Available online at www.sciencedirect.com

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 14 (2006) 5711–5720

Bioorganic &
Medicinal
Chemistry

Novel 2-substituted nitronyl nitroxides as free radical scavengers: Synthesis, biological evaluation and structure–activity relationship

Yihui Wu,[b] Lanrong Bi,[a] Wei Bi,[c] Zeng Li,[b] Ming Zhao,[b,*] Chao Wang,[b]
Jingfang Ju[d,*] and Shiqi Peng[a,*]

[a] *College of Pharmaceutical Sciences, Capital University of Medical Sciences, Beijing 100054, PR China*
[b] *College of Pharmaceutical Sciences, Peking University, Beijing 100083, PR China*
[c] *Department of Surgery, Second Affiliated Hospital of Hebei Medical University, Shijiazhuang 050000, PR China*
[d] *Mitchell Cancer Institute, USA, 307 N. University Blvd, Mobile, AL 36688-0002, USA*

Received 22 March 2006; revised 30 March 2006; accepted 6 April 2006
Available online 2 May 2006

Abstract—To develop more potent small molecules with enhanced free radical scavenger properties, we designed and synthesized a series of nitronyl nitroxide derivatives **4a–h**. A lead compound **4f** was discovered based on Ach-induced vascorelaxation assay. Further chemical modification based on this scaffold provided a new series of 2-substituted phenylnitronyl nitroxide derivatives **6a–s**. The newly synthesized compounds **6a–s** possess improved radical scavenger's activity based on PC12 cell survival assay. Compounds **6g**,**n**,**o**, and **s** are some of the most potent compounds in terms of NO, H_2O_2, and OH scavenging ability. 2-Substitued phenylnitronyl nitroxides had a higher radical scavenging activity with the electron-donating group (EDG). In contrast, the introduction of electron-withdrawing group (EWG) to the aromatic ring led to a dramatic decrease in its radical scavenging activity. These results suggest that the electron-donating group (EDG) of the aromatic ring may be an important factor influencing the radical scavenging behavior of these compounds, and the potency of free radical scavenging activity largely depended on the position and electronic properties of the phenyl ring substituents. The enhanced radical scavenging capacities of the novel 2-substituted nitronyl nitroxides may be potential drug leads against the deleterious action of ROS (reactive oxygen species)/RNS (reactive nitrogen species).

1. Introduction

Free radicals play important roles in many physiological and pathological conditions.[1] In general, the generation and scavenging of oxygen free radicals is balanced and any imbalance or excessive amounts of active radicals may contribute to disease development. It has been found that free radical reactions can produce deleterious modifications in membranes, proteins, enzymes, and DNA,[2] increasing the risk of diseases such as cancer,[3] Alzheimer's,[4] Parkinson's,[5] angiocardiopathy,[6] arthritis,[7] asthma,[8] diabetes,[9] and degenerative eye disease.[10] Therefore, it is important to find effective scavengers of free radicals for prevention and treatment of such disorders.

Nitronyl nitroxides, synthesized more than 30 years ago,[11] have received considerable attention recently because of their capability to trap NO.[12,13] More recently, nitronyl nitroxides were found to react with free radicals such as OH, H_2O_2, and O_2, protecting endothelial cells from the attack of free radicals.[14,15]

To develop small molecules with enhanced free radical scavenger properties, a series of new nitronyl nitroxide derivatives were designed and synthesized. In this study, free radical scavenger properties of the newly synthesized compounds were characterized to elucidate the relationship among their activity, chemical structure, and physicochemical parameters.

Keywords: Nitronyl nitroxide derivatives; Free radical scavenger; Structure–activity relationship.

* Corresponding authors. Tel.: +1 251 460 7393; fax: +1 251 460 6994 (J.J.); tel.: +86 10 8280 2482; fax: +86 10 8280 2482 (M.Z.); tel.: +86 10 8391 1528; fax: +86 10 8391 1528 (S.P.); e-mail addresses: jju@usouthal.edu; sqpeng@mail.bjum.edu.cn

0968-0896/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2006.04.016

2. Results and discussion

2.1. Synthesis of 2-substituted nitronyl nitroxides

The nitronyl nitroxide derivatives were synthesized according to Ullman's procedure with minor modification as shown in Scheme 1.[16] Synthesis was initiated with dinitro compound **1**, followed by the reduction with zinc in an ammonium chloride (Zn/NH_4Cl) buffered solution to yield the key intermediate bis(hydroxyamine) compound **2**, which was subsequently subjected to condensation with various aldehydes to generate the corresponding tetramethylimidazoline derivatives **3a**–**h** and **5a**–**p** with 51–85% yield, respectively. The final oxidation by PdO_2 gave the desired nitronyl nitroxide derivatives **4a**–**h** and **6a**–**p** with 38–90% yield, respectively. While the synthesis of **6s** and **q** was slightly different from the above-mentioned procedure. Treatment of **4g** with $BrCH_2COOC_2H_5$ followed by hydrolysis in the presence of NaOH (2 mol/L) provides **6q** in 89% yield. Similarly, the reaction of **4g** and $BrCH_2CH_2Br$ afforded **6s** in 64% yields.

The synthesis of nitronyl nitroxides relies almost exclusively on the condensation of 2,3-bis(hydroxyamino)-2,3-dimethylbutane with an aldehyde and oxidation of the condensation product.[16] The key intermediate **2** was prepared using the classical reduction process with zinc in ammonium chloride buffered solution of the dinitro analogue. Utilizing THF instead of ethanol according to the modified Lamchem–Mittag procedure can avoid the formation of undesired nitroxides and favor the reduction.[17] In addition, the best ratio of Zn and NH_4Cl was 1:8 to yield best results. This was perhaps due to the use of 8 equiv NH_4Cl resulting in a well-defined Zn complex, $[Zn(NH_3)_2Cl_2]$, which favors the reduction proceed smoothly and in good yield. The formation of the corresponding nitroxides requires only mild oxidants to introduce the free radical-generating moiety. Thus, PdO_2 was utilized to insure completion of the last oxidation step (Scheme 2) .

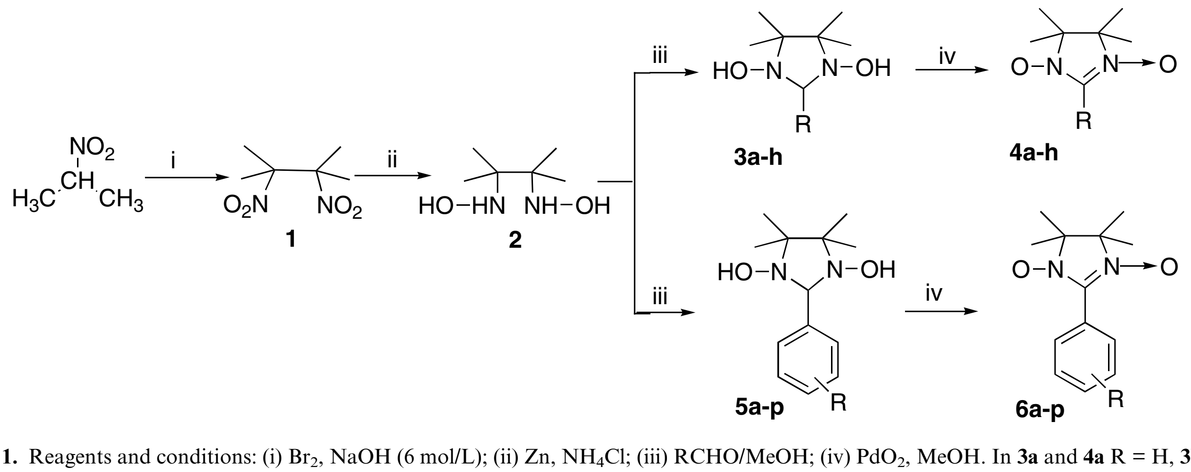
2.2. NO scavenging activity determined using rat aortic strip

A variety of 2-substituted nitronyl nitroxides **4a**–**h** were prepared and their NO scavenging activities were evaluated in the acetylcholine (Ach)-evoked, endothelium-mediated relaxation assay.[19]

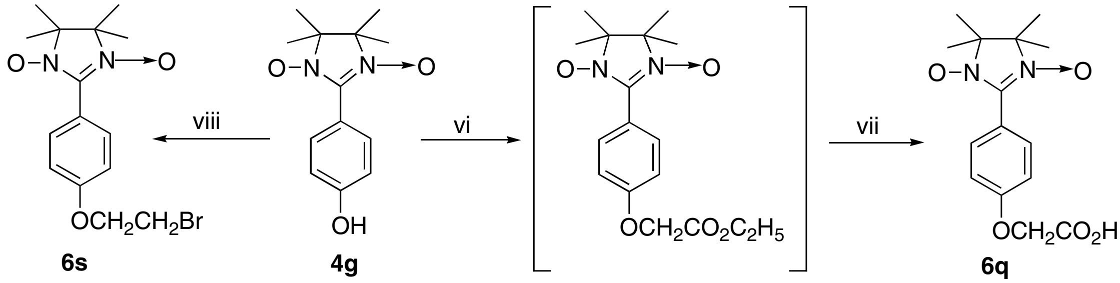
The endothelium controls the tone of the underlying vascular smooth muscle through the production of vasodilator mediators.[20–22] In this experimental model, Ach acts on the endothelium to release nitric oxide (NO), a potent vasodilating mediator. The decreased relaxation response in rat aortic strip could be attributed to a reduction in NO (synthesis by the endothelium), therefore, the present study was undertaken to assess the NO scavenging capability of these compounds.

