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Synthesis and Utilization of α -Substituted Nitroxides

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1. Introduction

Nitroxides are stable free radicals which have the $>N-O$ moiety. In most cases, nitroxides have a ring structure. For example, imidazoline, isoindoline, piperidine and pyrrolidine ring nitroxides (Fig. 1) have been used as agents for spin labeling, imaging, and as antioxidants. These nitroxides have four substituents at the α -position; two substituents on each α -carbon. All four substituents are needed for avoiding the disproportionation reaction of nitroxides except for the case of a bridgehead at the α -position. Methyl groups have been chosen as simple and inert substituents. However, it has been reported that other types of substituents, especially ethyl groups, showed unique characteristics that were unlike those of the conventional methyl group.

In this chapter, we will introduce the conventional as well as the latest synthetic methods used to introduce the various substituents to the α -position. Also, we will describe the structure–reactivity relationships of α -substituted nitroxides.

2. Synthetic methods of α -substituted nitroxides

2.1. Imidazoline

Imidazoline nitroxides have been synthesized from α -hydroxyaminoketone with carbonyl compounds (Scheme 1) (Volodarsky and Igor A, 1988). The R_1 groups of α -hydroxyaminoketone and R_2 groups of carbonyl compounds correspond to the α -position of the imidazoline ring. α -Hydroxyaminoketones are synthesized from appropriate olefines *via* nitrosylation, treatment with hydroxylamine, and hydrolysis (Kirilyuk et al., 2004). This is a practical method because this key compound can be synthesized on the gram scale. For R_2 groups, chain ketones or cyclic alkyl ketones are used as carbonyl

compounds (Bobko et al., 2007; Kirilyuk et al., 2004; Yan'shole et al., 2010; Zubenko et al., 2006). These give the 2,2,5,5-tetrasubstituted 2,5-dihydro-1*H*-imidazole-1-ol. After oxidation, they give the desired imidazole nitroxides. Imidazolidine nitroxides can be synthesized from imidazoline nitroxides by reduction (Zubenko et al., 2006). Therefore, α -substituted imidazoline nitroxides are also synthesized from α -substituted imidazolidine nitroxides.

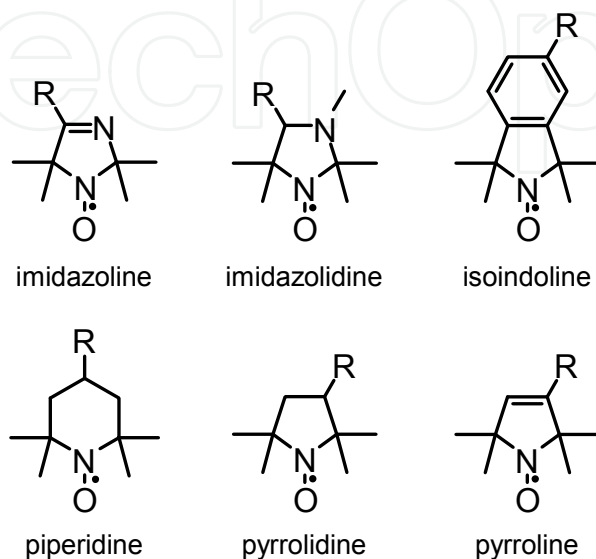
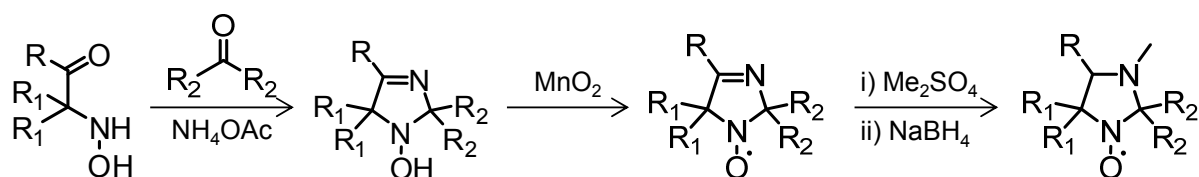
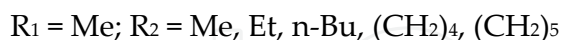


