Novel Piperidine Nitroxide Derivatives: Synthesis, Electrochemical and Antioxidative Evaluation

Hongli Zhao[1], Juhua Wu[1], Xianjiang Meng[1], Shaohua Zuo[1], Wentian Wang[1], Huihui Yuan[2], Minbo Lan[1,2*]

1. Research Center of Analysis and Test, East China University of Science and Technology, Shanghai 200237, P.R. China. 2. Key Laboratory for Ultrafine Materials of Ministry of Education, East China University of Science and Technology, Shanghai 200237, P.R. China.

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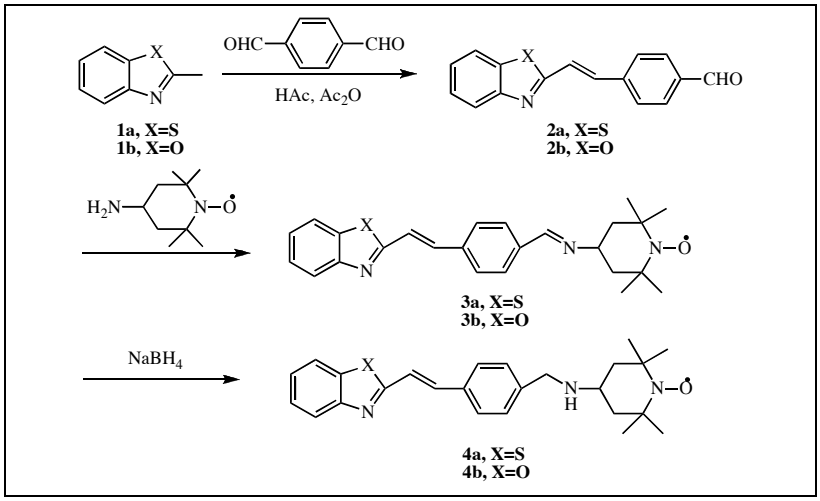

Novel paramagnetic materials (**3a**, **3b**, **4a** and **4b**) based on heterocyclic dyes (**2a** and **2b**) and one stable nitroxide radical (4-NH_2-TEMPO) were synthesized. EI-MS, elemental analysis and FT-IR spectral data confirmed the structures of the newly synthesized compounds. In addition, the cyclic voltammetry, the antioxidant activity and reactive oxygen species (ROS) scavenging properties of these compounds are discussed in this paper.

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INTRODUCTION

The stable nitroxide radicals, such as 2,2,6,6-tetra-methyl-1-piperidinyloxy (TEMPO) and its derivatives, are widely used in various biological and pathological areas as spin labels [1], electron paramagnetic resonance imaging (EPRI) [2], antioxidants and anticancer drugs [3,4,5], or synthetic studies [6,7]. Some of the chemical properties of nitroxides favor the design of new nitroxide compounds using in different biological and chemical systems. Nitroxide free radicals continue to be interesting compounds because they often exhibit a chemical inertness quite uncharacteristic of most other free radicals [8,9]. Now piperidine nitroxides and their derivatives are intensively investigated because of their versatile properties such as antioxidant and radioprotective [10,11]. They are less toxic stable free radicals and can be used in noninvasive methods to exert superoxide dimutase (SOD) mimic activity and scavenge reactive oxygen species (ROS) [12,13,14] as cycle antioxidants.

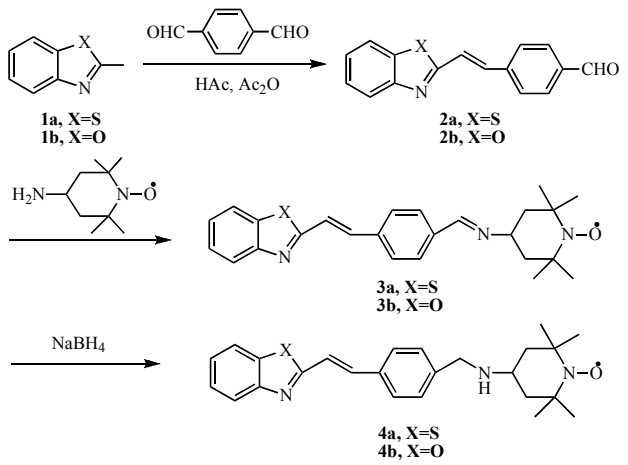
It is possible that organic molecular design and synthesis will yield other superior nitroxides with improved properties. To develop more potent compounds with enhanced free radical scavenger properties, we designed and synthesized a series of nitroxides combined with heterocyclic dyes and piperidine nitroxide free radical. Polymethine dyes, which represent a special class of charged π-electron organic compounds [15], could improve properties such as antioxidant activity of new nitroxides prepared. We wish to report in this paper our research about synthesis and characterization of novel nitroxides. The newly synthesized compounds exhibit improved antioxidant activity, ROS scavenger's activity and possess interesting electrochemical properties. Furthermore, the relationship between the antioxidant activity and electrochemical properties of the studied compounds is also discussed.

