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PAPER

Mechanistic investigations on the efficient catalytic decomposition of peroxynitrite by ebselen analogues†

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In this study, ebselen and its analogues are shown to be catalysts for the decomposition of peroxynitrite (PN). This study suggests that the PN-scavenging ability of selenenyl amides can be enhanced by a suitable substitution at the phenyl ring in ebselen. Detailed mechanistic studies on the reactivity of ebselen and its analogues towards PN reveal that these compounds react directly with PN to generate highly unstable selenoxides that undergo a rapid hydrolysis to produce the corresponding seleninic acids. The selenoxides interact with nitrite more effectively than the corresponding seleninic acids to produce nitrate with the regeneration of the selenenyl amides. Therefore, the amount of nitrate formed in the reactions mainly depends on the stability of the selenoxides. Interestingly, substitution of an oxazoline moiety on the phenyl ring stabilizes the selenoxide, and therefore, enhances the isomerization of PN to nitrate.

Introduction

Peroxynitrite (PN, $ONOO^-$), a strong biological oxidizing and nitrating species, is generated *in vivo* by the diffusion-controlled reaction of superoxide anion radical ($O_2^{\bullet-}$) and nitric oxide ($NO^\bullet$).[1] PN is a major component of the reactive nitrogen species (RNS) and reactive oxygen species (ROS) systems which are involved in nitrosative and oxidative stress.[2] The formation of PN has been detected in activated macrophages[3] and endothelial cells.[4] It has been shown that the overproduction of PN induces DNA damage[5] and initiates lipid peroxidation in biomembranes or low-density lipoproteins.[6] In addition, it can oxidize protein/non-protein thiols[7] and is known to inactivate a variety of enzymes by tyrosine nitration, and thereby, hampers signal transduction process.[8,9] As the PN-mediated nitration of tyrosine residues as well as the oxidation of bio-components are harmful to biological systems in general and proteins and peptides having tyrosyl residues in particular, it is important to develop synthetic compounds that can effectively inhibit the PN-mediated nitration and oxidation reactions.

A number of biologically occurring small-molecules such as CO_2, ascorbate, methionine, cysteine *etc.* have been shown to react with PN and thus protect the biomolecules from PN-mediated damage.[10] The antioxidant mammalian selenoenzymes such as glutathione peroxidase (GPx),[11] selenoprotein P[12] and thioredoxin reductase (TrxR)[13] also provide enzymatic defense against PN. Furthermore, Sies *et al.* have shown that a number of sulfur- and selenium-containing compounds can act as efficient inhibitors of PN-mediated oxidation and nitration reactions.[10,14] The GPx-like properties of these compounds have been shown to be responsible for their PN scavenging effects. It is well known that the organoselenium compound ebselen (**1**) acts as an efficient scavenger of PN and provides defense against PN-mediated toxicity.[15] However, the mechanism by which ebselen scavenges PN is still not clear. It has been shown that ebselen reacts with PN to produce the corresponding selenoxide (**4**) as the only selenium-containing product.[15] In this reaction, PN is converted to nitrite (NO_2^-) and the amount of NO_2^- produced in the reaction depends on the amount of ebselen used in the reaction. A number of studies reveal that some amount of NO_2^- is converted to NO_3^-, indicating that ebselen may catalyze the isomerization of PN to NO_3^-.[15a] A few years ago, Musaev *et al.* reported, with the help of theoretical calculations, that ebselen and its analogues (**1–3**) cannot catalyze the isomerization of PN to NO_3^- (Fig. 1).[16] This conclusion was based on the assumption that the reactions of compounds **1–3** with PN produce the corresponding selenoxides **4–6**, which are incapable of transferring oxygen from selenium to NO_2^- and the energy barrier for the conversion of these selenoxides

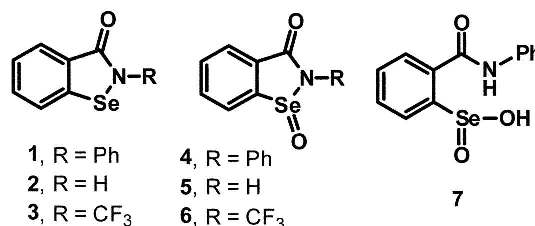

Fig. 1 Chemical structures of ebselen and related compounds.

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to the selenenyl amides is very high. Therefore, the dissociation of selenoxide-nitrite complex has been shown to be more favored than the transfer of oxygen from the selenoxide to nitrite.

Recently, we have shown that the reaction of ebselen with H_2O_2 does not produce the selenoxide (**4**) as previously reported, but that it produces the corresponding seleninic acid (**7**) as the only selenium-containing product.[17] As the reaction of ebselen with PN may also produce the seleninic acid **7**, a re-investigation of the mechanism is required for a better understanding of the antioxidant activity of ebselen. In this paper, we show that the reaction of ebselen with PN produces the seleninic acid **7** *via* selenoxide (**4**) and the rapid hydrolysis of the selenoxide **4** to the seleninic acid (**7**) is a deactivating pathway for the isomerization of PN to NO_3^-. We also show for the first time that any substituent capable of stabilizing the selenoxides can enhance the isomerization reaction by preventing the hydrolysis.