Figure 1. Basic structure of nitroxides

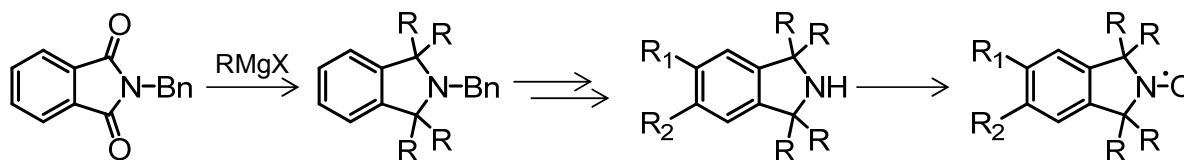


Scheme 1. α -Substitution synthesis of imidazoline and imidazolidine nitroxides



2.2. Isoindoline

Isoindoline nitroxides have been prepared by the addition of a greater than fourfold excess of a single Grignard reagent to *N*-benzylphthalimide (Scheme 2) (Griffiths et al., 1983). Therefore, according to the type of Grignard reagent, various substituents can be introduced to the α -position of the nitroxide moiety (Chan et al., 2010). Corresponding 2-benzyl-1,1,3,3-tetrasubstituted isoindoline derivatives are then deprotected and subsequent oxidation gives the α -substituted isoindoline nitroxides. The isoindoline skeleton has the potential to have low solubility in water; longer alkyl chains cause a decrease in water solubility. Hence, many studies have been conducted with ethyl groups with respect to biological applications (Fairfull-Smith et al., 2009; Marx et al., 2000).