RESULTS AND DISCUSSION

The studied compounds were synthesized according to **Scheme 1**. Original heterocyclic dyes 2-(*p*-formylstyryl)-benzothiazole (**2a**) and 2-(*p*-formylstyryl)benzoxazole (**2b**) were synthesized by 2-methylbenzothiazole (**1a**) and 2-methylbenzoxazole (**1b**) reacted with *p*-phthaldialde-hyde in

acetic anhydride and acetic acid solutions in about 80% yield, according to the process described by Huang, Z.L. *et al* [16]. The Schiff-base derivatives *N*-[2-(*p*-vinylbenzyl-idene)benzothiazole] 4-amino-2,2,6,6-tetra-methyl-1-piperi-dinyloxy (**3a**) and *N*-[2-(*p*-vinylbenzyl-idene)benzoxazole] 4-amino-2,2,6,6-tetramethyl-1-piper-idinyloxy (**3b**), in turn, were obtained by the reaction of **2a** and **2b** with 4-NH_2-TEMPO in alcohol solution containing a catalytic amount of acetic acid in 80-85% yield. The N-methylamine derivatives *N*-[2-(*p*-vinyl-benzyl)benzoxazole] 4-amino-2,2,6,6-tetra-methyl-1-pi-peridinyloxy (**4a**) and *N*-[2-(*p*-vinylbenzyl) benzoxazole] 4-amino-2,2,6,6-tetramethyl-1-piperidinyloxy (**4b**) of **3a** and **3b** were prepared by reducing compounds **3a** and **3b** with $NaBH_4$ in alcohol solution, and the purification were performed by silica-gel column chromatography using the solvent system of petroleum ether/ethyl acetate/acetone as eluent to afford **4a** and **4b** (35-45%) as orange crystals.

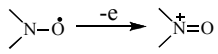
Scheme 1



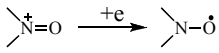
Cyclic voltammetry (CV). All the electrochemical measurements were carried out in dry acetonitrile (AN) solution containing 0.1 *M* sodium perchlorate ($NaClO_4.H_2O$) as supporting electrolyte at a scan rate of 50mV/s with a CHI440A Electrochemical Analyzer controlled by a personal computer. The conventional three-electrode setup was used with a platinum electrode as the working electrode, a platinum wire as the counter electrode, and a saturated calomel electrode (SCE) as the reference electrode.

Figure 1 and 2 show typical cyclic voltammograms of all studied compounds. Both polymethine dyes molecules **2a** and **2b** indicate a little charge-transfer character, no redox reaction (in Figure 2 a and b). Because they could be classified as neutral streptopolymethines similar to mero-cyanine dyes, no electrons transfer. Generally, piperidine nitroxides show reversible redox behavior such as 4-OH-TEMPO (Figuer 1 b), whose peak separation of cyclic voltammogram is 68 mV (Table 1) closed to the theoretical value (59.5 mV) based on a reversible redox reaction. When conjugated to 4-NH_2-TEMPO, the obtained compounds **3a** and **3b** display typical reversible redox behavior similar to that of the 4-OH-TEMPO. In Figure 1 a, 4-NH_2-TEMPO shows a little difference in redox signal with 4-OH-TEMPO because of the effect of the $-NH_2$ group. But, in the structures of the compounds **3a** and **3b**, new double bonds C=N were formed by the -NH_2 group of the 4-NH_2-TEMPO reacting with the -CHO of the polymethine matrix. And the C=N conjugates with phenyl ring, thus forming one whole conjugated system with the polymethine molecules. This also is the reason that the compounds **3a** and **3b** as Schiff bases could exist stably. The nitrogen atom of the C=N does not become an oxidizable center in the conjugated system within the range of the CV experimental scan because it is not easily oxidized, hence no electrons are transfer in this moiety of the molecule. The compounds **3a** and **3b** exhibit the typical reversible redox signals of the nitroxyl radical and the peak separations are 64 mV and 71 mV, respectively. The reaction followed as [17]:

Nitroxide free radical is oxidized into oxoammonium cation at the anode by losing an electron:



Oxoammonium cation is reduced into nitroxide free radical at the cathode by obtaining an electron:



When the C=N bonds in **3a** and **3b** structures were reduced, it is interesting that the CV curves of the compounds **4a** and **4b** exhibit two-electron transfers (Figure 2). The measurement results indicate that there are

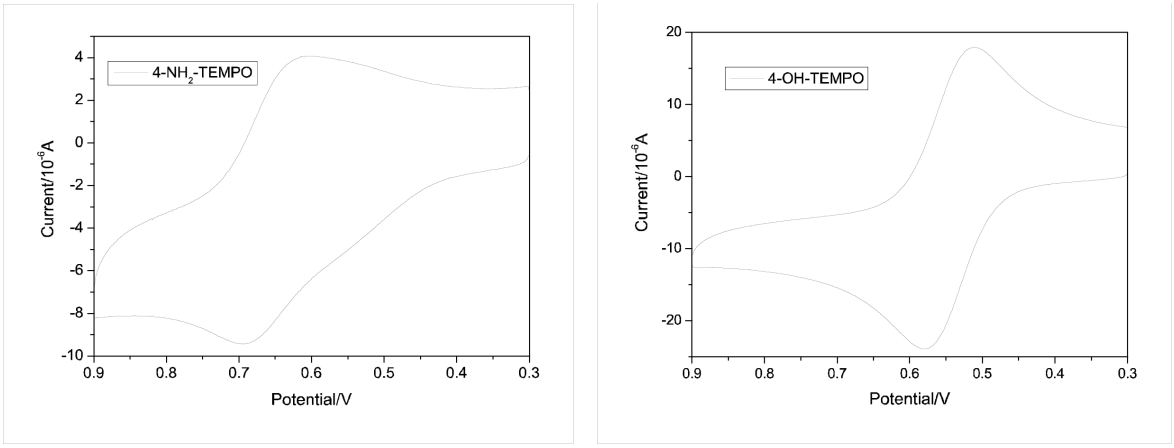
Table 1

Electrochemical parameters of the compounds.

	4-OH-TEMPO	4-NH_2-TEMPO	3a		4a	3b		4b
Epa/V	0.579	0.693	0.582	0.582	0.721	0.579	0.572	0.717
Epc/V	0.511	0.611	0.518	0.538	0.648	0.508	0.517	0.638
(Epa+Epc)/2,$E_{1/2}$/V	0.545	0.652	0.550	0.560	0.685	0.544	0.544	0.678
Epa-Epc (mV)	68	82	64	44	73	71	55	69

