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Development of novel nitroxyl radicals for controlling reactivity with ascorbic acid

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Abstract

Piperidine and pyrrolidine nitroxyl radicals (nitroxide) contain unpaired electrons and have been widely recognized as antioxidants, contrast agents, spin probes, radiation protective agents and polymerization mediators. Nitroxyl radicals can react with free radicals and reductants and their reactivities depend on the basic structure of the nitroxyl radicals themselves. However, reductants easily reduce these radicals and they lose their paramagnetic nature and function. Therefore, the aim of this study was to develop various functional nitroxyl radicals, particularly focusing on stability towards AsA through the improvement of the synthetic route for a series of 2,6-substituted nitroxyl radicals. Tetraethyl-substituted piperidine nitroxyl radical 8 exhibited resistance to AsA reduction and 2,6-dispiro-4′,4″-dipyrane-piperidin-4-one-N-oxyl 5 had a second-order rate constant 10-times greater than those of hydroxyl-TEMPO and oxo-TEMPO. The 2,6-substituted compound offers various reactivities towards AsA and the possibility to be used as a new antioxidant, contrast agent and radical polymerizer.

Keywords: *Nitroxyl radical, ascorbic acid, free radical, redox, oxidative stress*

Introduction

Stable piperidine and pyrrolidine nitroxyl radicals (nitroxide) have been widely used as antioxidants [1–3], contrast agents [4,5], radiation protective agents [6,7] and compounds that mediate radical polymerization [8,9]. Based on their reactivities with free radicals and redox enzymes, change in their paramagnetic nature occurs upon chemical reduction or oxidation by a biological species [10]. These unique characteristics have resulted in studies monitoring the redox status [11–15] and free radical reactions [16–18] of oxidative stress by electron spin resonance (ESR) and magnetic resonance imaging (MRI) in animal models. Administration of nitroxyl radicals to animals makes it possible to obtain images with useful spatial resolution and functional information using magnetic resonance systems [5,19,20]. However, the disadvantage of using existing nitroxyl radicals, such as ascorbic acid (AsA), in biological systems is their rapid reduction [10,21]. AsA is one of the bioreductants responsible for the reduction of nitroxyl radicals to hydroxylamine [21]. While a piperidine nitroxyl radical such as 4-hydroxy-TEMPO (Tempol) can be utilized as an antioxidant [1], superoxide dismutase mimic [22] and a radiation protective agent [6] in animals and humans, it possesses higher reactivity toward AsA compared to pyrrolidine nitroxyl radical [10]. Therefore, development of novel piperidine nitroxyl radicals having resistance toward AsA would greatly expand their general applications.

Recent reports indicate that tetraethyl-substituted isoindoline [23], imidazoline [24] and imidazolidine [24] nitroxyl radical compounds increase stability towards AsA reduction compared to tetramethyl analogues due to steric hindrance, suggesting that the introduction of a bulky alkyl substituent into a

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neighbouring position on piperidine derivatives induces resistance to reduction. Meanwhile, tetraethyl-substituted piperidine compounds have been synthesized to mediate radical polymerization [25]. They used bisphosphonate as an intermediate material after starting with commercially available methallyl dichloride to produce tetraethyl-substituted piperidine compounds via multiple steps. The reactivity of this compound towards AsA remains unclear. Miura et al. [9] reported that the reaction of intermediate acetonine with excess cyclohexanone in the presence of NH_4Br and H_2O gave 2,6-dicyclohexane-piperidone-4-one-N-oxyl via oxidation. The proposed mechanism is based on the breaking of the pyrimidine ring and its reassembly as a piperidine [26]. These results gave us the idea of synthesizing several new piperidine nitroxyl radicals, including the tetraethyl-substituted radical. Hence, the objective of this study was to synthesize 2,6-substituted piperidine-4-one derivatives and other nitroxyl radicals with various reactivities towards AsA. Tetraethyl-substituted piperidine nitroxyl radical exhibited resistance toward AsA reduction and the possibility for use as an image contrast agent to visualize the differences in AsA concentration. Furthermore, we succeeded in synthesizing 2,6-dispiro-4′,4″-dipyrane-piperidin-4-one-N-oxyl 5, which possessed high reactivity towards AsA.

