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Antioxidant Activity of the Anti-Inflammatory Compound Ebselen: A Reversible Cyclization Pathway via Selenenic and Seleninic Acid Intermediates

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Abstract: A revised mechanism that accounts for the glutathione peroxidase (GPx)-like catalytic activity of the organoselenium compound ebselen is described. It is shown that the reaction of ebselen with H_2O_2 yields seleninic acid as the only oxidized product. The X-ray crystal structure of the seleninic acid shows that the selenium atom is involved in a noncovalent interaction with the carbonyl oxygen atom. In the presence of excess thiol, the Se–N bond in ebselen is readily cleaved by the thiol to produce the corresponding selenenyl sulfide. The selenenyl sulfide thus produced undergoes a disproportionation in the presence of H_2O_2 to produce the diselenide, which upon reaction with H_2O_2, produces a mixture of selenenic and seleninic acids. The addition of thiol to the mixture containing selenenic and seleninic acids leads to the formation of the selenenyl sulfide. When the concentration of the thiol is relatively low in the reaction mixture, the selenenic acid undergoes a rapid cyclization to produce ebselen. The seleninic acid, on the other hand, reacts with the diselenide to produce ebselen as the final product. DFT calculations show that the cyclization of selenenic acids to the corresponding selenenyl amides is more favored than that of sulfenic acids to the corresponding sulfenyl amides. This indicates that the regeneration of ebselen under a variety of conditions protects the selenium moiety from irreversible inactivation, which may be responsible for the biological activities of ebselen.

Keywords: antioxidant activity · ebselen · gluthatione peroxidase mimics · selenium · selenoenzymes

Introduction

Ebselen (**1**, 2-phenyl-1, 2-benzisoselenazol-3(2*H*)one), a lipid-soluble organoselenium compound undergoing phase III clinical trials for a number of disease states, such as stroke and hearing loss, exhibits numerous biological activities both in vitro and in vivo systems.[1] Ebselen is an excellent scavenger of reactive oxygen species (ROS) such as peroxynitrite (PN) and the rate of the reaction between ebselen and PN is about three orders of magnitude higher than that of naturally occurring small molecules, such as ascorbate, cysteine, and methionine.[2] Ebselen and related derivatives effectively protect against lipid peroxidation induced by transition metals.[1a,3] Furthermore, ebselen has been shown to inhibit a number of enzymes, which include nitric oxide synthase (NOS),[4,5] the enzymes involved in inflammatory diseases, such as lipoxygenase (LOX) and cycloxygenase (COX),[6] NADPH oxidase,[7] protein kinase C (PKC),[7] glutathione-s-transferase (GST),[8] cytochrome P-450[9] and b-5 reductases,[10] H^+/K^+-ATPase,[11] the cysteine proteases, such as papain,[8] and prostaglandin H synthetase.[12] Ebselen also exhibits significant antitumor and immunomodulating activities and has been suggested to have a potential to protect ROS-mediated brain damage.[13] Interestingly, most of the biological activities of ebselen have been associated with its ability to mimic the enzymatic properties of glutathione peroxidase (GPx), a mammalian selenoenzyme that protects various organisms from oxidative damage by catalyzing the reduction of harmful hydroperoxides in the presence of glutathione (GSH) (Scheme 1) or other thiol cofactor systems.[14] In addition to the GPx activity, ebselen has been shown to enhance the antioxidant activity of the human thioredoxin (Trx) system by acting as a substrate for the selenium-containing enzyme, thioredoxin reductase (TrxR).[15] These studies indicate that the Trx system may serve as a better cofactor than GSH for the antioxidant activity of ebselen. Recent evidence also suggests that the dehydroascorbate reductase and thiol transferase (glutaredoxin) activities

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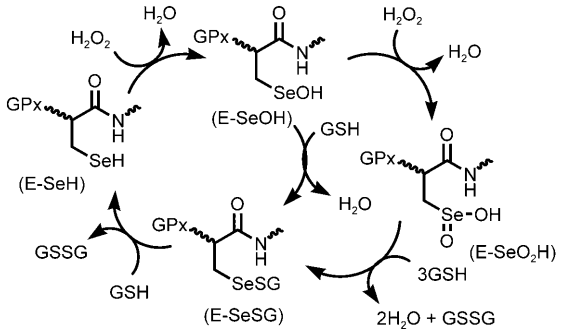
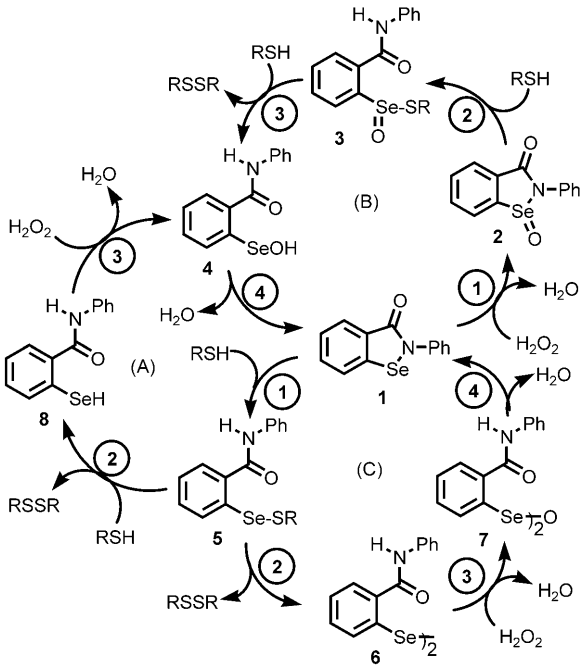


Scheme 1. The catalytic cycle of GPx involving selenol (E–SeH), selenenic acid (E–SeOH), and selenenyl sulfide (E–SeSG) as the key intermediates. At higher peroxide concentrations, the selenenic acid is oxidized to seleninic acid (E–SeO_2H), which may lie off the main catalytic pathway.

are important for the antioxidant and anti-inflammatory properties of ebselen.[16]

Although ebselen has been shown to be one of the major GPx mimics, the mechanism by which ebselen exerts its GPx activity and the importance of the cyclic selenazole moiety are still not clear. Recent studies have shown that ebselen is a relatively inefficient catalyst in the reduction of hydroperoxides due to a deactivation pathway involving thiol exchange reactions at the selenium center in the selenenyl sulfide intermediates, which hampers the regeneration of the selenol.[17] Furthermore, the selenol moiety in **8** is a relatively poor nucleophile due to the noncovalent Se···O interactions.[17b] Therefore, ebselen does not seem to follow the classical GPx catalytic cycle involving the selenol, selenenic acid, and selenenyl sulfide intermediates (Scheme 2, cycle A),[17a] although ebselen exhibits significant GPx activity when GSH is used as the cosubstrate[17b,c] and the formation of selenol has been observed during the reaction of ebselen with thiols.[18] According to the literature data,[19–22] the first step in the catalytic mechanism of ebselen is the formation of a selenoxide (cycle B) or a selenenyl sulfide (cycle A) depending upon the relative concentration of thiol and peroxide. It has been proposed that ebselen follows cycle B at higher peroxide concentrations, but under physiologically relevant conditions, that is, in the presence of an excess of thiol, cycle C may be operative.[19,20] However, none of the intermediates other than the selenenyl sulfides have been unambiguously confirmed.

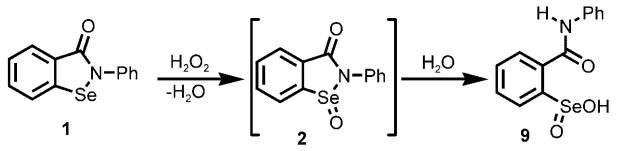


Scheme 2. Summary of possible key intermediates proposed for the catalytic cycle of ebselen.