Results and discussion

Inhibition of PN-mediated reactions

Ebselen (**1**) and its analogues **8–13** used in the present study were synthesized by treating 2-chloro-selenobenzoyl chloride with appropriate primary amines (Fig. 2).[18] The diselenides **15–17** were synthesized in almost quantitative yields by treating the corresponding selenenyl amides (**1**, **11** and **12**) with triphenylphosphine. Compound **14** was synthesized by following a lithiation route as previously described.[19] To understand the inhibition potency of these selenium compounds towards PN-mediated nitration reactions, we have chosen some representative compounds and carried out the inhibition of PN-mediated nitration of bovine serum albumin (BSA). The nitration of tyrosine residues in BSA has been used extensively as a biomarker for oxidative and nitrosative stress.[20] The nitration was studied by immunoblotting method and the nitrated tyrosine residues in the protein were detected using an antibody against 3-nitro-L-tyrosine.

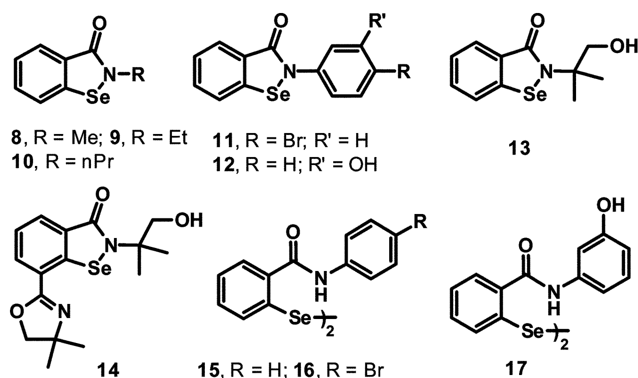

Fig. 2 Chemical structures of selenenyl amides (**8–14**) and diselenides (**15–17**).

As can be seen from Fig. 3, all the selenenyl amides and diselenides significantly inhibited the nitration reactions. The inhibitory activity of selenenyl amides **2**, **8** and **13** were found to be almost comparable to that of ebselen. This indicates that substitution at the nitrogen atom in selenenyl amides does not have a significant effect on the PN-mediated nitration of BSA. This is in contrast to the GPx-like antioxidant activities of these

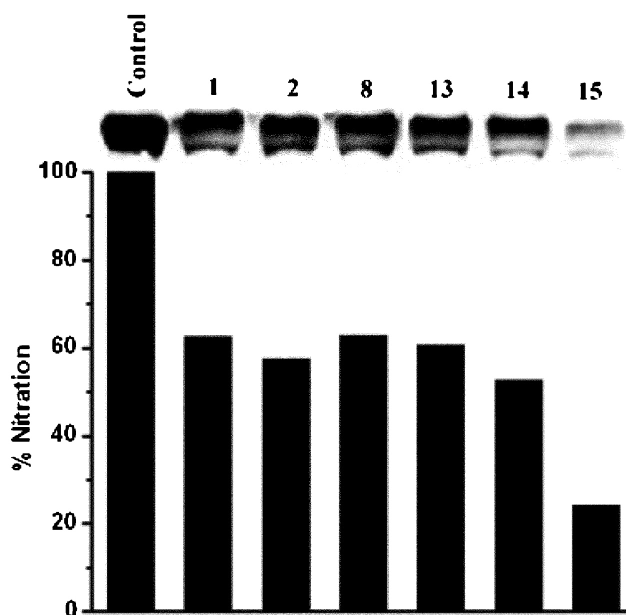

Fig. 3 Immunoblotting for the inhibition of PN-mediated nitration of BSA. BSA (133.3 μM) was incubated with PN (1.5 mM) and inhibitors (80.0 μM) in phosphate buffer (500 mM, pH: 6.9) at 20 °C for 7 min and then subjected to gel electrophoresis. The nitrated proteins were detected by immunoblotting using the antibody against 3-nitro-L-tyrosine.

ebselen analogues in which substitution at the nitrogen atom has a considerable effect on the GPx activity.[18] On the other hand, the 6-oxazoline substituted selenenyl amide **14** showed significantly higher inhibition activity than other selenenyl amides indicating some positive role of the additional oxazolyl group in **14** towards PN scavenging. The diselenide **15** has been found to be the strongest PN scavenger among all the compounds studied for the inhibition of PN-mediated nitration of BSA. As shown in Fig. 3, the diselenide **15** corresponding to ebselen is almost two times more active than ebselen. The higher activity of the diselenide can be ascribed to the availability of two selenium moieties in the diselenide unit for scavenging PN.

To understand the relative activities of all the selenenyl amides and diselenides towards PN-mediated reactions, we have carried out the PN-mediated nitration of free L-tyrosine. The nitration of L-tyrosine was followed by a reverse-phase HPLC method and the formation of 3-nitro-L-tyrosine was monitored at 275 nm. The IC_{50} values (concentration of the test compound required to inhibit 50% of the nitration) were calculated from the peak area of 3-nitro-L-tyrosine at various concentrations of selenium compounds (Table 1). In addition to the nitration, we have also studied the PN-mediated oxidation of dihydrorhodamine 123 (DHR) by fluorescence spectroscopy. In this assay, the IC_{50} values represent the concentrations of selenium compounds required to inhibit 50% of the conversion of DHR to rhodamine 123 (Table 1).