The parental nitronyl nitroxide **4a** was found to be a weak inhibitor of Ach-induced vasorelaxation. Consequently, nitronyl nitroxide ring was modified with a variety of substituents. The effect of different substitutions was then examined in Ach-induced vasorelaxtion assay as shown in Table 1. Introduction of the aromatic ring at the 2-position led to a significantly improved NO scavenging activity, suggesting that in the 2-substituted phenylnitronyl nitroxides series, the scaffold was optimized compared to other substitutions at the 2-position of nitronyl nitroxides.

Among these compounds tested, Ach-induced relaxation was significantly reversed by compounds **4f**–**h**. At the concentration of 10 μmol, compound **4f** showed good inhibition ability with 62.7 ± 5.5%, whereas compounds **4g** and **h** displayed even higher inhibition activity than **4f** in this assay, and their inhibition percentage of Ach-induced vasorelaxation was 73.1 ± 5.9% and 71.3 ± 8.8%, respectively. Clearly, compounds **4g** and **h** were more potent based on the inhibition assay. We speculate that the NO scavenging activity of compounds **4f**–**h** led to attenuation of the NO concentration in vitro,



Scheme 1. Reagents and conditions: (i) Br_2, NaOH (6 mol/L); (ii) Zn, NH_4Cl; (iii) RCHO/MeOH; (iv) PdO_2, MeOH. In **3a** and **4a** R = H, **3b** and **4b** R = $–CH_2Cl$, **3c** and **4c** R = –COOH, **3d** and **4d** R = fur-2-yl, **3e** and **4e** R = pyrid-3-yl, **3f** and **4f** R = phenyl, **3g** and **4g** R = 4′-hydroxylphenyl, **3h** and **4h** R = 4-nitrophenyl; in **5a** and **6a** R = 4′-Br, **5b** and **6b** R = 4′-Cl, **5c** and **6c** R = 4′-CH_3, **5d** and **6d** R = 4′-OCH_3, **5e** and **6e** R = 3′-NO_2, **5f** and **6f** R = 3′-OH, **5g** and **6g** R = 3′,4′-dimethoxyl, **5h** and **6h** R = 4′-OH–3′-OCH_3, **5i** and **6i** R = 2′,4′-dichloro, **5j** and **6j** R = 3′,4′-methylenedioxy, **5k** and **6k** R = 2′-F, **5l** and **6l** R = 2′-NO_2, **5m** and **6m** R = 3′-OH-4′-OCH_3, **5n** and **6n** R = 4′-$N(CH_3)_2$, **5o** and **6o** R = 2′,4′-dimethoxyl, **5p** and **6p** R = 3′-OH.



Scheme 2. Reagents and conditions: (vi) $BrCH_2COOC_2H_5$ and $NaOC_2H_5$; (vii) NaOH/MeOH; (viii) $BrCH_2CH_2Br$, $NaOC_2H_5$.

Table 1. Inhibition of ACh-induced vasorelaxation of **4a**–**h**

Compound	Inhibition percentage ($\overline{X}$ ± SD%)		
	100 µmol[b]	10 µmol[c]	1 µmol
NS		1.63 ± 1.45	
4a	31.2 ± 2.9[a]	8.6 ± 3.7[a]	1.7 ± 1.5
4b	15.0 ± 2.4[a]	7.7 ± 9.8	1.6 ± 1.5
4c	14.3 ± 6.5[a]	35.0 ± 17.8[a]	1.7 ± 1.6
4d	44.4 ± 5.9[a,b]	20.2 ± 4.2[a]	1.6 ± 1.5
4e	48.6 ± 2.9[a,b]	16.6 ± 2.8[a]	1.7 ± 1.7
4f	81.9 ± 8.9[a,c,d]	62.7 ± 5.5[a,c,d]	9.8 ± 7.8
4g	99.2 ± 1.4[a,c,d]	73.1 ± 5.9[a,c,d]	26.1 ± 7.0[a]
4h	98.0 ± 2.7[a,c,d]	71.3 ± 8.8[a,c,d]	14.5 ± 14.5

$n = 6$; NS, vehicle.

[a] Compare to NS, $P < 0.001$.

[b] Compare to **4a**–**c**, $P < 0.01$.

[c] Compare to **4a**–**c**, $P < 0.001$.

[d] Compare to **4d** and **e**, $P < 0.001$.

Scheme 3. ESR spectrum of **6g** at 10^{-5} mol/L in phosphate buffer (pH 7.4).

in return, the effect was also attributed to the inhibition of Ach-induced vasorelaxation.

2.3. Stability of compounds 4a–h in the aqueous solution

To test the stability of the compounds **4a**–**h** at room temperature, their pH value of aqueous solution (10^{-5} mol/L) was adjusted to 2.0, 7.4 and 11.5. TLC ($CHCl_3/CH_3OH$, 20:1) analysis indicated that, compounds **4a**–**c** started to decompose after 2 h, compounds **4d** and **e** started to decompose after 15 h, whereas no decomposition was observed for compounds **4f**–**h** after 200 h. Based on TLC analysis, ESR spectra, and the results of the rat aortic strip assay, compound **4f** was selected as the lead compound for further chemical modifications.

2.4. Electron spin resonance (ESR) spectra of 4f–h and 6a–s

ESR spectrometry is a widely accepted method for free radical detections and has been successfully used to study radical scavenging capacities of antioxidants.[18] The unpaired electron in a free radical results in a special ESR absorbance. This absorbance disappears when the free radical obtains an electron from antioxidants. The ESR spectra of **4f**–**h** and **6a**–**s** were obtained at two different concentrations of 10^{-7} and 10^{-5} mol/L, in water and phosphate buffer (pH 7.4), respectively. They all exhibited free radical characteristics with spectra showing a five-line pattern at 1:2:3:2:1 ratio, and there was no difference among the compounds tested. The ESR spectrum of **6g** in phosphate buffer (pH 7.4) is presented as an example in Scheme 3. The characteristic spectra resulting from the interaction of a free electron are the two nitrogens in the nitronyl nitroxides.

2.5. Scavenging activities determined by cell survival assay in PC12 cells

The chemical modification of lead compound **4f**, focusing on the benzene ring substituents, was carried out to further improve the free scavenging ability. A series of new 2-substituted phenyl nitronyl nitroxides derivatives were synthesized. The free radical scavenging properties of these **4f** derivatives were evaluated to elucidate structure–activity relationships.

Various free radicals are formed during normal cell metabolism. Therefore, the newly synthesized compounds can be analyzed for their functional scavenging capacity against the different active free radicals. The free radical scavenging activities of these compounds against NO, $H_2O_2^{\bullet}$, and $^{\bullet}OH$ were evaluated by cell survival assay in PC12 cells using a published method with minor modifications.[23] The EC_{50} (µM) values are summarized in Table 2.

The derivatives exhibited varying degrees of scavenging capacity on different active radicals. The scavenging capacity (EC_{50}) among all of the tested compounds ranged from 8.32 ± 0.25 to 114.82 ± 4.35 µM for NO, from 25.29 ± 0.72 to 107.86 ± 4.37 µM for H_2O_2, from 27.73 ± 1.32 to 167.06 ± 7.91 µM for $^{\bullet}OH$. Among them, compounds **6g**,**n**,**o**, and **s** exhibited the highest scavenging capacity of active radical species. For example, compound **6g** was the most potent compound in inhibiting NO free radical activity with EC_{50} 8.32 ± 0.25 µM,

Table 2. The EC$_{50}$ of **4f–h**, **6a–s** as NO, H$_2$O$_2$, and $^{\bullet}$OH scavenger

Compound:	**4f**	**4g**	**4h**	**6a**	**6b**	**6c**	**6d**
EC$_{50}$/NO	67.61 ± 4.22	39.81 ± 1.52	114.82 ± 4.35	46.77 ± 2.38	63.10 ± 3.57	33.11 ± 1.53	30.90 ± 1.28
EC$_{50}$/H$_2$O$_2$	51.63 ± 2.41	48.18 ± 2.44	107.86 ± 4.37	55.32 ± 2.05	58.88 ± 2.73	38.27 ± 1.91	42.94 ± 1.78
EC$_{50}$/$^{\bullet}$OH	66.51 ± 2.33	56.03 ± 2.47	167.06 ± 7.91	60.66 ± 2.97	74.62 ± 2.56	64.99 ± 3.15	59.28 ± 2.54
Compound:	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**
EC$_{50}$/NO	87.10 ± 2.81	61.66 ± 2.42	8.32 ± 0.25	25.70 ± 1.43	42.76 ± 1.97	19.50 ± 2.24	44.67 ± 1.75
EC$_{50}$/H$_2$O$_2$	62.07 ± 2.95	57.93 ± 2.86	30.40 ± 1.42	32.57 ± 1.63	64.99 ± 3.15	29.71 ± 1.46	76.36 ± 3.82
EC$_{50}$/$^{\bullet}$OH	63.52 ± 3.02	69.64 ± 2.88	29.71 ± 1.24	42.94 ± 2.03	78.14 ± 3.85	47.08 ± 2.11	85.68 ± 3.24
Compound:	**6l**	**6m**	**6n**	**6o**	**6p**	**6q**	**6s**
EC$_{50}$/NO	47.86 ± 2.10	27.54 ± 1.17	9.12 ± 0.14	9.33 ± 0.31	44.67 ± 0.82	91.20 ± 2.23	9.55 ± 0.24
EC$_{50}$/H$_2$O$_2$	60.66 ± 3.03	40.08 ± 2.05	29.03 ± 1.48	25.29 ± 0.72	52.83 ± 3.26	74.62 ± 4.12	27.73 ± 2.75
EC$_{50}$/$^{\bullet}$OH	74.62 ± 3.52	54.06 ± 2.48	31.11 ± 1.47	27.73 ± 1.32	55.32 ± 2.77	100.67 ± 4.56	39.16 ± 1.85

and the scavenging capacity of H$_2$O$_2$ and $^{\bullet}$OH was also quite effective with EC$_{50}$ 30.40 ± 1.42, 29.71 ± 1.24 μM, respectively.