In view of the complications associated with the catalytic mechanism of ebselen, we have studied the reactivity of ebselen with peroxides and thiols. In this paper, we report the first structural evidence that the seleninic acid **9**, which has never been proposed as an intermediate in the catalytic mechanism of ebselen, is the only stable and isolable product in the reaction of ebselen with peroxides. We also demonstrate that the disproportionation of the selenenyl sulfide to produce the corresponding diselenide (Scheme 2, cycle C, step 2) is more important than the generation of selenol (cycle A, step 2). In addition, we propose that the regeneration of ebselen by cyclization of the selenenic acid under a variety of conditions may be important for the biological activity of ebselen.

Results and Discussion

There are numerous reports in the literature indicating that the reactions of ebselen (**1**) with peroxides and peroxynitrite ($ONOO^-$) always produce the corresponding selenoxide **2** as the major oxidized product.[1h–j,19,22–24] Therefore, our first objective in this study was to confirm the formation of selenoxide **2**. For this purpose, we have extensively used ^{77}Se NMR spectroscopy. When ebselen was treated with H_2O_2 in $CDCl_3$, the ^{77}Se NMR signal at $\delta = 960$ ppm due to ebselen disappeared completely and the reaction produced a new signal at $\delta = 1130$ ppm.[25] The signal at $\delta = 1130$ ppm was shifted to $\delta = 1145$ ppm upon addition of 100 μL of acetonitrile. However, further analysis of the product obtained from this reaction suggested that the oxidized product is not the expected selenoxide **2**, but it is the corresponding seleninic acid **9**. The identity of this compound was unambiguously established by several methods including single-crystal X-ray diffraction studies. This indicates that the oxidation of ebselen by H_2O_2 produces the selenoxide **2**, which undergoes a facile hydrolysis to produce the seleninic acid **9** in quantitative yield (Scheme 3).[26] To understand whether the water present in the H_2O_2 solution is responsible for the hydrolysis, we carried out the oxidation with a solid H_2O_2/urea complex in anhydrous $CDCl_3$. However, this reaction also produced the seleninic acid **9**, which indicates that the water produced during the oxidation of ebselen by H_2O_2 is sufficient to hydrolyze the Se–N bond.



Scheme 3. The reaction ebselen (**1**) with H_2O_2 produces the seleninic acid **9** via the formation of a hydrolytically unstable selenoxide **2**.

The crystal structure of **9** (Figure 1a) shows an unusual Se···O nonbonded interaction between the tetravalent selenium atom and the amide carbonyl moiety. The Se···O distance (2.46 Å) is much shorter than the sum of the van der Waals radii of the selenium and oxygen atoms (3.35 Å). Because of this interaction, the Se–OH group adapts a position *trans* to the Se···O interaction, leading to an almost linear arrangement of the O···Se–O moiety (< O···Se–O: 168.2°). Although Se···O nonbonded interactions between a divalent selenium atom and other heteroatoms are very common,[27] to the best of our knowledge, there is no report on the Se···O nonbonded interaction involving tetravalent selenium and oxygen atoms. The crystal structures of the cytosolic GPx (cGPx, Figure 1b)[28] and human plasma GPx (pGPx)[29] indicate that the selenocysteine (Sec) residue at the active site exists as a seleninic acid (E–SeO_2H) in the purified form. Although the seleninic acid form of the enzyme is believed to lie off the main catalytic pathway, this species may be relevant as the reduction of this species with an excess amount of thiol readily affords the catalytically active selenol (E–SeH).[30] Interestingly, the amino acid residues glutamine (Gln) and tryptophan (Trp), which are proposed to be involved in a catalytic triad with the selenocysteine residue in both cGPx and pGPx, are conserved in the entire GPx superfamily; this indicates that these amino acid residues play functional roles in catalysis. The crystal structures show that the selenium atom in the seleninic acid form of GPx is located within hydrogen bonding distances to Gln and Trp (Se···N(Gln80): 3.3, Se···N(Trp158): 3.6 Å in cGPx enzyme;[28] Se···N(Gln79): 3.5, Se···N(Trp153): 3.6 Å in pGPx enzyme).[29]

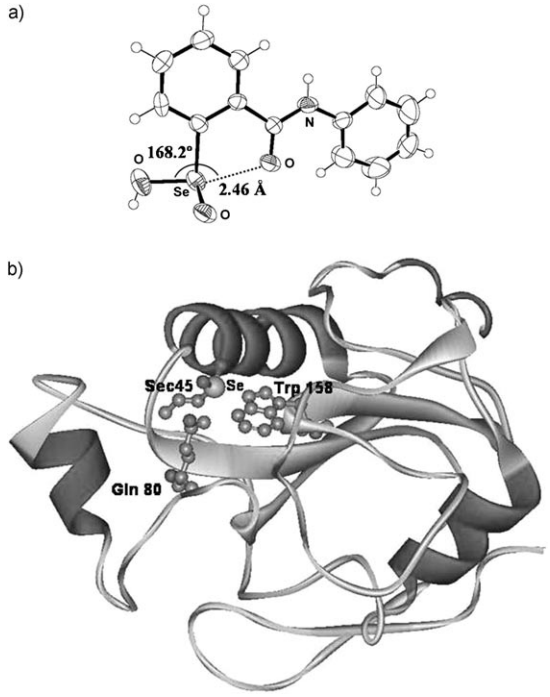
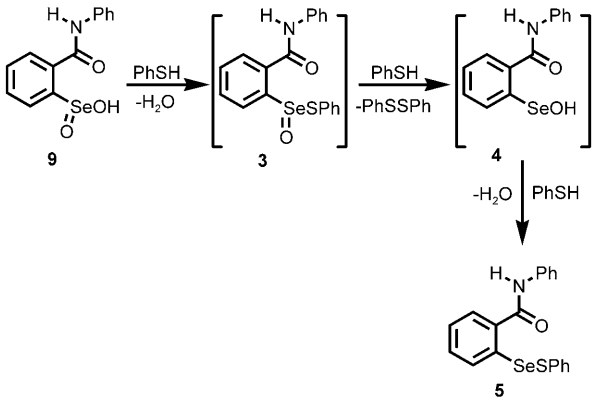


Figure 1. a) Single-crystal X-ray structure of the seleninic acid **9** (including 50% probability ellipsoid). b) X-ray crystal structure of cytosolic glutathione peroxidase (cGPx) showing the seleninic acid moiety and the Sec-Trp-Glu catalytic triad (PDB Code: 1 gp1).[28]

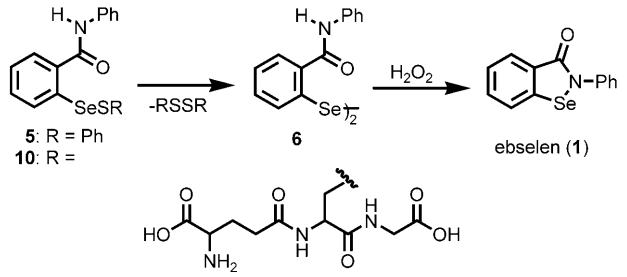
The reaction of seleninic acid **9** with an excess amount of PhSH produced the corresponding selenenyl sulfide (**5**, R = Ph) in a nearly quantitative yield, which was isolated and compared to an authentic sample. The selenenyl sulfide **5** could also be obtained directly by treating ebselen (**1**) with one equivalent of PhSH. The ^{77}Se NMR spectroscopic chemical shift for this compound ($\delta = 588$ ppm) indicates strong Se···O noncovalent interactions between the selenium and carbonyl oxygen atoms.[17b] It has been shown previously that the oxidation of ebselen by H_2O_2 generates the selenoxide **2**, which upon reaction with thiols produces the corresponding selenenic acid **4** presumably via the formation of a thiolseleninate **3** (Scheme 2, cycle B).[19] Similarly, the formation of selenenyl sulfide **5** from the reaction of **9** with PhSH may proceed via the formation of the thiolseleninate **3** (R = Ph) and selenenic acid **4** as shown in Scheme 4. When the concentration of thiol was not sufficient to perform all three steps, the selenenic acid **4** underwent a rapid cyclization to produce ebselen. The addition of an excess amount of PhSH to the reaction mixture containing ebselen led to the formation of **5**. Therefore, the selenenyl sulfide **5** was found to be the only selenium-containing product when a large excess (5 equiv) of thiol was added to the seleninic acid **9** (Scheme 4).

