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Synthesis and Antioxidant Activity of Peptide-Based Ebselen Analogues

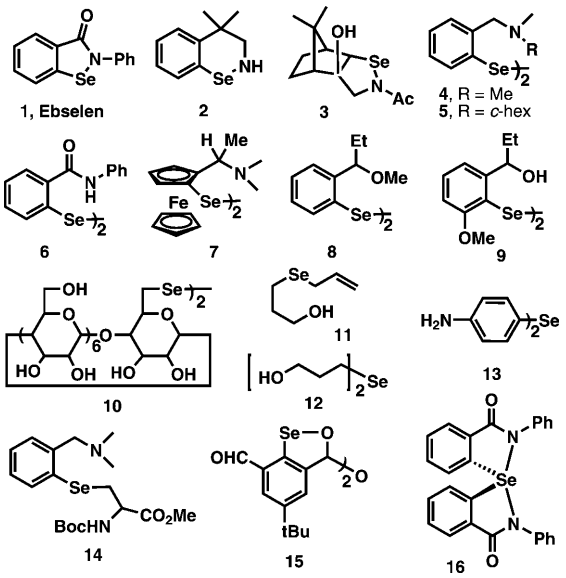
Kandhan Satheeshkumar and Govindasamy Mugesh*[a]

Abstract: A series of di- and tripeptide-based ebselen analogues has been synthesized. The compounds were characterized by ^{1}H, ^{13}C, and ^{77}Se NMR spectroscopy and mass spectral techniques. The glutathione peroxidase (GPx)-like antioxidant activity has been studied by using H_2O_2, *tert*-butyl hydroperoxide (*t*BuOOH), and cumene hydroperoxide (Cum-OOH) as substrates, and glutathione (GSH) as a co-substrate. Although all the peptide-based compounds have a selenazole ring similar to that of ebselen, the GPx activity of these compounds highly depends on the nature of the peptide moiety attached to the nitrogen atom of the selenazole ring. It was observed that the introduction of a phenylalanine (Phe) amino acid residue in the N-terminal reduces the activity in all three peroxide systems. On the other hand, the introduction of aliphatic amino acid residues such as valine (Val) significantly enhances the GPx activity of the ebselen analogues. The difference in the catalytic activity of dipeptide-based ebselen derivatives can be ascribed mainly to the change in the reactivity of these compounds toward GSH and peroxide. Although the presence of the Val-Ala-CO_2Me moiety facilitates the formation of a catalytically active selenol species, the reaction of ebselen analogues that has a Phe-Ile-CO_2Me residue with GSH does not generate the corresponding selenol. To understand the antioxidant activity of the peptide-based ebselen analogues in the absence of GSH, these compounds were studied for their ability to inhibit peroxynitrite (PN)-mediated nitration of bovine serum albumin (BSA) and oxidation of dihydrorhodamine 123. In contrast to the GPx activity, the PN-scavenging activity of the Phe-based peptide analogues was found to be comparable to that of the Val-based compounds. However, the introduction of an additional Phe residue to the ebselen analogue that had a Val-Ala dipeptide significantly reduced the potency of the parent compound in PN-mediated nitration.

Keywords: antioxidants · ebselen · glutathione · peptides · selenium

Introduction

Organoselenium compounds that mimic the function of the antioxidant selenoenzyme glutathione peroxidase (GPx) are currently attracting considerable interest.[1,2] The initial observations by Sies et al.[3] and Wendel et al.[4] that the cyclic selenazole-based compound ebselen (**1**) mimics the activity of GPx led to the development of several organoselenium compounds that can catalytically reduce hydroperoxides in the presence of thiols. These include compounds **2** and **3** that have cleavable Se–N bonds,[5] amine- and amide-based diselenides (**4**–**7**),[6] diaryl diselenides that have Se···O interactions (**8** and **9**),[7] the cyclodextrin-based diselenide (**10**),[8] several monoselenides (**11**–**14**),[9] cyclic selenelate ester (**15**),[10] and spirodiazaselenurane (**16**) (Scheme 1).[11] Although the catalytic mechanisms for the GPx-like activity of many of these compounds are considerably different from that of the natural enzyme or ebselen, the selenium center in these compounds undergoes redox reactions in the presence of peroxides and thiols.



Scheme 1. Some representative examples of small-molecule GPx mimics **1**–**16**.

Although ebselen (**1**) has been shown to be the first successful GPx mimic, the mechanism by which ebselen exerts

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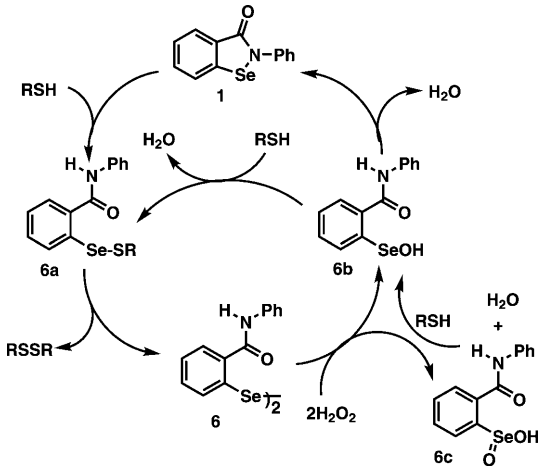


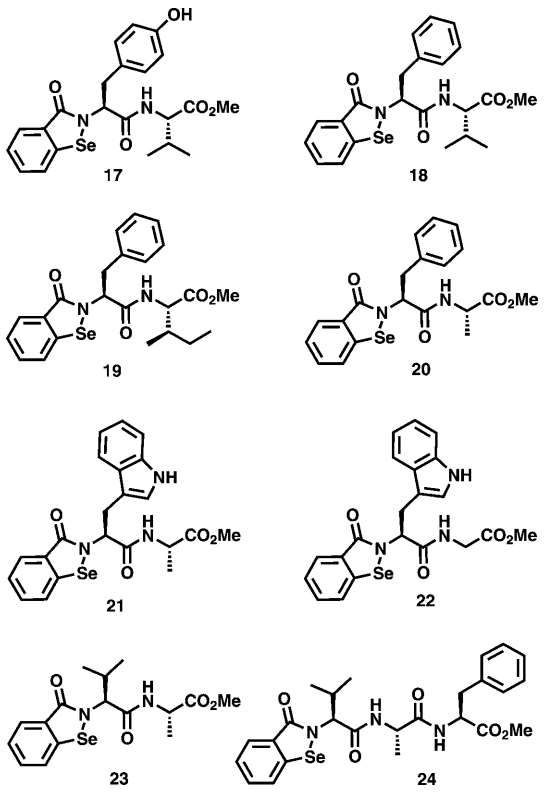
Scheme 2. Proposed mechanism for the GPx activity of ebselen.

its catalytic activity is completely different from that of GPx (Scheme 2). According to the revised catalytic mechanism,[12] the reaction of ebselen with a thiol produces the corresponding selenenyl sulfide intermediate (**6a**), which undergoes a disproportionation reaction to afford diselenide **6**. It has been shown that the formation of diselenide **6** is the rate-determining step, and this reaction depends on the nature of thiol used for the GPx assay.[12] The reaction of diselenide **6** with peroxides such as H_2O_2 produces the corresponding selenenic acid (**6b**) and seleninic acid (**6c**) both of which react with the thiol to regenerate **6a**. In the absence of thiols, the selenenic acid (**6b**) undergoes a facile cyclization to produce ebselen. The regeneration of ebselen from its catalytically active and inactive forms under a variety of conditions protects the selenium moiety in **6b** and **6c** from an overoxidation to other oxidized selenium species such as selenonic acid.

