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Structural Concept of Nitroxide As a Lipid Peroxidation Inhibitor

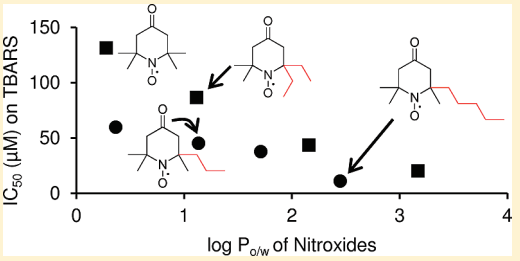
Toshihide Yamasaki,[†,△] Yuko Ito,[†,△] Fumiya Mito,[†] Kana Kitagawa,[†] Yuta Matsuoka,[†] Mayumi Yamato,[‡] and Ken-ichi Yamada[†,*]

[†]Department of Bio-functional Science, Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka 812-8582, Japan

[‡]Innovation Center for Medical Redox Navigation, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka 812-8582, Japan

Supporting Information

ABSTRACT: Nitroxides have antioxidative activities toward lipid peroxidation, but the influence of steric factors is not known. We synthesized alkyl-substituted nitroxides at the α-position of the N−O moiety to enhance lipophilicity and the bulk effect. There was good correlation between the IC_{50} and lipophilicity (log $P_{o/w}$) of nitroxides with use of the thiobarbituric acid-reactive substances (TBARS) assay. Furthermore, an inhibitory effect on the TBARS assay was dependent upon the number and length of alkyl groups, though nitroxides had almost identical lipophilicity.



Lipid radicals and peroxyl radicals produced by highly reactive oxygen species are involved in cell injury. The consecutively produced lipid peroxidation involved in this process is associated with the initiation of disorders associated with oxidative stress (e.g., cardiovascular diseases, cancer, chronic inflammation).[1] Moreover, lipid peroxide decomposes into lipid radicals, peroxyl radicals, and alkoxyl radicals,[2] and acts as a substrate for chain reactions. Once formed, peroxyl radicals can be rearranged via a cyclization reaction to malondialdehyde and 4-hydroxynonenal, which are the major aldehyde products,[3,4] followed by cytotoxic and genotoxic effects.[5] The products of lipid peroxidation also act as signaling molecules[6,7] and subsequently undergo secondary reactions as substrates of various enzymes. Therefore, to minimize and inhibit the progression of oxidative stress, lipid-derived free radicals are target molecules because they are the chain-reaction intermediates of lipid peroxidation. Here, nitroxide (>N−O$^{\bullet}$: nitroxyl radical or aminoxyl radical) with an unpaired electron has a direct reaction with a lipid-derived radical (R$^{\bullet}$) via radical−radical coupling to produce alkoxyamine derivates (>N−O−R)[8−10] as follows:

$$>N-O^{\bullet} + R^{\bullet} \rightarrow >N-O-R$$

This process results in termination of the radical chain reaction.[11] This feature of nitroxide would give it the function of an antioxidant and free-radical tracing agent.[12,13] We previously reported the synthetic method in which the 2,6 position of the piperidine nitroxide ring was substituted with cyclic ketone derivatives.[14] The synthesized compounds regulated the reactivity with free radicals and reducing agents.[15] For instance, the calculated Gibbs energy from the difference in redox potential between ascorbic acid and nitroxide showed a linear relationship with the rate of nitroxide reduction.[16] Conversely, tetraethyl-substituted nitroxide did not react with reducing agents, which can be explained by the lower reduction potential. However, this compound had an inhibitory effect on lipid peroxidation.[17] These results suggest that the feature of the N−O group surrounding the 2,6 position of piperidine nitroxide can be regulated with the reactivity of the lipid radical without undesirable reduction. Furthermore, confirmation of this hypothesis would further expand the application range of nitroxide as not only an antioxidant, but also as an antifatigue and photoprotecting agent in rubber and polymers[18,19] (which is based on the mechanism of the radical−radical coupling reaction). To find a good antioxidant, the lipophilicity of a compound must be considered. This is because lipophilicity is a good index for the permeability of the blood−brain barrier and as a basic index for drug design.[20] Here, the reaction with lipid-derived radicals would provide stability to alkyl-substituted piperidine nitroxide via van der Waal's forces. Hence, the aim of the present study was to clarify the effect of lipophilicity and steric factors near the N−O moiety of piperidine nitroxides to lipid peroxidation to find good candidates for antioxidants.