From Table 1, it is clear that ebselen and related compounds effectively inhibit both the PN-mediated nitration of free L-tyrosine and the oxidation of DHR. For example, at 1500 μM concentration of PN, ebselen showed an IC_{50} value of 63.1 ± 0.3 μM for the nitration reaction. Compound **2**, which lacks the phenyl group of ebselen, was found to be two times more active than ebselen. This is in contrast to the inhibition of PN-mediated

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Table 1 A comparison of IC_{50} values for the inhibition of PN-mediated nitration of L-tyrosine and oxidation of DHR by compounds **1–2** and **8–17**

Compound	IC_{50} value (μM)[a] Nitration of L-tyrosine[b]	Oxidation of DHR[c]
1, Ebselen	63.1 ± 0.3	0.8 ± 0.01
2	35.2 ± 0.1	49.2 ± 1.10
8	42.1 ± 0.1	1.3 ± 0.01
9	35.4 ± 0.1	1.2 ± 0.02
10	29.3 ± 0.3	1.6 ± 0.02
11	35.5 ± 0.3	1.5 ± 0.01
12	23.4 ± 0.3	0.7 ± 0.01
13	21.5 ± 0.4	1.8 ± 0.01
14	5.9 ± 0.1	15.5 ± 0.03
15	4.0 ± 0.2	2.8 ± 0.03
16	13.4 ± 0.3	16.3 ± 0.01
17	22.6 ± 0.1	1.2 ± 0.01

[a] The concentration of inhibitor required to inhibit 50% of the nitration or oxidation. [b] The test mixture with each concentration of inhibitor was incubated at 22 °C for 7 min for the nitration assay. Assay conditions: L-tyrosine (1.0 mM), PN (1.5 mM), inhibitors (variable) in phosphate buffer (100 mM, pH: 7.5). [c] Assay conditions: DHR (0.5 μM), PN (0.97 μM) and inhibitors (variable) with 100 μM DTPA in phosphate buffer (100 mM, pH: 7.5) at 22 °C. The fluorescence emission intensity due to rhodamine 123 was monitored immediately after the addition of PN at 526 nm.

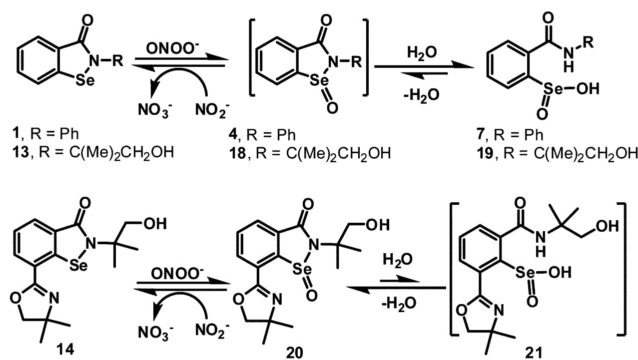
nitration of BSA in which the activities of ebselen and analogue **2** were comparable. Compounds **8–10** having alkyl substituents on the nitrogen were found to be more effective than ebselen. The activity of the bromo-substituted compound **11** was also found to be higher than that of ebselen. These observations indicate that the IC_{50} values for the nitration of L-tyrosine do not correlate well with the inhibition potencies toward PN-mediated nitration of BSA and also with the GPx-like antioxidant activities. However, compound **12** having an *m*-hydroxy substituent on the phenyl ring exhibited much better activity than ebselen, which is in agreement with the GPx activity.[18] Compound **13** having an aliphatic alcohol side chain also exhibited good PN-scavenging activity. Interestingly, compound **14** that contains an oxazoline moiety in the 6-position of the phenyl ring was found to be almost 10 times more active than ebselen. This compound was also more active than compound **13** that lacks the oxazoline group, which is in agreement with the inhibition of protein nitration. The diselenides **15–17** also exhibited good PN-scavenging activity, although the activity of compound **17** was almost identical to that of the corresponding selenenyl amide **12**. These observations indicate that the PN-scavenging activity of ebselen and related compounds not only depends on the nature of the selenium moiety, but also depends on the nature of the substrate used for nitration and the assay conditions. However, the inhibition of nitration by various ebselen analogues (Fig. 3) at 80 μM concentration of selenium compounds and 1500 μM concentration of PN indicates that these compounds effectively catalyze the isomerization of PN to NO_3^-.

A detailed analysis of the IC_{50} values (range from 4.0 ± 0.2 to 63.1 ± 0.1 μM) and the concentrations of PN used for the nitration reactions (1500 μM) indicates that these compounds inhibit the nitration reaction in a catalytic manner. For stoichiometric reactions, the IC_{50} values are expected to be in the mM range. As the concentration of 3-nitro-L-tyrosine increases steadily with an increase in the concentration of PN till 1500 μM (Figure S1, ESI†), ebselen at 64 μM concentration is expected to show ~1–2% inhibition. Similarly, an IC_{50} value of 4.0 ± 0.2 μM for compound **15** is remarkable as a concentration of ~500 μM is required for a stoichiometric inhibition. These observations indicate that all the selenenyl amides and diselenides catalytically scavenge PN. In other words, all these compounds catalyze the isomerization of PN to NO_3^- during the nitration of L-tyrosine.

In contrast to the catalytic nature of the inhibition of nitration reactions, the selenium compounds act as stoichiometric scavengers of PN in the oxidation of DHR (Table 1). The concentrations required for 50% inhibition by some selenium compounds (**2**, **14** and **16**) were found to be much higher than the PN concentration (0.97 μM). As the oxidation of DHR to rhodamine 123 by PN is extremely fast, the selenium compounds are unable to catalyze isomerization of PN to NO_3^-. Therefore, the oxidation of the selenium center in ebselen and related compounds alone is responsible for the observed IC_{50} values. It should be noted that the nitration reactions were relatively slow and there was sufficient time for the inhibitors to interact with PN. These observations indicate that the isomerization of PN to NO_3^- is important for slow reactions, but it does not contribute to the overall activity of selenium compounds for fast reactions.