The available data suggest that ebselen does not follow the classical GPx catalytic cycle that involves selenol, selenenic acid, and selenenyl sulfide intermediates, although the formation of selenol has been observed during the reaction of ebselen with thiols.[13] It is still not clear whether the facilitation of selenol formation by modification in the basic structure can enhance the GPx activity of ebselen derivatives. In this paper, we report the synthesis and GPx activity of a series of ebselen analogues with di- and tripeptide moieties. We also show that not only the overall catalytic activity of these compounds but also the formation of selenol highly depend on the nature of peptide moiety attached to the nitrogen atom of the selenazole ring. Although some ebselen analogues that have amino acid residues have been reported,[14] there is no report on the synthesis and GPx activity of peptide-based ebselen derivatives.

Results and Discussion

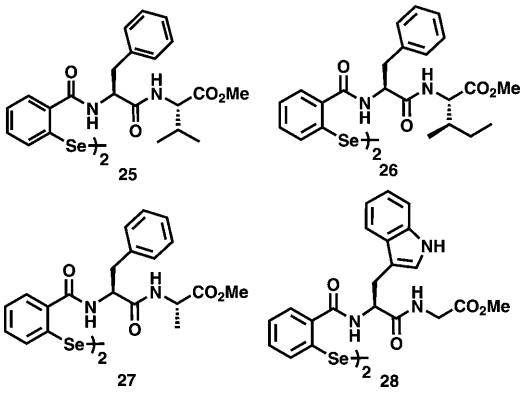
Synthesis of peptide-substituted ebselen analogues: A number of di- and tripeptide-containing ebselen analogues



Scheme 3. Ebselen analogues with different di- and tripeptide moieties.

17–**24** (Scheme 3) were synthesized in moderate yields by treating 2-(chloroseleno)benzoyl chloride with appropriate peptides that have free amino groups. Although 1:1 reactions of 2-(chloroseleno)benzoyl chloride with simple primary amines generally afford the corresponding cyclic selenazoles as the only products,[15] the nature of peptides in this study appears to affect the reaction products. Whereas the reactions of 2-(chloroseleno)benzoyl chloride with H_2N-Tyr-Val-CO_2Me, H_2N-Trp-Ala-CO_2Me, H_2N-Val-Ala-CO_2Me, and H_2N-Val-Ala-Phe-CO_2Me afforded only the cyclic compounds **17**, **21**, **23**, and **24**, respectively, similar reactions with H_2N-Phe-Val-CO_2Me, H_2N-Phe-Ile-CO_2Me, H_2N-Phe-Ala-CO_2Me, and H_2N-Trp-Gly-CO_2Me afforded considerable amounts of the diselenides **25**, **26**, **27**, and **28**, respectively (Scheme 4), in addition to the expected cyclic compounds (**18**–**20** and **22**).

The structure of compound **23** was further studied by single-crystal X-ray diffraction analysis. This compound crystallized in a non-centrosymmetric space group (*P*1) with a Flack parameter[16] of 0.01. This indicates that the crystals of compound **23** are enantiomerically pure. The ebselen moiety is almost perpendicular to the side chain of the peptide backbone as shown in Figure 1. The crystal packing indicates that there are significant hydrogen-bonding interactions between two molecules. The carbonyl oxygen atom of the selenazole ring forms hydrogen bonds with the peptidic NH group of the adjacent molecule. These hydrogen bonds may play an important role in stabilizing some of the intermediates in the catalytic cycle.



Scheme 4. Peptide-based diselenides with a Phe or Trp moiety.

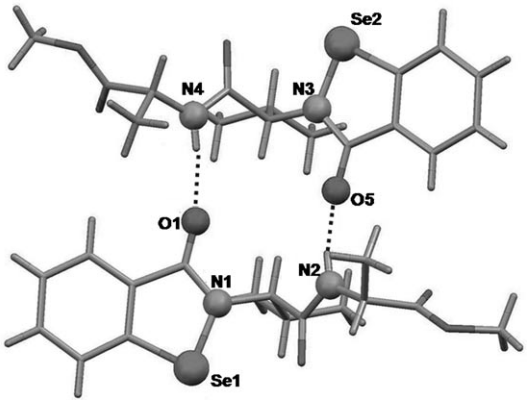
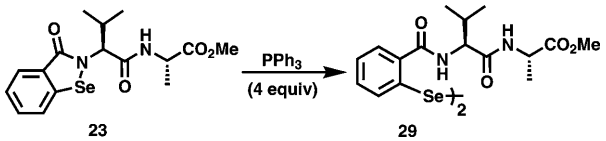


Figure 1. Crystal structure of compound **23** showing C=O···H–N hydrogen bonds.

It is known that the treatment of ebselen and its analogues with triphenylphosphine leads to the formation of the corresponding diselenides.[17] To check the reactivity of peptide-based ebselen analogues, compound **23** was treated with different concentrations of triphenylphosphine, and the reaction was followed by ^{77}Se NMR spectroscopy (Scheme 5). When four equivalents of triphenylphosphine



Scheme 5. Triphenylphosphine-mediated conversion of **23** to **29**.

was added, a complete conversion of **23** to the corresponding diselenide (**29**) was observed (Figure S41 in the Supporting Information). This indicates that the diselenide could be obtained in stable form even for the Val-Ala moiety, which appears to favor the formation of a cyclic compound. Similarly, the reaction of diselenide **26** with an excess amount (5 equiv) of H_2O_2 afforded the cyclic compound **19** in quantitative yield (Figures S42 and S42a in the Supporting Information). These observations indicate that the peptide-based cyclic compounds and diselenides can be readily interconverted with appropriate reagents.

Glutathione peroxidase (GPx) activity: The GPx-like catalytic activity of compounds **17–24** was studied using glutathione (GSH) as thiol cofactor and hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (*t*BuOOH), and cumene hydroperoxide (Cum-OOH) as substrates. Initial rates (v_0) were determined for the reduction of peroxides at 2 mM concentrations of the selenium catalysts. For comparison, the initial rates for the reduction of peroxides by ebselen were also determined. From Table 1, it is evident that the

Table 1. Initial rates for the reduction of peroxides by GSH in the presence of compounds **1** and **17–24**.

