

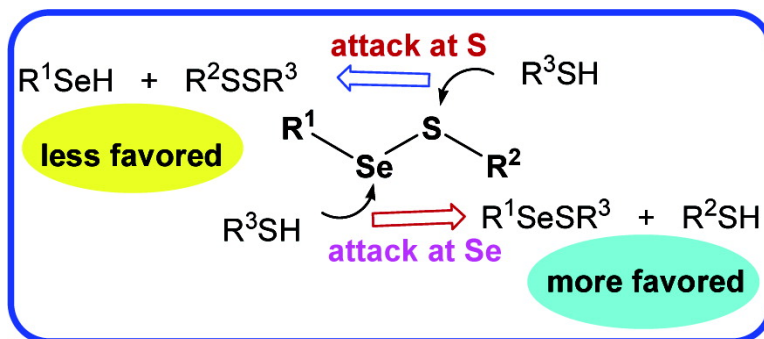
Article

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Glutathione Peroxidase (GPx)-like Antioxidant Activity of the Organoselenium Drug Ebselen: Unexpected Complications with Thiol Exchange Reactions

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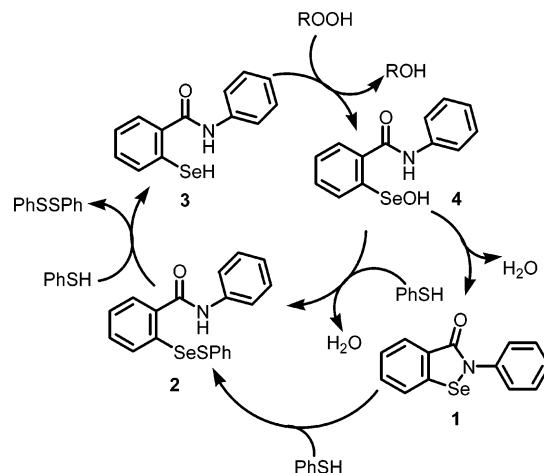
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Abstract: The factors that are responsible for the relatively low glutathione peroxidase (GPx)-like antioxidant activity of organoselenium compounds such as ebselen (**1**, 2-phenyl-1,2-benziselenazol-3(2*H*)-one) in the reduction of hydroperoxides with aromatic thiols such as benzenethiol and 4-methylbenzenethiol as cosubstrates are described. Experimental and theoretical investigations reveal that the relatively poor GPx-like catalytic activity of organoselenium compounds is due to the undesired thiol exchange reactions that take place at the selenium center in the selenenyl sulfide intermediate. This study suggests that any substituent that is capable of enhancing the nucleophilic attack of thiol at sulfur in the selenenyl sulfide state would enhance the antioxidant potency of organoselenium compounds such as ebselen. It is proved that the use of thiol having an intramolecularly coordinating group would enhance the biological activity of ebselen and other organoselenium compounds. The presence of strong S···N or S···O interactions in the selenenyl sulfide state can modulate the attack of an incoming nucleophile (thiol) at the sulfur atom of the –Se–S– bridge and enhance the GPx activity by reducing the barrier for the formation of the active species selenol.

Introduction

Ebselen (**1**, 2-phenyl-1,2-benziselenazol-3(2*H*)-one), a cyclic organoselenium compound with low toxicity, exhibits interesting therapeutic properties for a number of disease states including antiinflammatory activities.¹ These properties are apparently due to the ability of ebselen to catalyze the reduction of hydroperoxides by glutathione (GSH) or other thiols, mimicking the catalytic activity of one of the selenoenzymes, glutathione peroxidase (GPx).² Because of these properties, ebselen is being used as a standard for comparing the GPx activity of selenium compounds. Despite its importance in biology and medicine, the catalytic GPx cycle of ebselen is controversial probably due to major differences in working conditions such as solvents, pH, and the nature of the hydroperoxides that are used by many research groups to characterize the potential catalytic intermediates.³ Although several mechanisms have been proposed to explain the observed GPx activity of ebselen, the available information reveals a hypothetical

Scheme 1. GPx Cycle of Ebselen^a



^a GSH has been replaced by PhSH.

catalytic cycle as shown in Scheme 1. According to this model, the Se–N bond in ebselen is readily cleaved by thiols to produce the corresponding selenenyl sulfides, which upon reduction by excess thiols produce selenol **3**. The catalytically active selenol then reduces hydroperoxides to form a selenenic acid, which further reacts with thiols to regenerate the selenenyl sulfides.

