in-cell EPR spectroscopy by injection of nucleic acid samples into *Xenopus* oocytes: The alkyne spin label **66** can be introduced either during or before automated solid phase DNA synthesis;[54] the latter requires modification of DNA synthesis conditions to tolerate the label.

CuAAC is widely exploited as a means to attach chemical probes to biomolecules, but its application to spin labelling can be complicated by the potential for ascorbate-mediated reduction of the nitroxide. However, with appropriate reaction conditions, azide-functionalized spin labels can be attached to both the nucleobase and ribose framework. For example, the modification of 5-ethynyluridine provides a complementary site for successful click nitroxide attachment (**119**, Figure 5a), where the labelled oligonucleotide could serve as a probe for abasic sites in the complementary strand of the duplex, due to efficient intercalation of the nitroxide ring.[31] Our groups recently described the synthesis of a range of 2'-ethynyl

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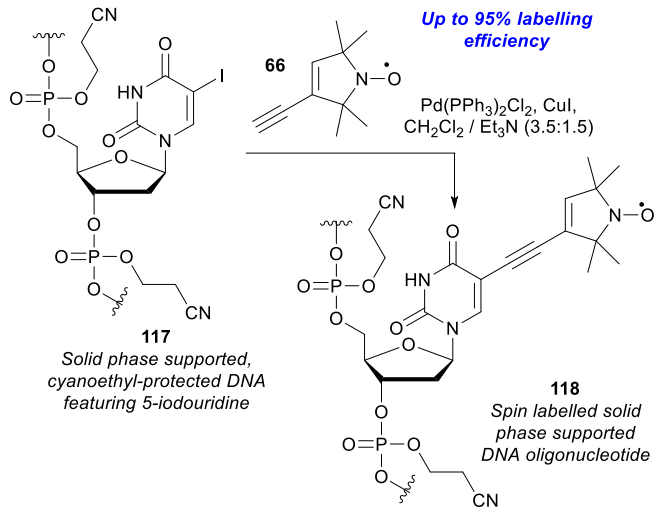

Scheme 17. Sonogashira coupling approach to oligonucleotide spin labelling.

oligonucleotides, which offer an alternative, base-independent method for DNA modification by post-DNA synthesis click chemistry (**120**).[13] Finally, bioorthogonal click spin labelling of cyclooctyne-functionalized proteins has recently been reported (Figure 5b),[55] where GFP modified with a bicyclononyne-lysine sidechain underwent strain-promoted azide-alkyne cycloaddition (SPAAC) with an azidopyrroline nitroxide (**121**). Despite a ~55% labelling efficiency after 2 h incubation, the authors concluded that CuAAC is superior, even when using reduction-susceptible labels.

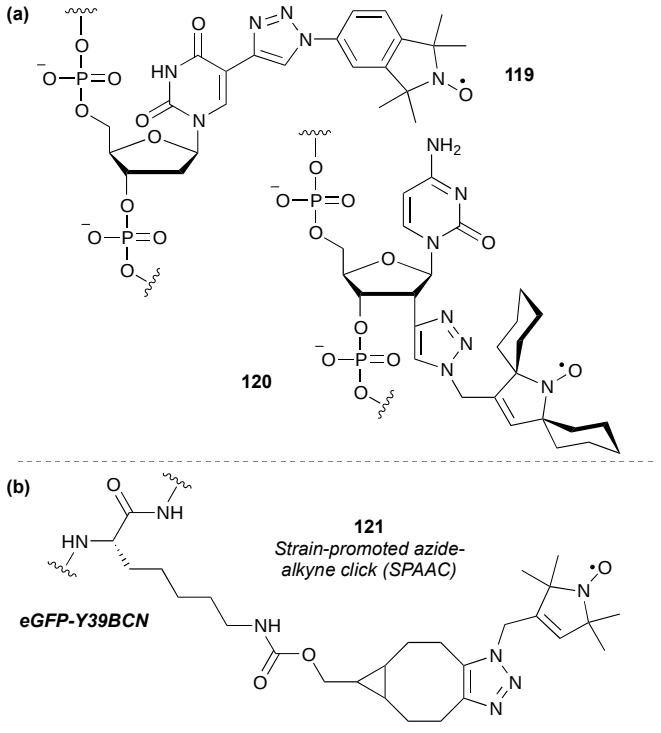

Figure 5. Azide-alkyne cycloadditions in spin labelling: **(a)** nucleobase and ribose modifications; **(b)** bioorthogonal click chemistry in a cellular environment.

Conclusions and Perspectives

The efficient synthesis of nitroxides equipped with functional groups that enable conformationally restricted spin labelling is key to applications of EPR spectroscopy in biomolecules. The plethora of strategies to label proteins and nucleic acids is arguably still challenged by the balance of flexibility against structural impact of nitroxides and their tethers, and by the need for concise syntheses of biostable nitroxides suitable for different spin labelling strategies. Many avenues for future developments in this field are apparent, including the labelling of other biomolecules (e.g. carbohydrates, natural products); the use of room temperature DEER experiments (enabling a true exploration of dynamic behaviour); the use of multiple labels or multiple conformations to enhance the accuracy of data interpretation; and the application of biomolecule EPR to more ambitious contexts including in-cell measurements. Such challenges will no doubt continue to inspire workers in the field, and promote the use of EPR as a valid analytical method to those who are not. Equally certain is that the synthetic chemistry required to develop new labels and new strategies will be driven by such ambitions.

Acknowledgements

EAA thanks the EPSRC (EP/K005391/1 and EP/M019195/1). JEL thanks the EPSRC (EP/L022044/1), and the Royal Society for a University Research Fellowship.

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