Compound	Initial rate v_0 [μM min^{-1}][a]		
	H_2O_2	*t*Bu–OOH	Cum–OOH
1	85.2±1.0	27.5±7.6	44.3±0.5
17	54.9±1.5	19.5±5.3	83.7±8.5
18	66.3±1.5	8.4±4.6	20.0±6.2
19	38.5±2.7	5.6±0.3	20.5±1.4
20	183.8±1.6	5.6±0.2	38.6±1.5
21	145.7±4.5	22.4±1.9	67.3±6.9
22	100.6±2.9	18.6±0.8	61.9±7.7
23	149.7±2.1	55.0±5.3	154.5±1.6
24	145.7±4.5	17.4±6.3	31.4±6.7

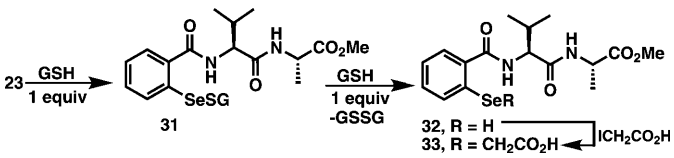
[a] The reactions were carried out in phosphate buffer (100 mM, pH 7.5) at 23 °C. Catalyst: 80.0 μM; glutathione reduced 2.0 mM; NADPH: 0.4 mM; glutathione disulfide reductase 1.7 unit mL^{-1}; peroxide: 1.6 mM. The initial rates were corrected for the background reaction between peroxide and thiol.

GPx activity of the ebselen analogues highly depends on the nature of peptides attached to the nitrogen atom. The nature of peroxide also has a significant effect on the reaction rates. Whereas compound **17**, which has a Tyr-Val dipeptide, was found to be a better catalyst than ebselen in the Cum-OOH assay, a significant decrease in the activity was observed when H_2O_2 or *t*BuOOH was used as the substrate. The introduction of a Phe residue appears to decrease the activity as compounds **18–20** with Phe-Val, Phe-Ile, or Phe-Ala exhibited poor activity when *t*BuOOH and Cum-OOH were used as substrates. In the H_2O_2 assay, only compound **20** exhibited higher activity compared to ebselen. The Trp-based compounds **21** and **22** were found to be much better catalysts than ebselen in both H_2O_2 or Cum-OOH systems, but the activity of these compounds in *t*BuOOH was slightly lower than that of ebselen. Compound **23**, which has a Val-Ala peptide, exhibited the highest activity in the series in all three peroxide assays. Interestingly, compound **24** with an additional Phe residue exhibited much lower catalytic activity than compound **23** when *t*BuOOH or Cum-OOH was employed. However, there was no significant change in the activity upon the introduction of the Phe residue when H_2O_2 was used as the substrate. These observations indicate that the GPx activity of the peptide-based ebselen analogues depends not only on the nature of

peptide moiety, but also the nature of peroxide used for the assay.

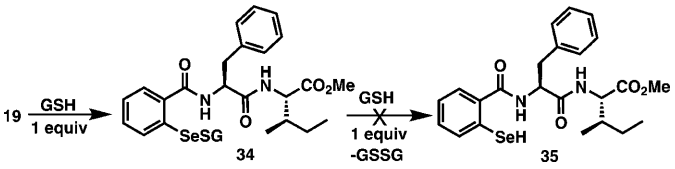
Mechanistic studies: To understand the reason for the difference in the activity of various peptide-based ebselen analogues, we have studied the reactivity of compound **19** (the least active compound) and **23** (the most active compound) toward GSH and peroxide. We have shown previously that the reaction of ebselen with thiols such as PhSH produces the corresponding selenenyl sulfide (**6a**, R = Ph), which does not produce any selenol upon further treatment with an excess amount (5 equiv) of PhSH. Alternatively, compound **6a** has been shown to undergo a disproportionation reaction to produce diselenide **6** and PhSSPh. This is due to the presence of a strong Se···O interaction in compound **6a**, which enhances a thiol exchange reaction at selenium.[18] Therefore, the rate of disproportionation depends on the nature of thiols and strength of the Se···O interactions. When GSH was employed instead of PhSH, the disproportionation selenenyl sulfide that led to the formation of diselenide **6** was significantly faster than that of **6a**.[12] The facile disproportionation has been shown to be responsible for the higher activity of ebselen in the presence of GSH relative to that of PhSH.[12]

Treatment of **23** with one equivalent of GSH afforded the expected selenenyl sulfide **31**, which upon treatment with another equivalent of GSH produced selenol **32**. Although **32** was too unstable to isolate and characterize, this species could be trapped by treating the reaction mixture with iodoacetic acid. When selenenyl sulfide **31** was treated with one equivalent of GSH in the presence of iodoacetic acid, a quantitative conversion of **31** to **33** was observed (Scheme 6). The reactivity of compound **23** towards GSH appears to be completely different from that of the selenenyl sulfide obtained from the reaction of ebselen with GSH. This may account for the higher GPx activity of compound **23** compared to that of ebselen.



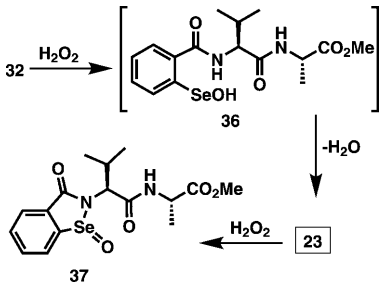
Scheme 6. Reaction of compound **23** with GSH.

The reaction of compound **19** with GSH generated the corresponding selenenyl sulfide **34**, which is similar to the reaction of **23** with GSH. However, further reaction of **34** with GSH did not produce the expected selenol **35**, but it produced diselenide **26** in nearly quantitive yield (Scheme 7). As only the diselenide was observed even in the presence of iodoacetic acid, the disproportionation of **34** to **26** appears to be more favored than the formation of selenol. The disproportionation of **34** to **26** was found be slower than that observed for the selenenyl sulfide derived from ebselen. This may account for the lower GPx activity of compound **19** compared to that of ebselen in different peroxide systems. The selenenyl sulfides derived from other ebselen analogues may undergo different degrees of disproportionation reactions depending upon the nature of peptide moiety. These observations indicate that the Val-Ala moiety in compound **31** facilitates the formation of selenol and therefore increases the GPx activity of compound **23**.



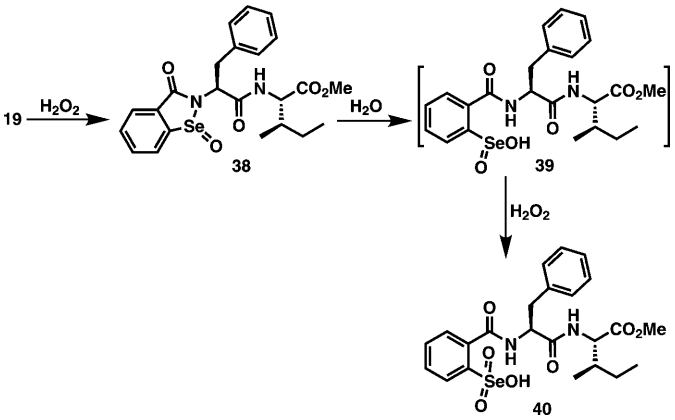
Scheme 7. Reaction of compound **19** with GSH.