We have shown previously that the nucleophilic attack of an additional thiol at the selenenyl sulfide linkage does not produce the selenol **8** due to a thiol exchange reaction.[17b]



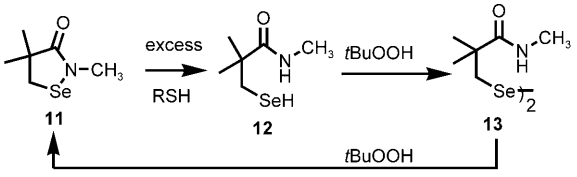
Scheme 4. The reaction of seleninic acid **9** with PhSH to produce the selenenyl sulfide **5**.

Therefore, it was considered to be of interest to test the reactivity of **5** towards H_2O_2. In the presence of H_2O_2, the selenenyl sulfide **5** underwent a disproportionation reaction in $CDCl_3$, leading to the formation of diselenide **6** and PhSSPh (Scheme 5). The diselenide **6**, precipitated out as a white



Scheme 5. Disproportionation of the selenenyl sulfides **5** and **10** in the presence of H_2O_2 affording ebselen via the formation of diselenide **6**.

solid, was isolated and characterized by ^{77}Se NMR spectroscopy. When the reaction mixture was stirred or the white solid was mixed thoroughly with the remaining solution, ebselen was isolated in quantitative yield. Furthermore, the disproportionation of **5** to **6** takes place even in the absence of H_2O_2. In this case, compound **6** was found to be a dead-end product and the formation of ebselen was not observed. This indicates that the formation of diselenide **6** is responsible for the generation of ebselen in the presence of H_2O_2. The mechanism for the conversion of diselenide **6** to ebselen proceeds via the selenenic acid intermediate **4** (vide infra). When glutathione (GSH) was used instead of an aromatic thiol, the disproportionation of the resulting selenenyl sulfide **10** leading to the formation of diselenide **6** was considerably faster than that of **5**. This may account for the higher GPx activity of ebselen in the presence of GSH relative to that of aromatic thiols, such as PhSH. Reich and Jasperse have shown that the isoselenazolidine-3-one **11** reacts with thiols to produce the corresponding selenol **12**, which upon oxidation by *t*BuOOH, regenerates **11** (Scheme 6).[31] In this reaction, the oxidation of **12** by peroxide produces the diselenide **13**, which disproportionates in solution to produce compound **11**. However, it is not clear whether the selenenyl sulfide derived from **11** can undergo any such disproportionation reaction in the presence of *t*BuOOH to produce the diselenide **13**. Based on these results, the formation of diselenide **6** from the selenenyl sulfides **5** and **10** appears to be very important for the catalytic activity of ebselen, because

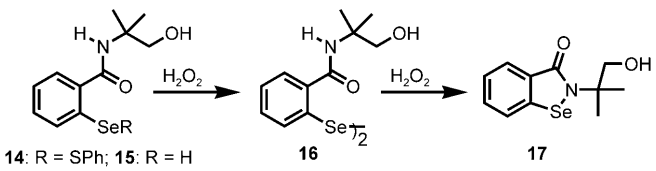


Scheme 6. The formation of diselenide **13** and the cyclic selenenamide **11**.

the selenenyl sulfides **5** and **10** fail to produce any catalytically active selenol **8**.

The formation of diselenide **6** was also observed in the reaction of selenol **8** with H_2O_2. This is due to the high reactivity of the selenenic acid **4** produced in the reaction towards the unreacted selenol **8** and the relatively low reactivity of the selenol towards H_2O_2.[32] These observations suggest that the diselenide **6** may become the predominant species even in the presence of a thiol system that is capable of producing the selenol **8**.[33] This is in agreement with the catalytic cycle proposed by Fischer and Dereu (Scheme 2, cycle C)[19] in which the diselenide **6** has been shown to be crucial for the catalytic activity. The involvement of the diselenide **6** as one of the key intermediates in the catalytic mechanism of ebselen is further supported by the observations of Zhao and Holmgren that the diselenide **6** forms a part of ebselen antioxidant action in the presence of thioredoxin.[34] We have also observed that the diselenide **6** reacts with H_2O_2 initially to produce a mixture of ebselen and seleninic acid **9**. However, the reaction of compound **6** with seleninic acid **9** leads to the formation of ebselen as the final product.

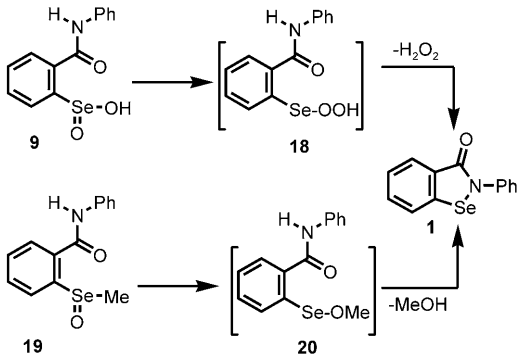
To understand whether the replacement of the phenyl group attached to nitrogen in ebselen by another substituent alters the mechanism, we have studied the reactions of compound **14** with PhSH and H_2O_2. Similar to the reactivity of selenenyl sulfide **5** towards PhSH, compound **14** does not react with PhSH to generate the selenol **15**. This is due to the presence of a strong Se···O interaction in compound **14**, which enhances a thiol exchange reaction at selenium.[35] On the other hand, the reaction of **14** with H_2O_2 afforded the selenenyl amide **17** in nearly quantitative yield, which indicates that the disproportionation of **14** to produce **16** is similar to that of **5**. The reaction of selenol **15** with H_2O_2 afforded the diselenide **16**. When the concentration of H_2O_2 was high, only a trace amount of **16** was detected as the diselenide **16** underwent further reactions with H_2O_2 to produce the selenenyl amide **17** (Scheme 7). These observations indicate that the replacement of the phenyl ring attached to the nitrogen atom may not have any effect on the mechanism of reaction with H_2O_2 and thiol.



Scheme 7. The formation of diselenide **16** and the cyclic selenenyl amide **17** from compounds **14** and **15**.