According to a previously described method,[14,16] we used chain ketone compounds and synthesized alkyl-substituted piperidine nitroxides **2b**−**i** (Table 1). However, the yield of the product was <18%. In the case of cyclic ketones, we reported that we obtained the 2,2,6,6-tetrasubstituted piperidin-4-one derivatives (precursor compounds of nitroxides (**1**)) in ∼30% yield.[14] The reason for this low yield was thought to be because the chain

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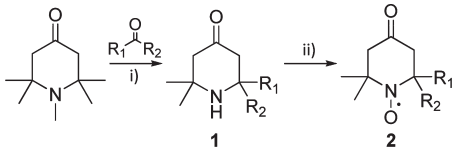
Table 1. Synthesized Nitroxides (2b−i)[a] and Their Partition Coefficients



compd 2	R_1	R_2	yield (1)	log $P_{o/w}$ (2)	IC_{50} (μM) (2)[b]
a	Me	Me	−[c]	0.277	131.4 ± 19.2
b	Et	Et	11	1.115	86.6 ± 15.4
c	*n*-Pr	*n*-Pr	17	2.158	43.6 ± 4.3
d	*n*-Bu	*n*-Bu	4	3.169	20.2 ± 3.3
e	Me	Et	8	0.366	59.8 ± 6.5
f	Me	*n*-Pr	18	1.133	45.2 ± 5.4
g	Me	*n*-Bu	12	1.711	37.7 ± 3.4
h	Me	*n*-C_5H_{11}	15	2.449	11.1 ± 1.5
i	Me	*n*-C_8H_{17}	5	n.d.[d]	5.6 ± 0.1

[a] Conditions: (i) NH_4Cl, Triton B, DMSO, 60°C; (ii) H_2O_2, $Na_2WO_4 \cdot 2H_2O$. [b] Values are means ± standard deviations (n = 3). [c] Nitroxide **2a** is commercially available. [d] n.d.: not determined. The ESR signal in the water phase was not detected because of high lipophilicity.

ketone compound was not adequate for the aldol reaction because of intermolecular condensation and hindrance of the carbonyl group by the alkyl chain. So far, to introduce the alkyl groups into the α-position of piperidine nitroxide, it has been synthesized in a stepwise fashion. However, synthetic methods to substitute the α-position of **1a** by alkyl substituents in one step are lacking. Here, we could readily obtain α-substituted piperidin-4-one derivatives substituted with alkyl groups.

The partition coefficients of nitroxides between *n*-octanol and phosphate buffer ($P_{o/w}$) were determined by electron spin resonance (ESR) spectrometry (Table 1). The logarithm of $P_{o/w}$ (log $P_{o/w}$) was increased along with the length of the alkyl chain at the 2- or 2,6-position of the piperidine ring. The inhibitory effect on lipid peroxidation was then measured by the thiobarbituric acid-reactive substances (TBARS) assay. The half-maximal inhibitory concentration (IC_{50}) was calculated (i.e., the nitroxide concentration at 50% inhibition of lipid peroxidation (Table 1)). IC_{50} values were dependent upon the structures of the substituent. The nitroxides **2h** and **2i**, which had *n*-pentyl and *n*-octyl groups, respectively, had the highest inhibitory effects.

The correlation of IC_{50} and log $P_{o/w}$ is shown in Figure 1. IC_{50} values decreased with an increase in the log $P_{o/w}$. Therefore, nitroxides which had a large value of log $P_{o/w}$ had a good protecting effect upon lipid peroxidation. In addition, the IC_{50} values of nitroxides **2e**−**h** (which had three methyl and one alkyl groups at the 2,6-position) were relatively smaller than those of nitroxides **2a**−**d** (which had two methyl and two alkyl groups at the same position). These results suggested that the bulky alkyl substituent that approximated to the N−O moiety induced resistance against lipid radicals. With respect to nitroxides **2b** and **2f**, although these compounds had the same number of carbon atoms and similar lipophilicity, the IC_{50} value of nitroxide **2f** was lower than that of **2b**. This indicated that the protecting effect was involved with the steric hindrance surrounding the N−O moiety. Conversely, nitroxide **2e** (which substituted the one methyl group of nitroxide **2a** with an ethyl group) had a