Reaction of ebselen analogues with PN

To understand the reactivity of ebselen analogues towards PN, we have analyzed the reactions by ^{77}Se NMR spectroscopy. When ebselen was treated with an excess amount of PN (10–12 equiv), the peak at 964 ppm corresponding to ebselen disappeared completely and a new signal was observed at 1137 ppm. Similarly, the reaction of **13** with PN produced a new signal at 1136 ppm. In this reaction, the peak corresponding to the starting material (885 ppm) disappeared completely. The isolation and characterization of products from these reactions of ebselen and **13** with PN indicate the formation of the corresponding seleninic acids **7** and **19**, respectively. These observations suggest that the hydrolysis of selenoxides **4** and **18** leads to the formation of the corresponding seleninic acids **7** and **19**, respectively (Scheme 1). It should be noted that the formation of the seleninic acid **7** as the only selenium-containing compound has been observed previously in the reaction



Scheme 1 Reaction of ebselen (**1**) and its analogue **13** with PN to produce the corresponding seleninic acids **7** and **19**, respectively. A similar reaction of compound **14** with PN produces the corresponding selenoxide **20** as the only stable product.

of ebselen with H_2O_2.[17] In contrast, the reaction of oxazoline-based compound **14** with PN did not produce the seleninic acid **21**, but it produced the selenoxide **20** in nearly quantitative yield (Scheme 1).

The reaction of **14** with PN produced a new signal at 1102 ppm, which can be ascribed to the selenoxide **20**. The isolation and characterization of this compound by single crystal X-ray diffraction studies† indicate that the oxazoline nitrogen strongly interacts with the selenium center (rSe1 ··· N2 = 2.775 Å). This interaction is slightly weaker than that observed for the selenenyl amide **14**.[19a] However, the non-bonded Se ··· N interaction does not alter the hydrolysis of Se1–N1 bond in compound **20**. In the crystal structure, the selenoxide (–Se=O) moiety lies perpendicular to the selenazole ring plane (Fig. 4). The steric hindrance of the oxazoline moiety may also play an important role in the stabilization of selenoxide **20**. Although the Se1 ··· N2 interaction in compound **20** may activate the Se1–N1 bond towards hydrolysis, similar interaction in the seleninic acid (**21**) is expected to destabilize the compound. Therefore, a rapid elimination of a water molecule would regenerate the selenoxide (**20**). In the absence of such interactions in compounds **7** and **19**, the regeneration of selenoxides **4** and **18** appears to be very slow.

Fig. 4 ORTEP diagram of compound **20**. Displacement ellipsoids are drawn at the 50% probability level and hydrogen atoms are shown as small spheres of arbitrary radii.

Interestingly, when the solution of ebselen with PN was kept for a few hours, the regeneration of ebselen was observed. The intensity of the peak for ebselen increased with time, and after ~24 h around 50% of the seleninic acid **7** was converted to ebselen (Figure S48, ESI†). This indicates the possibility that the hydrolysis of selenoxide **4** is reversible and a considerable amount of seleninic acid **7** may undergo cyclization to produce the selenoxide **4** (Scheme 1). Similarly, compound **13** was regenerated almost quantitatively when the solution of seleninic acid **19** was kept for a long time. In solution, the selenoxides **4** and **18** react with NO_2^- to regenerate the selenenyl amides **1** and **13** with an elimination of NO_3^- anion and thereby catalyze the conversion of NO_2^- to NO_3^- (Scheme 1). Therefore, the stability of the selenoxides determines the conversion of NO_2^- to NO_3^-. In other words, the isomerization of PN to NO_3^- depends on the stability of the selenoxides produced in the reactions. The seleninic acids such as **7** and **19** may also catalyze the conversion of NO_2^- to NO_3^- by interacting directly with NO_2^-. In this case, the seleninic acids may generate the corresponding selenenic acids (Ar-SeOH) with the formation of NO_3^-. The selenenic acids thus produced may eliminate a water molecule to regenerate the corresponding selenenyl amides. However, our experimental results suggest that selenoxides are more efficient than the seleninic acid for the conversion of NO_2^- to NO_3^-. As the hydrolysis of selenoxides disfavors the isomerization process, it is important to prevent the hydrolysis to enhance the catalytic isomerization. In agreement with this, the selenoxide **20**, which does not undergo any hydrolysis, rapidly regenerates the selenenyl amide **14** under identical conditions. While ~50% regeneration of ebselen from the seleninic acid **7** was observed after ~24 h, a complete regeneration of selenenyl amide **14** from the corresponding selenoxide was observed (Figures S48 and S51, ESI†). Therefore, the isomerization of PN to NO_3^- is favored in the presence of compound **14**. This is also reflected in the IC_{50} values obtained for this compound in the nitration reactions (Table 1, Fig. 3).

To further confirm the isomerization of PN to NO_3^- in the presence of selenium compounds, we have determined the concentration of NO_3^- after keeping the reaction mixture of PN and selenium compounds for 30 min in the assay buffer. The amount of NO_3^- formed in the reaction was then determined by measuring the concentration of nitro-resorcinol in the presence of concentrated H_2SO_4 by following the literature procedure.[21] In the presence of concentrated H_2SO_4, the NO_3^- produced by the isomerization of PN nitrates resorcinol and the measurement of the concentration of nitro-resorcinol at 505 nm by UV–vis spectrophotometry allowed the quantification of NO_3^- in the solution. A significant enhancement in the absorbance due to nitro-resorcinol was observed when resorcinol was treated with PN in the presence of selenium compounds (Table S25, ESI†). Although there is no clear correlation between the amount of nitro-resorcinol formed and the IC_{50} values for the nitration reactions, these observations suggest that the catalytic PN-scavenging activity of selenium compounds is mainly due to the isomerization of PN to NO_3^-.