Materials and methods

Chemicals

1,2,5,6-Tetrahydro-2,2,4,6,6-pentamethylpyrimidine (acetonin monohydrate 1) was prepared according to the previously reported method [9,26]. We purchased 4-oxo-2,2,6,6-tetramethylpiperidine-N-oxyl (oxo-TEMPO), 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (hydroxy-TEMPO) and 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-N-oxyl (carbamoyl-PROXYL) from Sigma-Aldrich Co. (MO, USA). We used ultrapure water in all experiments (Millipore Co., MA, USA). All other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Synthesis of 2,6-substituted piperidine-4-one (general procedure)

Acetonin monohydrate 1 (58.1 mmol) and anhydrous NH_4Cl (58.1 mmol) were added to DMSO (10 mL). We then added a cyclic ketone compound (348.6 mmol) to the solution and stirred it for 10 h at 60°C, followed by the addition of 10% HCl (200 mL) on ice. Later, we washed the mixture with hexane. The water phase was adjusted to pH 10 using 10% K_2CO_3 and extracted with diethyl ether. We dried the ether extracts and then evaporated them. The residue was chromatographed on a silica gel column using chloroform as the eluent. 2,6-Dispiro-1′,1″-dicyclohexyl-piperidin-4-one 2 was recrystallized from isopropyl ether on colourless plates (45% yield): mp 99.7°C, ^{1}H NMR: δ_H (400 MHz; $CDCl_3$): 1.21 (1H, s), 1.37–1.41 (8H, m), 1.46–1.5 (8H, m), 1.61–1.64 (4H, m), FABMS 236.3, Calculated for $C_{15}H_{25}NO$: C 76.55; H 10.71; N 5.95, Found: C 76.51; H 10.68; N 5.93. 2,6-dispiro-4′,4″-dipyrane-piperidin-4-one 4 was recrystallized from isopropyl ether on colourless plates (35% yield): mp 167°C, ^{1}H NMR: δ_H (400 MHz; $CDCl_3$): 1.21 (1H, s), 1.6–1.67 (8H, m), 2.4 (4H, s), 3.54–3.6 (4H, m), 3.81–3.86 (4H, m), FABMS 240.1, Calculated for $C_{13}H_{21}NO_3$: C 65.25; H 8.84; N 5.85, Found: C 65.17; H 8.85; N 5.86. 2,6-dispiro-4′,4″-dithiopyrane-piperidin-4-one 6 was recrystallized from isopropyl ether on colourless plates (40% yield): mp 155.7–157.1°C, ^{1}H NMR: δ_H (400 MHz; $CDCl_3$): 0.8 (1H, s), 1.76–1.9 (8H, m), 2.27 (4H, s), 2.44–2.5 (4H, m), 2.88–2.95 (4H, m), FABMS 272.2, Calculated for $C_{13}H_{21}NOS_2$: C 57.52; H 7.80; N 5.16, Found: C 57.54; H 7.78; N 5.11.

2,2,6,6-Tetraethyl-piperidin-4-one 5

We dissolved 1.8 mmol of 2,6-dispiro-4′,4″-dithiopyran-4-piperidin-4-one 6 in ethanol (15 mL). Then, 8 mL of active Raney-Ni was added to the solution, followed by 2 h of stirring at 60°C. After the solution was filtered carefully with celite, the solvent was removed by evaporation. The residue was chromatographed on a silica gel column using chloroform as the eluent and 2,2,6,6-tetraethyl-piperidine-4-one 7 was synthesized. The obtained yield was ~15%. ^{1}H NMR: δ_H (400 MHz; $CDCl_3$): 0.8–0.9 (12H, m), 1.35–1.55 (8H, m), 2.27 (4H, s), FABMS 212.2, Calculated for $C_{13}H_{25}NO$: C 73.88; H 11.92; N 6.63, Found: C 73.81; H 11.99; N 6.25.