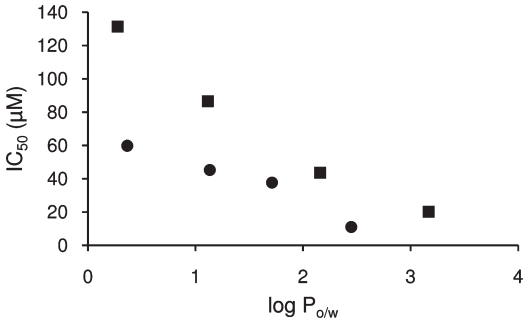


Figure 1. Correlation of the values of IC_{50} and log $P_{o/w}$ of nitroxides with use of the thiobarbituric acid-reactive substances assay: ■, **2a**−**d**; ●, 2e−h.

better protecting effect than **2a**, although these two nitroxides had a similar value of log $P_{o/w}$. These results suggested that the protecting effect against lipid peroxidation was affected not only by the steric effect, but also by the affinity of nitroxides with lipid radicals.

Tetraethyl-substituted piperidine nitroxide bound with lipid-derived radicals did not decompose the nitroxide and lipid-derived radical upon heating (100 °C), but the tetramethyl type did. These observations were confirmed by LC/MS using the linoleic acid/lipoxygenase system. The alkoxyamine adducts (>N−O−R) of nitroxides **2b**−**i** were supposed to be stable because their ESR signals were recovered with only 10−30% intensity by heating of the reaction mixture. The reactivity of these nitroxides in a rat microsomal lipid peroxidation system was found to be dependent upon the lipophilicity in the present study. Here, other factors such as steric hindrance and the stability of >N−O−R must also be considered. The *mono*-alkyl-substituted nitroxides **2e**−**i** tended to be more efficient in inhibiting lipid peroxidation than *di*-alkyl-substituted nitroxides **2a**−**d**. The stability of >N−O−R was supposed to be almost identical, but *di*-alkyl-substituted nitroxides **2b**−**d** had slightly higher stability than *mono*-alkyl-substituted nitroxides. Therefore, these alkyl substrate nitroxides are thought to be effective inhibitors of lipid peroxidation via thermally stabilized alkoxyamine derivatives. Nitroxides are also used to identify the structure of protein-based carbon-centered radicals combined with LC/MS techniques.[21] These results suggest that nitroxides controlling the reactivity of the target molecules would be useful not only for antioxidant and photoprotecting agents, but also as detection agents of protein- and lipid-derived radicals.

■ EXPERIMENTAL SECTION

Reagents and solvents from commercial suppliers were used without further purification. Silica gel (100−200 mesh) was used for column chromatography. Compound **2b** was synthesized as previously described.[16] TLC was detected with iodine vapor.

2,2-Diethyl-6,6-dimethylpiperidin-4-one (1b). NH_4Cl (5.35 g, 100 mmol) and Triton B (4 mmol) were added portion-wise to a stirred solution of 1,2,2,6,6-pentamethylpiperidin-4-one (3.38 g, 20 mmol) and 3-pentanone (5.17 g, 60 mmol) in DMSO (32 mL) at room temperature. The mixture was then heated for 4 h at 50 °C. It was diluted with H_2O, acidified with 7% aq HCl, and extracted with ether (3×) to remove the neutral fraction. The reaction mixture was adjusted to pH 9 with use of K_2CO_3 and then extracted with AcOEt (3×). The AcOEt extract was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was separated by column chromatography

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with hexane/AcOEt (80:20) to afford **1b** (413 mg, 11%). The analytical data were in agreement with a previous study.[16]

2,2-Diethyl-6,6-dimethylpiperidin-4-one-1-oxyl (2b). Compound **2b** was synthesized from compound **1b** according to a previously described method.[16]