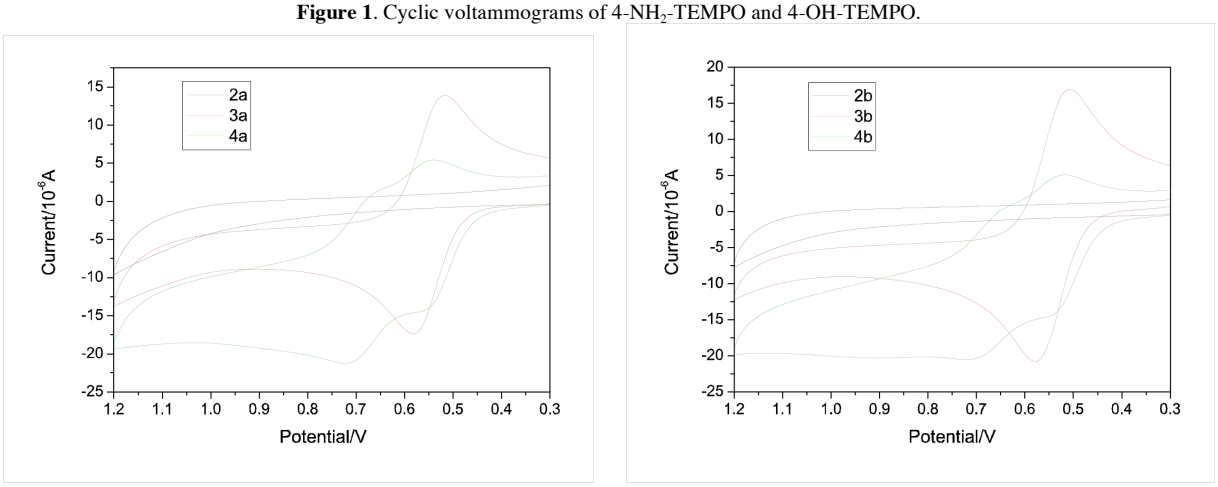
Note: : Epa: anodic peak potential; Epc: cathodic peak potentials; (Epa+Epc)/2 ($E_{1/2}$): the half of the sum of anodic and cathodic peak potentials; Epa-Epc: the peak separation of cyclic voltammogram.

two redox reaction processes within the molecular structures of compounds **4a** and **4b**. Because the cyclic voltammograms of the heterocyclic matrix **2a** and **2b** show no redox signals, and the CV curves of the compounds **3a** and **3b** only give the redox signals of the nitroxyl radical (Figure 2), which suggested that none of those two-electron transfers of the novel compounds **4a** and **4b** could take place on the heterocyclic units. And the C-N bonds reduced from the C=N bonds in the molecular structures of the Schiff bases **3a** and **3b** show charge-transfer character. The new single bond C-N could be one new oxidizable nitrogen center breaking away from the original neutral conjugated systems, and form the two oxidizable nitrogen centers of the compounds **4a** and **4b** together with the nitroxyl radical. Novel compounds **4a** and **4b** exhibit two distinct oxidation stages, respectively, corresponding to the two oxidizable centers (Figure 2 and Table 1).



a b

Figure 1. Cyclic voltammograms of 4-NH_2-TEMPO and 4-OH-TEMPO.



a b

Figure 2. Cyclic voltammograms of the studied compounds.

The total antioxidant activity (TAA). Nitroxides have been recognized as a class of antioxidant compounds for a long time. To evaluate the total antioxidant activity (TAA) of the materials, one of the most commonly used methods is the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$) assay. Here, we used this method to evaluate the TAA of all our studied compounds. The $ABTS^+$ radical has absorption maxima in the near-infrared region at 645, 734 and 815 nm. The antioxidant suppresses the A734 to an extent and on a time scale dependent on the antioxidant activity, and tests were carried out at 6 minutes after initial mixing because the system had been stable at that time [18].

The 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical was generated through reacting ABTS with potassium persulfate in our assay according to the method described by Roberta Re *et al* [18]. A 1 mL $ABTS^+$ solution was added to 1 mL of standard or sample (diluted as required). The decolourisation caused by reduction of the cation through antioxidants from the sample was measured at 734 nm, with a UV–Vis recording spectrophotometer, at 6 minutes after initial