Oxidation of piperidine compounds to nitroxyl radicals

We combined the piperidine compound (4.18 mmol) and $Na_2WO_4 \cdot 2H_2O$ (2.43 mmol) in ethanol. H_2O_2 (30%) was then slowly added to the solution followed by stirring for 24 h at room temperature. After stirring, the solution was saturated with K_2CO_3 and extracted with ether. We dried the ether extracts by evaporation. The residue was chromatographed on a silica gel column using hexane/ethyl acetate (1:3) as the eluent. 2,6-Dispiro-1′,1″-dicyclohexyl-piperidin-4-one-N-oxyl 3 was recrystallized from ethyl acetate on yellow plates (50% yield): mp 114.2°C, FABMS 250.3, Calculated for $C_{15}H_{24}NO_2$: C 71.96; H 9.66; N 5.59, Found: C 71.81; H 9.62; N 5.57. 2,6-Dispiro-4′,4″-dipyrane-piperidin-4-one-N-oxyl 5 was recrystallized from ethyl acetate on yellow plates (85% yield): mp 149.5°C, FABMS 255.2, Calculated for $C_{13}H_{20}NO_4$: C 61.4; H 7.93; N 5.51, Found: C 61.32; H 7.91; N 5.41. We obtained 2,2,6,6-tetraethyl-piperidin-4-one-N-oxyl 8 as red oil (86% yield): FABMS 226.3, Calculated for $C_{13}H_{24}NO_2$: C

68.99; H 10.69; N 6.19, Found: C 68.87; H 10.62; N 6.08.

Reaction with nitroxyl radicals and AsA

The ESR spectra of nitroxyl radicals in phosphate buffered saline (PBS) solution were obtained using an X-band ESR spectrometer (JEOL, Akishima, Japan). The mixture of nitroxyl radicals and AsA was immediately introduced into an ESR tube and the area under the low-field peak of the first derivative spectrum was measured as a function of time. The second-order rate constant between nitroxyl radicals and AsA in PBS was calculated from the first-order decay of the ESR spectra in the presence of 10–50 μM nitroxyl radicals under conditions of pseudo-first-order reactions (AsA concentration, 1–100 mM) according to the procedure of Vianello et al. [27]. We plotted the logarithms of AsA and nitroxyl radical concentration vs the logarithm of the initial rate and found that the slope was 1 for all nitroxyl radicals tested in this study. We then calculated the second-order rate constants of each nitroxyl radical. ESR experimental conditions were: frequency, 9.4 GHz; power, 10 mW; magnetic field, 334 mT; modulation amplitude, 0.032 mT; and time constant, 0.03 s.

Chemical reaction of nitroxyl radicals in H_2O_2 solution caused by UV irradiation

The hydroxyl radical was generated by UV photolysis of H_2O_2 as described previously [28]. Nitroxyl radicals (25 μM) were dissolved in 10 mM phosphate buffer solution, which was mixed with H_2O_2 (10 mM). We immediately introduced the mixture into an ESR tube and recorded X-band ESR spectra as a function of time with the irradiation of UV light (100–120 mW/cm^2) in the ESR cavity to generate ·OH. The UV irradiation was performed using an SX-UI251HQ irradiation unit equipped with a 250 W extra-high pressure UV lamp (USH-250SC) (Ushio Co., Ltd., Tokyo, Japan). We used a UV filter with a cut-off wavelength of 300 nm to prevent UV-induced destruction of nitroxyl radicals, which have a maximum absorbance of 230 nm.

Overhauser-enhanced MRI (OMRI) measurement

The OMRI experiments were performed on a custom built (Philips Research Laboratories, Hamburg, Germany) whole body scanner operating in field-cycled mode to avoid excess power deposition during the ESR cycle. The nuclear magnetic resonance (NMR) field strength of the scanner was 15 mT. At this field, the NMR frequency was 625 kHz using a saddle transmission coil. The receiving coil was a solenoid coil tuned to 625 kHz. Maximum transmission power was 100 W (peak). The ESR irradiation frequency was 226 MHz and a saddle coil was used for transmission.

We used eight tubes containing 2 mM nitroxyl radicals (hydroxy-TEMPO or compound 8) with different concentrations of AsA (0, 0.3, 1 and 3 mM) for the phantom experiments with OMRI. Typical scan conditions in OMRI were: repetition time (T_R)/echo time (T_E)/ESR irradiation time (T_{ESR}): 1200 ms/25 ms/600 ms; no. of averages = 2; 64 phase-encoding steps. The image field of view (48 mm) was represented by a 64 × 64 matrix.