2,2-Dimethyl-6,6-dipropylpiperidin-4-one (1c). NH_4Cl (2.68 g, 50 mmol) and Triton B (40 wt % soln in MeOH, 2 mL) were added portion-wise to a stirred solution of 1,2,2,6,6-pentamethylpiperidin-4-one (1.69 g, 10 mmol) and 4-heptanone (3.43 g, 30 mmol) in DMSO (16 mL) at room temperature. The mixture was then heated for 4.5 h at 50 °C. It was diluted with H_2O, acidified with 7% aq HCl, and extracted with ether (3×) to remove the neutral fraction. The reaction mixture was adjusted to pH 12 with K_2CO_3 and then extracted with AcOEt (3×). The AcOEt extract was dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was separated by column chromatography with hexane/AcOEt to afford 2,2-dimethyl-6,6-dipropylpiperidin-4-one (**1c**) (368 mg, 17%) as a pale-yellow needle after recrystallization: mp 53.4−55.0 °C (hexane); MS (FAB^+):212.2 ($M^+ + 1$); ν_{max}/cm^{-1} 1690 (C═O); ^1H NMR (400 MHz; $CDCl_3$) δ (ppm) 0.886 (6H, t, J = 6.8 Hz), 1.218 (6H, s), 1.23−1.49 (8H, m), 2.235 (2H, s), 2.264 (2H, s); ^{13}C NMR (125 MHz; $CDCl_3$) δ (ppm) 14.4, 16.8, 32.2, 42.9, 51.3, 54.4, 54.5, 59.6, 211.3; HRMS (ESI^+) calcd for $C_{13}H_{26}NO$ $[M + H]^+$ 212.2014, found 212.2030. Anal. Calcd for $C_{13}H_{25}NO$: C, 73.88; H, 11.92; N, 6.63. Found: C, 73.74; H, 11.88; N, 6.67.

2,2-Dimethyl-6,6-dipropylpiperidin-4-one-1-oxyl (2c). Compound **1c** (59 mg, 0.28 mmol) and $Na_2WO_4 \cdot 2H_2O$ (50 mg, 0.15 mmol) were added in methanol (5 mL); H_2O_2 (30%, 1 mL) was slowly added to the solution. The mixture was stirred at room temperature, and H_2O_2 added (monitoring by TLC). After stirring, the solution was saturated with K_2CO_3 and extracted with chloroform. The chloroform extracts were dried over anhydrous sodium sulfate and evaporated. The residue was separated by column chromatography with hexane/AcOEt to afford compound **2c** (38 mg, 60%) as an orange oil: MS (FAB^+) 226.3 (M^+); ν_{max}/cm^{-1} 1720 (C═O); A_N = 1.568 mT. Anal. Calcd for $C_{13}H_{24}NO_2$: C, 68.99; H, 10.69; N, 6.19. Found: C, 68.95; H, 10.76; N, 6.28.

2,2-Dibutyl-6,6-dimethylpiperidin-4-one (1d). 5-Nonanone (4.27 g, 30 mmol) was used in place of 4-heptanone according to the method for compound **1c**. The mixture was stirred for 4 h at 60 °C. The product was separated by column chromatography with hexane/AcOEt (85:15) to afford compound **1d** (96 mg, 4%) as a pale-yellow oil: MS (FAB^+) 240.3 ($M^+ + 1$); ν_{max}/cm^{-1} 1705 (C═O); ^1H NMR (400 MHz; $CDCl_3$) δ (ppm) 0.900 (6H, t, J = 6.8 Hz), 1.223 (6H, s), 1.15−1.52 (12H, m), 2.239 (2H, s), 2.267 (2H, s); ^{13}C NMR (125 MHz; $CDCl_3$) δ (ppm) 14.0, 23.0, 25.7, 32.2, 40.1, 51.4, 54.4, 54.6, 59.5, 211.3; HRMS (ESI^+) calcd for $C_{15}H_{30}NO$ $[M + H]^+$ 240.2327, found 240.2314. Anal. Calcd for $C_{15}H_{29}NO$: C, 75.26; H, 12.21; N, 5.85. Found: C, 75.15; H, 12.11; N, 5.75.

2,2-Dibutyl-6,6-dimethylpiperidin-4-one-1-oxyl (2d). Compound **1d** (100 mg, 0.42 mmol) was oxidized according to the method for compound **2c**. The crude product was separated by column chromatography with hexane/AcOEt to afford compound **2d** (53 mg, 50%) as an orange oil: MS (FAB^+) 254.3 (M^+); ν_{max}/cm^{-1} 1720 (C═O); A_N = 1.575 mT. Anal. Calcd for $C_{15}H_{28}NO_2$: C, 70.82; H, 11.09; N, 5.51. Found: C, 70.55; H, 11.05; N, 5.24.