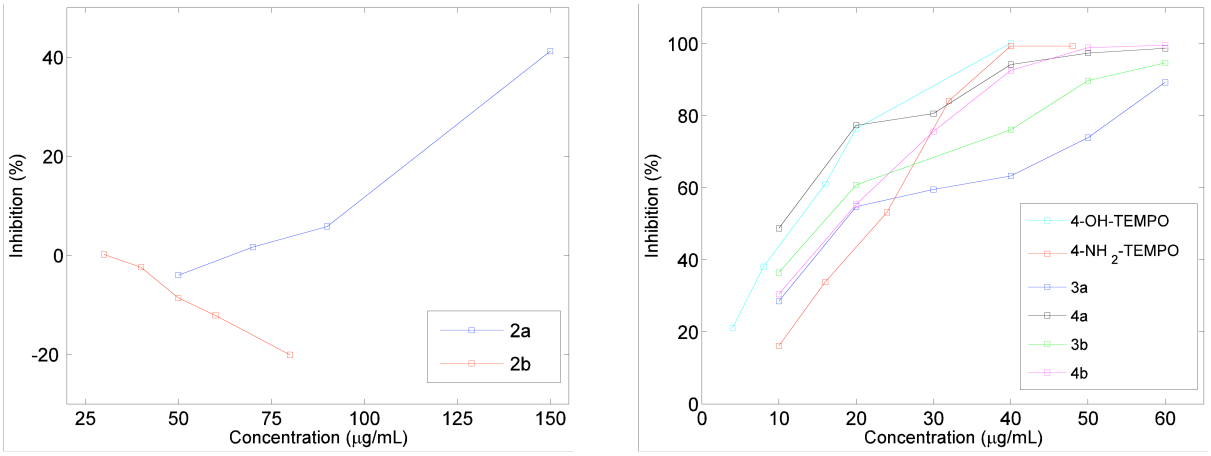


Figure 3 The antioxidant activity of the compounds

mixing. Assays were performed with five or six different concentrations per sample, and each concentration test had 3 replicates. Figure 3 shows the TAA of the studied compounds. The results indicate that the polymethine matrix have a little antioxidative activity and piperdine nitroxides play a main role in the antioxidant action, while the polymethine moieties in the para-position of the nitroxyl group could influence the antioxidative effect of the compounds. All compounds with the nitroxyl radical exhibit high TAA.

Scavenging ROS properties of the compounds. Synthetic nitroxide antioxidants could attenuate oxidative damage and nitroxides are a class of more stable agents than other traditional antioxidants [12]. As shown in Scheme 1, because the structural modification occurs only in the heterocycle-based part, where the S atom has been replaced by an O atom, such a modification only leads to negligible changes in the π-conjugated length and the characterization of the compounds. According to the measurements, the properties of the compounds **a** and **b** series in the cyclic voltammetry and the total antioxidant activity are similar. We selected the compounds **a** series to study the scavenging ROS properties. The scavenging activities for superoxide radicals $O_2^{\bullet-}$ of the compounds were assayed by the reduction of nitroblue tetrazolium (NBT). The superoxide radical was generated in the *N*-methylphenazonium (PMS) and nicotinamide adenine dinucleotide (reduced form, NADH) (PMS-NADH) system and assayed by the reduction of NBT according to the method described by the F. Liu, V.E.C. Ooi and S.T. Chang [19]. And the color reaction of superoxide radicals and NBT was detected at 560 nm with a UV–Vis spectrophotometer. The blank sample with no PMS had no absorbance at 560 nm. The scavenging activities for hydroxyl radical •OH of the compounds were measured by salicylic acid method according to the literatures [20,21]. The hydroxyl radicals were generated in the reaction of H_2O_2-$FeSO_4$ and colored substance produced by reacting •OH with salicylic acid had absorption at 510nm, which measured by a UV–Vis spectrophotometer. Assays were performed with five different concentrations per sample, and each concentration test had 3 replicates, with a UV–Vis recording spectrophotometer (UNICO UV-2102PC spectrophotometer). A linear relationship between samples' concentration and inhibition was established.

According to the assays, a linear relationship between samples' concentration and inhibition could be established with high correlation coefficients, R^2>0.97. Further according to the linear relationship, 50% inhibition concentration (IC50) values of the scavenging ROS activity of the compounds were obtained. Table 2 shows the IC50 values of the studied compounds. Polymethine matrix **2a** shows very weak hydroxyl radicals scavenging

Table 2

The ROS scavenging activity of the compounds (n=5)

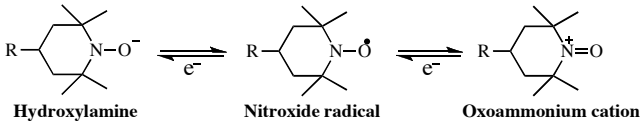
Compound	superoxide radical scavenging activity IC_{50}(mg/ml)	hydroxyl radical scavenging activity IC_{50}(mg/ml)
OH-TEMPO	0.766	2.34
NH_2-TEMPO	1.36	2.24
2a	Precipitate	No hydroxyl radical scavenging activity
3a	Precipitate	2.44
4a	0.271	2.57

Note: 50% inhibition concentration (IC_{50}) values were calculated by linear relationship, R^2>0.97; "Precipitate" means the assay systems were

activity. And the $O_2^{\bullet-}$ scavenging assay systems of the polymethine moiety **2a** and the novel compounds **3a** were unable to be measured with the UV–Vis spectrophotometer because of precipitate formed. The compound 4a exhibits high superoxide radical scavenging activity at low concentration.