Scheme 2. α -Substitution synthesis of isoindoline nitroxides

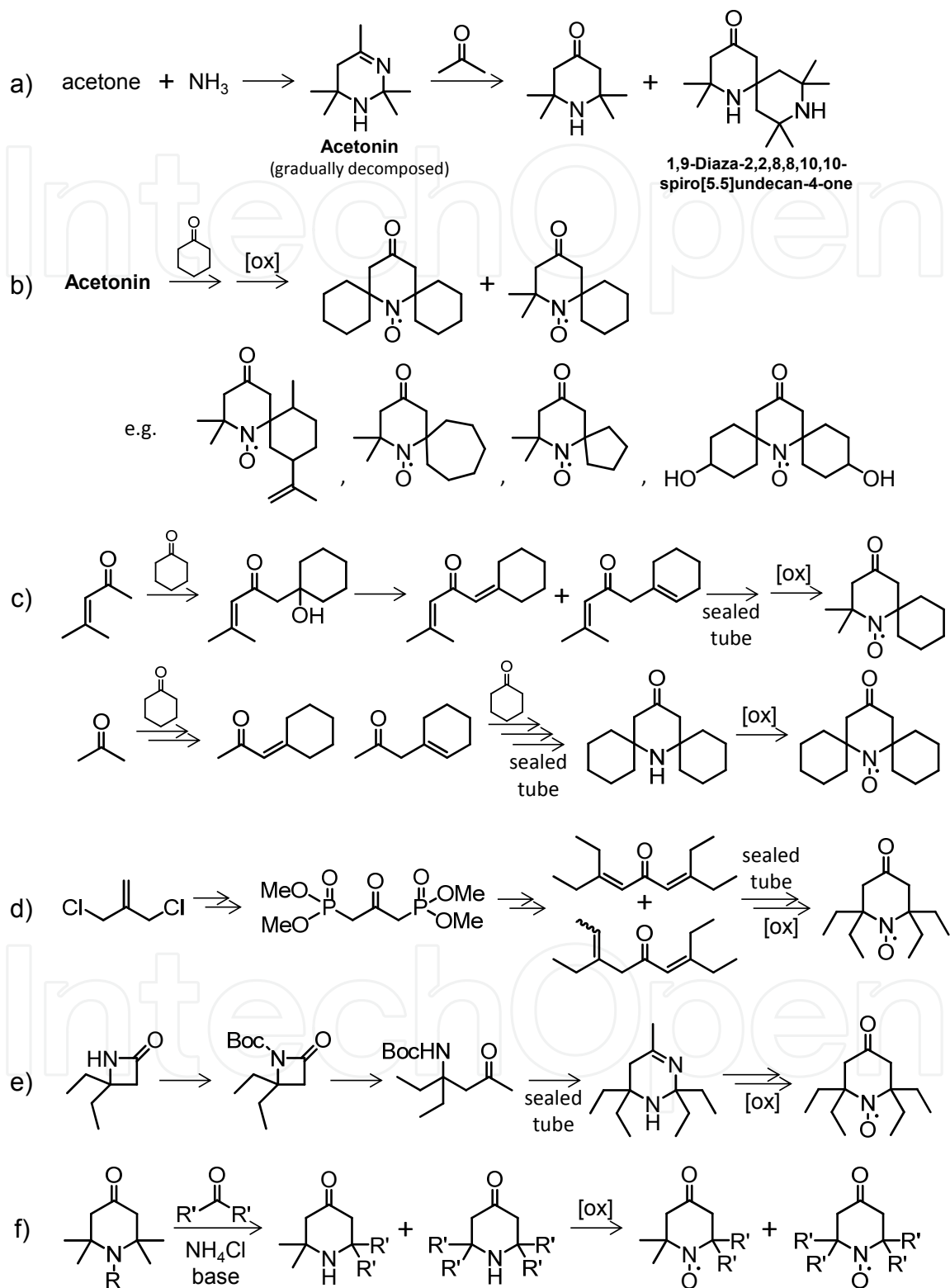
R = Me, Et, n-Pr, n-Bu, Ph

2.3. Piperidine

Piperidine nitroxides have been synthesized by two main approaches. One is the synthesis from acetonin (2,2,4,4,6-pentamethyl-2,3,4,5-tetrahydropyrimidine) with carbonyl compounds (Schemes 3a and b). Murayama *et al.* reported the isolation of 1,9-diaza-2,2,8,8,10,10-hexamethylspiro[5.5]undecan-4-one as a byproduct in the method development for 2,2,6,6-tetramethyl-4-piperidone (Murayama *et al.*, 1969). Subsequently, using acetonin, various α -substituted piperidine derivatives were reported by Ma *et al.* (Ma *et al.*, 1993) and Miura *et al.* (Scheme 3b) (Mannan *et al.*, 2007; Miura *et al.*, 2003; Miura *et al.*, 2001; Okazaki *et al.*, 2007). These methods have the advantage of synthesis in a few steps, but the key compound used at the start of the synthesis, acetonin, lacks stability.

The other main approach is a stepwise synthesis from the appropriate starting material (Schemes 3c, d and e). For example, Yoshioka *et al.* reported on the synthesis for piperidine nitroxides having a spirocyclohexyl group *via* α , β -unsaturated ketone derivatives (Yoshioka *et al.*, 1972). In addition, focusing on substitution of the tetraethyl group, Studer *et al.* reported a stepwise synthesis *via* bisphosphonates (Wetter *et al.*, 2004), and also β -lactams for obtaining piperidine nitroxides on a large scale (Schulte *et al.*, 2005) which involved several synthetic steps and a high-pressure reactor.

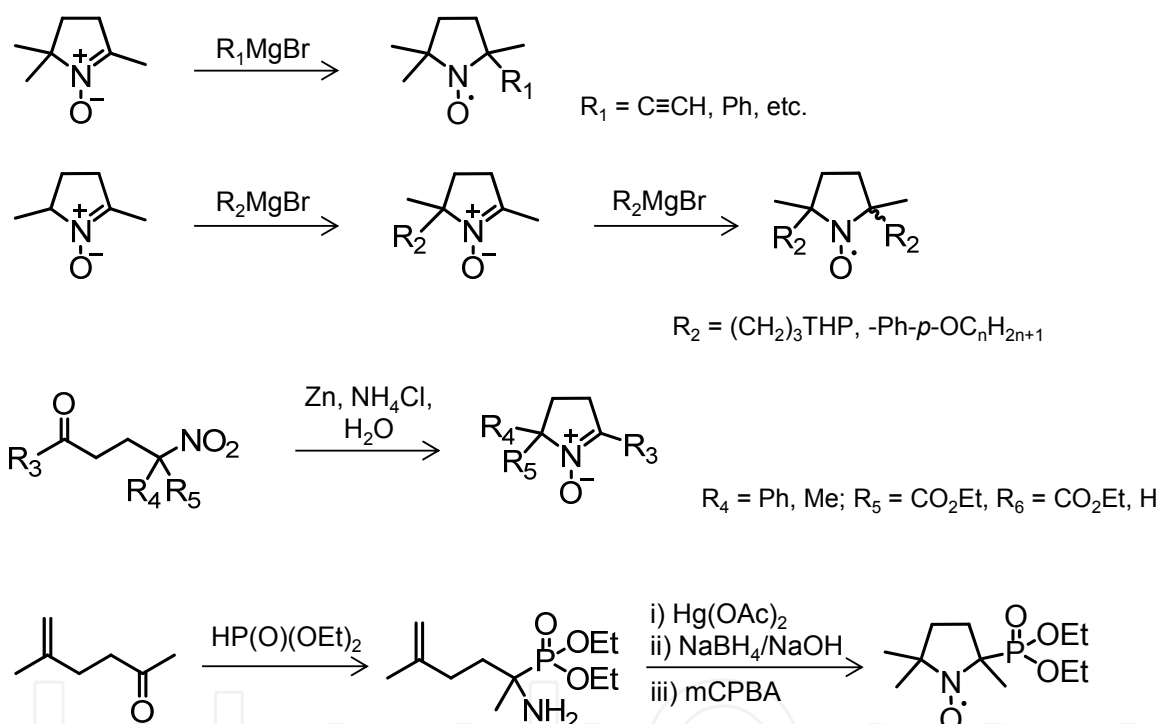
Recently, an alternative synthetic method has been developed (Scheme 3f) (Sakai *et al.*, 2010). This method involves 2,2,6,6-tetramethyl-4-piperidone as a starting compound; this compound is more stable than acetonin and is available commercially. This compound with cyclohexanone directly gave the piperidone derivative having spirocyclohexyl groups at the 2,6-position under a mild reaction condition. Moreover, the reaction yield was increased by using 1,2,2,6,6-pentamethyl-4-piperidone and a base. With this starting compound, various substituents have been introduced to the α -position (Yamasaki *et al.*, 2011; Yamasaki *et al.*, 2010). From the investigation of the reaction mechanism, the nitrogen derived from ammonium chloride was introduced to the piperidone ring. Therefore, using ^{15}N -labeled NH_4Cl instead of $^{14}\text{NH}_4\text{Cl}$, ^{15}N -labeled 2,2,6,6-tetrasubstituted piperidin-4-one-1-oxyls can be produced with high (>98%) ^{15}N content. Thus, the external NH_4X compound seems to be the source of nitrogen during this reaction.