2-Ethyl-2,6,6-trimethylpiperidin-4-one (1e). NH_4Cl (8.02 g, 150 mmol) and Triton B (40 wt % soln in MeOH, 15 mL) were added portion-wise to a stirred solution of 1,2,2,6,6-pentamethylpiperidin-4-one (5.08 g, 30 mmol) and 2-butanone (6.48 g, 90 mmol) in DMSO (25 mL) at room temperature. The mixture was then heated for 4.5 h at 50 °C. It was diluted with H_2O, acidified with 7% aq HCl, and extracted with ether (4×) to remove the neutral fraction. The reaction mixture was adjusted to pH 12 with K_2CO_3 and then extracted with AcOEt (7×). The AcOEt extract was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was separated by column chromatography with hexane/AcOEt (8:2) to afford 2-ethyl-2,6,6-trimethylpiperidin-4-one (**1e**) (400 mg, 8%) as an orange oil: MS (FAB^+) 170.2 ($M^+ + 1$); ν_{max}/cm^{-1} 1704 (C═O); ^1H NMR (400 MHz; $CDCl_3$) δ (ppm) 0.912 (3H, t, J = 7.6 Hz), 1.157 (3H, s), 1.233 (3H, s), 1.237 (3H, s), 1.40−1.59 (2H, m), 2.229 (1H, s), 2.247 (1H, s), 2.268 (1H, s), 2.278 (1H, s); ^{13}C NMR (125 MHz; $CDCl_3$) δ (ppm) 8.1, 28.4, 31.7, 32.4, 36.8, 52.3, 54.3, 54.9, 57.4, 211.0; HRMS (ESI^+) calcd for $C_{10}H_{20}NO$ $[M + H]^+$ 170.1545, found 170.1562. Anal. Calcd for $C_{10}H_{19}NO$: C, 70.96; H, 11.31; N, 8.28. Found: C, 70.85; H, 11.11; N, 8.14.

2-Ethyl-2,6,6-trimethylpiperidin-4-one-1-oxyl (2e). Compound **1e** (400 mg, 2.36 mmol) was oxidized according to the method for compound **2c**. The crude product was separated by column chromatography with $CHCl_3$/MeOH to afford compound **2e** (173.2 mg, 40%) as an orange oil: MS (FAB^+) 184.2 (M^+); ν_{max}/cm^{-1} 1719 (C═O); HRMS (ESI^+) calcd for $C_{10}H_{18}NNaO_2$ $[M + Na]^+$ 207.1235, found 207.1247; A_N = 1.513 mT. Anal. Calcd for $C_{10}H_{18}NO_2$: C, 65.19; H, 9.85; N, 7.60. Found: C, 64.97; H, 9.61; N, 7.42.

2,2,6-Trimethyl-6-propylpiperidin-4-one (1f). 2-Pentanone (5.17 g, 60 mmol) was used in place of 3-pentanone according to the method for compound **1b**. The product was separated by column chromatography with hexane/AcOEt (8:2) to afford **1f** (700 mg, 18%) as a pale-yellow oil: MS (FAB^+) 184.2 ($M^+ + 1$); ν_{max}/cm^{-1} 1703 (C═O); ^1H NMR (400 MHz; $CDCl_3$) δ (ppm) 0.910 (3H, t, J = 6.8 Hz), 1.170 (3H, s), 1.229 (6H, br s), 1.29−1.52 (4H, m), 2.231 (1H, s), 2.256 (1H, s), 2.266 (1H, s), 2.273 (1H, s); ^{13}C NMR (125 MHz; $CDCl_3$) δ (ppm) 14.4, 17.0, 28.9, 31.8, 32.4, 46.9, 52.8, 54.3, 54.9, 57.4, 211.0; HRMS (ESI^+) calcd for $C_{11}H_{22}NO$ $[M + H]^+$ 184.1701, found 184.1696. Anal. Calcd for $C_{11}H_{21}NO$: C, 72.08; H, 11.55; N, 7.64. Found: C, 71.73; H, 11.32; N, 7.58.

2,2,6-Trimethyl-6-propylpiperidin-4-one-1-oxyl (2f). Compound **1f** (500 mg, 2.53 mmol) was oxidized according to the method for compound **2c**. The crude product was separated by column chromatography with hexane/AcOEt (98:2) to afford compound **2f** (253 mg, 50%) as an orange oil: MS (FAB^+) 198.2 (M^+); ν_{max}/cm^{-1} 1720 (C═O); HRMS (ESI^+) calcd for $C_{11}H_{20}NNaO_2$ $[M + Na]^+$ 221.1392, found 221.1409; A_N = 1.589 mT. Anal. Calcd for $C_{11}H_{20}NO_2$: C, 66.63; H, 10.17; N, 7.06. Found: C, 66.30; H, 9.88; N, 6.96.