Although the seleninic acid **9** reacts with the diselenide **6** to produce ebselen, compound **9** was found to be stable in its pure form for several months without any noticeable decomposition or conversion to any other species. The direct cyclization of the seleninic acid to ebselen (**1**) was also not

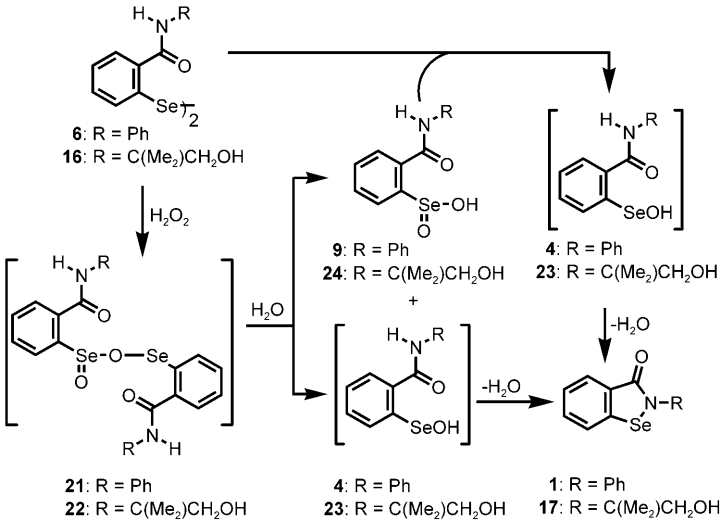
observed at room temperature. In acetonitrile, compound **9** underwent an unusual cyclization to produce ebselen in ≈5% yield after 15 months. However, when the seleninic acid **9** was heated in CH_3CN/CH_3OH, a complete conversion of **9** to ebselen was observed within 24 h without any decomposition. The mechanism of cyclization may involve the conversion of **9** to an unstable selenium–peroxo species **18** followed by an attack of the amide nitrogen atom at the selenium center leading to an elimination of H_2O_2 (Scheme 8). This reaction is expected be irreversible as the



Scheme 8. The formation of ebselen from the seleninic acid **9** and methyl selenoxide **19**.

H_2O_2 produced in the reaction may get decomposed at higher temperatures. A similar reaction involving the elimination of methanol has been proposed for the formation of ebselen from the methyl selenoxide **19** (Scheme 8).[36] To confirm the formation of ebselen from compound **19** at higher temperatures, we have synthesized the selenoxide **19** from 2-methyl selenobenzanilide, which is one of the metabolites of ebselen in vivo. The crystal structure of **19** shows the existence of noncovalent interactions between the selenium and carbonyl oxygen atoms (Se···O: 2.69 Å), although the interaction is found to be weaker than that of **9**. While the seleninic acid **9** did not produce any ebselen at room temperature, the selenoxide **19** produced a small amount of ebselen within a week. However, when selenoxide **19** was heated in CH_3CN/MeOH it produced ebselen in a good yield.

The conversion of **9** to ebselen in the reaction of diselenide **6** with H_2O_2 is surprising, as the seleninic acid does not undergo any cyclization at room temperature. The most striking feature we have observed in the ebselen antioxidant cycle is the facile reaction of the diselenide **6** with the seleninic acid **9** to produce ebselen in quantitative yield (Scheme 9). Although diselenides and seleninic acids are known to exist in equilibrium with the corresponding selenenic acids,[37] the rapid cyclization of **4** to ebselen drives the complete conversion of the diselenide **6** to ebselen in the presence of peroxides. Interestingly, when pure seleninic acid **9** was treated with diselenide **6** in the absence of peroxide, the formation of ebselen was clearly observed. The conversion of **16** to the selenenyl amide **17** follows a similar

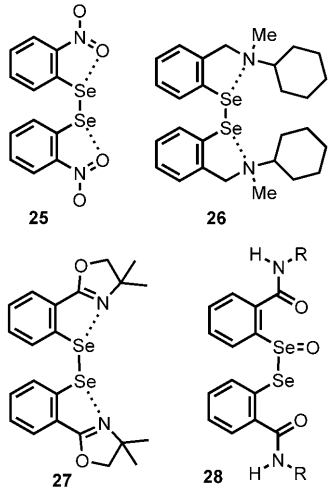


Scheme 9. Proposed mechanism for the conversion of the diselenides **6** and **16** to the corresponding selenenyl amides **1** and **17**, respectively, in the presence of H_2O_2.

route. The rapid reaction of **16** with H_2O_2 produces a mixture of the selenenic acid **23** and seleninic acid **24**, both of which are finally converted to the selenenyl amide **17**. Similar to ebselen, the selenenyl amide **17** reacts with H_2O_2 to produce the corresponding selenoxide, which undergoes a rapid hydrolysis to produce the seleninic acid **24**. As the reactions of diselenides **6** and **16** with one equivalent of H_2O_2 produce the corresponding seleninic acids (**9** and **24**) and selenenyl amides (**1** and **17**) in a 1:1 ratio, we presume that the oxidation of **6** and **16** produces compounds **21** and **22**, respectively, which upon hydrolysis generates the corresponding selenenic and seleninic acids (Scheme 9).

The facile reaction of the diselenides **6** and **16** with H_2O_2 to produce the selenenyl amides **1** and **17**, respectively, is somewhat intriguing because the selenium atoms in the diselenides are electrophilic centers. The single-crystal X-ray structures indicate that both the selenium atoms in compounds **6** and **16** are involved in noncovalent interactions with the carbonyl oxygen atoms (see Figures S43 and S46 in the Supporting Information). The average Se···O distances in compounds **6** (2.85 Å) and **16** (2.85 Å) are much shorter than the sum of the van der Waals radii of selenium and oxygen atoms (3.35 Å). Although the final product of the oxidation of an aryl diselenide (ArSeSeAr) by either peracids or H_2O_2 is normally the seleninic acid ($ArSeO_2H$),[33b] the Se···O interactions should increase the possibility of a nucleophilic attack at the selenium atom. Therefore, the reactions of the diselenides **6** and **16** with H_2O_2 are expected to be much slower than that with thiols. In agreement with this, the reactions of the diselenides **25** and **26** with either Se···O or Se···N interactions with H_2O_2 have been shown to be extremely slow.[27b,33b] Furthermore, we have found that the reaction of the oxazoline-based diselenide **27**[38] containing very strong Se···N interactions with H_2O_2 is extremely slow, which indicates that the Se···O interactions in com-

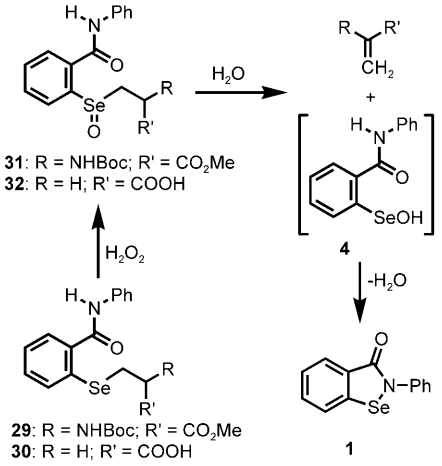
pounds **6** and **16** are not responsible for the higher reactivity of these compounds towards H_2O_2.



In the first mechanistic study on the GPx activity of ebselen, Fischer and Dereu have shown that the rapid reaction of the diselenide **6** with H_2O_2 affords the corresponding selenenic acid anhydride (**7**, Scheme 2, cycle C, step 3) as the only oxidized product.[19] However, there is no experimental evidence available for the existence of the selenenic acid anhydride **7**. Recent computational studies by Boyd et al. have shown that the oxidation of the diselenide **6** by H_2O_2 to produce the selenoxide **28** is more favored than the formation of the anhydride **7**, as the selenenic acid anhydride **7** is $\approx$7.7 kcal mol^{-1} higher in energy than the selenoxide **28**.[39] It has also been proposed that the fast isomerization of the selenoxides (ArSe(=O)SeAr) to the corresponding anhydrides (ArSeOSeAR) proposed by Kice and Chiou[33b] is not a favored process in the case of **28**.[39] The free-energy barrier for the oxidation of one of the selenium atoms in **6** (38.4 kcal mol^{-1}) has been shown to be comparable with that of ebselen (36.8 kcal mol^{-1}).[39] However, it is not clear whether the Se···O interactions, which have not been included in the calculations, can alter the energy barrier for the conversion.