We initially examined the effect of halogen of the phenyl ring on the radical scavenging activity. Introduction of different halogen at the 2- or 4-position of the phenyl ring resulted in improved potency compared to unmodified **4f**. For example, 4-bromine derivative **6a** (EC$_{50}$/NO: 46.77 ± 2.38 μM) or 2′-fluorine derivative **6k** (EC$_{50}$/NO: 44.67 ± 1.75 μM) exhibited better NO scavenging activity than that of **4f** (EC$_{50}$/NO: 67.61 ± 4.22 μM). It appeared that either a bromine or fluorine atom at the 2- or 4-position on the phenyl ring was beneficial for the enhanced NO scavenging activity. In the case of substitution with a chlorine atom, that is, 4′-Cl derivative **6b** (EC$_{50}$/NO: 63.10 ± 3.57 μM) showed less improved activity compared to 4′-Br **6a** or 2′-F derivative **6k**. Interestingly, the effect of dichloro substitution on the radical scavenging activity was quite different from that of single-chlorine derivative. 2′,4′-Dichloro derivative **6i** (EC$_{50}$/NO: 42.76 ± 1.97 μM) exhibited higher NO scavenging potency than that of 4′-Cl derivative **6b**. Furthermore, it was noticed that the more distant bromine (OCH$_2$CH$_2$Br) group on the phenyl ring furnishing compound **6s** yield remarkable NO scavenging activity (EC$_{50}$/NO: 9.55 ± 0.24 μM). The enhanced NO radical scavenging activity of bromoethoxy functionality in **6s** on the phenyl ring remained to be determined.

Next, we continued to investigate the effect of the substituents of the phenyl ring on radical scavenging capability in P12 cell viability assay. As shown in Table 2, lead compound **4f** showed moderate radical inhibitory potency (EC$_{50}$/NO: 67.61 ± 4.22; EC$_{50}$/H$_2$O$_2$: 51.63 ± 2.41; and EC$_{50}$/$^{\bullet}$OH: 66.51 ± 2.33 μM). When an electron-withdrawing nitro group was introduced at the 4-position of the phenyl ring, 4′-nitro derivative, **4h**, a notable decrease of activity was observed (EC$_{50}$/NO: 114.82 ± 4.35; EC$_{50}$/H$_2$O$_2$: 107.86 ± 4.37; and EC$_{50}$/$^{\bullet}$OH: 167.06 ± 7.91 μM). Similarly, when an electron-withdrawing carboxymethyl group was introduced at the 4-position of the phenyl ring of 4-carboxymethyl derivative, **6q**, a marked decrease of potency was observed (EC$_{50}$/NO: 91.20 ± 2.23; EC$_{50}$/H$_2$O$_2$: 74.62 ± 4.12; and EC$_{50}$/$^{\bullet}$OH: 100.67 ± 4.56). In contrast, introduction of a small, slightly electron-donating methyl group at the 4-position of the phenyl ring in **6c** resulted in 2- to 3-fold enhancement of radical scavenging activity (EC$_{50}$/NO: 33.11 ± 1.53; EC$_{50}$/H$_2$O$_2$: 38.27 ± 1.91; and EC$_{50}$/$^{\bullet}$OH: 64.99 ± 3.15).

Interestingly, although introduction of a nitro group at the 4-position of the phenyl ring resulted in a notable decrease in NO free radical scavenging potency, when a nitro group was introduced at the 2-position of the phenyl ring in **6l**, the NO scavenging activity was greatly improved (EC$_{50}$/NO: 47.86 ± 2.10 μM). Based on this observation, we speculated that the position of the nitro group in the phenyl ring might be crucial for its NO inhibitory activity. Furthermore, we assumed that, the nonplanar torsional conformation of the nitro group in relation to the phenyl ring had an effect on radical stabilization.

Finally, we focused on elucidating the impact of the electron-donating group of the phenyl ring. Replacement of the methyl group in compound **6c** with a methoxy group led to compound **6d** (EC$_{50}$/NO: 30.90 ± 1.28; EC$_{50}$/H$_2$O$_2$: 42.94 ± 1.78; and EC$_{50}$/$^{\bullet}$OH: 59.28 ± 2.54 μM), and its free radical scavenging potency was effective and comparable to that of **6c**. Substitution with hydroxyl group at the 3-position of the phenyl ring led to **6f** (EC$_{50}$/NO: 61.66 ± 2.42; EC$_{50}$/H$_2$O$_2$: 57.93 ± 2.86; and EC$_{50}$/$^{\bullet}$OH: 69.64 ± 2.88 μM) with moderate radical scavenging activity. While the electron-donating hydroxyl group was introduced at the 2-position of the phenyl ring in **6p** (EC$_{50}$/NO: 44.67 ± 0.82; EC$_{50}$/H$_2$O$_2$: 52.83 ± 3.26; and EC$_{50}$/$^{\bullet}$OH: 55.32 ± 2.77 μM) leading to a higher radical scavenging activity compared to 3′-OH derivative **6f**, suggesting the hydroxyl group is favorable for radical scavenging activity specially at the 2-position of the phenyl ring. This may be due to the *ortho*-hydroxyl group of the phenyl ring increasing the stability of the radical form and participating in electron delocalization. In addition, other electron-donating group such as –N(CH$_3$)$_2$ also plays an important role on the enhanced radical scavenging activity of nitronyl nitroxide derivative as shown in compound **6n** with highly potent radical scavenging activity (EC$_{50}$/NO: 9.12 ± 0.14; EC$_{50}$/H$_2$O$_2$: 29.03 ± 1.48; and EC$_{50}$/$^{\bullet}$OH: 31.11 ± 1.47 μM).

Furthermore, it was revealed that the combined presence of hydroxyl and methoxy group on the phenyl ring produced substances with higher activity as shown in 3′-OCH$_3$–4′-OH derivative **6h**, 3′-OH–4′-OCH$_3$ derivative

6m with good radical scavenging profiles. (**6h**: EC_{50}/NO: 25.70 ± 1.43; EC_{50}/H_2O_2: 32.57 ± 1.63; and $EC_{50}/^{\cdot}OH$: 42.94 ± 2.03 μM; **6m**: EC_{50}/NO: 27.54 ± 1.17; EC_{50}/H_2O_2: 40.08 ± 2.05; and $EC_{50}/^{\cdot}OH$: 54.06 ± 2.48 μM). This increased activity due to the combined existence of the OCH_3 and OH most likely could be attributed to its positive effect on the stabilization of the phenoxy free radical and hence increases the free radical scavenging effect.

More importantly, compounds **6g** and **o** have an identical number of the methoxy groups, with 3′,4′- and 2′,4′-dimethoxyl groups, respectively, on the phenyl ring. Compared to lead compound **4f**, they exhibited most potent radical scavenging capacity with 8-fold increase in NO scavenging activity (**6g** EC_{50}/NO: 8.32 ± 0.25; EC_{50}/H_2O_2: 30.40 ± 1.42; and $EC_{50}/^{\cdot}OH$: 29.71 ± 1.24 μM; **6o** EC_{50}/NO: 9.33 ± 0.31; EC_{50}/H_2O_2: 25.29 ± 0.72; and $EC_{50}/^{\cdot}OH$: 27.73 ± 1.32 μM). It was important to notice that the dimethoxy groups on the phenyl ring significantly enhanced the free radical scavenging activity of the nitronyl nitroxide derivatives.

The radical scavenging capacity of the 2-substituted phenylnitronyl nitroxide derivatives depends on several factors such as the electron-donating and electron-withdrawing substituents on the phenyl ring, the number of methoxy groups or the chemical stability. It seemed that the structural feature responsible for the highest free radical scavenging activity is the dimethoxy moiety on the phenyl ring. In addition, the involvement of other electron-donating groups ($–N(CH_3)_2$ and $–OCH_2CH_2Br$) may also play crucial roles on the radical scavenging activity.

Clearly, the presence of electron-donating hydroxyl or methoxy groups attached to the aromatic ring is favorable for the free radical scavenging activity. This observation was consistent with the literature reported results.[24,25] Currently, it is well recognized that phenolic compounds in foods possess several interesting biological and chemical properties such as antioxidant activity and the ability to scavenge reactive oxygen species. As a consequence, they may prevent various diseases associated with oxidative stress, such as cancers, cardiovascular diseases, and inflammation.[25,26] The newly synthesized compounds containing phenolic substituents displayed good radical scavenger's activities and that might be of further significance for the development of synthetic antioxidants. Further in vivo studies will be needed to confirm these findings.