Results

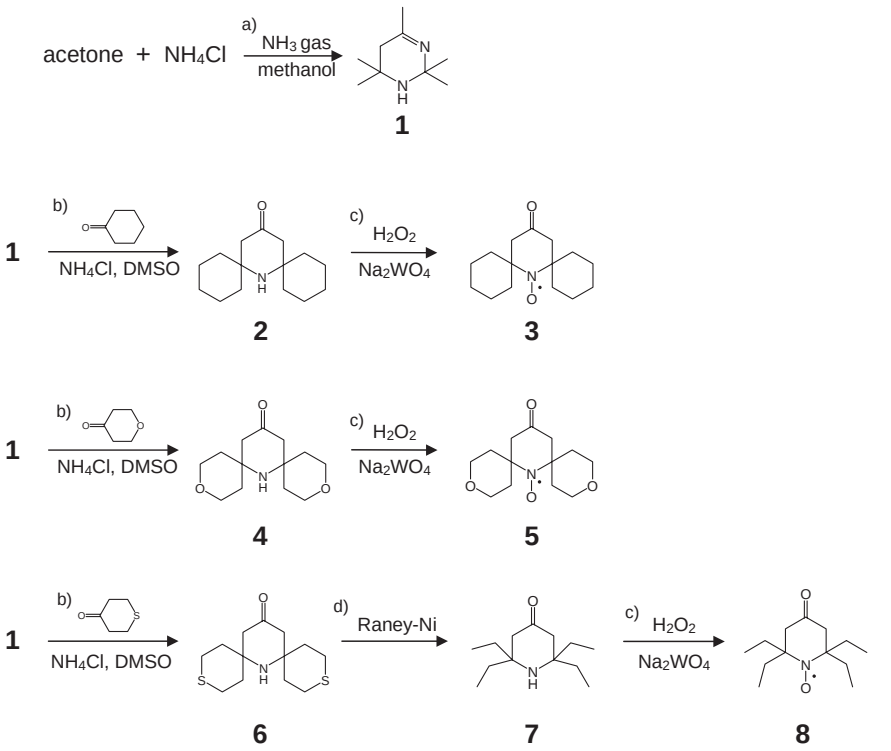
Synthesis of 2,6-substituted piperidine nitroxyl radicals

Scheme 1 shows the preparation of nitroxyl radicals 3, 5 and 8. We prepared acetonin monohydrate according to previous reports. Reaction of 1 with excess cyclic ketones in the presence of NH_4Cl and DMSO gave 2, 4 and 6 in 45, 35 and 40% yields, respectively. Several solvents were tested, but DMSO yielded the best results. Next, the oxidation of 2 and 4 with H_2O_2 in the presence of Na_2WO_4 gave 3 and 5 in 50 and 85% yields, respectively. With regard to compound 6, dissolving 6 in ethanol afforded 7 due to the desulfurization reaction in the presence of Raney-Ni. Next, the oxidation process gave 8 in 85% yield. This synthetic route made it possible to reduce the number of reaction steps and increase the overall yield.

Reactivity of 2,6-substituted piperidine nitroxyl radicals

We also examined the stability of synthesized nitroxyl radicals towards AsA. The changes in the ESR signal intensities of nitroxyl radicals in PBS at pH 7.4 were plotted as a function of time after AsA addition. The ESR spectra of hydroxy-TEMPO disappeared 10 min after the addition of AsA, while there was no change in the ESR signal intensity of compound 8. We calculated the decay rate from the slope of the ESR signal intensities as a function of time after AsA addition. The ESR signal intensities of compound 8 remained 97%, in contrast with the signal of carbamoy-PROXYL remaining 52% 30 min after AsA addition. The reaction rate of compound 8 was 10-times lower than that of carbamoyl-PROXYL, which has a greater resistance to reaction with AsA than the piperidine nitroxyl radical (see Figure 1). Furthermore, compound 8 did not react with glutathione or NADH under the same conditions (data not shown). Interestingly, we observed that the ESR signal intensity of 2,6-dispiro-4′,4″-dipyrane-piperidine-4-one-N-oxyl, compound 5, decreased much more rapidly in the presence of AsA than that of hydroxy-TEMPO. The decreased ESR signal intensities were recovered by the addition of potassium ferricyanide.