The reaction of selenol **32** with two equivalents of H_2O_2 produced selenoxide **37** as the major product (Scheme 8). However, at lower concentrations of H_2O_2 a considerable amount of selenenyl amide **23** was produced possibly through the formation of selenenic acid **36** as previously shown for ebselen. The reactions of compounds **19** and **23** with H_2O_2 led to some interesting observations. When H_2O_2 (1 equiv) was added to compound **23**, the formation of **37** was observed as the major product. An independent HPLC analysis of the reaction of **23** with H_2O_2 indicated that this reaction initially produces selenoxide **37**, which finally leads to the formation of selenenyl amide **23** (Figures S48 and S49 in the Supporting Information). It should be noted that the selenoxide produced from the reaction of ebselen with H_2O_2 is highly unstable and it undergoes a rapid hydrolysis to produce the corresponding seleninic acid (**6c**).[12] This indicates that the introduction of Val-Ala moiety leads to the stabilization of selenoxide **37**.



Scheme 8. Formation of selenoxide **37** from the selenol **32**.

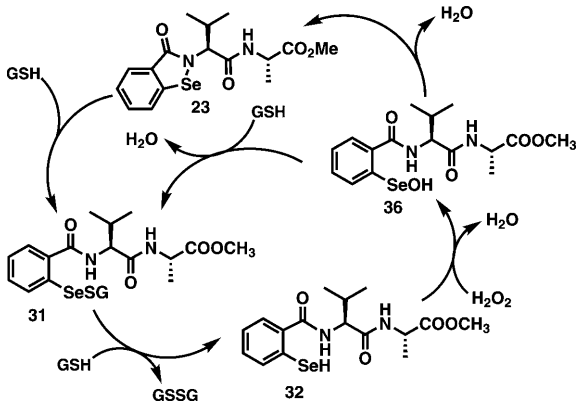
In contrast, reaction of the Phe-Ile-based selenenyl amide **19** with H_2O_2 produced the corresponding selenonic acid **40** as the major product (Scheme 9). Although the formation of selenoxide **38** was detected by mass spectroscopy, the rapid hydrolysis of this compound led to the formation of seleninic acid **39**, which upon reaction with H_2O_2 produced selenonic acid **40**. Interestingly, the regeneration of **19** was not observed in these reactions. The HPLC analysis of the reac-



Scheme 9. Reaction of **19** with H_2O_2 to produce the corresponding selenoxide, selenenic, and seleninic acids.

tion mixture indicated the formation of diselenide **26** as the major product (Figure S52 in the Supporting Information). As previously mentioned, the formation of diselenide **26** was also observed during the synthesis of compound **19**. However, no such diselenide formation was observed during the synthesis of ebselen or compound **23**. These observations indicate that the Phe-Ile moiety may disfavor the cyclization process by preventing the interactions between the −NH− and selenium moieties. Therefore, the selenium center in compound **39** undergoes a further oxidation to produce selenonic acid **40**, which may also account for the lower GPx activity of compound **19** compared to that of **23**.

On the basis of above experimental observations, a mechanism for the GPx activity of compound **23** can be proposed (Scheme 10). According to this mechanism, the cleavage of



Scheme 10. Proposed mechanism for the GPx activity of compound **23**.

the Se−N bond in selenenyl amide **23** by GSH produces selenenyl sulfide **31**, which upon reaction with a second equivalent of GSH generates the corresponding selenol **32**. Similar to the selenol moiety in GPx, selenol **32** reacts with H_2O_2 to produce the selenenic acid **36**, which further reacts with GSH to regenerate selenenyl sulfide **31**. When the concentration of GSH is much lower than that of H_2O_2, compound **36** undergoes a facile cyclization to regenerate **23**. Although the cyclization of compound **36** is very similar to that of **6b**, the reactivity of selenenyl sulfide **31** toward GSH is strikingly different from that of compound **6a**, which does not produce any selenol upon reaction with PhSH or GSH.[12] In contrast to compound **23**, the ebselen analogues that have a Phe residue such as compound **19** follow a mechanism similar to that of ebselen (Scheme 2). In this case, the disproportionation of selenenyl sulfides to the corresponding diselenides is the rate-determining step.

Inhibition of peroxynitrite-mediated nitration and oxidation: Peroxynitrite (PN) is a highly reactive oxygen and nitrogen species that plays a key role in a variety of inflammatory, allergic, and other diseases.[19] In biological medium, PN is generated by the diffusion-controlled reaction of superoxide anion radical ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$).[20] It is known that PN can induce DNA damage[21] and initiate lipid peroxidation in biomembranes or low-density lipoproteins.[22] In addition, PN can deactivate a variety of enzymes by tyrosine nitration.[23] To understand the protective effects of peptide-based ebselen analogues against PN, we have used the PN-mediated nitration of bovine serum albumin (BSA).[24] The inhibition of tyrosine nitration was followed by immunoblotting methods using antibody against 3-nitro-L-tyrosine. In a typical experiment, BSA (100 mM) was incubated with PN (1.2 mM) and inhibitor (133 μM), and the reaction was incubated at 20 °C for 20 min. At 133 μM concentration, the effect of various ebselen analogues on protein tyrosine nitration is given in Figure 2. From the percentage of nitration, it is clear that all the ebselen analogues significantly inhibit the PN-mediated nitration reaction. Compound **19**, which exhibited poor GPx activity, was found to be the most effective compound in inhibiting the PN-mediated nitration. The Val-Ala-based compound **23** also strongly inhibited the ni-

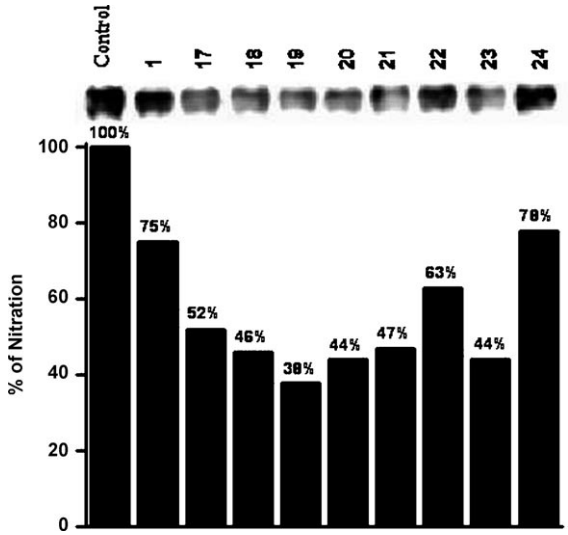
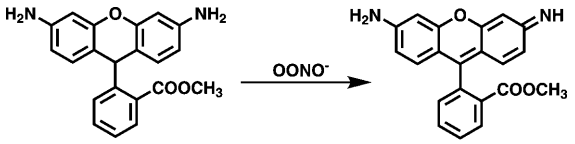


Figure 2. Immunoblots for the inhibition of PN-mediated nitration of BSA. BSA (100 mM) was incubated with inhibitor (133 μM) and PN (1.2 mM) for 30 min at 20 °C and then subjected to gel electrophoresis.

tration, thereby indicating that this compound is a good candidate for both GPx and PN-scavenging activities. In agreement with the effects of Phe residue on GPx activity, the introduction of an additional Phe residue to compound **23** significantly reduced its PN-scavenging activity.