The present study shows that the formation of the selenenic acids (**4** and **23**) and seleninic acids (**9** and **24**) and the subsequent cyclization of the selenenic acids to the selenenyl amides **1** and **17** are responsible for the complete oxidation of the diselenides **6** and **16**. The seleninic acids **9** and **24**, acting as oxidizing agents, transfer the oxygen atom to the diselenides, which leads to the formation of the corresponding selenenic acids. This process continues until all the diselenides are converted to the corresponding selenenyl amides. The cyclization also prevents the formation of the diselenides from the selenenic and seleninic acids. Because of these unusual transformations, the reactions of the diselenides **6** and **16** with H_2O_2 produce the selenenyl amides **1** and **17**, respectively, in quantitative yields. The oxidation of the diselenides **6** and **16** by the respective seleninic acids **9** and **24** is, however, not surprising since arylseleninic acids, such as benzeneseleninic acids, have been commonly used in various oxidation reactions.[40] Furthermore, ebselen and its analogues have been shown to be efficient catalysts for the oxidation of aromatic aldehydes, azomethine compounds, and sulfides in the presence of H_2O_2 or *t*BuOOH.[41]

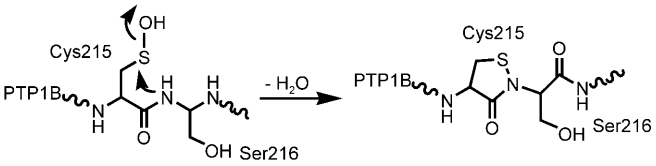
Further, to investigate whether the selenenic acid **4** is competent to undergo cyclization to produce ebselen, we have studied the cyclization of **4**, generated in situ from the corresponding selenocysteine derivative **29** by a β-hydrogen elimination reaction (Scheme 10). It is known that selenoxides containing a β-hydrogen atom can undergo olefin elimination to produce the corresponding selenenic acids.[37b,42] Therefore, the decomposition of aryl selenoxides is generally used for the generation of selenenic acid at room temperature. When compound **29** was treated with H_2O_2 in aqueous buffer (phosphate buffer, pH 7.5), ebselen (**1**) was obtained in good yield. Although the formation of the selenenic acid **4** was not observed during the reaction, we presume that the oxidation of the selenium center in **29** by H_2O_2 affords the corresponding selenoxide, which would undergo a β-hydrogen-atom elimination to produce the selenenic acid **4**. Compound **4** then undergoes cyclization by eliminating a water molecule to produce ebselen. The propionic acid derivative **30** also undergoes a similar reaction to produce ebselen. This indicates that the selenenic acid **4** can produce ebselen under physiological conditions. This is in agreement with a recent report that the sulfur analogue of **30** undergoes such a cyclization to produce the corresponding 3-isothiazolidinone heterocycle.[43]



Scheme 10. Cyclization of selenides **29** and **30** in the presence of H_2O_2 via an in situ generation of the selenenic acid **4**.

The cyclization of the selenenic acid **4** to produce the selenenyl amide (ebselen) is an unusual transformation as the amide moieties are generally considered poor nucleophiles. Although the conversion of a selenenic acid to a selenenyl amide species has not yet been confirmed in proteins, there are indications that such a transformation may occur in sele-

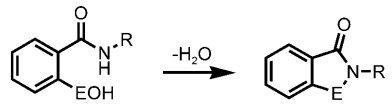
noenzymes. Ursini et al have shown that in GPx the selenenic acid (E–SeOH), produced in the presence of a peroxide substrate, reacts instantly with a yet undefined X–H group with elimination of H_2O to produce an oxygen-free Se^{II} compound that reacts equally fast with thiols.[44] Flohé has proposed that the selenenic (E–SeOH) acid in GPx may react with the nearby glutamine (Gln) residue to form an Se–N bond.[33c] It is also possible that the selenenic acid may react with the backbone amide to form a cyclic selenenyl amide, which could be a precursor for the selenenyl sulfide intermediate. This assumption is further supported by the recent discovery that the redox regulation of protein tyrosine phosphatase 1B (PTP1B) involves a sulfenyl–amide intermediate.[45] In this particular case, the protein sulfenic acid (PTP1B-SOH) produced in response to PTP1B oxidation by H_2O_2 is rapidly converted into a sulfenyl–amide species, in which the sulfur atom of the catalytic cysteine is covalently linked to the main-chain nitrogen atom of an adjacent serine residue (Ser216) (Scheme 11).[45] This novel and



Scheme 11. The reversible inactivation pathway involving the conversion of sulfenic acid to sulfenyl amide at the active site of PTP1B.

unusual protein modification has been shown to protect the active-site cysteine from irreversible oxidation to sulfinic (E–SO_2H) or sulfonic (E–SO_3H) acids. The formation of sulfenyl amide is reversible and the cleavage of the S–N bond by cellular thiols, such as glutathione (GSH), converts the inactivated protein back to its catalytically active form.[45] Furthermore, the inactivated PTP1B can also be reactivated enzymatically by cysteine-containing proteins, such as thioredoxin or glutaredoxin.[46]

As the selenium moiety in selenenic acids is generally more electrophilic than the sulfur atom in sulfenic acids, the attack of the amide nitrogen at the selenium center is expected to be more favored than a similar attack at the sulfur atom. On the other hand, the Se···O noncovalent interactions in selenenic acids is expected to be stronger than S···O in the corresponding sulfenic acids. This leads to an assumption that the formation of selenenyl amides from selenenic acids is probably more difficult than the formation of sulfenyl amides from the corresponding sulfenic acids. To verify this hypothesis, we carried out DFT calculations on compounds **4**, **23**, and **33**–**38** at the B3LYP level by using 6-31G(d) as the basis set (Scheme 12).[47] These calculations indicate the presence of strong intramolecular Se···O interactions in the selenenic acids (Table 1). The Natural Bond Orbital (NBO) analysis[48] unambiguously confirmed the presence of Se···O interactions in the selenenic acids. The



33: E = S, R = Ph
34: E = S, R = $C(Me_2)CH_2OH$
35: E = Se, R = H
36: E = S, R = H
37: E = Se, R = CH_3
38: E = S, R = CH_3

39: E = S, R = Ph
40: E = S, R = $C(Me_2)CH_2OH$
41: E = Se, R = H
42: E = S, R = H
43: E = Se, R = CH_3
44: E = S, R = CH_3

Scheme 12. Selenenic acids and sulfenic acids undergo cyclization to form selenenyl amides and sulfenyl amides, respectively.

Table 1. Structural parameters, second-order perturbation energy ($E_{E\cdots O}$), the energy ($E'_{E\cdots O}$) required to break the E···O interaction and a comparison of energy (E) barriers (cyclization step) for the conversion of sulfenic/selenenic acids **4**, **23**, and **33**–**38** calculated at the B3LYP/6-31G(d) level of theory.[49]

R–E–OH	$d_{E\cdots O}$ [Å]	$E_{E\cdots O}$ [kcal mol^{-1}]	$E'_{E\cdots O}$ [kcal mol^{-1}]	E (cyclization) [kcal mol^{-1}]
4	2.375	28.05	11.74	40.28
33	2.457	14.00	7.27	44.79
23	2.373	28.73	12.69	42.66
34	2.448	14.75	7.76	49.29
35	2.385	27.60	9.63	43.24
36	2.471	13.69	7.58	50.10
37	2.376	28.63	8.70	41.61
38	2.462	14.11	8.01	51.90

NBO analysis at the B3LYP/6-31G(d) level of theory on **4**, **23**, and **33**–**38** suggests that the stabilization energy due to a $n_O \rightarrow \sigma^*_{Se-OH}$ orbital interaction ($E_{Se\cdots O}$) in the selenium compounds is about 13 kcal mol^{-1} higher than that of the sulfur analogues (Table 1). Because of this interaction, the OH group adopts a position *trans* to the Se···O interaction, which leads to an almost linear arrangement of the O···Se–O moiety. As a result of this arrangement, the cyclization partners, that is, the NH and OH functionalities are positioned in opposite directions, which may hamper a facile cyclization of the selenenic acids. However, the activation energy calculations for the cyclization of the selenenic and sulfenic acids indicate that the energy required to break the Se···O interactions is only slightly higher than that of the S···O interactions in the sulfenic acids.