Table I lists the second-order rate constants (K_{PBS}) for the reactions between AsA and the nitroxyl



Scheme 1. Preparation of 2,6-substituted piperidine nitroxyl radicals. (A) Anhydrous ammonia (31.2 g), methanol (32.16 g), NH_4Cl (4.06 g), acetone (135.44 g), 23–28°C, 6 h; (B) Cyclic ketone compounds (6 equiv.), NH_4Cl (1 equiv.), DMSO, 60°C, 10 h; (C) $NaWO_4$ (0.25 equiv.), H_2O_2, ethanol, 24 h; and (D) Raney-Ni, ethanol, 60°C, 2 h.

radicals. The rate constant of compound 5 was ~10-times greater than those of hydroxyl-TEMPO and oxo-TEMPO. Because compound 8 was barely reduced by AsA, we could not evaluate its second-order rate constant.

Furthermore, we measured the changes in the ESR signal intensity of the nitroxyl radical caused by the hydroxyl radical. The hydroxyl radical was generated by the UV-dependent haemolytic cleavage of H_2O_2. The decay rates of oxo-TEMPO, hydroxy-TEMPO and compounds 5 and 8 with the hydroxyl radical were 0.028 ± 0.0028, 0.031 ± 0.0008, 0.031 ± 0.0036 and $0.047 \pm 0.0071\ min^{-1}$, respectively. Although the reactivities with AsA varied greatly by altering the 2,6-substituents, the effect of the hydroxyl radical on 2,6-substituents of piperidine nitroxyl radicals was not significant. The absence of H_2O_2 and UV irradiation did not alter the ESR signal intensities of these nitroxyl radicals.

OMRI imaging of nitroxyl radical reduction with AsA

To examine the potential use of AsA-resistant nitroxyl radicals as contrast agents for OMRI experiments, we tested a phantom containing nitroxyl probes in different tubes by OMRI modality (see Figure 2). The OMRI system, based on a double resonance technique, can produce images of free radical distributions in small animals by enhancing the water-proton signal intensity via the Overhauser effect [29,30]. We performed OMRI scanning as a function of time after AsA addition. Image data showed that compound 8 enhanced the image intensity similar to hydroxy-TEMPO, while a phantom containing PBS could not show a good image contrast due to the lack of nitroxyl radicals. The image intensity from tubes containing hydroxy-TEMPO in the presence of AsA gradually decreased with time, whereas the intensity of those containing compound 8 remained stable. These results suggested that the tetraethyl-substituted nitroxyl radical, compound 8, had the ability to function as an image contrast agent due to its AsA resistance. Therefore, appropriate selection of nitroxyl radicals for instance, based on their dependence on the difference of AsA concentration and their ability to generate free radicals, will provide functional images.

Discussion

Piperidine and pyrrolidine nitroxyl radicals have various uses in a wide range of fields and the creation of new functional compounds is eagerly anticipated. Recently, researchers reported 2,6-disubstituted imidazoline [24] and imidazolidine [24], which are other types of nitroxyl radicals [19]. Studies of imidazoline and imidazolidine nitroxyl radicals focus on pH measurements based on the hyperfine splitting change in ESR spectra at various pH solutions. They reported that tetraethyl-substituted compounds had other