To confirm that the selenoxide **20** is the main species that catalyzes the isomerization of PN to NO_3^- by interacting with the eliminated NO_2^- ion from PN, we have carried out a series of experiments with the isolated selenoxide **20** and $NaNO_2$ in the assay buffer. Interestingly, similarly to the reaction of selenenyl amide **14** with PN, the reaction of pure selenoxide **20** with $NaNO_2$ produced nitro-resorcinol under identical experimental conditions. This indicates the direct reactivity of the selenoxide **20** towards NO_2^- leading to the formation of NO_3^-. To understand whether the conversion of NO_2^- to NO_3^- by selenoxide **20** is catalytic or not, the formation of nitro-resorcinol was measured with an increasing concentration of selenoxide **20**. An enhancement in the absorbance due to nitro-resorcinol was observed with increasing concentration of selenoxide **20** (Table S26 and Figure S26, ESI†), indicating the catalytic nature of the isomerization of PN to NO_3^-.

To analyze the catalytic intermediates involved in the isomerization process, we have studied the reactivity of pure selenoxide **20** with $NaNO_2$ and/or PN. The intermediates and products of the reactions were analyzed by ^{77}Se NMR spectroscopic and ESI-MS spectrometric techniques. Treatment of **20** with $NaNO_2$ produced a new signal at 1115 ppm in the ^{77}Se NMR experiment along with the peak for the selenoxide at 1100 ppm. The new signal can be ascribed to the corresponding selenoxide–nitrite complex **24**. Formation of complex **24** was further confirmed by ESI-MS

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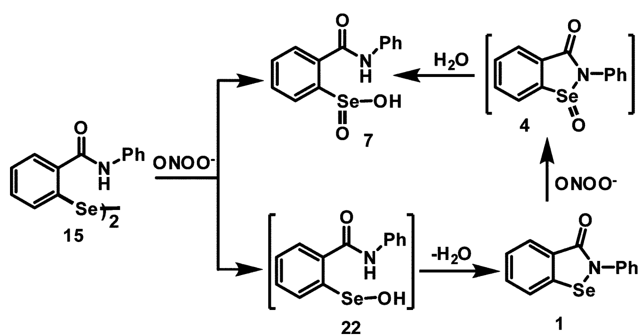
spectrometric analyses (Figures S55 and S56, ESI†). The reaction mixture slowly regenerated the selenenyl amide **14** upon keeping the solution for a longer time. Interestingly, the peak at 1115 ppm due to complex **24** disappeared completely upon addition of PN. This observation indicates that the selenoxide–nitrite complex **24** reacts with PN to regenerate the selenoxide **20**, which can further react with NO_2^- in a catalytic fashion. As the regeneration of selenenyl amide **14** is observed only upon keeping the reaction mixture for at least 24 h (Figure S59, ESI†), the selenenyl amide **14** may lie off the main catalytic cycle.

Although a number of ebselen analogues and related monoselenides have been studied as PN-scavengers,[10,14] the reactivity of the corresponding diselenides towards PN has not been studied. Therefore, we treated the diselenide **15** with PN and the reaction was followed by ^{77}Se NMR spectroscopy. In this reaction, a peak at 1133 ppm corresponding to the seleninic acid **7** was obtained along with a peak for ebselen. When a large excess of PN (~10–15 equiv) was used, a quantitative conversion of diselenide **15** to the seleninic acid **7** was observed (Figures S51 and S52, ESI†). This indicates that the reaction of diselenides with PN produces a mixture of selenenic and seleninic acids (**22** and **7**) as shown in Scheme 2. The formation of ebselen in the reaction of diselenide with PN is due to a rapid cyclization of **22** (Scheme 2). In the presence of an excess amount of PN, ebselen undergoes further reaction with PN to give the seleninic acid **7** *via* the formation of selenoxide **4**. As these processes utilize a greater amount of PN, the diselenide **15** was found to be more active than ebselen (Table 1). The generation of ebselen in the reaction of **15** with H_2O_2 has been reported earlier.[17]

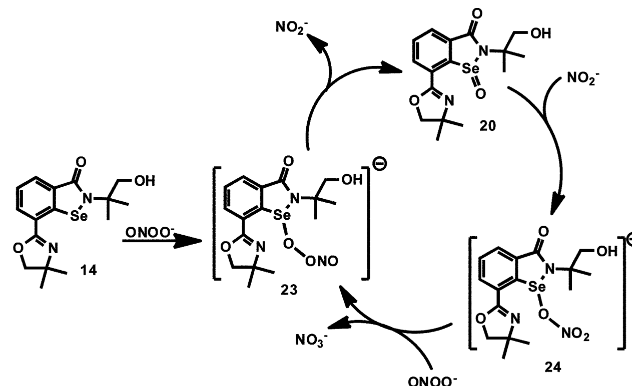


Scheme 2 The proposed reaction pathways of ebselen diselenide **15** with PN to produce the corresponding seleninic acid **7** as the main oxidation product.