The piperidine nitroxide derivatives have an unpaired electron on the piperidine ring, resulting in paramagnetic properties, the antioxidant activity and scavenging ROS properties. The different structural parameters such as ring size and ring substituents would influence the characterization of the compounds [12, 22]. The antioxidative effects and scavenging ROS properties of the stable nitroxides are similar to those of the SOD with a one-electron exchange among their reduced and oxidized states. They can be reduced to hydroxylamines or oxidized to oxoammonium cations (Scheme 2) [23]:

Scheme 2



Hydroxylamine **Nitroxide radical** **Oxoammonium cation**

Therefore, in theory, the nitroxides with superior reversible redox reaction could be improved antioxidant activity, which are validated by the experimental results of the cyclic voltammetry measurements. 4-OH-TEMPO shows reversible redox behavior, while because of the affect of amino group, the redox signal of 4-NH_2-TEMPO varied slightly. According to the CV measurements, half of the sum of anodic and cathodic peak potentials [(Epa+Epc)/2, $E_{1/2}$] is 0.545V and 0.652V (Table 1), respectively. Lower values of $E_{1/2}$ should correspond to higher antioxidant activity because the compounds could be oxidized comparatively easier. The assays of the antioxidant activity and the superoxide radical scavenging activity indicate that the 4-OH-TEMPO is superior to the 4-NH_2-TEMPO. For the novel compound **4a**, the CV measurements show there are two redox reaction processes in their molecular structures, and its superoxide radical scavenging activity precede other studied compounds clearly. The results confirm that the process of the compounds scavenging superoxide radicals is a cycle redox reaction. While the mechanism of the scavenging hydroxyl radicals is different, and the results of the assays show there is no obvious difference among the studied compounds. The nitroxyl radical play a major role in the scavenging hydroxyl radicals.

Conclusion. The scavenging effects of the compounds on superoxide were measured and the result is consistent with that of the ABTS assay. The results confirm that the superoxide radical scavenging of the nitroxides is a redox reaction process and the novel compounds exhibit improved antioxidant activity and the superoxide radical scavenging activity. Furthermore, according to the measurements, the superoxide radical scavenging activity of the nitroxides is related with redox property of the compounds, and the nitroxides can be used as preferable cycle antioxidants.

EXPERIMENTAL

Melting points were determined by an X-5 micro-melting point apparatus and uncorrected. EI-Mass spectra were obtained on a Micromass GCT TOF GC/MS Spectrometer. Elemental analyses were realized with an elementar vario EL III analyser. IR spectra were measured on Nicolet AVATAR360 FT-IR Spectrophotometer. The UV-Visible spectra were recorded on a UNICO UV-2102PC spectrophotometer. EPR measurements were performed at X-band, 9.85 GHz, using a Bruker EMX EPR spectrometer and the measurement of g-factors of the compounds was carried out in contrast to a standard g-marker valued 1.9800 provided by Bruker Company. And the traditional ^{1}H NMR spectra can do very limited help in detecting the final compounds because the spectra of the nitroxide-containing molecules are broad.

Materials. 4-Amino-2,2,6,6-tetramethyl-1-piperidinyloxy (4-NH_2-TEMPO, 97%) radical used as building blocks in this study and 4-hydroxyl-2,2,6,6-tetramethyl-1-piperidinyloxy (4-OH-TEMPO, 99.9%) radical are commercially available and were bought from Sigma Comp. 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) nitroblue tetrazolium (NBT), *N*-methyl-phenazonium (PMS) and nicotinamide adenine dinucleo-tide (reduced form, NADH) were bought from Sinopharm Chemical Reagent Co.Ltd. and all the reagents used in the experiments were of AR.