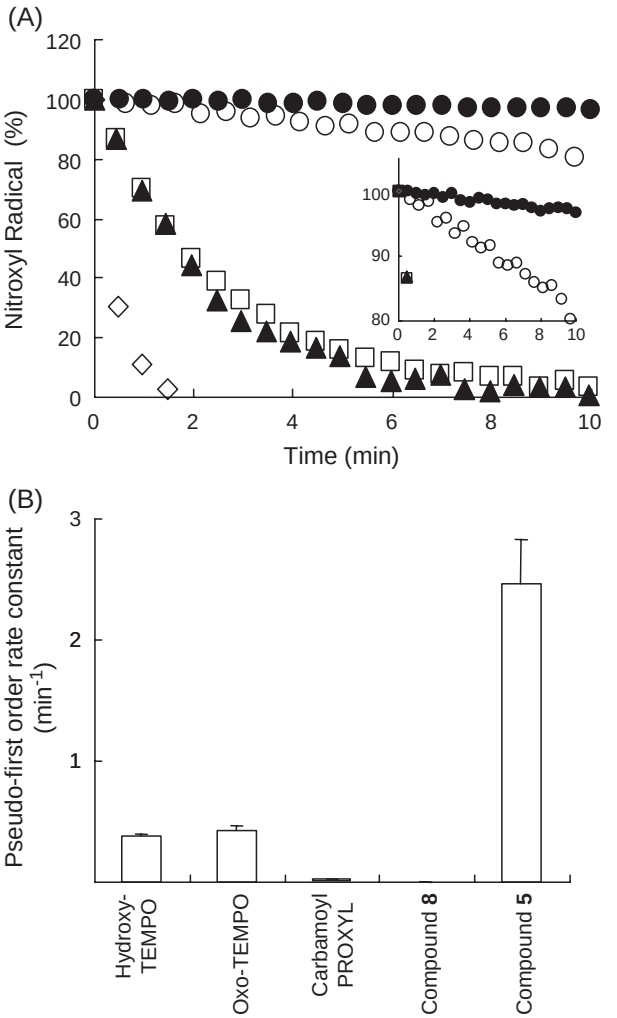


Figure 1. Nitroxyl radicals (100 μM) were mixed with AsA (1 mM) in PBS and their ESR spectra were measured as a function of time. (A) Effect of AsA on ESR signal decay of nitroxyl radicals. ●: compound **8**, ○: carbamoyl-PROXYL, ▲: hydroxy-TEMPO, □: oxo-TEMPO, ◇: compound 5. (B) The pseudo-first-order decay rate constants of nitroxyl radicals due to reaction with AsA. The values given are the averages of three repeated experiments and the bars indicate standard deviations.

Table I. Second-order rate constant (K_{PBS}; $M^{-1}s^{-1}$) of the reaction between nitroxyl radicals and ascorbic acid in PBS solution.

Nitroxyl radical	K_{PBS} ($M^{-1}s^{-1}$)
TEMPOL	3.0±0.41
Oxo-TEMPO	4.1±0.79
Carbamoyl-PROXYL	0.37±0.05
Compound 5	31.0±1.10
Compound 8	ND

The experiments were carried out in PBS at pH 7.4 in the presence of 10–50 μM nitroxyl radicals, under pseudo-first-order conditions (ascorbic acid concentration in the range of 0.1–2 mM). The second-order kinetic rate constants were calculated from the first-order decay rates of the nitroxyl radical ESR signals. The kinetic constant values are the average values derived from three repeated experiments.

functions such as AsA resistance. The development of 2,6-disubstituted piperidine and pyrrolidine nitroxyl radicals creates the possibility of the expansion of the wide range of fields. The synthetic routes to 2,6-disubstituted piperidine and pyrrolidine nitroxyl radicals have been reported by a few groups [9,26]. In this study, we used the reaction of this synthetic route to produce 2,6-disubstituted piperidine nitroxyl radicals. The use of DMSO as the reaction solvent enabled us to introduce cyclohexanone, tetrahydropyrane, and thiopyran at the 2,6-position of the piperidine nitroxyl radical. Following the reductive desulfurization of the functional group transformation of compound 6 using Raney-Ni, we obtained tetraethyl-substituted piperidine nitroxyl radical 8. This reaction process gave a good yield and reduced the number of synthetic steps as compared with earlier reports [25].

We examined the reactivity of nitroxyl radicals with AsA and hydroxyl radicals. Compound 8 was barely reduced by AsA. Ethyl substitutions at the 2 and 6 positions of piperidine derivatives showed enhanced stability towards reduction in biological systems, similar to other ring-type nitroxyl radicals reported previously [23,24]. In general, bulky substituents at the 2,6-position increase the stability with regards to reduction [23,24,31]. However, the ESR signal intensity of compound 5 decreased rapidly in the presence of AsA. The reaction product was a corresponding hydroxylamine, confirmed by using HPLC coupled to ECD/UV. Also, we confirmed that both compound 5 and hydroxy-TEMPO were reduced to hydroxylamine stoichiometrically (data not shown). These findings suggested that the 2,6-substitution of nitroxyl radicals can potentially regulate reactivity toward AsA reduction. Researchers have reported the protective effect due to the steric hindrance of the radical fragment of tetraethyl substituents of imidazoline and imidazolidine nitroxyl radicals on AsA reduction [23,24]. Although the detailed reaction mechanism between the nitroxyl radicals and AsA has yet to be elucidated, a comparison of functionality between compounds 5 and 8 will offer a solution to the difference through the measurement of redox potentials and the distribution of unpaired electrons.