In addition to the nitration of tyrosine residues in peptides and proteins, PN is also known to oxidize biomolecules. PN mediates the oxidation of dihydrorhodamine 123 (DHR) to rhodamine 123 (Scheme 11) in a linear fashion



Dihydrorhodamine 123 Rhodamine 123

Scheme 11. Oxidation of dihydrorhodamine 123 to rhodamine 123 by peroxynitrite.

over the range of 0–1000 nm, which is generally monitored by fluorescence spectrophotometric methods. To understand the effect of ebselen analogues on PN-mediated oxidation reactions, we have studied the conversion of DHR to rhodamine 123 in the presence of various concentrations of selenium compounds. The IC_{50} values (the concentration of test compounds required to inhibit 50% of the control activity) obtained for ebselen and compounds **17**–**24** are summarized in Table 2. Although there is no correlation between the

Table 2. Inhibition of PN-mediated oxidation of dihydrorhodamine 123 by ebselen and the peptide-based analogues.[a]

Compound	IC_{50} [μM]	Compound	IC_{50} [μM]
1	0.9±0.01	**21**	3.5±0.18
17	2.6±0.02	**22**	1.9±0.03
18	0.9±0.08	**23**	2.0±0.02
19	1.7±0.07	**24**	1.1±0.04
20	1.0±0.03		

[a] Assay conditions: dihydrorhodamine 123 (DHR; 0.1 mM), peroxynitrate (0.69 mM), and inhibitors (variable) in sodium phosphate buffer (100 mM, pH 7.5) with DTPA (100 μM) at 23°C.

effect of these compounds on protein tyrosine nitration and the IC_{50} values for the oxidation of DHR, all the compounds inhibited the oxidation of DHR in the low micromolar range. Interestingly, compounds **18**, **20**, and **24**, which exhibited poor GPx activity in the *t*BuOOH and Cum-OOH assays, were found to be very effective as inhibitors of PN-mediated oxidation. On the other hand, compound **23**, which exhibited much higher GPx activity, was found to be almost two times less active than ebselen in the DHR assay. Compound **19** was also found to be a good scavenger of PN as this compound effectively inhibited both the nitration and oxidation reactions.

Conclusion

In this study, we have shown for the first time that the GPx activity of peptide-based ebselen analogues depends on the nature of peptide moiety attached to the nitrogen atom of the selenazole ring. Whereas the introduction of a Phe amino acid residue reduces the GPx activity, the introduction of aliphatic amino acid residues such as Val significantly enhances the activity. The difference in the antioxidant activity of peptide-substituted selenenyl amides is due to the difference in the reactivity of these compounds toward GSH and peroxide. Although dipeptides such as Val-Ala that have aliphatic amino acid residues facilitates the formation of a catalytically active selenol, the introduction of aromatic amino acid such as Phe appears to prevent the generation of selenol. Furthermore, the Phe-based peptides prevent the cyclization of selenenic acids to produce the corresponding selenenyl amides, which leads to the overoxidation of selenenic and seleninic acids to selenonic acids. In addition to the GPx activity, the peptide-based ebselen analogues effectively inhibit the peroxynitrite-mediated nitration of protein tyrosine residues and oxidation of dihydrorhodamine 123. In contrast to the GPx activity, the PN scavenging activity of the Phe-based peptide analogues was found to be comparable to that of the Val-based compounds.

Experimental Section

General procedure: All the chemical reactions were carried out under nitrogen atmosphere using standard vacuum-line techniques. Due to the unpleasant odors of several of the reaction mixtures involved, most manipulations were carried out in a well-ventilated fume hood. Thin-layer chromatography (TLC) analyses were carried out on precoated silica gel plates (Merck), and spots were visualized by UV irradiation. Column chromatography was performed on glass columns loaded with silica gel or using an automated flash chromatography system (Biotage) by using preloaded silica cartridges. ^{1}H (400 MHz), ^{13}C (100.56 MHz), and ^{77}Se (76.29 MHz) NMR spectra were obtained using a Bruker 400 MHz NMR spectrometer. Chemical shifts are cited with respect to $SiMe_4$ as internal (^{1}H and ^{13}C) and Me_2Se as external (^{77}Se) standard. Mass spectral studies were carried out using a Q-TOF micro mass spectrometer or Bruker Daltonics Esquire 6000plus mass spectrometer with ESI-MS mode analysis. The melting point was determined in open capillary using an ANALAB melting-point apparatus.

Synthesis of 17: A solution of NH_2-Tyr-Val-OMe (0.12 g, 0.39 mmol) in dry acetonitrile (5 mL) was added dropwise with stirring over a period of 10 min to a solution of 2-(chloroseleno)benzoyl chloride (0.10 g, 0.39 mmol) in dry acetonitrile (5 mL). Triethylamine (0.10 mL, 0.78 mmol) was added, and the reaction mixture was stirred at 25°C for about 3 h. Solvent was evaporated in vacuo to give a yellow oil, which was purified on a silica gel column using ethyl acetate and petroleum ether as eluent to give the desired compound as a yellow oil. Yield: 0.091 g (49%); $[\alpha]_D^{25}=-15$ ($c=0.2$ in MeOH); ^{1}H NMR ($CDCl_3$): $\delta=$ 0.76–0.77 (d, $J=5.6$ Hz, 6H), 1.38–1.52 (m, 1H), 2.97–3.21 (m, 2H), 3.67 (s, 3H), 4.50–4.53 (t, $J=13.6$ Hz 1H), 5.49–5.53 (t, $J=14.4$ Hz, 1H), 6.66–6.68 (d, $J=7.6$ Hz, 2H), 6.99–7.01 (d, $J=6.4$ Hz, 3H), 7.32–7.36 (t, $J=$ 15.4 Hz, 1H), 7.53–7.56 (t, $J=14.8$ Hz, 1H), 7.62–7.64 (d, $J=8$ Hz, 1H), 7.96–7.98 ppm (d, $J=7.6$ Hz, 1H); ^{13}C NMR ($CDCl_3$) $\delta=22.2$, 22.9, 25.2, 39.2, 41.5, 51.5, 52.9, 59.0, 116.1, 124.2, 126.6, 127.1, 127.6, 128.9, 130.8, 132.7, 141.1, 155.9, 168.4, 170.2, 173.1 ppm; ^{77}Se NMR ($CDCl_3$): $\delta=$

896 ppm; HRMS: *m/z*: calcd for $C_{22}H_{24}N_2O_5Se$: 498.3862 $[M+Na]^+$; found: 497.0955.