3. Conclusion

In this study, screening for new small molecule inhibitors of Ach-induced vascorelaxation led to the discovery of the lead compound **4f**. Chemical modification on this scaffold resulted in a series of 2-substituted phenylnitronyl nitroxide derivatives **6a–s**. Our results indicated the newly synthesized compounds **6a–s** exhibit good to moderate radical scavenger's activity as shown in PC12 cell survival assay. The free radical scavenging activity of the compounds increased significantly with the number of the methoxy groups and also with the presence of other electron-donating groups in the phenyl ring. Compounds **6g**,**n**,**o**, and **s** are the most active compounds in the PC12 radical scavenging assay. More generally the results suggest that radical scavenging ability from 2-substituted phenylnitronyl nitroxide should be strongly dependent on the nature and the position of the substituents at the aromatic ring. The results suggested that 2-substituted phenylnitronyl nitroxides were potential useful free radical scavenger agents and further in vivo evaluations will be necessary.

4. Experimental

The purity of all the compounds was confirmed by TLC (Merck silica gel plates of type 60 F_{254}, 0.25 mm layer thickness) and HPLC (waters, C_{18} column 3.9 × 150 mm). Melting points were measured on a XT5 hot stage microscope (Beijing keyi electro-optic factory). The infrared spectra were recorded with a Perkin-Elmer 983 instrument. The EI-MS was determined by Trace MS System instrument (American Thermo Finnigan company). The ^1H NMR was determined by Varian INOVA-300 MHz spectrometer. The ESR spectra were obtained from 10^{-5} mol/L phosphate-buffered saline, using a BRUKER 300-E spectrometer. The conditions of measurements are as follows: center field: 3440 G, sweep width: 100 G, sweep time: 60 s, modulation amplitude: 1.1 G, time constant: 8.2×10^{-2} s, modulation frequency: 100 kHz, microwave frequency: 9.68 GHz, and microwave power: 20 MW.

4.1. 2,3-Dimethyl-2,3-dinitrobutane (1)

At −5 °C, 10 mL (0.19 mol) Br_2 were added dropwise within 1 h to the solution of 34.5 g (0.39 mol) of 2-nitropropane in 65 mL (6.0 mol/L) aqueous NaOH. Ethanol (128 mL) was added into the solution via stirring. The reaction mixture was stirred at 84 °C for 3 h. The hot reaction mixture was transferred into 400 mL of ice water. The formed colorless crystals were collected by filtration to yield 25 g (73%) of the title compound, mp 110–112 °C.

4.2. 2,3-Bis(hydroxylamino)-2,3-dimethylbutane (2)

2,3-Dimethyl-2,3-dinitrobutane (17.6 g, 0.1 mol) was dissolved in a mixture of THF (300 mL) and water (50 mL). Zn powder (27 g) was added all at once to this solution cooled to 8–10 °C in an ice bath. A solution of NH_4Cl (43 g, 08 mol) in H_2O (150 mL) was added dropwise to this slurry within 2 h, with continued stirring for 1 h at 10 °C, and the flask was stored in a fridge (4–6 °C) for 16 h. The slurry was filtered, and the precipitate was carefully washed with THF (4× 100 mL). The precipitate was then dried by three washings with diethyl ether and carefully collected (59 g). The solution was evaporated under vacuum until THF ceased to distill off. Then the solution was protected from air, and sodium carbonate (50 g) and sodium chloride (30 g) were added with cooling. Continuous extraction with chloroform (400 mL) was performed over 18 h. A white powder was obtained (9.4 g, 63% yield), mp 182 °C.

4.3. General procedure for the preparation of 3a–h

A solution of 296 mg (2 mmol) of 2,3-bis(hydroxylamino)-2,3-dimethylbutane and 2 mmol of various aldehyde in 6 mL of methanol was stirred at room temperature for 1 h. Compounds **3a**–**h** were obtained by filtration. The filtrate was evaporated under reduced pressure to obtain the second crop of the title compound. Compounds **3a**–**h** were directly used for the next reaction without further purification.

4.3.1. 1,3-Dihydroxy-4,4,5,5-tetramethylimidazolidine (3a). Yield: 53%. IR (KBr): 3338, 1600, 1480, 1384 cm^{-1}; ^{1}H NMR (DMSO-d_6) $\delta = 0.966$ (s, 12H), 3.873 (s, 2H), 7.644 (s, 2H); ESI-MS (*m/e*) = 160 [M]$^+$; Anal. Calcd for $C_7H_{16}N_2O_2$: C, 52.48; H, 10.07; N, 17.48. Found: C, 52.29; H, 10.22; N, 17.68.

4.3.2. 1,3-Dihydroxy-2-chloromethyl-4,4,5,5-tetramethylimidazolidine (3b). Yield: 69%. IR (KBr): 3460, 1460, 1381 cm^{-1}; ^{1}H NMR (DMSO-d_6) $\delta = 0.951–0.985$ (s, 12H), 3.82 (s, 1H), 7.998 (s, 2H), 3.609 (s, 2H); ESI-MS (*m/e*) = 208 [M]$^+$; Anal. Calcd for $C_8H_{17}N_2O_2Cl$: C, 46.04; H, 8.21; N, 13.42. Found: C, 46.29; H, 8.25; N, 13.38.

4.3.3. 1,3-Dihydroxy-2-carboxyl-4,4,5,5-tetramethylimidazolidine (3c). Yield: 83%. Mp: 103–105 °C. IR (KBr): 3340, 2701–2402, 1460, 1381 cm^{-1}; ^{1}H NMR (DMSO-d_6) $\delta = 0.99$ (s, 12H), 4.064 (s, 1H), 8.05 (s, 2H), 12.248 (s, 1H); ESI-MS (*m/e*) = 204 [M]$^+$; Anal. Calcd for $C_8H_{16}N_2O_4$: C, 47.05; H, 7.90; N, 13.72. Found: C, 47.19; H, 7.85; N, 13.72.

4.3.4. 1,3-Dihydroxy-2-(fur-2-yl)-4,4,5,5-tetramethylimidazolidine (3d). Yield: 73%. Mp: 93–95 °C. IR (KBr): 3440, 1601, 1503, 1450, 1386 cm^{-1}; NMR (DMSO): $\delta = 1.04$ (s, 12H), 4.59 (s, 1H), 6.37 (d, $J = 6.5$ Hz, 1H), 6.44 (d, $J = 6.3$ Hz, 1H), 7.58 (t, $J = 6.6$ Hz, 1H); ESI-MS (*m/e*) = 226 [M]$^+$; Anal. Calcd for $C_{11}H_{18}N_2O_3$: C, 58.39; H, 8.02; N, 12.38. Found: C, 58.35; H, 7.96; N, 12.37.

4.3.5. 1,3-Dihydroxy-2-(pyrid-3-yl)-4,4,5,5-tetramethylimidazolidine (3e). Yield: 69%. Mp: 90-92 °C. IR (KBr): 3440, 1601, 1505, 1450, 1383 cm^{-1};NMR (DMSO): $\delta = 1.04$ (s, 12H), 4.55 (s, 1H), 7.35 (s, 1H), 7.81 (s, 1H) 8.46 (s, 1H) 8.62 (s, 1H); ESI-MS (*m/e*) = 237 [M]$^+$; Anal. Calcd for $C_{12}H_{19}N_3O_2$: C, 60.74; H, 8.07; N, 17.71. Found: C, 60.49; H, 7.99; N, 17.65.

4.3.6. 1,3-Dihydroxy-2-phenyl-4,4,5,5-tetramethylimidazolidine (3f). Yield: 85%. Mp: 168–169 °C. IR (KBr): 3340, 1606, 1500, 1450, 1381 cm^{-1}; ^{1}H NMR ($CDCl_3$) $\delta = 1.14$ (s, 12H), 4.78 (s, 1H), 7.31–7.51 (m, $J = 6.9$ Hz, 5H), 7.71 (s, 2H); ESI-MS (*m/e*) = 236.3 [M]$^+$; Anal. Calcd for $C_{13}H_{20}N_2O_2$: C, 66.07; H, 8.53; N, 11.85. Found: C, 66.12; H, 8.61; N, 11.93.

4.3.7. 1,3-Dihydroxy-2-(4′-hydroxyl)phenyl-4,4,5,5-tetramethylimidazolidine (3g). Yield: 51%. Mp: 161–162 °C. IR (KBr) 3455, 1610, 1506 1450, 1382, 830 cm^{-1}; ^{1}H NMR (DMSO) $\delta = 1.03$ (s, 12H), 4.39 (s, 1H), 6.70 (d, $J = 7.0$ Hz, 2H), 7.23 (d, $J = 7.0$ Hz, 2H), 7.63 (s, 1H), 7.85 (s, 1H); ESI-MS (*m/e*) = 252 [M]$^+$; Anal. Calcd for $C_{13}H_{20}N_2O_3$: C, 61.88; H, 7.99; N, 11.10. Found: C, 61.92; H, 8.04; N, 11.17.

4.3.8. 1,3-Dihydroxy-2-(4′-nitro)phenyl-4,4,5,5-tetramethylimidazolidine (3h). Yield: 63%. Mp: 154–156 °C. IR (KBr) 3425, 1625, 1520, 1384, 1350, 835 cm^{-1}; ^{1}H NMR (DMSO) $\delta = 1.09$ (s, 12H), 4.71 (s, 1H), 7.70 (d, $J = 7.2$ Hz, 2H), 8.21 (d, $J = 7.2$ Hz, 2H), 8.40 (s, 2H); ESI-MS (*m/e*) = 281 [M]$^+$; Anal. Calcd for $C_{13}H_{19}N_3O_4$: C, 55.51; H, 6.81; N, 14.94. Found: C, 55.64; H, 6.93; N, 14.89.

4.4. General procedure for the preparation of 4a–h

At room temperature the suspension of 1 mmol of **3a**–**h** and 0.7 g of lead dioxide in 10 mL methanol was stirred for 3 h. After filtration, the filtrate was evaporated under reduced pressure, compounds **4a**–**h** were obtained as dark blue crystals.