Tetraethyl-substituted nitroxyl radicals are good candidates for use as spin probes and contrast agents. OMRI image data clearly demonstrated their significant protection from AsA. OMRI modality is a good candidate for the measurement of *in vivo* redox status [5,20], pO_2 [19], and pH [32]. However, the reactivities of nitroxyl radicals with AsA could cloud the evaluation of accurate information of redox status and free radical generation *in vivo*. These results suggest that the use of these nitroxyl radicals may solve this concern and yield good information regarding the position of free radical production in

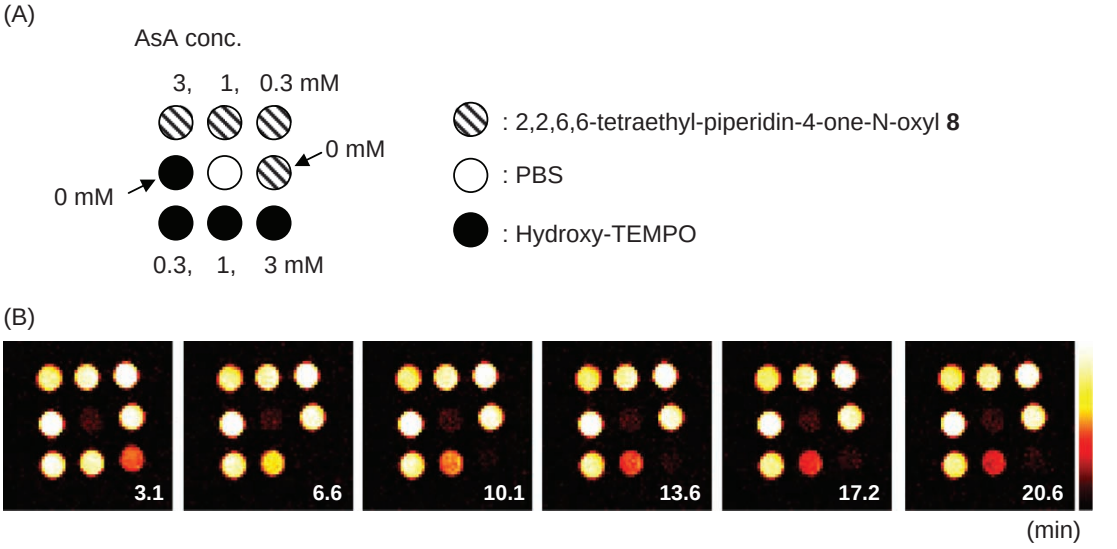


Figure 2. (A) Schematic representation of the nine tubes containing hydroxyl-TEMPO and compound 8 (2 mM) with various concentrations of AsA (0, 0.3, 1 and 3 mM). Among the eight tubes, four contained hydroxyl-TEMPO with varying concentrations of AsA (shown as closed circles) and the remaining four (shown as hatched circles) contained compound 8. The central white tube contained PBS. (B) Time-dependent OMRI images of nitroxyl radicals. Typical scan conditions in OMRI were $T_R/T_E/T_{ESR}$: 1200 ms/25 ms/600 ms; no. of averages = 2; 64 phase-encoding steps. Pixel size was 1 mm × 1 mm with a slice thickness of 10 mm. The image field of view (48 mm) was represented by a 64 × 64 matrix.

non-invasive animal disease models related to oxidative stress.

In conclusion, we succeeded in synthesizing 2,6-disubstituted piperidine nitroxyl radicals that possessed various AsA reactivities. Further development of new synthetic methods is expected to create various di- or mono-substituted piperidine or pyrrolidine nitroxyl radicals, which cannot be synthesized by conventional methods. Functional nitroxyl probes involving substitution of the piperidine nitroxyl radicals can be valuable for monitoring *in vivo* free radical reactions and redox statuses and are promising as new antioxidants that can counteract the pro-oxidant effect of nitroxyl radicals.

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