Although ebselen analogues catalyze the formation of NO_3^- from NO_2^-, the isomerization reactions do not play an important role in the inhibition of PN-mediated oxidation of DHR. As the oxidation of DHR by PN is extremely fast, the rapid oxidation of the selenium centers in the selenenyl amides and diselenides alone is responsible for the inhibition. The lower activity of compound **14** as compared to that of compound **13** in the inhibition of PN-mediated oxidation of DHR is due to the presence of a strong Se···N interaction in compound **14**, which significantly decreases the reactivity of selenium towards PN. Therefore, the oxazoline substituent plays important roles in the nitration reactions than the oxidation of DHR.

Catalytic isomerization of PN

Based on our experimental evidences, a catalytic mechanism for the isomerization of PN to NO_3^- by compound **14** can be proposed (Scheme 3). According to this mechanism, the selenenyl amide **14** reacts with PN to produce the corresponding selenoxide **20** *via* the selenenyl amide-(O-ONO) complex **23**. The oxidation of the selenium center in compound **14** is less favored as compared to that of ebselen and **13**. This is due to the presence of a Se···N interaction that considerably reduces the reactivity of selenium towards PN. The elimination of NO_2^- from complex **23** produces the selenoxide **20**. The Se···N interaction is retained in the selenoxide structure and is, therefore, responsible for the stability of selenoxide **20**. The next step is the interaction between selenoxide **20** and NO_2^- to produce the selenoxide–NO_2^- complex **24**. In the presence of an excess amount of PN, complex **24** reacts further with PN to regenerate the selenoxide **20** with the elimination of NO_2^- and NO_3^-. When PN is depleted in the reaction mixture, a slower transfer of oxygen from selenium to NO_2^- in complex **24** leads to the elimination of NO_3^- with the regeneration of compound **14** (Scheme 3). It should be noted that the first half of the reaction is probably responsible for the inhibition of PN-mediated oxidation of DHR and the second half of the reaction *i.e.*, NO_3^- formation, is important for the inhibition of PN-mediated nitration reactions. The presence of the oxazoline moiety at the 6-position of the phenyl ring favors the formation of NO_3^-.



Scheme 3 Proposed mechanism for the catalytic isomerization of PN to NO_3^- by the 6-oxazoline-substituted selenenyl amide **14**.

The catalytic mechanism shown in Scheme 3 is similar to the one proposed for an iron–porphyrin complex.[21] In this case, the Fe^{III}–L cationic complex reacts with PN to produce an L–Fe^{III}–O–ONO species, which undergoes homolysis to produce an O=Fe^{IV}–L intermediate. The recombination of this intermediate with nitrite generates a L–Fe^{III}–O–NO_2 complex, which upon elimination of nitrate regenerates the Fe^{III}–L cationic complex. Therefore, the two electron redox processes at the selenium centers in the selenenyl amides appear to be quite similar to those of the Fe^{III}–L cationic complex.

Conclusions

In this study, we show that the reactions of selenenyl amides with peroxynitrite (PN) produce the corresponding seleninic acids *via* selenoxides. Hydrolysis of selenoxides to the seleninic acids is a

deactivating pathway, which is probably due to the lower reactivity of seleninic acids towards NO_2^- as compared to selenoxides. This study also reveals that the introduction of an oxazoline substituent at the 6-position of the phenyl ring in ebselen derivative can prevent the hydrolysis by stabilizing the selenoxide. The stabilization of selenoxide leads to an efficient catalytic isomerization of PN to nitrate. The overall activity of ebselen and its analogues on PN-mediated reactions depends on the nature of substrate used for the oxidation or nitration reaction. While the isomerization plays an important role in the inhibition of PN-mediated tyrosine nitration, such isomerization does not have any effect on PN-mediated oxidation of dihydrorhodamine 123. These observations indicate that the selenium compounds that can enhance the PN $\leftrightarrow$ nitrate isomerization would provide a better protection against PN-mediated reactions.

Experimental section

General procedure

Compounds **2**, **8–12**,[18] **13**,[17] **14**[19a] and **15**[17] were synthesized by following literature methods. ^{1}H (400 MHz), ^{13}C (100 MHz) and ^{77}Se (76 MHz) NMR spectra were obtained on a Bruker 400 MHz NMR spectrometer. Chemical shifts are cited with respect to Me_4Si (^{1}H and ^{13}C) as internal, and Me_2Se (^{77}Se) as external standards. Mass spectral studies were carried out on a Bruker Daltonics Esquire 6000plus mass spectrometer with ESI-MS mode analysis. UV–vis spectroscopic experiments were carried out on a Varian CARY 300 Bio spectrophotometer. Peroxynitrite (PN) was synthesized in the laboratory following the literature procedure.[20f,22]

Synthesis of compound 7

To a stirred solution of ebselen (0.02 g, 0.073 mmol) in methanol was added an alkaline solution of peroxynitrite in excess amount (10–12 equiv) and the reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated to obtain crude solid which was purified by a reverse phase C_{18} column using water and methanol as eluent. The solvent was evaporated *in vacuo* to obtain the pure compound **7** as white solid. Yield: 9.4 mg (42%). ^{1}H NMR (MeOH-d$_4$) δ (ppm): 7.12 (t, 1H, $J = 8.0$ Hz), 7.33 (t, 2H, $J = 8.0$ Hz), 7.57 (t, 1H, $J = 8.0$ Hz), 7.71 (d, 3H, $J = 8.0$ Hz), 7.87 (d, 1H, $J = 8.0$ Hz), 8.33 (d, 1H, $J = 8.0$ Hz). ^{13}C NMR (MeOH-d$_4$) δ (ppm): 123.7, 127.1, 127.8, 130.1, 131.2, 132.7, 134.3, 137.1, 141.2, 156.2, 169.5. ^{77}Se NMR (MeOH-d$_4$) δ (ppm): 1144. ESI-MS *m/z* calcd. for $C_{13}H_{11}NO_3Se$ [M – H]$^-$ 307.98; found: 307.70.