Scheme 3. α -Substitution synthesis of piperidine nitroxides. a), b) from acetonin, c), d), e) stepwise synthesis, f) from piperidone

2.4. Pyrrolidine

α -Substituted pyrrolidine nitroxides has been synthesized *via* the nucleophilic addition of organometallic reagents such as 2,2,5-trimethyl-3,4-dihydro-2*H*-pyrrole-1-oxide or 2,5-dimethyl-3,4-dihydro-2*H*-pyrrole-1-oxide to nitrone (Ikuma et al., 2004; Keana et al., 1983). Grignard reagents are also used as nucleophiles. These moieties are introduced to the α -position of the nitroxide. Therefore, various substituents could be introduced depending on the organometallic reagent. Moreover, substitution of the starting 3,4-dihydro-2*H*-pyrrole-1-oxide at the 2 and 5 positions has been carried out (Black et al., 2000). This also led to α -substituted pyrrolidine nitroxides. These derivatives have been synthesized *via* intramolecular reductive amination from 5-nitrohexan-2-one derivatives. Additionally, the stepwise synthesis from 5-methylhex-5-en-2-one to introduce the phosphonate group has been reported (Le Moigne et al., 1991).



Scheme 4. α -Substitution synthesis of isoindoline nitroxides

$R = \text{Me}, \text{Et}, n\text{-Pr}, n\text{-Bu}, \text{Ph}$

3. Evaluation of α -substituted nitroxides

3.1. Common reactivity of nitroxides

Nitroxides have several potential advantages as spin probes (Kuppusamy et al., 2002; Yamada et al., 2006), spin labels (Borbat et al., 2001), contrast agents (Soule et al., 2007) and antioxidants (Wilcox and Pearlman, 2008). These applications are based on the complementary nature of the radical moieties in nitroxides; paramagnetism allows them to react with free radicals and interact with nuclear spin. For instance, these properties allow

nitroxides to be used as contrast agents for magnetic resonance imaging (MRI) to give images of the morphological nature and redox imbalance in animal models of oxidative stress.

In biological systems, understanding of biophysical properties is helpful to promote effective utilization and control of the reactivity of nitroxides. Nitroxides are readily oxidized to oxoammonium cations or reduced to hydroxylamines by various *in vivo* oxidants or reductants (Fig. 2) (Kocherginsky and Swartz, 1995). Upon reaction with superoxides, nitroxides undergo one-electron oxidation and subsequent two-electron reduction with reductants, which is driven forward by the redox potential of the nitroxide redox couple (Krishna et al., 1992). Conversely, nitroxides are reduced by ascorbic acid and converted into hydroxylamines (Saphier et al., 2003). This effect is one of the limitations of using nitroxides for monitoring *in vivo* redox status because the lifetime *in vivo* is shortened. The reduction rate by ascorbic acid is dependent mainly upon their ring structures and substituent groups. The rate constants decrease in the order oxazolidines > piperidines > pyrrolines > pyrrolidines, and the increasing inductive effects by the substituent group at the β - or γ -positions of pyrrolidine and piperidine nitroxide also affect the rate (Kocherginsky and Swartz, 1995). In this section, the effect of α -substituted nitroxides (especially piperidine nitroxide) on reduction by ascorbic acid was summarized and its *in vivo* application discussed.