To investigate the overall energy required for the conversion of the sulfenic/selenenic acids to the corresponding sulfenyl/selenenyl amides, we have performed detailed DFT calculations. According to these calculations, the major steps for the cyclization of sulfenic/selenenic acids include: 1) breaking of the intramolecular S/Se···O interaction and rotation of the amide NH group towards the sulfur/selenium atom, 2) rotation of the OH group towards the NH group of the amide moiety, and 3) elimination of a water molecule to form the expected cyclic sulfenyl/selenenyl amide. Therefore, we have computed the relative electronic energies (including the ZPVE correction) in the gas phase for the cyclization of **4**, **23**, and **33**–**38** (Table 1). The nature of the potential-energy surfaces (PES) for the cyclization of the sele-

nenic acids is identical to that of the sulfenic acids (Figure 2). The most important and rate-determining step is the ring-closing step with an elimination of a water molecule. The B3LYP/6-31G(d) level optimized geometries of different intermediates and transition states involved in the cyclization of **4** to **1** are shown in Figure 3. A comparison of the energy barriers for the cyclization step indicates that the energy required for the cyclization of the selenenic acids is $\approx$5–10 kcal mol^{-1} lower than that of the sulfenic acids. This suggests that the rate of formation of a selenenyl amide can be identical with or even faster than that of a sulfenyl amide. Furthermore, the substitution of one of the hydrogen atoms in compound **35** with a phenyl group (compound **4**) brings down the energy barrier by $\approx$3 kcal mol^{-1}. These observations suggest that the phenyl ring attached to the nitrogen atom in ebselen is important for the regeneration of ebselen during the GPx cycle. This is in agreement with our previous experimental observations that the presence of a phenyl substituent on the nitrogen atom is important for the GPx activity of ebselen.[17d]

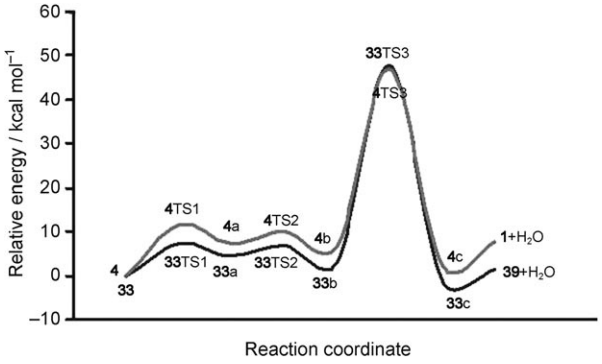


Figure 2. Energy (ΔE_{elec}+ZPVE) profiles for the cyclization of selenenic acid **4** and sulfenic acid **33** to the corresponding selenenyl amide **1** and sulfenyl amide **39**.[50]

Based on our experimental and theoretical data, we propose a revised mechanism for the GPx activity of ebselen as shown in Scheme 13. According to this cycle, ebselen (**1**) rapidly reacts with a thiol to produce the corresponding selenenyl sulfide **5** (step 1), which undergoes a disproportionation reaction in the presence of peroxide to produce the diselenide **6** (step 2). The rapid reaction of the diselenide **6** with peroxide produces the selenenic acid **4** and seleninic acid **9** (step 3). In the presence of an excess of thiol, compounds **4** and **9** react with the thiol to produce the selenenyl sulfide **5** (steps 5 and 6, respectively). When the thiol is depleted in the reaction, the seleninic acid **9** reacts with the diselenide **6** to produce the selenenic acid (step 7), which undergoes cyclization to produce ebselen (step 4). The conversion of compound **9** to ebselen may not require the diselenide **6** in vivo. As the seleninic acid **9** is an efficient oxygen transfer agent, this compound can be easily reduced to the selenenic acid **4** by a number of meth-

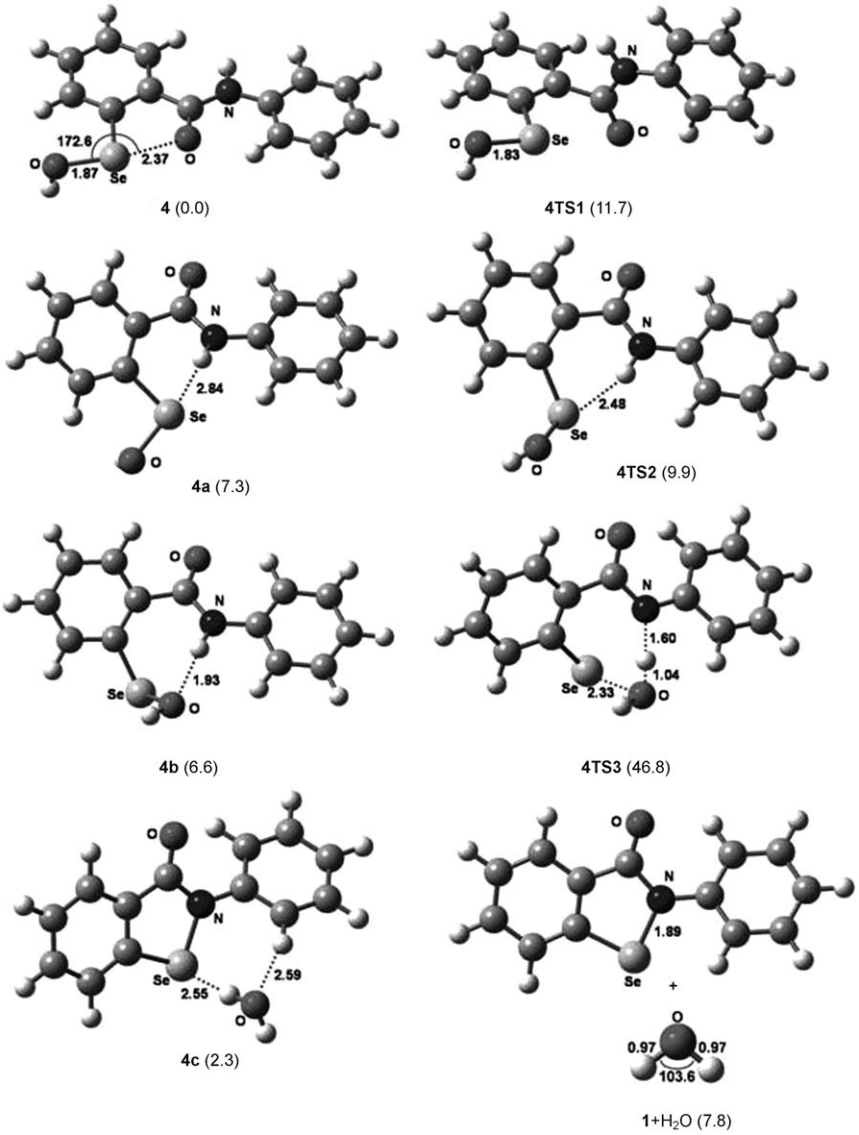
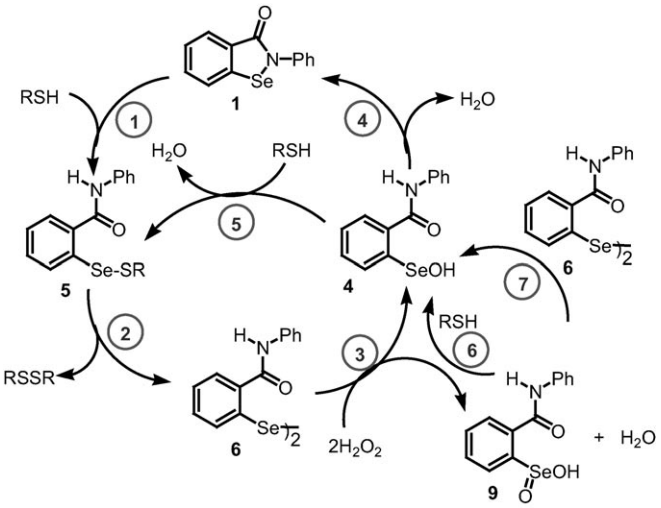


Figure 3. B3LYP/6-31G(d) level optimized geometries of different intermediates and transition states involved in the cyclization of the selenenic acid **4** to ebselen (**1**).