2-Butyl-2,6,6-trimethylpiperidin-4-one (1g). 2-Hexanone (3.00 g, 30 mmol) was used in place of 4-heptanone according to the method for compound **1c**. The product was separated by column chromatography with hexane/AcOEt (9:1) to afford **1g** (239 mg, 12%) as a pale-yellow oil: MS (FAB^+) 198.3 ($M^+ + 1$); ν_{max}/cm^{-1} 1703 (C═O); ^1H NMR (400 MHz; $CDCl_3$) δ (ppm) 0.909 (3H, t, J = 6.4 Hz), 1.170 (3H, s), 1.231 (6H, br s), 1.24−1.54 (6H, m), 2.230 (1H, s), 2.255 (1H, s), 2.265 (1H, s), 2.274 (1H, s); ^{13}C NMR (125 MHz; $CDCl_3$) δ (ppm) 14.0, 23.0, 26.0, 29.0, 31.8, 32.5, 44.3, 52.8, 54.3, 54.9, 57.4, 211.0; HRMS (ESI^+) calcd for $C_{12}H_{24}NO$ $[M + H]^+$ 198.1858, found 198.1845. Anal. Calcd for $C_{12}H_{23}NO$: C, 73.04; H, 11.75; N, 7.10. Found: C, 72.83; H, 11.63; N, 7.02.

2-Butyl-2,6,6-trimethylpiperidin-4-one-1-oxyl (2g). Compound **1g** (199 mg, 1.01 mmol) was oxidized according to the method for compound **2c**. The crude product was separated by column chromatography with hexane/AcOEt (95:5) to afford compound **2g** (68 mg, 32%) as an orange oil: MS (FAB^+) 212.3 (M^+); ν_{max}/cm^{-1} 1720 (C═O); A_N = 1.598 mT. Anal. Calcd for $C_{12}H_{22}NO_2$: C, 67.89; H, 10.44; N, 6.60. Found: C, 68.01; H, 10.44; N, 6.53.

2,2,6-Trimethyl-6-pentylpiperidin-4-one (1h). 2-Heptanone (3.43 g, 30 mmol) was used in place of 4-heptanone according to the method for compound **1c**. The product was separated by column chromatography with hexane/AcOEt to afford 2,2,6-trimethyl-6-pentylpiperidin-4-one (**1h**) (316 mg, 15%) as an orange oil: MS (FAB^+) 212.3 ($M^+ + 1$); ν_{max}/cm^{-1} 1690 (C═O); ^1H NMR (400 MHz; $CDCl_3$) δ (ppm) 0.888 (3H, t, J = 6.8 Hz), 1.170 (3H, s), 1.229 (3H, s),

1.232 (3H, s), 1.24−1.53 (8H, m), 2.230 (1H, s), 2.253 (1H, s), 2.266 (1H, s), 2.273 (1H, s); ^{13}C NMR (125 MHz; $CDCl_3$) δ (ppm) 14.0, 22.5, 23.4, 29.0, 31.8, 32.2, 32.4, 44.5, 52.8, 54.3, 54.9, 57.4, 211.0; HRMS (ESI$^+$) calcd for $C_{13}H_{26}NO$ $[M + H]^+$ 212.2014, found 212.2005. Anal. Calcd for $C_{13}H_{25}NO$: C, 73.88; H, 11.92; N, 6.63. Found: C, 73.61; H, 11.70; N, 6.56.

2,2,6-Trimethyl-6-pentylpiperidin-4-one-1-oxyl (2h). Compound **1h** (316 mg, 1.5 mmol) was oxidized according to the method for compound **2c**. The crude product was separated by column chromatography with hexane/AcOEt to afford compound **2h** (226 mg, 70%) as an orange oil: MS (FAB$^+$) 226.3 (M^+); ν_{max}/cm^{-1} 1720 (C═O); HRMS (ESI$^+$) calcd for $C_{13}H_{24}NNaO_2$ $[M + Na]^+$ 249.1705, found 249.1720; A_N = 1.498 mT. Anal. Calcd for $C_{13}H_{24}NO_2$: C, 68.99; H, 10.69; N, 6.19. Found: C, 68.75; H, 10.47; N, 6.10.