4.4.1. 4,4,5,5-Tetramethylimidazoline-3-oxide-1-oxyl (4a). Yield: 90%. Mp: 92–93 °C. IR (KBr) 1533, 1384, 889 cm^{-1}; ESI-MS (*m/e*) = 157 [M]$^+$; Anal. Calcd for $C_7H_{16}N_2O_2$: C, 53.49; H, 8.34; N, 17.82. Found: C, 53.29; H, 8.42; N, 17.68.

4.4.2. 2-Chloromethyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (4b). Yield: 38%. Mp: 73–75 °C. IR (KBr) 1510, 1482, 1380 cm^{-1}; ESI-MS (*m/e*) = 205 [M]$^+$; Anal. Calcd for $C_8H_{14}N_2O_2Cl$: C, 46.72; H, 6.86; N, 13.62. Found: C, 46.89; H, 6.42; N, 13.48.

4.4.3. 2-Carboxyl-4,4,5,5-tetramethylimidazolidine-3-oxide-1-oxyl (4c). Yield: 88%. Mp: 84–86 °C. IR (KBr) 2705–2400, 1709, 1500, 1462, 1380 cm^{-1}; ESI-MS (*m/e*) = 201 [M]$^+$; Anal. Calcd for $C_8H_{13}N_2O_4$: C, 47.76; H, 6.51; N, 13.92. Found: C, 47.69; H, 6.33; N, 13.80.

4.4.4. 2-(Fur-2-yl)-4,4,5,5-tetramethylimidazolidine-3-oxide-1-oxyl (4d). Yield: 83%. Mp: 80–82 °C. IR (KBr) 1619, 1505, 1450, 1382 cm^{-1}; ESI-MS (*m/e*) = 223 [M]$^+$; Anal. Calcd for $C_{11}H_{15}N_2O_3$: C, 59.18; H, 6.77; N, 12.55. Found: C, 59.30; H, 6.55; N, 12.68.

4.4.5. 2-(Pyrid-3-yl)-4,4,5,5-tetramethylimidazolidine-3-oxide-1-oxyl (4e). Yield: 79%. Mp: 81–83 °C. IR (KBr) 1609, 1500, 1452, 1380 cm^{-1}; ESI-MS (*m/e*) = 234 [M]$^+$; Anal. Calcd for $C_{12}H_{16}N_3O_2$: C, 61.52; H, 6.88; N, 17.94. Found: C, 61.33; H, 6.69; N, 18.08.

4.4.6. 2-Phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (4f). Yield: 80%. Mp: 84–85 °C. IR (KBr) 1610, 1450, 1381, 1365 cm^{-1}; ESI-MS (*m/e*) = 233 [M]$^+$; Anal. Calcd for $C_{13}H_{17}N_2O_2$: C, 66.93; H, 7.34; N, 12.01. Found: C, 67.02; H, 7.41; N, 12.09.

4.4.7. 2-(4′-Hydroxyl)phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (4g). Yield: 52% Mp: 134–135 °C. IR (KBr) 3250, 1500, 1490, 1355, 840 cm^{-1}; ESI-MS (*m/e*) = 249 [M]$^+$; Anal. Calcd for $C_{13}H_{17}N_2O_3$: C, 62.64; H, 6.87; N, 11.24. Found: C, 62.75; H, 6.93; N, 11.21.

4.4.8. 2-(4′-Nitrophenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (4h). Yield: 73%. Mp: 175-176 °C. IR (KBr) 1360, 1600, 1450, 830 cm^{-1}; ESI-MS (*m/e*) = 278 [M]$^+$; Anal. Calcd for $C_{13}H_{16}N_3O_4$: C, 56.11; H, 5.79; N, 15.10. Found: C, 56.15; H, 5.82; N, 15.18.

4.5. General procedure for the preparation of 5a–p

A solution of 296 mg (2 mmol) of 2,3-bis(hydroxylamino)-2,3-dimethylbutane and 2 mmol of various aldehydes in 3 mL of methanol was stirred at room temperature for 16 h. The title compound was collected by filtration. The filtrate was evaporated under reduced pressure to obtain the second crop of the title compound. The title compound was directly used for the next reaction without further purification.

4.5.1. 1,3-Dihydroxy-2-(4′-bromophen-1′-yl)-4,4,5,5-tetramethylimidazolidine (5a). Mp: 205–207 °C. $R_f = 0.69$ ($CHCl_3/CH_3OH$, 10:1). EI-MS (*m/z*) 314 [M]$^+$. IR (KBr) 3310, 1590, 1450, 1075, 830 cm^{-1}. ^{1}H NMR ($CDCl_3$) $\delta = 1.14$ (s, 12H), 4.77 (s, 1H), 7.37 (d, $J = 8.4$ Hz, 2H), 7.47 (d, $J = 7.8$ Hz, 2H), 7.73 (s, 2H). Anal. Calcd for $C_{13}H_{19}N_2O_2Br$: C, 49.54; H, 6.08; N, 8.89. Found: C, 49.62; H, 6.14; N, 8.93.

4.5.2. 1,3-Dihydroxy-2-(4′-chlorophen-1′-yl)-4,4,5,5-tetramethylimidazolidine (5b). Mp: 213–215 °C. $R_f = 0.73$ ($CHCl_3/CH_3OH$, 10:1). EI-MS (*m/z*) 270 [M]$^+$. IR (KBr) 3325, 1600, 1500, 1085, 825 cm^{-1}. ^{1}H NMR ($CDCl_3$) $\delta = 1.03$ (s, 6H), 1.07 (s, 6H), 4.50 (s, 1H), 7.38 (d, $J = 8.7$ Hz, 2H), 7.48 (d, $J = 8.4$ Hz, 2H), 7.80 (s, 2H). Anal. Calcd for $C_{13}H_{16}N_2O_2Cl$: C, 57.67; H, 7.07; N, 10.35. Found: C, 57.72; H, 7.12; N, 10.43.

4.5.3. 1,3-Dihydroxy-2-(4′-methylphen-1′-yl)-4,4,5,5-tetramethylimidazolidine (5c). Mp: 199–201 °C. $R_f = 0.61$ ($CHCl_3/CH_3OH$, 10:1). EI-MS (*m/z*) 250 [M]$^+$. IR (KBr) 3335, 2985, 1600, 815 cm^{-1}. ^{1}H NMR ($CDCl_3$) $\delta = 1.14$ (s, 6H), 1.19 (s, 6H), 1.37 (s, 3H), 4.90 (s, 1H), 7.68 (d, $J = 9.0$ Hz, 2H), 8.22 (d, $J = 8.4$ Hz, 2H), 8.35 (s, 2H). Anal. Calcd for $C_{14}H_{22}N_2O_2$: C, 67.17; H, 8.86; N, 11.19. Found: C, 67.23; H, 8.81; N, 11.25.

4.5.4. 1,3-Dihydroxy-2-(4′-methoxylphen-1′-yl)-4,4,5,5-tetramethylimidazolidine (5d). Mp: 179–181 °C. $R_f = 0.52$ ($CHCl_3/CH_3OH$, 10:1). EI-MS (*m/z*) 266 [M]$^+$. IR (KBr) 3340, 2835, 1500, 825 cm^{-1}. ^{1}H NMR (DMSO-d_6) $\delta = 0.99$ (s, 6H), 1.03 (s, 6H), 3.73 (s, 3H), 4.56 (s, 1H), 6.88 (d, $J = 4.2$ Hz, 2H), 7.38 (d, $J = 5.7$ Hz, 2H), 7.77 (s, 2H). Anal. Calcd for $C_{14}H_{22}N_2O_3$: C, 63.14; H, 8.33; N, 10.52. Found: C, 63.19; H, 8.38; N, 10.49.

4.5.5. 1,3-Dihydroxy-2-(3′-nitrophen-1′-yl)-4,4,5,5-tetramethylimidazolidine (5e). Mp: 179–181 °C. $R_f = 0.51$ ($CHCl_3/CH_3OH$, 10:1). EI-MS (*m/z*) 281 [M]$^+$. IR (KBr) 3315, 1530, 1360, 1600, 875, 790, 685 cm^{-1}. ^{1}H NMR (DMSO-d_6) $\delta = 0.52$ (s, 12H), 4.19 (s, 1H), 7.04 (dd, $J = 8.7$ Hz, $J = 8.1$ Hz, 1H), 7.34 (d, $J = 6.0$ Hz, 1H), 7.57 (d, $J = 7.5$ Hz, 1H), 7.77 (s, 1H), 8.03 (s, 2H). Anal. Calcd for $C_{13}H_{19}N_3O_4$: C, 55.51; H, 6.81; N, 14.94. Found: C, 55.61; H, 6.90; N, 14.87.

4.5.6. 1,3-Dihydroxy-2-(3′-hydroxyphen-1′-yl)-4,4,5,5-tetramethylimidazolidine (5f). Mp: 244–246 °C. $R_f = 0.56$ ($CHCl_3/CH_3OH$, 6:1). EI-MS (*m/z*) 252 [M]$^+$. IR (KBr) 3320, 1500, 880, 795, 695 cm^{-1}. ^{1}H NMR (DMSO-d_6) $\delta = 1.03$ (s, 6H), 1.05 (s, 6H), 4.51 (s, 1H), 6.89 (dd, $J = 8.4$ Hz, $J = 8.0$ Hz, 1H), 6.92 (d, $J = 6.2$ Hz, 1H), 7.01 (d, $J = 7.3$ Hz, 1H), 7.71 (s, 1H), 7.86 (s, 1H), 7.95 (s, 2H). Anal. Calcd for $C_{13}H_{20}N_2O_3$: C, 61.88; H, 7.99; N, 11.10. Found: C, 61.85; H, 8.05; N, 11.18.