Synthesis of *N*-[2-(*p*-vinylbenzylidene)benzothiazole] 4-amino-2,2,6,6-tetramethyl-1-piperidinyloxy (3a) and *N*-[2-(*p*-vinylbenzylidene)benzoxazole] 4-amino-2,2,6,6-tetramethyl-1-piperidinyloxy (3b). An ethyl acetate solution (40 ml) of the intermediate **2a** (0.53 g, 0.002 mol) and 4-amino-TEMPO (0.34 g, 0.002 mol) with a small amount of acetic acid was heated to reflux and the reaction was continued for 2 h. The reaction mixture was concentrated *in vacuo* to give a crude solid that was recrystallized from the solvent of ethyl acetate and *n*-hexane to give the corresponding product as orange powdery solid (0.72 g, 86%): mp 182-184°C.

Using the alcohol solution, under similar conditions also gave the **3b** as orange powdery in 81% yield: mp 163-164°C **3a**.EI-MS *m*/*z*: [M^+]=418. IR: γ_{max}^{KBr} (cm^{-1}) 1640 (C=N), 1601 (C=C), 1375 (N-O). UV-Vis. λ^{max} (nm) (in CH_3OH): 231, 354. Anal. Calcd. for $C_{25}H_{28}N_3O\cdot S$: C, 71.74; H, 6.74; N, 10.04. Found: C, 71.70; H, 6.95; N, 9.83. EPR: α_N in CH_2Cl_2:15.64G; g value=2.0075

3b EI-MS *m*/*z*: [M^+]=402.2. IR: γ_{max}^{KBr} (cm^{-1}) 1640 (C=N), 1602 (C=C), 1375 (N-O). UV-Vis. λ^{max} (nm) (in CH_3OH): 233, 340. Anal. Calcd. for $C_{25}H_{28}N_3O_2$: C, 74.60; H, 7.01; N, 10.44. Found: C, 74.35; H, 7.12; N, 10.28. EPR: α_N in CH_2Cl_2:15.64G; g value=2.0071

Synthesis of *N*-[2-(*p*-vinylbenzyl)benzoxazole]4-amino-2,2,6,6-tetramethyl-1-piperidinyloxy (4a) and *N*-[2-(*p*-vinyl-

benzyl)benzoxazole]4-amino-2,2,6,6-tetramethyl-1-piperidinyloxy (4b). To the stirred solution of the **3a** (0.84 g, 0.002 mol) in alcohol (60 mL) was added $NaBH_4$ (0.23 g, 0.006 mol) divided three times. The reaction mixture was stirred at room temperature over night, and then it was concentrated and extracted with dichloromethane. The CH_2Cl_2 layer was dried over anhydrous $MgSO_4$ and then concentrated *in vacuo* to give a solid that was purified by column chromatography on silica gel by using the solvent system of petroleum ether/ethyl acetate/acetone and recrystallized from the mixed solvent of *n*-hexane/dichloromethane. The product **4a** was obtained as orange crystals (0.41g, 49%): mp 129-131°C

Under similar conditions also gave the **4b** as orange crystals in 34% yield: mp 119-120°C

4a EI-MS *m/z*: $[M^+]$=420.2. IR: γ_{max} KBr (cm^{-1}) 3318 (N-H), 1621(C=N), 1605 (C=C), 1375 (N-O). UV-Vis. λ_{max} (nm) (in CH_3OH): 232, 340. Anal. Calcd. for $C_{25}H_{30}N_3O\cdot S$: C,71.39; H, 7.19; N, 9.99. Found: C, 71.39; H, 7.37; N, 9.95. EPR: α_N in CH_2Cl_2: 15.64G; g value=2.0074

4b EI-MS *m/z*: $[M^+]$=404.2. IR: γ_{max} KBr (cm^{-1}) 3305(N-H),1642(C=N), 1605 (C=C), 1375 (N-O). UV-Vis. λ_{max} (nm) (in CH_3OH): 232, 326. Anal. Calcd. for $C_{25}H_{30}N_3O_2$: C, 74.23; H, 7.47; N, 10.39. Found: C, 74.21; H, 7.65; N, 10.13. EPR: α_N in CH_2Cl_2: 15.64G; g value=2.0072

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