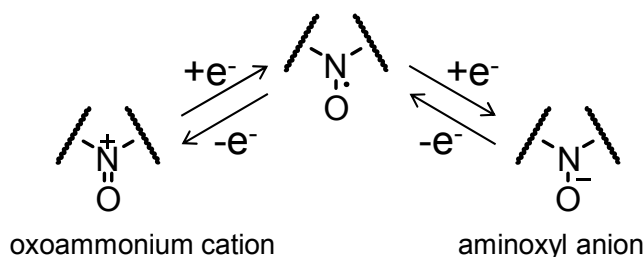


Figure 2. Redox couples of nitroxide

3.2. Reduction stability

As well as at β - or γ -positions, substituent groups at α -positions in a nitroxide ring can change their reactivity. For instance, phosphorylated pyrrolidinyl nitroxide showed moderate increase toward ascorbate reduction compared with the tetramethyl pyrrolidine nitroxide (Mathieu et al., 1997). On the other hands, the tetraethyl-substituted isoindoline (Marx et al., 2000), imidazoline (Kirilyuk et al., 2004), and imidazolidine (Kirilyuk et al., 2004) nitroxides showed high resistivity to ascorbate reduction than the corresponding tetramethyl compounds. Furthermore, Kirilyuk *et al.* reported that tetraethyl-substituted imidazoline nitroxides had significantly longer half-lives in rat blood samples as compared with the corresponding tetramethyl-nitroxides (Kirilyuk et al., 2004). Although piperidine nitroxides with spirocyclohexyl groups at α -positions showed no difference in reactivity with ascorbic acid, the enzymatic reaction was efficiently protected (Okazaki et al., 2007). Recently, tetraethyl-substituted piperidine nitroxide was also reported to exhibit resistance

to ascorbate reduction (Kinoshita et al., 2009). The electron spin resonance (ESR) or electron paramagnetic resonance (EPR) signal intensities of tetraethyl-substituted piperidine nitroxide remained 97% after ascorbate addition (Fig. 3). These reports indicated that the reduction rates of nitroxides vary significantly depending on their structure and microenvironment resulting from the α -substitution of nitroxides. Tetraethyl-type nitroxides could be potentially useful for monitoring *in vivo* free-radical reactions, pH changes, and redox status without undesirable reduction by ascorbic acid.

Tetraethyl-nitroxides, having higher lipophilicity than tetramethyl compounds, have been reported to be less toxic to cells (Kinoshita et al., 2010) although the toxicity is reported to be correlated with the structure and lipophilicity of nitroxides (Ankel et al., 1987). Furthermore, single-dose administration of tetraethyl piperidine nitroxide has been shown to have lower blood pressure-lowering effects compared with that of Tempol (Kinoshita et al., 2010).

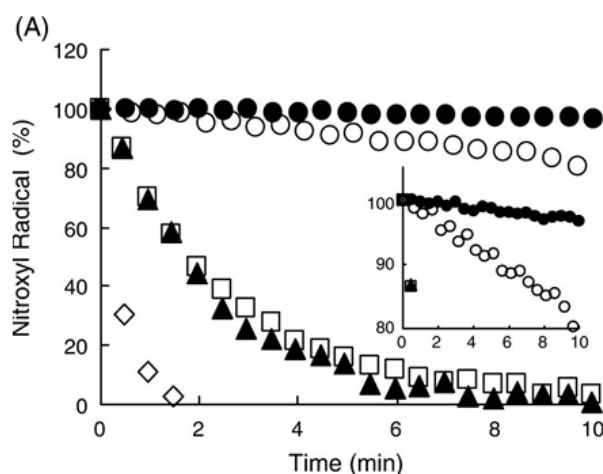


Figure 3. Effect of AsA on the decay of ESR signals of nitroxides. Nitroxides (100 μ M) were mixed with AsA (1 mM) in phosphate buffered saline and their ESR spectra measured as a function of time. •: 4-oxo-2,2,6,6-tetraethyl-piperidine-1-oxyl; ○: carbamoyl-PROXYL; ▲: hydroxy-TEMPO (TEMPOL); □: oxo-TEMPO (TEMPONE); ◇: 7-Aza-3,11-dioxa-15-oxodispiro[5.1.5.3]hexadec-7-yl-7-oxyl. (Kinoshita et al., *Free Radic Res* 2009;43: 565, copyright© 2012, Informa Healthcare. Reproduced with permission of Informa Healthcare)

3.3. Electrochemical behaviours

The change in nitroxide reactivity due to the presence of tetraethyl substituents suggests that introduction of bulky alkyl groups at α -positions in a nitroxide ring are responsible for their reduction stability. Steric hindrance around the radical moiety is one of the most important factors inhibiting access to reductants. However, the ESR signal intensities of 7-Aza-3,11-dioxa-15-oxodispiro[5.1.5.3]hexadec-7-yl-7-oxyl (which has also bulky spirocyclohexyl rings at α -positions) decrease rapidly in the presence of ascorbate

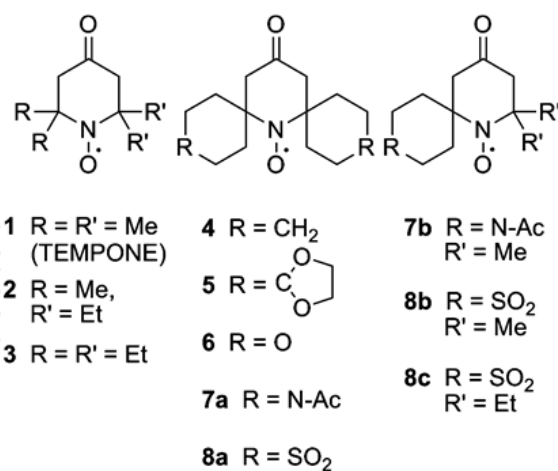
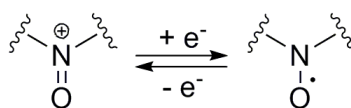