Synthesis of compounds 16–17

To a CH_2Cl_2 solution of the ebselen derivatives (**11** and **12**) (0.10 mmol), triphenylphosphine (0.20 mmol) was added and the reaction mixture was stirred at room temperature for 3 h to obtain a white precipitate of the corresponding diselenide compounds. The solvent was evaporated and the solid was washed 3–4 times with petroleum ether and CH_2Cl_2 mixture to remove the unreacted triphenylphosphine and its by-products. The residue was dried under vacuum to obtain the products as white powder in quantitative yields.

Compound **16**: ^{1}H NMR (DMSO-d$_6$) δ (ppm): 7.12–7.20 (m, 2H), 7.31 (d, 2H, $J = 8.0$ Hz), 7.45 (d, 2H, $J = 8.0$ Hz), 7.50 (d, 1H, $J = 8.0$ Hz), 7.65 (d, 1H, $J = 8.0$ Hz), 10.44 (s, 1H). ^{13}C NMR (DMSO-d$_6$) δ (ppm): 117.0, 123.5, 127.6, 129.5, 131.2, 132.5, 132.8, 133.1, 134.4, 138.7, 167.4. ^{77}Se NMR (DMSO-d$_6$) δ (ppm): 441. ESI-MS *m/z* calcd. for $C_{26}H_{19}Br_2N_2O_2Se_2$ [M+H]$^+$ 708.81; found: 708.78.

Compound **17**: ^{1}H NMR (DMSO-d$_6$) δ (ppm): 6.52 (d, 3H, $J = 8.0$ Hz), 7.07–7.14 (m, 2H), 7.27 (s, 1H), 7.33–7.41 (m, 2H), 7.72 (d, 1H, $J = 8.0$ Hz), 7.84 (d, 1H, $J = 8.0$ Hz), 10.42 (s, 1H). ^{13}C NMR (DMSO-d$_6$) δ (ppm): 108.6, 112.4, 127.6, 129.4, 130.4, 131.1, 132.7, 132.9, 134.9, 140.4, 158.3, 167.3. ^{77}Se NMR (DMSO-d$_6$) δ (ppm): 443. ESI-MS *m/z* calcd. for $C_{26}H_{21}N_2O_4Se_2$ [M+H]$^+$ 584.98; found: 584.81.

Synthesis of compound 19

To a stirred solution of **13** (0.025 g, 0.092 mmol) in methanol was added an alkaline solution of peroxynitrite in excess amount (10–12 equiv) and the reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated to obtain crude solid which was purified by a reverse phase C_{18} column using water and methanol as eluent. The solvent was evaporated *in vacuo* to obtain the pure compound **19** as white solid. Yield: 9.7 mg (37%). ^{1}H NMR (MeOH-d$_4$) δ (ppm): 1.29 (s, 6H), 3.66 (s, 2H), 7.40 (t, 1H, $J = 8.0$ Hz), 7.51–7.61 (m, 2H), 7.81 (s, 1H), 8.10 (d, 1H, $J = 8.0$ Hz) ^{13}C NMR (MeOH-d$_4$) δ (ppm): 23.4, 56.3, 67.3, 125.2, 127.7, 130.3, 131.1, 135.9, 152.3, 169.3. ^{77}Se NMR (MeOH-d$_4$) δ (ppm): 1145. ESI-MS *m/z* calcd. for $C_{11}H_{15}NO_4Se$ [M+2H]$^{2+}$ 307.01; found: 307.60.

Synthesis of compound 20

To a solution of selenazole **14** (0.10 g, 0.272 mmol) in MeOH was added an alkaline solution of an excess amount of PN (10–12 equiv). The reaction mixture was stirred for 12 h at room temperature and then extracted with dichloromethane (3 × 50 mL) to remove the unreacted starting materials and other non-polar impurities. The aqueous solution was then neutralized with 1 N HCl and the solvent was evaporated by lyophilization. The white solid obtained was dissolved in a minimum amount of methanol to exclude the excess amount of NaCl. The solution was filtered and the solvent was evaporated to obtain the product **20** as white powder. Yield: 44 mg (43%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.41 (s, 3H), 1.47 (s, 3H), 1.69 (s, 3H), 1.73 (s, 3H), 3.58 (d, 1H, $J = 11.6$ Hz), 4.00 (d, 1H, $J = 11.6$ Hz), 4.28– 4.44 (m, 2H), 7.75 (t, 1H, $J = 7.2$ Hz), 7.97 (d, 1H, $J = 7.6$ Hz), 8.02 (d, 1H, $J = 7.6$ Hz). ^{13}C NMR ($CDCl_3$) δ (ppm): 25.2, 26.3, 28.0, 28.5, 63.3, 67.8, 68.2, 81.0, 125.3, 130.2, 131.2, 133.4, 134.0, 143.8, 160.2, 168.3. ^{77}Se NMR ($CDCl_3$) δ (ppm): 1102. ESI-MS *m/z* calcd. for $C_{16}H_{20}N_2O_4Se$ [M+Na]$^+$: 407.0486; found: 407.0506.