4.5.7. 1,3-Dihydroxy-2-(3′,4′-dimethoxylphen-1′-yl)-4,4,5,5-tetramethylimidazolidine (5g). Mp: 147–149 °C.$R_f = 0.52$ ($CHCl_3/CH_3OH$, 6:1). EI-MS (*m/z*) 296 [M]$^+$. IR (KBr) 3310, 2825, 1610, 865, 815 cm^{-1}. ^{1}H NMR (DMSO-d_6) $\delta = 1.05$ (s, 12H), 3.74 (s, 6H), 4.53 (s, 1H), 6.85 (s, 1H), 6.87 (d, $J = 6.6$ Hz, 1H), 6.92 (d, $J = 6.4$ Hz, 1H), 7.85 (s, 2H). Anal. Calcd for $C_{15}H_{24}N_2O_4$: C, 60.79; H, 8.16; N, 9.45. Found: C, 60.82; H, 8.23; N, 9.54.

4.5.8. 1,3-Dihydroxyl-2-(3′-methoxyl-4′-hydroxyphen-1′-yl)-4,4,5,5-tetramethylimidazolidine (5h). Mp: 235–237 °C, $R_f = 0.47$ ($CHCl_3/CH_3OH$, 6:1), EI-MS (*m/z*) 282 [M]$^+$, IR (KBr) 3310, 2840, 1600, 870, 815 cm^{-1}. ^{1}H NMR (DMSO-d_6) $\delta = 1.00$ (s, 6H), 1.03 (s, 6H), 3.74 (s, 3H), 4.41 (s, 1H), 6.70 (d, $J = 7.8$ Hz, 1H), 6.85 (d, $J = 8.1$ Hz, 1H), 7.02 (s, 1H), 7.68 (s, 1H), 8.05 (s, 2H). Anal. Calcd for $C_{14}H_{22}N_2O_4$: C, 59.56; H, 7.85; N, 9.92. Found: C, 59.61; H, 7.92; N, 9.85.

4.5.9. 1,3-Dihydroxyl-2-(2′,4′-dichlorophenyl)-4,4,5,5-tetramethylimidazolidine (5i). Mp: 201–203 °C. $R_f = 0.69$ ($CHCl_3/CH_3OH$, 10:1). EI-MS (*m/z*) 305 [M]$^+$. IR (KBr) 3315, 1590, 1450, 995, 820, 865 cm^{-1}. ^{1}H NMR (DMSO-d_6) $\delta = 1.11$ (s, 12H), 5.01 (s, 1H), 7.50 (d, $J = 7.6$ Hz, 1H), 7.52 (d, $J = 8.1$ Hz, 1H), 7.58 (s, 1H), 7.79 (s, 2H). Anal. Calcd for $C_{13}H_{18}N_2O_2Cl_2$: C, 51.16; H, 5.94; N, 9.18. Found: C, 51.24; H, 5.99; N, 9.23.

4.5.10. 1,3-Dihydroxyl-2-(3′,4′-methylendioxyphen-1′-yl)-4,4,5,5-tetramethylimidazolidine (5j). Mp: 188–190 °C, $R_f = 0.47$ ($CHCl_3/CH_3OH$, 10:1), EI-MS (*m/z*) 280 [M]$^+$, IR (KBr) 3315, 1600, 915, 1235, 1105 cm^{-1}. ^{1}H NMR (DMSO-d_6) $\delta = 1.05$ (s, 12H), 3.74 (s, 6H), 4.53 (s, 1H), 6.82 (d, $J = 7.7$ Hz, 1H), 6.84 (d, $J = 8.0$ Hz, 1H), 6.87 (s, 1H) 7.85 (s, 2H). Anal. Calcd for $C_{14}H_{20}N_2O_4$: C, 59.99; H, 7.19; N, 9.99. Found: C, 60.04; H, 7.25; N, 10.09.

4.5.11. 1,3-Dihydroxy-2-(2′-fluorophen-1′-yl)-4,4,5,5-tetramethylimidazolidine (5k). Mp: 172–173 °C, $R_f = 0.69$ ($CHCl_3/CH_3OH$, 6:1), EI-MS (*m/z*) 254 [M]$^+$, IR (KBr) 3310, 1600, 1130, 1235, 775 cm^{-1}. ^{1}H NMR (DMSO-d_6) $\delta = 1.07$ (s, 12H), 4.95 (s, 1H), 7.18 (t, $J = 8.4$ Hz, 1H), 7.30 (d, $J = 8.1$ Hz, 1H), 7.68 (d, $J = 8.7$ Hz, 2H), 8.14 (s, 2H). Anal. Calcd for $C_{13}H_{19}N_2O_4F$: C, 61.40; H, 7.53; N, 11.02. Found: C, 61.49; H, 7.61; N, 11.09.

4.5.12. 1,3-Dihydroxy-2-(2′-nitrophen-1′-yl)-4,4,5,5-tetramethylimidazolidine (5l). Mp: 189–190 °C. $R_f = 0.54$ ($CHCl_3/CH_3OH$, 10:1). EI-MS (*m/z*) 281 [M]$^+$. IR (KBr) 3325, 1600, 1535, 1365, 750 cm^{-1}. ^{1}H NMR (DMSO-d_6) $\delta = 1.00$ (s, 6H), 1.04 (s, 6H), 5.37 (s, 1H), 7.52 (d, $J = 6.6$ Hz, 1H), 7.60 (t, $J = 7.8$ Hz, 1H), 7.92 (t, $J = 7.6$ Hz, 1H), 8.05 (d, $J = 7.2$ Hz, 1H), 8.23 (s, 2H).

Anal. Calcd for $C_{13}H_{19}N_3O_4$: C, 55.51; H, 6.81; N, 14.94. Found: C, 55.46; H, 6.90; N, 14.86.

4.5.13. 1,3-Dihydroxyl-2-(3′-hydroxyl-4′-methoxyl)phenyl-4,4,5,5-tetramethylimidazolidine (5m). Mp: 245–247 °C. $R_f = 0.52$ ($CHCl_3/CH_3OH$, 6:1). EI-MS (*m/z*) 282 $[M]^+$. IR (KBr) 3340, 2825, 1500, 1450, 825 cm^{-1}. ^1H NMR (DMSO-d_6) $\delta = 1.03$ (s, 6H), 1.04 (s, 6H) 3.72 (s, 3H), 4.53 (s, 1H), 6.82 (s, 1H), 6.89 (d, $J = 6.6$ Hz, 1H), 6.94 (d, $J = 6.4$ Hz, 1H), 7.65 (s, 1H), 8.04 (s, 2H). Anal. Calcd for $C_{14}H_{22}N_2O_4$: C, 59.56; H, 7.85; N, 9.92. Found: C, 59.65; H, 7.93; N, 9.89.

4.5.14. 1,3-Dihydroxyl-2-(4′-*N,N*-dimethylphen-1′-yl)-4,4,5,5-tetramethylimidazolidine (5n). Mp: 168–169 °C. $R_f = 0.53$ ($CHCl_3/CH_3OH$, 6:1). EI-MS (*m/z*) 279 $[M]^+$. IR (KBr) 3345, 2840, 1595, 1450, 840 cm^{-1}. ^1H NMR (DMSO-d_6) $\delta = 1.03$ (s, 6H), 1.05 (s, 6H), 2.85 (s, 6H), 4.01 (s, 1H), 6.68 (d, $J = 8.7$ Hz, 2H), 7.25 (d, $J = 8.7$ Hz, 2H), 7.60 (s, 2H). Anal. Calcd for $C_{15}H_{25}N_3O_2$: C, 64.49; H, 9.02; N, 15.04. Found: C, 64.58; H, 9.12; N, 15.17.

4.5.15. 1,3-Dihydroxyl-2-(2′,4′-dimethoxylphenyl)-4,4,5,5-tetramethylimidazolidine (5o). Mp: 140–141 °C. $R_f = 0.49$ ($CHCl_3/CH_3OH$, 10:1). EI-MS (*m/z*) 296 $[M]^+$. IR (KBr) 3295, 2825, 1500, 865, 810 cm^{-1}. ^1H NMR (DMSO-*d*6) $\delta = 1.03$ (s, 12H), 3.72 (s, 6H), 4.99 (s, 1H), 7.02 (s, 1H), 7.42 (d, $J = 6.7$ Hz, 1H), 7.45 (d, $J = 6.5$ Hz, 1H), 7.79 (s, 2H). Anal. Calcd for $C_{15}H_{24}N_2O_4$: C, 60.79; H, 8.16; N, 9.45. Found: C, 60.82; H, 8.21; N, 9.51.

4.5.16. 1,3-Dihydroxy-2-(2′-hydroxylphen-1′-yl)-4,4,5,5-tetramethylimidazolidine (5p). Mp: 139–141 °C. $R_f = 0.73$ ($CHCl_3/CH_3OH$, 6:1). EI-MS (*m/z*) 252 $[M]^+$. IR (KBr) 3325, 1600, 1500, 765 cm^{-1}. ^1H NMR (DMSO-d_6) $\delta = 1.08$ (s, 12H), 4.63 (s, 1H), 6.70 (d, $J = 6.3$ Hz, 1H), 6.74 (t, $J = 6.5$ Hz, 1H), 7.14 (t, $J = 6.6$ Hz, 1H), 7.20 (d, $J = 6.4$ Hz, 1H), 8.12 (s, 1H), 8.35 (s, 2H). Anal. Calcd for $C_{13}H_{20}N_2O_3$: C, 61.88; H, 7.99; N, 11.10. Found: C, 61.93; H, 8.05; N, 11.16.