Figure 4. Structure of α -substituted piperidine nitroxides



Nitroxide	E_{pa}^b	E_{pc}^c	$E^{1/2}^d$	ΔE^e	i_{pa}/i_{pc}^f
1	0.939	0.869	0.904	0.070	2.30
2	0.926	0.860	0.893	0.065	1.56
3	0.806	0.747	0.776	0.058	2.64
4	0.878	0.811	0.845	0.067	2.97
5	1.013	0.874	0.944	0.139	3.29
6	1.053	0.940	0.997	0.113	3.06
7a	1.113	- ^g	-	-	-
7b	1.019	0.902	0.961	0.117	3.34
8a	1.303	- ^g	-	-	-
8b	1.122	1.012	1.067	0.110	3.51
8c	1.116	1.020	1.068	0.096	3.65

^a glassy carbon electrode, Ag/AgCl, Pt, sweep rate: 0.1 V s⁻¹. Potentials were shown as vs. SHE. ^b anodic peak potential. ^c cathodic peak potential. ^d $E^{1/2} = (E_{pa} + E_{pc})/2$. ^e $\Delta E = E_{pa} - E_{pc}$. ^f The peak currents (i_{pa} and i_{pc}) were measured from the respective baseline currents. ^g The current of the cathodic peak was too low to determine the potential value. Adapted with permission from (Yamasaki *et al.*, *J Org Chem* 2011;76:435). Copyright (2012) American Chemical Society.

Table 1. Experimental redox potentials of oxoammonium cation / nitroxide redox couple^a

Nitroxide	E_{pa}^b	E_{pc}^c	$E^{1/2d}$	ΔE^e	i_{pa}/i_{pc}^f
1	0.524	-0.371	0.076	0.895	0.20
2	0.453	-0.401	0.026	0.854	0.20
3	0.226	-0.298	-0.036	0.524	2.12
4	0.244	-0.141	0.051	0.385	0.99
5	0.515	-0.364	0.076	0.879	0.09
6	0.644	-0.386	0.129	1.029	0.19
7a	0.613	-0.247	0.183	0.860	0.27
7b	0.585	-0.381	0.102	0.967	0.31
8a	0.653	-0.357	0.148	1.010	2.18
8b	0.694	-0.494	0.100	1.189	0.16
8c	0.416	-0.427	-0.006	0.844	0.22

^a glassy carbon electrode, Ag/AgCl, Pt, sweep rate: 0.1 V s⁻¹. Potentials were shown as vs. SHE. ^b anodic peak potential. ^c cathodic peak potential. ^d $E^{1/2} = (E_{pa} + E_{pc})/2$. ^e $\Delta E = E_{pa} - E_{pc}$. ^f The peak currents (i_{pa} and i_{pc}) were measured from the respective baseline currents. Adapted with permission from (Yamasaki *et al.*, *J Org Chem* 2011;76:435). Copyright (2012) American Chemical Society.

Table 2. Experimental redox potentials of nitroxide / deprotonated hydroxylamine redox couple^a

(Kinoshita *et al.*, 2009). This suggests that the electronic environment around the N-O moiety also influences its reduction stability. In fact, the rate of reduction of β - or γ -substituted nitroxides by ascorbate has been reported to be primarily dependent upon their structure and correlation with $E_{1/2}$ (Blinco *et al.*, 2008; Kocherginsky and Swartz, 1995). The reactivity of α -substituted nitronyl nitroxides is also dependent upon the electronic properties of the substituent groups (Wu *et al.*, 2006). The α -substitution of piperidine nitroxide has been reported to change dramatically their redox potentials for one-electron oxidation and reduction (Yamasaki *et al.*, 2011). In the oxidation step, electron-donating substituents are likely to stabilize oxoammonium cations, and substituents with heteroatoms destabilize them because of the electron-withdrawing inductive effect (Fig. 4, Table 1). The redox potentials for one-electron reduction are listed in Table 2. The electron-withdrawing groups at the α -positions of the piperidine ring destabilize the reduced form of nitroxides, whereas electron-donating substituents stabilize them.

3.4. Structure–reactivity relationships

As described above, ascorbate can readily convert nitroxides into the corresponding hydroxylamines. The reduction rate is correlated with the inductive effects from the β -position in the piperidine ring and the γ -position in the pyrrolidine ring (Morris *et al.*, 1991).

Also, nitroxides with heteroatoms in their ring are unstable for the reduction (Couet et al., 1985).