PN-mediated nitration of L-tyrosine

High performance liquid chromatography (HPLC) experiments were carried out on a Waters Alliance System (Milford, MA) consisting of a 2695 separation module, a 2996 photodiode-array detector and a fraction collector. The assays were performed in 1.8 mL sample vials and a built-in autosampler was used for sample injection. The Alliance HPLC System was controlled with

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EMPOWER software (Waters Corporation, Milford, MA). The nitration assay of L-tyrosine were analyzed by reverse-phase HPLC (Lichrosphere-100 RP-C18 60A 5 μm 250 × 4.6 mm) using isocratic elution with 100% water containing 0.1% TFA and methanol (8 : 2). In the PN-mediated nitration of L-tyrosine, we employed a mixture containing L-tyrosine (1 mM) and PN (1.5 mM) in sodium phosphate buffer (100 mM) of pH 7.5 without and with increasing concentration of the inhibitor. It was then incubated for 7 min before injection. The formation of 3-nitro-L-tyrosine was monitored at the wavelength of 275 nm. The inhibition plots were obtained using Origin 6.1 software utilizing sigmoidal curve fitting and these plots were used for the determination of IC_{50} values.

Inhibition of protein nitration

For bovine serum albumin (BSA), the nitration was performed by the addition of PN (1.5 mM) to BSA (133.3 μM) in 0.5 M phosphate buffer of pH 6.9 at 20 °C. After the addition of PN, the final pH was maintained below 7.5. The reaction mixture was incubated for 7 min at 20 °C. Similarly, the reactions of BSA with PN were performed in the presence of different selenium compounds (80 μM) as inhibitors. After 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 immunoblot analyses.

Electrophoretic analysis

Gel was prepared with 10% polyacrylamide with 6% stacking gel for BSA. The gel was run in the running buffer of pH 8.3 with glycine and SDS. After separating the proteins, the gel was analyzed by immunoblotting experiments. The proteins were transferred to a PVDF membrane and the non-specific binding sites were blocked by 5% non-fat skimmed milk in PBST (blocking solution) for 1 h. Then the membrane was probed with rabbit polyclonal primary antibody against 3-nitro-tyrosine (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 1 h. The probed membrane was washed three times with blocking solution with 0.1% Tween 20 after each of the above steps and the immunoreactive protein was then detected by luminol-enhanced chemiluminescence (ECL, Amersham).

PN-mediated oxidation assay

PN-mediated oxidation of dihydrorhodamine 123 (DHR) was studied using fluorescence spectroscopy. Fluorescence intensity was measured using Horiba Jobin Yvon, USA Fluoromax-4 Luminescence spectrometer with excitation and emission wavelengths of 500 nm and 526 nm, respectively. The stock solution of DHR in dimethylformamide was purged with nitrogen and stored at −20 °C. The working solution of DHR and PN were kept on ice bath. The assay mixture contained DHR (0.50 μM), PN (0.97 μM) in 100 mM phosphate buffer of pH 7.4 with 100 μM DTPA and variable inhibitor concentrations. The fluorescence intensity from the reaction of DHR with PN was set as 100% and the intensity after the addition of various inhibitors was expressed as the percentage of the intensity observed in the absence of inhibitors. The final fluorescence intensities were corrected for background reactions. The inhibition plots were obtained using Origin 6.1 software utilizing sigmoidal curve fitting and these plots were used for the determination of IC_{50} values.

Determination of nitrate (NO_3^-) concentration

The isomerization of PN to NO_3^- was determined qualitatively by measuring the formation of nitro-resorcinol by following the literature procedure with minor modifications.[23] The mixture of PN (0.26 mM) and selenium compounds (73 μM) was incubated for 30 min in the assay buffer to ensure the isomerization of PN to NO_3^- by the selenium compounds. The nitration of resorcinol (11.4 mM) by the NO_3^- produced was initiated by the addition of concentrated H_2SO_4 (1 mL) in water. The resultant mixture was further incubated in the dark for another 30 min. The formation of nitro-resorcinol was measured by UV–vis spectrophotometric method at the wavelength of 505 nm with the molar extinction coefficient (ε) of 1.7×10^4 M^{-1} cm^{-1} due to nitro-resorcinol. The control reaction was carried out under identical experimental conditions but in the absence of selenium compounds.

X-Ray crystallography

Single crystal X-ray diffraction data was collected on a Bruker AXS SMART APEX CCD diffractometer. The X-ray generator was operated at 50 KV and 35 mA using Mo-Kα radiation (λ = 0.71073 Å). The data was collected using SMART software package.[24] The data were reduced by SAINTPLUS,[24] an empirical absorption correction was applied using the package SADABS[25] and XPREP[24] was used to determine the space group. The crystal structure was solved by direct methods using SIR92[26] and refined by full-matrix least-squares method using SHELXL97.[27] All non-hydrogen atoms were refined anisotropically and hydrogen atoms were assigned at idealized locations.

Crystal data for compound 20[28]

$C_{16}H_{20}N_2O_4Se$; M_r = 383.30; triclinic; space group: $P\bar{1}$; a = 6.9670(14) Å; b = 10.517(2) Å; c = 11.826(2) Å; α = 87.388(3)°; β = 73.485(3)°; γ = 85.092(3)°; V = 827.5(3) $Å^3$; ρ_c = 1.538 g m^{-3}; Z = 2; μ Mo-Kα radiation (λ = 0.71370 Å); T = 291(2)K; R_{int}: 0.024; R (observed data): R_1 = 0.040; wR_2 = 0.108; R (all data): R_1 = 0.045; wR_2 = 0.112; GOF = 1.078; $\Delta\rho_{min}$ and $\Delta\rho_{max}$ ($eÅ^{-3}$): −0.407 and 1.361.

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28 CCDC-765980 (**20**) 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.

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