4.6. General procedure for the preparation of compounds 6a–p

The mixture of 250 mg (0.8 mmol) of **5a**–**p** and 0.5 g of lead dioxide in 80 mL methanol was stirred at room temperature for 20 min and TLC ($CHCl_3/CH_3OH$, 20:1) indicated the complete disappearance of **5a**–**p**. The reaction mixture was filtrated and the filtrate was evaporated under vacuum to provide 230 mg (93%) of the title compound as dark blue crystals.

4.6.1. 2-(Phen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6a). Mp: 89–91 °C. $R_f = 0.85$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 311 $[M]^+$. IR (KBr) 1600, 1450, 1357, 1070, 825 cm^{-1}. Anal. Calcd for $C_{13}H_{16}N_2O_2Br$: C, 50.02; H, 5.17; N, 8.97. Found: C, 50.12; H, 5.23; N, 9.05. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.16$ G, $g = 2.00997$.

4.6.2. 2-(4′-Chlorophen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6b). Mp: 103–105 °C. $R_f = 0.67$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 267 $[M]^+$. IR (KBr) 1590, 1500, 1365, 1090, 825 cm^{-1}. Anal. Calcd for $C_{13}H_{13}N_2O_2Cl$: C, 58.32; H, 6.02; N, 10.46. Found: C, 58.41; H, 6.14; N, 10.54. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.12$ G, $g = 2.00997$.

4.6.3. 2-(4′-Methylphen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6c). Mp: 86–88 °C. $R_f = 0.88$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 247 $[M]^+$. IR (KBr) 2980, 1610, 1500, 1365, 810 cm^{-1}. Anal. Calcd for $C_{14}H_{19}N_2O_2$: C, 67.99; H, 7.74; N, 11.33. Found: C, 67.92; H, 7.83; N, 11.38. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.22$ G, $g = 2.00993$.

4.6.4. 2-(4′-Methoxylphen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6d). Mp: 89–92 °C. $R_f = 0.88$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 263 $[M]^+$. IR (KBr) 2830, 1600, 1355, 835 cm^{-1}. Anal. Calcd for $C_{14}H_{19}N_2O_3$: C, 63.86; H, 7.27; N, 10.64. Found: C, 63.92; H, 7.35; N, 10.59. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.28$ G, $g = 2.00991$.

4.6.5. 2-(3′-Nitrophen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6e). Mp: 161–162 °C. $R_f = 0.69$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 278 $[M]^+$. IR (KBr) 1530, 1350, 1600, 1450, 880, 790, 680 cm^{-1}. Anal. Calcd for $C_{13}H_{16}N_3O_4$: C, 56.11; H, 5.79; N, 15.10. Found: C, 56.19; H, 5.84; N, 15.16. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.10$ G, $g = 2.00998$.

4.6.6. 2-(3′-Hydroxyphen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6f). Mp: 137–139 °C. $R_f = 0.33$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 249 $[M]^+$. IR (KBr) 3340 (OH); 1590, 1450, 1380, 880, 800, 690 cm^{-1}. Anal. Calcd for $C_{13}H_{17}N_2O_3$: C, 62.64; H, 6.87; N, 11.24. Found: C, 62.71; H, 6.94; N, 11.18. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.18$ G, $g = 2.00994$.

4.6.7. 2-(3′,4′-Dimethoxylphen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6g). Mp: 95–97 °C. $R_f = 0.34$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 293 $[M]^+$. IR (KBr) 2830, 1595, 1355, 870, 820 cm^{-1}. Anal. Calcd for $C_{15}H_{21}N_2O_4$: C, 61.42; H, 7.22; N, 9.55. Found: C, 61.56; H, 7.29; N, 9.61. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.28$ G, $g = 2.00991$.

4.6.8. 2-(3′-Methoxyl-4′-hydroxyphen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6h). Mp: 83–85 °C. $R_f = 0.38$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 279 $[M]^+$. IR (KBr) 3340, 2830, 1590, 1345, 875, 820 cm^{-1}. Anal. Calcd for $C_{14}H_{19}N_2O_4$: C, 60.20; H, 6.86; N, 10.03. Found: C, 60.23; H, 6.91; N, 10.08. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.29$ G, $g = 2.00990$.

4.6.9. 2-(2′,4′-Dichlorophen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6i). Mp: 123–125 °C. $R_f = 0.54$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 302 $[M]^+$. IR (KBr) 1590, 1450, 1365, 1000, 825, 870 cm^{-1}. Anal. Calcd for $C_{13}H_{15}N_2O_2Cl_2$: C, 51.67; H, 5.00; N, 9.27. Found: C, 51.73; H, 5.08, N 9.35. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.15$ G, $g = 2.00997$.

4.6.10. 2-(3′,4′-Methylendioxyphen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6j). Mp: 97–99 °C.

$R_f = 0.75$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 277 $[M]^+$. IR (KBr) 1600, 1365, 910, 1240, 1100 cm^{-1}. Anal. Calcd for $C_{14}H_{17}N_2O_4$: C, 60.64; H, 6.18; N, 10.10. Found: C, 60.72; H, 6.24; N, 10.15. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.28$ G, $g = 2.00990$.

4.6.11. 2-(2′-Fluorophen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6k). Mp: 112–114 °C. $R_f = 0.52$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 251 $[M]^+$. IR (KBr) 1610, 1450, 1370, 1135, 770 cm^{-1}. Anal. Calcd for $C_{13}H_{16}N_2O_4$: C, 62.14; H, 6.42; N, 11.15. Found: C, 62.23; H, 6.58; N, 11.09. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.15$ G, $g = 2.00996$.

4.6.12. 2-(2′-Nitrophen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6l). Mp: 145–147 °C. $R_f = 0.49$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 278 $[M]^+$. IR (KBr) 1530, 1360, 1450, 740 cm^{-1}. Anal. Calcd for $C_{13}H_{16}N_3O_4$: C, 56.11; H, 5.79; N, 15.10. Found: C, 56.21; H, 5.85; N, 15.04. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.14$ G, $g = 2.00997$.

4.6.13. 2-(3′-Hydroxyl-4′-methoxylphen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6m). Mp: 117–119 °C. $R_f = 0.34$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 279 $[M]^+$. IR (KBr) 3320, 2830, 1595, 1500, 1350, 820 cm^{-1}. Anal. Calcd for $C_{14}H_{19}N_2O_4$: C, 60.20; H, 6.86; N, 10.03. Found: C, 60.31; H, 6.79; N, 10.15. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.25$ G, $g = 2.00991$.

4.6.14. 2-(4′-*N*,*N*-dimethylphen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6n). Mp: 151–154 °C. $R_f = 0.51$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 276 $[M]^+$. IR (KBr) 2850, 1595, 1500, 1360, 845 cm^{-1}. Anal. Calcd for $C_{15}H_{22}N_3O_2$: C, 65.19; H, 8.02; N, 15.21. Found: C, 65.25; H, 8.13; N, 15.16. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.27$ G, $g = 2.00992$.

4.6.15. 2-(2′,4′-Dimethoxylphen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6o). Mp: 75–77 °C. $R_f = 0.37$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 293 $[M]^+$. IR (KBr) 2830, 1610, 1365, 805, 870 cm^{-1}. Anal. Calcd for $C_{15}H_{21}N_2O_4$: C, 61.42; H, 7.22; N, 9.55. Found: C, 61.49; H, 7.31; N, 9.62. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.32$ G, $g = 2.00989$.

4.7. 2-(2′-Hydroxylphen-1′-yl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (6p)

Mp: 83–85 °C. $R_f = 0.75$ ($CHCl_3/CH_3OH$, 20:1). EI-MS (*m/z*) 249 $[M]^+$. IR (KBr) 3345, 1610, 1340, 760 cm^{-1}. Anal. Calcd for $C_{13}H_{17}N_2O_3$: C, 62.64; H, 6.87; N, 11.24. Found: C, 62.58; H, 6.93; N, 11.35. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.21$ G, $g = 2.00993$.

4.7.1. 2-(4′-Oxymethylcarboxylphen-1′-yl)-4,4,5,5-tetramethylimidazolidine-3-oxide-1-oxyl (6q). The mixture of 250 mg(1.0 mmol) of **4g**, 0.32 mL of ethyl bromoacetate, and 100 mg of sodium ethylate in 5 mL of anhydrous tetrahydrofuran was stirred at 60 °C for 5 h and TLC ($CHCl_3/CH_3OH$, 20:1) indicated the complete disappearance of **4g**. The reaction mixture was evaporated under vacuum and the residue was purified on silica gel chromatograph ($CHCl_3$) to give 300 mg (90%) of 2-(4′-ethoxycarbonylmethoxyphen-1′-yl)-4,4,5,5-tetramethylimidazolidine-3-oxide-1-oxyl. To the solution of 33 mg (1.0 mmol) of 2-(4′-ethoxycarbonylmethoxyphen-1′-yl)-4,4,5,5-tetramethylimidazolidine-3-oxide-1-oxyl in 3 mL of methanol 0.5 mL of NaOH aqueous solution (2 mol/L) was added and the mixture was stirred at room temperature for 30 min and TLC ($CHCl_3/CH_3OH$, 10:1) indicated the complete disappearance of the materials. The reaction mixture was evaporated under vacuum and the residue was diluted with 2 mL of saturated NaCl aqueous solution. The solution was adjusted to pH 5–6 with HCl (2 mol/L) and extracted with $CHCl_3$ (3× 3 mL). The organic phase was dried with anhydrous $MgSO_4$ and filtered. The filtrate was evaporated in vacuum to give 30 mg (89%) of title compound as blue acicular crystals. Mp: 155–157 °C. EI-MS (*m/z*) 307 $[M]^+$. IR (KBr) 1760; 1605, 1490, 1450, 1260, 830 cm^{-1}. Anal. Calcd for $C_{15}H_{19}N_2O_5$: C, 68.62; H, 6.23; N, 9.12. Found: C, 68.69; H, 6.31; N, 9.08. ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.15$ G, $g = 2.00998$.