Imidazole, isoindoline and piperidine nitroxides have a common feature: tetraethyl-nitroxides at α -positions adjacent to the radical moiety have high resistance to reduction by ascorbate compared with the widely used tetramethyl-nitroxides (see above). The rate of decay of the ESR signals of nitroxides seems to be inversely proportional to the number of ethyl groups (Yamasaki et al., 2010). Nitroxides containing four ethyl groups are more resistant to the reduction than those with two ethyl groups. The reduction rates of nitroxides which have heteroatoms in their spirocyclohexyl ring have been found to be higher than tetramethyl nitroxides. Electron-withdrawing groups at spirocyclohexyl rings decrease the electron density around the N-O moiety, thereby favoring the reduction reaction. The trend of redox potentials for nitroxide reduction from electrochemical experiments is likely to be exactly the same as that of the nitroxide reduction rate by ascorbate. The ESR signal decay rate and the electromotive force between nitroxide and ascorbate (ΔE_{N-A}) or the change in Gibbs free energy (ΔG) demonstrates very good correlations with ΔG in the negative ΔG region ($r^2 = 0.988$) (Fig. 5) (Yamasaki et al., 2010). This indicates that reduction of the nitroxide by ascorbate occurred spontaneously if the ΔG value is negative, and that the reduction is not spontaneous if the ΔG value is positive. The factors influencing the reduction process of the nitroxide are dependent not only upon steric hindrances but also on redox potentials. The α -substitutions of piperidine nitroxides would be an effective approach to control the reactivity of nitroxides as a function of their applications.

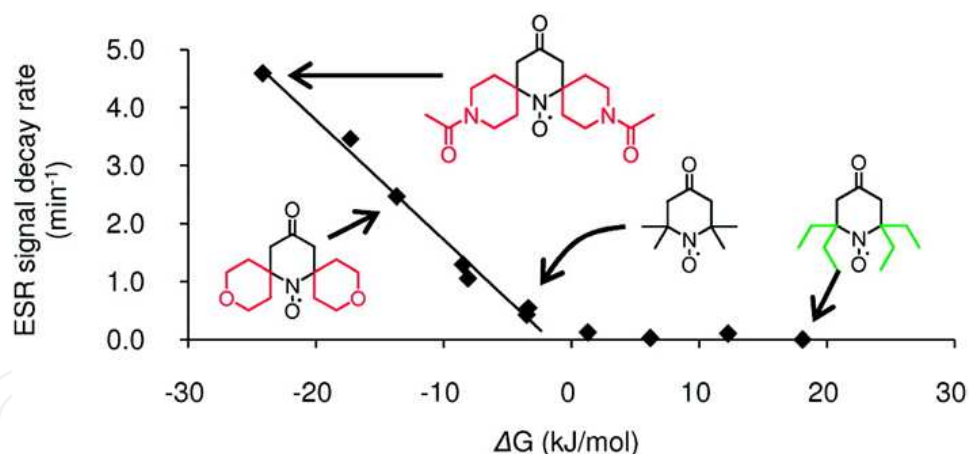


Figure 5. Relationship between the rates of decay of ESR signals and ΔG . ΔG was calculated from ΔE_{N-A} with use of the standard expression $\Delta G = -nF\Delta E_{N-A}$, where ΔE_{N-A} is a subtraction of the redox potential of ascorbate from that of nitroxides, n is the number of electrons per mole of product, and F is the Faraday constant. The correlation coefficient when ΔG was negative was 0.988. Reprinted with permission from (Yamasaki et al., *J Org Chem* 2011;76:435). Copyright (2012) American Chemical Society)

3.5. *In vivo* evaluation and imaging

Nitroxides are reduced to mainly the hydroxylamine form *in vivo* by non-enzymatic processes involving glutathione, the reduced form of nicotinamide adenine dinucleotide

phosphate (NADPH), and ascorbic acid. Tetramethyl-nitroxides are widely used as biological reporters such as superoxide-dismutase mimics (Krishna et al., 1996), antioxidants (Wilcox and Pearlman, 2008) and radiation-protecting agents (Metz et al., 2004). However, non-specific reactions limit their applications to monitor changes in redox status. To increase their general versatility, improvement of the stability of nitroxides *in vivo* is important.

In general, the stability of nitroxide is reflected by their type of ring, substituent groups, and lipophilicity. Piperidine-nitroxides show a short half-life compared with that of pyrrolidine-nitroxides. A typical tetramethyl-piperidine nitroxide, Tempone (oxo-TEMPO), has a short life-time (≈ 2 min) in blood due to rapid reduction (Ishida et al., 1989; Schimmack et al., 1976). However, piperidine nitroxides with spirocyclohexyl groups show resistance to enzymatic reduction in mouse liver homogenates (Okazaki et al., 2007). Conversely, tetraethyl nitroxides show resistance to reduction by ascorbic acid and seem to be stable *in vivo*. In fact, the half-lives of 4-oxo- and 4-hydroxy-2,2,6,6-tetraethyl-piperidine-1-oxyl in the mouse tail were 16.4 ± 1.3 min and 20.0 ± 3.0 min, respectively, although that of carbamoyl-PROXYL was 8.5 ± 2.7 min (Kinoshita et al., 2010). The factor influencing the reactivity of nitroxides to reductants is thought to be their redox potential. Also, higher inductive substitution constants at the 4-position of piperidine nitroxides were found to confer higher rate constants for ascorbic acid reduction, emphasizing the importance of the electronic effects of the substituents of nitroxides.