2,2,6-Trimethyl-6-octylpiperidin-4-one (1i). 2-Decanone (4.69 g, 30 mmol) was used in place of 4-heptanone according to the method for compound **1c**. The mixture was heated for 4 h at 50 °C. The product was separated by column chromatography with hexane/AcOEt to afford compound **1i** (127 mg, 5%) as a pale-yellow oil: MS (FAB$^+$) 254.3 ($M^+ + 1$); ν_{max}/cm^{-1} 1708 (C═O); ^{1}H NMR (400 MHz; $CDCl_3$) δ (ppm) 0.879 (3H, t, J = 6.8 Hz), 1.168 (3H, s), 1.230 (6H, br s), 1.24−1.53 (14H, m), 2.228 (1H, s), 2.251 (1H, s), 2.265 (1H, s), 2.272 (1H, s); ^{13}C NMR (125 MHz; $CDCl_3$) δ (ppm) 14.0, 22.6, 23.8, 29.0, 29.2, 29.5, 30.0, 31.8, 32.5, 44.6, 52.8, 54.3, 55.0, 57.4, 211.0; HRMS (ESI$^+$) calcd for $C_{16}H_{32}NO$ $[M + H]^+$ 254.2484, found 254.2489. Anal. Calcd for $C_{16}H_{31}NO$: C, 75.83; H, 12.33; N, 5.53. Found: C, 75.77; H, 11.99; N, 5.54.

2,2,6-Trimethyl-6-octylpiperidin-4-one-1-oxyl (2i). Compound **1i** (100 mg, 0.39 mmol) was oxidized according to the method for compound **2c**. The crude product was separated by column chromatography with hexane/AcOEt to afford compound **2i** (78 mg, 74%) as an orange oil: MS (FAB$^+$): 268.3 (M^+); ν_{max}/cm^{-1} 1721 (C═O); A_N = 1.596 mT. Anal. Calcd for $C_{16}H_{30}NO_2$: C, 71.59; H, 11.27; N, 5.22. Found: C, 71.45; H, 11.13; N, 5.10.

Thiobarbituric Acid-Reactive Substances Assay

a. Animals. Animal experiments and all procedures were approved by the Committee on the Ethics of Animal Experiments, Faculty of Pharmaceutical Sciences, Kyushu University (Kyushu, Japan). Male Wistar rats were purchased from Kyudo Company Limited (Saga, Japan). Rats were allowed free access to water and food (MF, Oriental Yeast Company, Tokyo, Japan).

b. Preparation of Microsomal Suspensions. Liver samples from male Wistar rats were obtained after killing under deep anesthesia. They were washed with chilled 0.9% NaCl and homogenized with a Potter-type homogenizer in three volumes (w/v) of chilled phosphate-buffered saline (PBS). The homogenate from each sample was centrifuged at 700 × g for 10 min at 4 °C, and the supernatant centrifuged at 7 000 × g for 10 min at 4 °C. The resulting supernatant was centrifuged at 105 000 × g for 60 min at 4 °C. Protein concentrations of microsomes were determined with use of a BCA protein assay kit (Pierce, Rockford, IL, USA).

c. Protective Effect of Nitroxides on Lipid Peroxidation. Peroxidation of a microsomal suspension (0.5 mg/mL) was initiated by the addition of cysteine (500 μM) and ferrous sulfate (5 μM) with or without nitroxide (5−1000 μM). A 96-well culture plate was covered with tape and incubated at 37 °C for 30 min.[22] Each well contained 40 μL of 20% acetic acid, 60 μL of 1.3% (w/v) thiobarbituric acid dissolved in 0.3% NaOH, and 15 μL of 10% (w/v) sodium dodecyl sulfate. The plate was covered with tape and gently shaken at 60 °C for 40 min. The plate was cooled and fluorescence measured (532 nm for excitation, 553 nm for emission).[23] The half-maximal inhibitory concentration (IC_{50}), i.e., the nitroxide concentration at which 50% inhibition of lipid peroxidation is achieved, was calculated with use of Sigma Plot 11.2 software (Systat Software Incorporated, San Jose, CA) with the 4−8 parameter exponential function standard curve analysis for dose response.

d. Partition Coefficients between *n*-Octanol and Phosphate Buffer (PB). The nitroxide solution was prepared in 10 mM phosphate buffer (PB) (pH 7.4; treated with Chelex100). A 50-μL aliquot of the nitroxide solution was vigorously mixed with 500 μL of *n*-octanol, and the mixture was centrifuged at 3000 rpm for 5 min. The ESR spectra of *n*-octanol and PB were measured with an X-band ESR spectrometer (JES-FA100; JEOL, Tokyo, Japan). The amount of nitroxide was calculated from the double-integrated ESR signal intensity by using Mn^{2+} as an external standard for correcting the sensitivities of *n*-octanol and PB. The conditions for the ESR measurements were as follows: power, 10 mW; frequency, 9.4 GHz; magnetic field, 337 mT; modulation amplitude, one-third line width or less; and time constant, 0.3 s.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. ^{1}H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: yamada@pch.phar.kyushu-u.ac.jp.