Synthesis of 18: A solution of NH_2-Phe-Val-OMe (0.11 g, 0.39 mmol) in dry acetonitrile (5 mL) was added dropwise with stirring over a period of 10 min to a solution of 2-(chloroseleno)benzoyl chloride (0.10 g, 0.39 mmol) in dry acetonitrile (5 mL). Triethylamine (0.10 mL 0.78 mmol) was added to this, and the reaction mixture was stirred at 25 °C for about 3 h. Removal of the solvent under reduced pressure afforded the crude product, which was purified on a silica gel column using ethyl acetate and petroleum ether as eluent to give the desired compound as a yellow solid. Yield: 0.082 g (45 %); m.p. 135–137 °C; $[\alpha]_D^{25} = -35$ ($c = 0.2$ in MeOH); ^{1}H NMR ($CDCl_3$): $\delta = 0.84$–0.85 (d, $J = 2.4$ Hz, 7H), 2.06 (m, 1H), 3.11–3.31 (m, 2H), 3.67 (s, 3H), 4.43–4.45 (t, $J = 8.2$ Hz, 1H), 5.44–5.48 (t, $J = 14.8$ Hz, 1H), 6.42–6.44 (d, $J = 8.4$ Hz, 1H), 7.20–7.31 (m, 5H), 7.41–7.43 (t, $J = 7.6$ H, 1Hz), 7.60–7.64 (m, 1H), 8.02–8.04 ppm (d, $J = 7.6$ Hz, 1H); ^{13}C NMR ($CDCl_3$): $\delta = 18.2$, 19.3, 25.4, 31.8, 39.4, 52.7, 57.8, 58.9, 124.3, 126.5, 127.3, 127.5, 129.1, 129.7, 132.6, 136.4, 140.5, 168.2, 169.9, 171.7 ppm; ^{77}Se NMR ($CDCl_3$): $\delta = 899$ ppm; HRMS: *m/z*: calcd for $C_{22}H_{24}N_2O_4Se$: 482.3868 $[M+Na]^+$; found: 483.0799.

Synthesis of 19: A solution of NH_2-Phe-Ile-OMe (0.12 g, 0.39 mmol) in dry acetonitrile (5 mL) was added dropwise with stirring over a period of 10 min to a solution of 2-(chloroseleno)benzoyl chloride (0.10 g, 0.39 mmol) in dry acetonitrile (5 mL). Triethylamine (0.10 mL 0.78 mmol) was added to the reaction mixture, and it was stirred at 25 °C for about 3 h. Removal of solvent under reduced pressure afforded yellow residue, which was purified on a silica gel column using ethyl acetate and petroleum ether as eluent to yield compound **19** as a yellow powder. Yield: 0.074 g (40 %); m.p. 146–148 °C; $[\alpha]_D^{25} = -35$ ($c = 0.2$ in MeOH); ^{1}H NMR ($CDCl_3$): $\delta = 0.77$–0.78 (d, $J = 5.2$ Hz, 6H), 1.43–1.54 (m, 3H), 3.15–3.20 (m,2H), 3.64 (s, 3H), 4.47–4.47 (d, $J = 4.8$ Hz, 1H), 4.89–4.91 (q, $J = 7.2$ Hz, 1H), 6.41–6.43 (d, $J = 8.0$ Hz, 1H), 7.06–7.25 (m, 7H), 7.37–7.37 (d, $J = 1.2$ Hz, 1H), 7.70–7.72 ppm (d, $J = 7.6$ Hz, 1H); ^{13}C NMR ($CDCl_3$): $\delta = 22.4$, 23.2, 25.2, 38.8, 41.9, 51.6, 52.8, 55.3, 126.7, 127.5, 127.7, 129.2, 129.9, 131.7, 132.6, 133.7, 136.8, 168.4, 170.9, 173.1 ppm; ^{77}Se NMR ($CDCl_3$): $\delta = 904.346$ ppm; HRMS: *m/z*: calcd for $C_{23}H_{26}N_2O_4Se$: 496.4133 $[M+Na]^+$; found: 497.0955.

Synthesis of 20: A solution of 2-(chloroseleno)benzoyl chloride (0.10 g, 0.39 mmol) in dry acetonitrile (5 mL) was added dropwise with stirring a solution of NH_2-Phe-Ala-OMe (0.09 g, 0.39 mmol) in dry acetonitrile (5 mL) over a period of 10 min. After addition of triethylamine (0.10 mL 0.78 mmol), the reaction mixture was stirred at 25 °C for about 3 h. The solvent was removed under reduced pressure. Purification of the crude compound on a silica gel column using ethyl acetate and petroleum ether as eluent gave the expected compound as yellow oil. Yield: 0.090 g (53 %); $[\alpha]_D^{25} = -55$ ($c = 0.2$ in MeOH); ^{1}H NMR ($CDCl_3$): $\delta = 1.31$–1.33 (d, 3H, $J = 7.2$ Hz), 3.09–3.30 (m, 2H), 3.68 (s, 3H), 4.46–4.50 (t, $J = 14.4$ Hz, 1H), 5.52–5.56 (t, $J = 14.8$ Hz, 1H), 6.90–6.91 (d, $J = 6.8$ Hz, 1H), 7.16–7.20 (m, 5H), 7.26–7.30 (t, $J = 13.6$ Hz, 1H), and 7.37–7.40 ppm (t, $J = 14.4$ Hz, 1H); ^{3}C NMR ($CDCl_3$): $\delta = 18.7$, 30.2, 39.0, 48.9, 53.1, 55.3, 126.7, 127.5, 127.7, 129.3, 129.9, 131.7, 132.7, 136.7, 168.3, 170.6, 173.1 ppm; ^{77}Se NMR ($CDCl_3$): $\delta = 901$ ppm; HRMS: *m/z*: calcd for $C_{20}H_{20}N_2O_4Se$: 454.3336 $[M+Na]^+$; found: 455.0489.