4.7.2. 2-(4′-Bromoethoxyphen-1′-yl)-4,4,5,5-tetramethylimidazolidine-3-oxide-1-oxyl (6s). The mixture of 250 mg (1.0 mmol) of **4g**, 0.5 mL of 1,2-dibromoethane, and 150 mg of sodium ethylate in 5mL of anhydrous tetrahydrofuran was stirred at 60 °C for 8 h and TLC ($CHCl_3/CH_3OH$, 10:1) indicated the complete disaperance of **5f**. The reaction mixture was filtered and the filtrate was evaporated in vacuum. The residue was purified with silica gel chromatograph ($CHCl_3$/methanol, 50:1) to give 162 mg (64%) of **6s** as blue acicular crystals. Mp: 117–118 °C. EI-MS (*m/z*) 356 $[M]^+$. IR (KBr) 1600, 1500, 1450, 1250, 840 cm^{-1}. Anal. Calcd for $C_{15}H_{20}N_2O_2Br$: C, 50.57; H, 5.66; N, 7.86. Found: C, 50.52; H, 5.71; N, 7.93 ESR: a five-line pattern with intensity ratios of 1:2:3:2:1, $a_N = 8.30$ G, $g = 2.00990$.

4.8. Determination of ESR

A center field of 3480 G, sweep width of 100 G, sweep time of 100 s, modulation amplitude of 1.0×10^{-1} G, time constant of 1.6×10^{-1} s, modulation frequency of 100 kHz, microwave frequency of 9.72 GHz, and microwave power of 10 MW were all used for the ESR measurement. The ESR spectra of **6a–s** at two different concentrations, 10^{-7} and 10^{-5} mol/L, and in desecrated water and phosphate buffer (pH 7.4), respectively, were recorded.

4.9. NO scavenging activity determined using rat aortic strip[19]

The NO scavenging activity of **4a–h** in the rat aortic strip was examined according to a published method.[19] In brief, immediately after decapitation rat aortic strips were obtained and put in a perfusion bath with 5 mL of warmed (37 °C), oxygenated (95%O_2, 5%CO_2) Krebs solution (pH 7.4). The aortic strips were mounted to the tension transducers and the relaxation–contraction curves were recorded. Noradrenaline (NE, final concentration 10^{-9} mol/L) solution was added to induce contraction. When the hypertonic contraction reached to the maximum level, NE was washed and the vessel strips were stabilized for 30 min. After the renewal of the solution, NE (final concentration 10^{-9} mol/L) was added. When the hypertonic contraction value of aortic strips reached the peak, 15 L of NS or the solution of **4a–h** in 15 L of water (final concentration 10^{-6} mol/L) was added, respectively. Upon stabilization, 1.5 L ACh (final concentration 10^{-6} mol/L) was added and the percentage inhibition of ACh-induced vasorelaxation by test compounds was determined.

4.10. Evaluation of 4f–h and 6a–s as scavengers of NO, $H_2O_2^{\cdot}$, and $^{\cdot}OH$ in PC12 cells[23,27,28]

Free radical scavenging activities were evaluated in PC12 cells using a method of Dawson with minor modifications. In brief, PC12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% of heat-inactivated horse serum (Hyclone), 5% of fetal bovine serum (GIBCO), 1.0 mM sodium pyruvate, 100 U/mL penicillin, and 100 g/mL streptomycin at 37 °C, in 5% CO_2 atmosphere. PC12 cells were seeded in 96-well plates coated with poly-L-lysine at a density of 20,000 cells per well during the exponential phase of growth. After 24 h, attachment period fresh media containing 12.5, 25, 50, 100, or 200 M of **4f–h** and **6a–s**, respectively, were added to each well and were incubated for 1 h. NO damage was then induced by adding 2 mM of sodium nitroprusside followed by 2 h of incubation. The media were replaced with fresh media and cells were incubated for 14 h, following which cell survival was measured by a colorimetric assay with MTT according to the method of Mosmann. Similarly, H_2O_2 damage was induced by 1 mM H_2O_2 followed by 1 h of incubation, while OH damage was induced by 1 mM H_2O_2/30 M Fe(II) followed by 1 h of incubation.

Acknowledgments

This work was supported by Beijing area major laboratory of peptide and small molecular drugs, National Natural Scientific Foundation of China (30472071, 20232020), and National Natural Scientific Foundation of Beijing (7052010).

References and notes

1. Vendemiale, G.; Grattagliano, I.; Altomare, E. *Int. J. Clin. Lab. Res.* **1999**, *29*, 49–55.
2. Valko, M.; Rhodes, C. J.; Moncol, J.; Izakovic, M.; Mazur, M. *Chem. Biol. Interact.* **2006**, *160*, 1–40.
3. Cooke, M. S.; Evans, M. D.; Dizdaroglu, M.; Lunec, J. *FASEB J.* **2003**, *17*, 1195–1214.
4. Terranova, R.; Sorace, R.; Romeo, A.; Mauro, C. D.; Romeo, R.; Luca, S. *Minerva Med.* **2001**, *92*, 405–410.
5. Olanow, C. W. *Ann. Neurol.* **1992**, *32*, S2–S9.
6. Bankson, D. D.; Kestin, M.; Rifai, N. *Clin. Lab. Med.* **1993**, *13*, 463–480.
7. Jaswal, S.; Mehta, H. C.; Sood, A. K.; Kaur, J. *Clin. Chim. Acta* **2003**, *338*, 123–129.
8. Mak, J. C.; Chan, Y. M. M *Curr. Opin. Pulm. Med.* **2006**, *12*, 7–11.
9. Sheu, S. S.; Nauduri, D.; Anders, M. W. *Biochim. Biophys. Acta* **2006**, *1762*, 256–265.
10. Anderson, R. E.; Kretzer, F. L.; Rapp, L. M *Adv. Exp. Med. Biol.* **1994**, *366*, 73–86.
11. Osiecki, J. H.; Ullman, E. F. *J. Am. Chem. Soc.* **1968**, *90*, 1078.
12. Akaike, T.; Yoshida, M.; Miyamoto, Y.; Sato, K.; Kohno, M.; Sasamoto, K.; Miyazaki, K.; Ueda, S.; Maeda, H. *Biochemistry* **1993**, *32*, 827–832.
13. Koshland, D. E. *Science* **1992**, *258*, 1861.
14. Haseloff, R. F.; Zollner, S.; Kirilyuk, I. A.; Grigor'ev, I. A.; Reszka, R.; Bernhardt, R.; Mertsch, K.; Roloff, B.; Blasig, I. E. *Free Radical Res.* **1997**, *26*, 7–17.
15. Blasig, I. E.; Mertsch, K.; Haseloff, R. F. *Neuropharmacology* **2002**, *43*, 1006–1014.
16. Ullman, E. F.; Osiecki, J. H.; Boocock, D. G. B; Darcy, R. *J. Am. Chem. Soc.* **1972**, *94*, 7049–7059.
17. Lamchen, M.; Mittag, T. W. *J. Chem. Soc.* **1966**, *24*, 2300.
18. Kopani, M.; Celec, P.; Danixovi, L.; Michalk, P.; Biro, C. *Clin. Chim. Acta* **2006**, *364*, 61–66.
19. Cowart, M.; Kowaluk, E. A.; Daanen, J. F.; Kohlhaas, K. L.; Alexander, K. M.; Wagenaar, F. L.; Kerwin, J. F. *J. Med. Chem.* **1998**, *41*, 2636.
20. Komori, K.; Vanhoutte, P. M. *Blood Vessels* **1990**, *27*, 238–245.
21. Moncada, S.; Palmer, R. M. J.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109–142.
22. Calles-Escandon, J.; Cippola, M. *Endocr. Rev.* **2001**, *22*, 36–52.
23. Desole, M. S.; Sciola, L.; Sircana, S.; Godani, C.; Migheli, R.; Delogu, M. R.; Piras, G.; De, N. G.; Miele, E. *Neurosci. Lett.* **1998**, *247*, 1–4.
24. Badeau, M.; Adlercreutz, H.; Kaihovaara, P.; Tikkanen, M. J. *J. Steroid. Biochem. Mol. Biol.* **2005**, *96*, 271–278.
25. Hsu, L. Y.; Lin, C. F.; Hsu, W. C.; Hsu, W. L; Chang, T. C. *Biol. Pharm. Bull.* **2005**, *28*, 1211–1215.
26. Yilmaz, Y.; Toledo, R. T. *Trends Food Sci. Technol.* **2004**, *15*, 422–433.
27. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55–63.
28. Kanski, J.; Aksenova, M.; Stoyanova, A.; Butterfield, D. A. *J. Nutr. Biochem.* **2002**, *13*, 273–281.