Author Contributions

△These authors contributed equally.

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■ REFERENCES

(1) Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T. D.; Mazur, M.; Telser, J. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 44–84.
(2) Minotti, G. *Chem. Res. Toxicol.* **1993**, *6*, 134–46.
(3) Esterbauer, H.; Schaur, R. J. g.; Zollner, H. *Free Radical Biol. Med.* **1991**, *11*, 81–128.
(4) Spiteller, P.; Kern, W.; Reiner, J.; Spiteller, G. *Biochim. Biophys. Acta* **2001**, *1531*, 188–208.
(5) Esterbauer, H. *Am. J. Clin. Nutr.* **1993**, *57*, 779S−785S (discussion 785S−786S).
(6) Yang, Y.; Sharma, R.; Sharma, A.; Awasthi, S.; Awasthi, Y. C. *Acta Biochim. Pol.* **2003**, *50*, 319–36.
(7) Forman, H. J.; Fukuto, J. M.; Miller, T.; Zhang, H.; Rinna, A.; Levy, S. *Arch. Biochem. Biophys.* **2008**, *477*, 183–195.
(8) Chateauneuf, J.; Lusztyk, J.; Ingold, K. U. *J. Org. Chem.* **1988**, *53*, 1629–1632.
(9) Beckwith, A. L. J.; Bowry, V. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1992**, *114*, 4983–4992.
(10) Bowry, V. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1992**, *114*, 4992–4996.
(11) Samuni, A. M.; Barenholz, Y. *Free Radical Biol. Med.* **1997**, *22*, 1165–1174.
(12) Cimato, A. N.; Piehl, L. L.; Facorro, G. B.; Torti, H. B.; Hager, A. A. *Free Radical Biol. Med.* **2004**, *37*, 2042–2051.
(13) Johnson, C. G.; Caron, S.; Blough, N. V. *Anal. Chem.* **1996**, *68*, 867–872.

(14) Sakai, K.; Yamada, K. i.; Yamasaki, T.; Kinoshita, Y.; Mito, F.; Utsumi, H. *Tetrahedron* **2010**, *66*, 2311–2315.
(15) Kinoshita, Y.; Yamada, K.; Yamasaki, T.; Sadasue, H.; Sakai, K.; Utsumi, H. *Free Radical Res.* **2009**, *43*, 565–71.
(16) Yamasaki, T.; Mito, F.; Ito, Y.; Pandian, S.; Kinoshita, Y.; Nakano, K.; Murugesan, R.; Sakai, K.; Utsumi, H.; Yamada, K.-i. *J. Org. Chem.* **2011**, *76*, 435–440.
(17) Kinoshita, Y.; Yamada, K.; Yamasaki, T.; Mito, F.; Yamato, M.; Kosem, N.; Deguchi, H.; Shirahama, C.; Ito, Y.; Kitagawa, K.; Okukado, N.; Sakai, K.; Utsumi, H. *Free Radical Biol. Med.* **2010**, *49*, 1703–9.
(18) Lau, W. W. Y.; Lee, C. S.; Goh, S. H.; Lee, S. Y.; Pan, J.; Song, Y. *J. Appl. Polym. Sci.* **1993**, *50*, 403–409.
(19) Hawker, C. J.; Bosman, A. W.; Harth, E. *Chem. Rev.* **2001**, *101*, 3661–3688.
(20) Van De Waterbeemd, H.; Camenisch, G.; Folkers, G.; Chretien, J. R.; Raevsky, O. A. *J. Drug Targeting* **1998**, *6*, 151–165.
(21) Wright, P. J.; English, A. M. *J. Am. Chem. Soc.* **2003**, *125*, 8655–8665.
(22) Malvy, C.; Paoletti, C.; Searle, A. J.; Willson, R. L. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 734–7.
(23) Ohkawa, H.; Ohishi, N.; Yagi, K. *Anal. Biochem.* **1979**, *95*, 351–8.