Scheme 13. A revised mechanism for the GPx activity of ebselen.

ods.[51] The selenenyl sulfide and seleninic acid are the predominant species in the presence of large amounts of thiol and peroxide, respectively.

In this catalytic cycle, the formation of the diselenide **6** is the rate-determining step and this reaction depends on the nature of the thiol used for the GPx assay. When an unconventional thiol, such as dihydrolipoic acid, is employed,[33] the diselenide is probably produced via the formation of selenol **8**. With aromatic thiols, such as PhSH, the selenenyl sulfide **5** would never produce the selenol **8** (Scheme 2, cycle A, step 1) due to the thiol exchange reactions. Therefore, the rate of the overall reaction depends on the rate of the disproportionation reaction (Scheme 13, step 2), because the nature of the peroxide normally does not affect the GPx activity of ebselen.[17d] Fisher and Dereu have shown that *meta*-chloroperbenzoic acid is as effective an oxidizing agent as H_2O_2 in converting the diselenide derived from *N*-benzylbenzamide to the corresponding selenenyl amide.[19] We have recently shown that the nature of the peroxide has little effect on the GPx activity, whereas the nature of the thiols has a dramatic effect on the catalytic activity of ebselen and its analogues.[17d] This indicates that the discrepancies observed in the outcomes of different studies may be ascribed to the differences in the thiols, and not to the peroxides employed for the assays.

Conclusion

In this study, we have re-evaluated the GPx-like catalytic mechanism of ebselen and found that the reversible cyclization of the selenenic acid to ebselen is a likely chemical mechanism for the antioxidant and anti-inflammatory activities of ebselen. The reaction of ebselen with peroxides does not afford the selenoxide as previously postulated, but it produces the corresponding seleninic acid, which undergoes reaction with a thiol to produce the selenenyl sulfide or interacts with the diselenide to regenerate ebselen. The disproportionation of the selenenyl sulfide to the diselenide is crucial for the catalytic activity of ebselen. The regeneration of ebselen from its catalytically active and inactive forms under different conditions may also be responsible for the relatively low toxicity of ebselen in vivo. The cyclization of open-chain intermediates to ebselen is important to protect the selenium moiety from irreversible inactivation. As a result, the selenium atom is not released during the biotransformations, which prevents the ebselen-derived selenium atom from entering into the selenium metabolism of the organism. We have also demonstrated that the cyclization of the selenenic acid to the corresponding selenenyl amide is similar to the formation of a sulfenyl amide at the active site of protein tyrosine phosphatase 1B (PTP1B). This comparison, however, leads to an assumption that the formation of a selenenyl amide is feasible in selenoproteins, which should be the focus of future studies.

Experimental Section

General procedure: *n*BuLi was purchased from Acros. Selenium powder, $NaBH_4$, and benzanilide were obtained from Aldrich. All other chemicals were of the highest purity available. All experiments were carried out under anhydrous and anaerobic conditions by using standard Schlenk techniques for the synthesis. Due to the unpleasant odors of several of the reaction mixtures involved, most manipulations were carried out in a well-ventilated fume hood. MS studies were carried out on a Q-TOF Micro mass spectrometer with electrospray ionization MS mode analysis. In the case of isotopic patterns, the value given is for the most intense peak. Elemental analyses were performed on a ThermoFinigan FLASH EA 1112 CHNS analyzer. Liquid-state NMR spectra were recorded in $CDCl_3$, $[D_4]$MeOH, or $[D_6]$DMSO as a solvent. ^1H (400 MHz), ^{13}C (100.56 MHz), and ^{77}Se (76.29 MHz) NMR spectra were obtained on a Bruker 400 MHz NMR spectrometer. Chemical shifts are cited with respect to $SiMe_4$ as internal (^1H and ^{13}C) and Me_2Se as external (^{77}Se) standards. 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 on an automated flash chromatography system (Biotage) by using preloaded silica cartridges. HPLC experiments were carried out on a Waters Alliance System (Milford, MA) consisting of a 2690 separation module, a 2690 photodiode-array detector, and a fraction collector. HPLC studies 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 EMPOWER software (Waters Corporation, Milford, MA). Anthranilic acid was first diazotized and was added to disodium diselenide to produce diselenosalicylic acid, which was treated with freshly distilled thionyl chloride to produce the 2-(chloroseleno)benzoyl chloride as a yellow solid. The cyclic selenenyl amide **17** was prepared by treating 2-(chloroseleno)benzoyl chloride with 2-amino-2-methylpropan-1-ol in dry acetonitrile. The selenoxide **19** was prepared by following the literature method.[36]

Synthesis of 9: Aqueous 30% H_2O_2 (41 μL, 0.36 mmol) was added to a stirred solution of ebselen (0.1 g, 0.36 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was stirred for 24 h at ambient temperature. The precipitate formed was filtered, washed with CH_2Cl_2, and dried in vacuo to give **9** as a white solid in 70% yield.[52] ^1H NMR ($CDCl_3$): $\delta = 8.14$ (d, $J = 8.0$ Hz, 3H), 7.94 (d, $J = 7.2$ Hz, 1H), 7.79–7.88 (m, 2H), 7.40 (d, $J = 7.6$, 1H), 7.42–7.55 ppm (m, 4H); ^{13}C NMR ($CDCl_3$): $\delta = 165.9$, 144.2, 134.7, 133.7, 132.3, 129.4, 128.8, 128.0, 127.6, 126.0, 125.2 ppm; ^{77}Se NMR ($CDCl_3$): $\delta = 1122$ ppm; HRMS (TOF MS ES$^+$): *m/z*: 331.9769 $[M+Na]^+$.

Synthesis of 10: Glutathione (GSH) (112 mg, 0.36 mmol) in water (2 mL) was added to a stirred solution of ebselen (0.1 g, 0.36 mmol) in MeOH (2 mL). The reaction mixture was stirred for 0.5 h at ambient temperature. The precipitate formed was filtered, washed repeatedly with water and CH_2Cl_2, and dried in vacuo to give **10** as a white solid in 70% yield. ^{77}Se NMR ($CDCl_3$): $\delta = 545$ ppm; elemental analysis calcd (%) for $C_{23}H_{26}N_4O_7SSe$: C 47.51, H 4.51, N 9.63; found: C 45.53, H 3.91, N 9.52.

Synthesis of 14: Benzenethiol (230 μL, 2.24 mmol) was added to a stirred solution of **17** (0.6 g, 2.22 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was stirred for 24 h at ambient temperature. The solvent was then removed in vacuo and **14** was then purified by flash chromatography by using petroleum ether/ethyl acetate as the eluent to give **14** as a colorless oil in 60% yield. ^{1}H NMR ($CDCl_3$): $\delta = 8.15$ (d, $J = 8.0$ Hz, 1H), 7.50 (d, $J = 7.6$ Hz, 3H), 7.43 (t, $J = 7.6$ Hz, 3H), 7.21–7.28 (m, 3H), 7.15 (t, $J = 6.8$, 7.2 Hz, 1H), 6.36 (s, 1H), 3.73 (s, 2H), 1.44 ppm (s, 6H); ^{13}C NMR ($CDCl_3$): $\delta = 168.4$, 136.8, 136.6, 132.1, 131.5, 128.9, 126.6, 126.4, 126.0, 70.1, 56.5, 24.5 ppm; ^{77}Se NMR ($CDCl_3$): $\delta = 585$ ppm; HRMS (TOF MS ES$^+$): m/z: 404.0200 $[M+Na]^+$.