Synthesis of 21: a solution of NH_2-Trp-Ala-OMe (0.11 g, 0.39 mmol) in dry acetonitrile (5 mL) was added dropwise with stirring over a period of 10 min to a solution of 2-(chloroseleno)benzoyl chloride (0.10 g, 0.39 mmol) in dry acetonitrile (5 mL). After the addition of triethylamine (0.10 mL 0.78 mmol), the reaction mixture was stirred at 25 °C for about 3 h. Solvent was evaporated under reduced pressure and the crude compounds was purified on a silica gel column using ethyl acetate and petroleum ether as eluent to give the desired compound as a yellow solid. Yield: 0.110 g (60 %); m.p. 193–196 °C; $[\alpha]_D^{25} = -10$ ($c = 0.2$ in MeOH); ^{1}H NMR ($CDCl_2$): $\delta = 1.25$–1.26 (d, $J = 4$ Hz, 3H), 3.25–3.39 (m, 2H), 3.58 (s, 3H), 4.38–4.44 (q, 1H), 5.62–5.63 (t, $J = 4$ Hz, 1H), 6.88–6.89 (d, $J = 4$ Hz, 1H), 7.15–7.20 (m, 3H), 7.30–7.37 (m, 2H), 7.56–7.99 (m, 3H), 7.99–8.01 (d, $J = 4$ Hz, 1H), 8.42 ppm (s, 1H); ^{3}C NMR ($CDCl_3$): $\delta =$ 18.43, 29.9, 48.8, 52.9, 58.0, 110.4, 111.6, 119.1, 119.9, 122.4, 123.9, 124.3, 126.4, 127.4, 127.9, 128.8, 132.5, 136.6, 141.3, 168.2, 170.4, 172.9 ppm; ^{77}Se NMR ($CDCl_3$): $\delta = 884$ ppm; HRMS: *m/z*: calcd for $C_{22}H_{21}N_3O_4Se$: 493.3696 $[M+Na]^+$; found: 494.0595.

Synthesis of 22: A solution of NH_2-Trp-Gly-COOMe (0.11 g, 0.39 mmol) in dry acetonitrile (5 mL) was added dropwise with stirring over a period of 10 min to a solution of 2-(chloroseleno)benzoyl chloride (0.10 g, 0.39 mmol) in dry acetonitrile (5 mL). After addition of triethylamine (0.10 mL 0.78 mmol), the reaction mixture was stirred at room temperature for about 3 h, and then the solvent was evaporated reduced pressure. Purification of the crude compound on a silica gel column using ethyl acetate and petroleum ether (1:3) as eluent afforded the desired compound as white solid. Yield: 0.086 g (48 %); m.p. 183–185 °C; $[\alpha]_D^{25} = -25$ ($c = 0.2$ in MeOH); ^{1}H NMR ($CDCl_3$): $\delta = 3.27$–3.44 (m, 2H), 3.58 (s, 3H), 3.82–3.90 (m, 2H), 5.62–5.59 (t, $J = 12$ Hz, 1H), 7.03–7.13 (m, 3H), 7.26–7.39 (m, 2H), 7.55–7.69 (m, 3H), 7.99–8.02 (d, $J = 4$ Hz, 1H), 8.36 ppm (s, 1H); ^{13}C NMR ($CDCl_3$): $\delta = 29.7$, 41.7, 52.8, 57.9, 110.4, 111.7, 119.1, 120.0, 122.6, 123.8, 124.3, 126.5, 127.3, 127.8, 128.9, 132.5, 136.6, 141.0, 168.2, 170.1, 170.9 ppm; ^{77}Se NMR ($CDCl_3$): $\delta = 900.03$ ppm; HRMS: *m/z*: calcd for $C_{21}H_{19}N_3O_4Se$: 479.3431 $[M+Na]^+$; found: 480.0438.

Synthesis of 23: A solution of NH_2-Val-Ala-COOMe (0.081 g, 0.04 mmol) in dry acetonitrile (5 mL) was added dropwise with stirring over a period of 10 min to a solution of 2-(chloroseleno)benzoyl chloride (0.10 g, 0.39 mmol) in dry acetonitrile (5 mL). Triethylamine (0.10 mL 0.78 mmol) was added and the reaction mixture was stirred at 25 °C for about 3 h. Removal of solvent under reduced pressure afforded the desired compound, which was purified by flash chromatography on a silica gel column by using ethyl acetate and petroleum ether as eluent to give a yellow solid. Yield : 0.08 g (51 %); m.p. 188–191 °C; $[\alpha]_D^{25} = -70$ ($c = 0.2$ in MeOH); ^{1}H NMR ($CDCl_3$): $\delta = 0.89$–0.90 (d, $J = 4$ Hz, 3H), 1.13–1.15 (d, $J = 8.0$ Hz, 4H), 1.42–1.44 (d, $J = 8$ Hz, 3H), 2.30–2.34, (m, 1H), 3.78 (s, 3H) 4.61–4,65 (q, 1H), 5.31–5.33 (d, $J = 10$ Hz, 1H),7.39–7.43 (t, $J = 7.2$ Hz, 1H), 7.56–7.60 (d, $J = 8$ Hz, 1H), 8.07–8.09 (d, $J = 7.6$ Hz, 1H), 8.23–8.24 ppm (d, $J = 6.8$ Hz, 1H); ^{13}C NMR ($CDCl_3$): $\delta = 18.1$, 19.3, 19.6, 33.25, 48.5, 52.8, 62.9, 124.2, 126.3, 127.2, 129.0, 132.4, 141.2, 168.2, 170.6, 173.5 ppm; ^{77}Se NMR ($CDCl_3$): $\delta = 900$ ppm; HRMS: *m/z*: calcd for $C_{17}H_{22}N_2O_4Se$: 406.2898 $[M+Na]^+$; found: 407.0486.

Synthesis of 24: A solution of NH_2-Val-Ala-Phe-COOMe (0.14 g, 0.39 mmol) in dry acetonitrile (5 mL) was added dropwise with stirring to a solution of 2-(chloroseleno)benzoyl chloride (0.10 g, 0.39 mmol) in dry acetonitrile (5 mL). After addition of triethylamine (0.10 mL 0.78 mmol), the reaction mixture was stirred at 25 °C for about 3 h, and then the solvent was evaporated under reduced pressure. Purification of the crude compound on a silica gel column using ethyl acetate and petroleum ether as eluent gave a yellow solid. Yield: 0.120 g (56 %); m.p. 126–128 °C; $[\alpha]_D^{25} = -20$ ($c = 0.2$ in MeOH); ^{1}H NMR ($CDCl_3$): $\delta = 0.85$–0.86 (d, $J =$ 4 Hz, 3H), 0.96–0.98, (d, $J = 8$ Hz, 4H), 1.66 (s, 1H), 2.10–2.30 (m, 1H), 3.12–3.13 (t, $J = 4$ Hz, 2H), 3.72 (s, 3H), 4.48–4.50, (t, $J = 8$ Hz, 1H), 4.86–4.89 (t, $J = 12$ Hz, 2H), 6.55–6.56 (d, $J = 4$ Hz, 1H), 7.04–7.06 (q, $J =$ 8 Hz, 3H), 7.24–7.31 (m, 3H), 7.41–7.43 (t, $J = 8$ Hz, 1H), 7.43–7.65 (m, 1H), 8.05 ppm (d, $J = 4$ Hz, 1H); ^{13}C NMR ($CDCl_3$): $\delta = 18.3$, 32.9, 38.4, 49.4, 52.9, 54.1, 63.1, 124.2, 126.5, 127.6, 129.1, 129.8, 132.5, 136.2, 140.9, 168.3, 170.5, 171.9, 172.2 ppm; ^{77}Se NMR ($CDCl_3$): $\delta = 894$ ppm; HRMS: *m/z*: calcd for $C_{25}H_{29}N_3O_5Se$: 553.4647 $[M+Na]^+$; found: 554.1170.