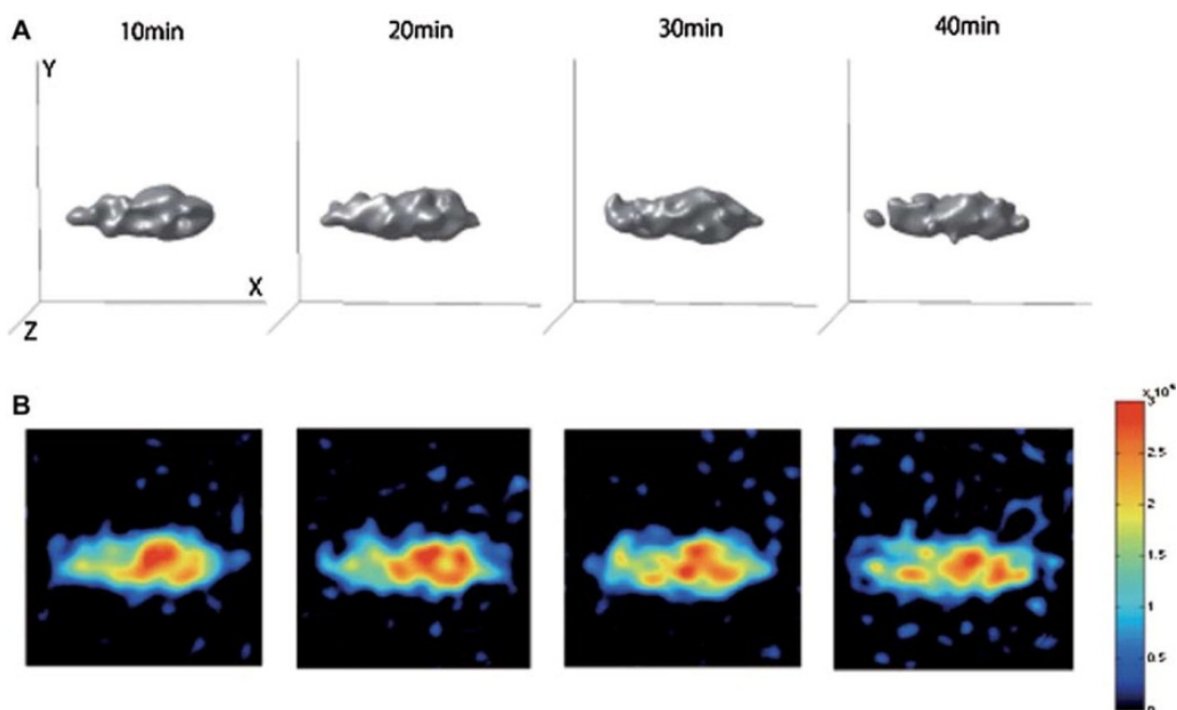


Figure 6. Temporal changes in three-dimensional surface-rendered EPR images (A) and two-dimensional slice images (B) of TEEPONE in mouse brains. EPR images were obtained 10, 20, 30, and 40 min after intravenous injection of TEEPONE. (Emoto *et al.*, *Free Radic Res* 2011;45: 1325, copyright© 2012, Informa Healthcare. Reproduced with permission of Informa Healthcare)

A nitroxide with a long half-life can also be a candidate *in vivo* imaging agent. *In vivo* evaluations have revealed that tetraethyl-nitroxides have several advantages for application as contrast agents for monitoring redox status: non-toxicity, lack of blood pressure-lowering effects, and stability *in vivo*. Recently, it has been reported that the half-life of 4-oxo-2,2,6,6-tetraethyl-piperidine-1-oxyl (TEEPONE) obtained in mice brains is 81.1 ± 0.7 min, indicating that TEEPONE is more stable compared with other nitroxides that are readily converted into hydroxylamines in tissues (Fig. 6) (Emoto et al., 2011). The decay rate of TEEPONE has no relation with ascorbic acid reduction: this compound can effectively scavenge free radicals such as carbon- and oxygen-centered radicals.

4. Conclusions

Recently, various types of α -substituted nitroxides have been synthesized and their *in vivo* and *in-vitro* characteristics evaluated. Introducing α -substituent groups can modify and control their nature according to usage as spin probes, antioxidants, and contrast agents. Although tetramethyl-nitroxides have been widely used in physiological and biological systems, the unique properties of α -substituted nitroxides will extend the possibilities of their applications. Development of α -substituted nitroxides creates the possibility of expansion of their use in a wide range of fields.

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