Synthesis of 16: Excess benzenethiol (1 mL, 9.7 mmol) was added to a stirred solution of **17** (0.6 g, 2.22 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was stirred for 48 h at ambient temperature and the reaction mixture was kept for aerial oxidation for another 48 h. The solvent was then removed in vacuo and the diselenide was purified by column chromatography by using petroleum ether/ethyl acetate 1:1 as the eluent to give **16** as a yellow solid in 60% yield. ^{1}H NMR ([D$_4$]MeOH): $\delta = 7.84$ (d, $J = 7.6$ Hz, 1H), 7.62 (d, $J = 8.4$ Hz, 1H), 7.26–7.35 (m, 2H), 3.75 (s, 2H), 1.46 ppm (s, 6H); ^{13}C NMR ([D$_4$]MeOH): $\delta = 168.4$, 134.3, 130.0, 129.7, 126.2, 124.9, 66.7, 54.5, 21.57 ppm; ^{77}Se NMR ([D$_4$]MeOH): $\delta = 429$ ppm; HRMS (TOF MS ES$^+$): m/z: 567.0295 $[M+Na]^+$.

Synthesis of 17: 2-(Chloroseleno)benzoyl chloride (1.23 g, 4.83 mmol) in acetonitrile (15 mL) was added dropwise to a stirred solution of 2-amino-2-methylpropan-1-ol (480 μL, 5 mmol) in dry acetonitrile (50 mL). The reaction mixture was stirred for 5 h at 25°C and then the precipitate formed was filtered off. The filtrate was evaporated under reduced pressure to give a colorless oil, which was then purified by column chromatography by using petroleum ether/ethyl acetate 5:1 as the eluent to give **17** as a white solid in 80% yield. ^{1}H NMR ($CDCl_3$): $\delta = 7.89$ (d, $J = 8.0$ Hz, 1H), 7.52 (d, $J = 3.2$ Hz, 2H), 7.34–7.37 (m, 1H), 5.50 (br s, 1H), 3.85 (s, 2H), 1.52 ppm (s, 6H); ^{13}C NMR ($CDCl_3$): $\delta = 167.0$, 137.0, 131.0, 128.1, 127.6, 125.2, 122.2, 69.2, 62.9, 24.9 ppm; ^{77}Se NMR ($CDCl_3$): $\delta = 885$ ppm; HRMS (TOF MS ES$^+$): m/z: 294.0032 $[M+Na]^+$.

Computational methods: All calculations were performed by using the Gaussian 98 suite of quantum chemical programs.[53] The hybrid Becke 3-Lee-Yang-Parr (B3LYP) exchange correlation functional was applied for DFT calculations.[47] Geometries were fully optimized at the B3LYP level of theory by using the 6-31G(d) basis sets. Transition states were located by using Schlegel's synchronous transit-guided quasi-Newton (STQN) method.[54,55] Transition states were searched by using the QST3 keyword and the resultant conformation was optimized by using the TS keyword. Furthermore, the transition state and the stable conformers were characterized by the presence or absence of a single imaginary mode. In all cases, intrinsic reaction coordinate (IRC)[56] calculations were performed to confirm that the transition states connect the reactant and the product molecules. The activation energies are the difference in the zero-point vibrational energy corrected electronic energy between the transition state and the stable conformations. Orbital interactions were analyzed by using the NBO method at the B3LYP/6-31G(d) level.[48]

X-ray crystallography: X-ray crystallographic studies were carried out on a Bruker CCD diffractometer with graphite-monochromatized $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å) controlled by a Pentium-based PC running on the SMART software package.[57] 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 by using the SHELXTL software package.[58] All non-hydrogen atoms were refined anisotropically and hydrogen atoms were assigned idealized locations. Empirical absorption corrections were applied to all structures by using SADABS.[59–60] The structures were solved by the direct method (SIR-92) and refined by the full-matrix least-squares procedure on F^2 for all reflections (SHELXL-97).[61]

Crystal data for ***6***: $C_{26}H_{20}N_2O_2Se_2$; $M_r = 550.4$; monoclinic; space group $C2/c$; $a = 24.4233$, $b = 5.0510(21)$, $c = 19.2476(79)$ Å; $\beta = 109.618(6)°$; $V = 2236.59(53)$ Å^3; $Z = 4$, $\rho_{calcd} = 1.63$ g cm^{-1}; $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å); $T = 291(2)$ K; $R_1 = 0.031$, $wR_2 = 0.068$ ($I > 2\sigma(I)$); $R_1 = 0.044$, $wR_2 = 0.072$ (all data).

Crystal data for ***9***: $C_{13}H_{11}NO_3Se$; $M_r = 308.2$; orthorhombic; space group $Pna2(1)$; $a = 19.0020(39)$, $b = 14.1292(29)$, $c = 4.5882(9)$ Å; $V = 1231.85(4)$ Å^3; $Z = 4$; $\rho_{calcd} = 1.66$ g cm−1; $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å); $T = 291(2)$ K; $R_1 = 0.037$, $wR_2 = 0.065$ ($I > 2\sigma(I)$); $R_1 = 0.059$, $wR_2 = 0.071$ (all data).

Crystal data for ***16***: $C_{22}H_{28}N_2O_4Se_2$; $M_r = 542.4$; monoclinic; space group $P2(1)/n$; $a = 8.4090(50)$, $b = 15.3440(50)$, $c = 18.1000(50)$ Å; $\beta = 95.268(5)°$; $V = 2325.54(20)$ Å^3; $Z = 4$; $\rho_{calcd} = 1.55$ g cm^{-1}; $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å); $T = 291(2)$ K; $R_1 = 0.060$, $wR_2 = 0.118$ ($I > 2\sigma(I)$); $R_1 = 0.126$, $wR_2 = 0.142$ (all data).

Crystal data for ***19***: $C_{14}H_{13}NO_2Se$; $M_r = 306.2$; monoclinic; space group $P2(1)/C$; $a = 9.5949(12)$, $b = 7.2494(9)$, $c = 19.7972(24)$ Å; $\beta = 98.011(2)°$; $V = 1363.60(5).16)$ Å^3; $Z = 4$; $\rho_{calcd} = 1.49$ g cm^{-1}; $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å); $T = 291(2)$ K; $R_1 = 0.032$, $wR_2 = 0.077$ ($I > 2\sigma(I)$); $R_1 = 0.046$, $wR_2 = 0.083$ (all data).

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[50] For B3LYP/6-31G(d) level optimized geometries and relative electronic $\Delta(E+ZPE)$ and Gibbs free energies $\Delta(G+ZPE)$ of different intermediates and transition states involved in the cyclization of the sulfenic acid **33** to sulfenyl amide **39** see, Table S1 (**3**–**3c**, **5**) and S2 in the Supporting Information of reference **33d**.
[51] Seleninic acids may transfer one of the oxygen atoms to disulfides or thiols to produce the corresponding selenenic acids. The protein thiols may be preferred targets in vivo.
[52] It should be noted that when the reaction of ebselen with H_2O_2 was carried out for longer time ($\approx$2 days) complete conversion of ebselen to seleninic acid **9** was observed, which was confirmed by ^{77}Se NMR spectroscopy.
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