Measurement of GPx activity: The GPx activity of ebselen and all the peptide-based compounds was studied spectrophotometrically. The test mixture contained glutathione (2.0 mM), EDTA (1 mM), glutathione disulfide reductase (1.7 units mL^{-1}), and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH; 0.4 mM) in 0.1 M potassium phosphate buffer of pH 7.5. The samples (80 μM) were added to the test mixture at room temperature (25 °C), and the reaction was started by addition of H_2O_2, *t*BuOOH, or Cum-OOH (1.6 mM). The initial reduction rates (v_0) were calculated from the rate of NADPH oxidation at 340 nm. Each initial rate was measured at least 3 times and calculated from the first 5–10 % of the reaction by using the molar extinction coefficient (6.22 $mM^{-1}cm^{-1}$) for NADPH. For the peroxidase activity, the rates were corrected for a background reaction between peroxide and glutathione.

Single-crystal X-ray structure determination: X-ray crystallographic studies were carried out using a Bruker CCD diffractometer with graphite-

monochromatized $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å) controlled by a Pentium-based PC running the SMART software package.[25] Single crystals were mounted at room temperature on the ends of glass fibers, and data were collected at room temperature. The structures were solved by direct methods and refined using the SHELXTL software package.[26] All non-hydrogen atoms were refined anisotropically and hydrogen atoms were assigned idealized locations. Empirical absorption corrections were applied to all structures using SADABS.[27] The structures were solved by direct method (SIR-92) and refined by full-matrix least-squares procedure on F^2 for all reflections (SHELXL-97).[28]

Crystal data for 23: $C_{16}H_{20}N_2O_4Se_2$; $M_r = 921.7$; triclinic; space group $P1$; $a = 8.3945(18)$, $b = 9.470(2)$, $c = 12.166(3)$ Å; $\alpha = 85.632(4)$, $\beta = 75.894(3)$, $\gamma = 79.468(4)°$; $V = 921.7(3)$ Å^3; $Z = 2$; $\rho_{calcd} = 1.38$ g cm^{-3}; $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å); $T = 293(2)$ K; $R_1 = 0.069$, $wR_2 = 0.118$ ($I > 2\sigma(I)$); $R_1 = 0.045$, $wR_2 = 0.106$ (all data). CCDC-791202 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Synthesis of peroxynitrite (PN): Peroxynitrite was synthesized by following the literature method with minor modifications.[29] A solution of 30% (≈ 8.8 M) H_2O_2 (5.7 mL) was diluted to 50 mL with water, cooled to about 4 °C in an ice/water mixture, added to NaOH (5 N, 30 mL), and diethylene triamine pentaacetic acid (DTPA; 0.04 M, 5 mL) in NaOH (0.05 N) with gentle mixing, and then diluted to a total volume of 100 mL. The concentration of H_2O_2 in the final solution was 0.5 M; the pH ranged from 12.5 to 13.0. The buffered H_2O_2 was stirred vigorously with an equimolar amount of isoamyl nitrite (0.05 M or 6.7 mL) for 3–4 h at room temperature. The reaction was monitored by withdrawing aliquots at an interval of 15 or 30 min and assaying for peroxynitrite at 302 nm using a UV/Vis spectrophotometer. When the yield of peroxynitrite reached a maximum, the aqueous phase was washed with dichloromethane, chloroform, and hexane (3 × 100 mL) in a separating funnel to remove the contaminating isoamyl alcohol and isoamyl nitrite. The unreacted H_2O_2 was removed by passing the aqueous phase through a column filled with granular MnO_2 (25 g). The concentration of the stock solution of peroxynitrite was measured after 500 times dilution with 0.1 N NaOH solution and then assaying for peroxynitrite at 302 nm ($\varepsilon = 1670$ M^{-1} cm^{-1}) by the UV/Vis spectrophotometric method.

Inhibition of protein nitration of BSA: For bovine serum albumin (BSA), the nitration was performed by the addition of PN (1.2 mM) to BSA (100 mM) in 0.5 M phosphate buffer of pH 7.0 with DTPA (0.1 mM) at 20 °C. After the addition of PN, the final pH was maintained below 7.5. The reaction mixture was incubated for 20 min at 20 °C. Similarly, the reactions of BSA with PN were performed in the presence of different peptide-based ebselen analogues (133 μM) as inhibitors. Upon performing the reactions, the mixture was denatured by boiling at 100 °C for 5 min in the presence of sample loading dye and subjected to polyacrylamide gel electrophoresis and Western blot analyses.

Electrophoretic analysis: Gel was prepared with 10% and 15% polyacrylamide with 6% stacking gel for BSA. The gel was run in the running buffer of pH 8.3 with glycine and sodium dodecyl sulfate (SDS). After separating the proteins, the gel was analyzed by Western blot experiments. The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and the nonspecific binding sites were blocked by 5% nonfat skimmed milk in PBST (blocking solution) for 2 h. Then the membrane was probed with rabbit polyclonal primary antibody against nitrotyrosine (1:20000 dilutions) in blocking solution for 2 h followed by incubation with horseradish peroxidase conjugated donkey polyclonal anti-rabbit IgG (1:20000 dilutions) for another 2 h. The probed membrane was then washed three times with blocking solution with 0.1% Tween 20, and the immunoreactive protein was detected by luminol-enhanced chemiluminiscence (ECL, Amersham).

Inhibition of PN-mediated oxidation: PN-mediated oxidation of dihydrorhodamine 123 (DHR) was measured by fluorometric techniques by following the literature report with minor modifications. Fluorescence intensity was measured using a Horiba Jobin Yvon Fluromax-4 spectrometer with excitation and emission wavelengths of 500 and 526 nm, respectively. The stock solution for DHR in dimethylformamide was purged with nitrogen and stored at −20 °C. The working solution of DHR and peroxinitrite were kept in an ice bath. The assay mixture contained DHR (0.1 μM), peroxynitrite (0.69 μM) in 100 mM phosphate buffer of pH 7.5 with 100 μm DTPA and variable inhibitor concentrations. The fluorescence intensity from the reaction of DHR with peroxynitrite was set as 100%, and the intensity after the addition of various inhibitors was expressed as the percentage of the observed in the absences of inhibitors. The final fluorescence intensity was corrected for background reactions. The inhibition plots were obtained by using Origin 6.1 software that utilized sigmoidal curve fitting, and these plots were used for the calculation of IC_{50} values.

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