However, recent studies have shown that ebselen, irrespective of the reaction pathway, is a relatively inefficient catalyst in the reduction of hydroperoxides with aryl and benzylic thiols such as PhSH and BnSH as cosubstrates,⁴ and the reason for the relatively low activity is still not clear. Back et al. have noted a similar lack of reactivity with certain other selenenyl

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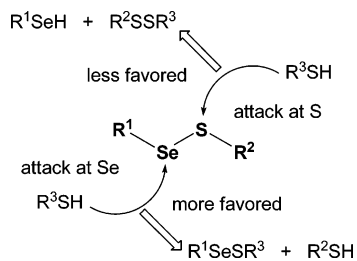


Figure 1. Nucleophilic attack of thiol at selenium or sulfur.

sulfides and have shown that these compounds undergo a deactivation pathway that competes with the main catalytic cycle.^{4b} The use of 3-carboxy-4-nitrobenzenethiol, which has been shown to enhance the GPx activity of the semisynthetic enzyme selenosubtilisin,⁵ does not increase the catalytic efficiency of ebselen.^{4c} Because the therapeutic effect of ebselen has been linked to its peroxidase activity and the peroxidase activity of this compound depends on the reduction of the selenenic acid **4** to the corresponding selenol **3** by thiols, it is important to study the effect of the nature of the thiols on GPx-like catalytic activity. Although the effect of various substituents attached to the selenium moiety on the GPx cycle of selenium compounds has been extensively studied,^{3d,6} the effect of such substituents attached to the thiol cosubstrates is very poorly understood. It has been reported that dithiols such as dihydro-lipoate may serve better as cofactors than GSH in the peroxidase activity of ebselen.^{7,8}

It is known that basic amino groups in the active site of GPx or similar groups in model compounds interact with selenium to modulate its reactivity.^{9,10} Interestingly, although such interactions have been shown to increase the electrophilic reactivity of selenium, these interactions in some of the intermediates become detrimental to the biological activity of synthetic selenium compounds. For example, strong Se \cdots N interactions in the selenenyl sulfide intermediate in the GPx cycle enhance a nucleophilic attack of thiol at selenium instead of the desired attack at sulfur, leading to a thiol exchange reaction that would hamper the regeneration of the active species selenol. Recent studies on model compounds show that the reduction of selenenyl sulfides to selenol requires surmounting a substantial barrier (~ 50 kcal/mol),¹¹ and the nucleophilic attack of thiol at selenium is both kinetically and thermodynamically more favorable than at sulfur (Figure 1).¹²

Table 1. Initial Rates (v_0) for the Reduction of H₂O₂ (7 mM) by PhSH (5 mM), 4-MeC₆H₄SH (5 mM), or GSH in the presence of Ebselen (0.47 mM) at 23 °C

	thiol	method	v_0 ($\mu\text{M}\cdot\text{min}^{-1}$)
control	GSH	GSH–GSSG ^a	15.11 \pm 0.69
	PhSH	HPLC	2.46 $\times 10^{-3}$
	4-MeC ₆ H ₄ SH	HPLC	1.85 $\times 10^{-3}$
ebselen	GSH	GSH–GSSG	91.68 \pm 2.37
	PhSH	HPLC	0.38 $\times 10^{-3}$
	4-MeC ₆ H ₄ SH	HPLC	0.56 $\times 10^{-3}$

^a Reactions were carried out in 0.1 M phosphate buffer, pH 7.24, with EDTA (1 mM), GSH (1 mM), NADPH (0.2 mM), GSSG reductase (0.6 unit/mL), ebselen (0.025 mM), and H₂O₂ (2 mM).

Because the selenium center in GPx readily changes its oxidation states during the catalytic cycle, several weak interactions between selenium and nearby amino acid moieties have been shown to modulate such changes. The objective of this work is not to study the nature of Se \cdots X (X = heteroatom) interactions in selenium compounds^{10,13,14} but to describe, with the help of ⁷⁷Se NMR spectroscopic studies, HPLC methods, and theoretical calculations, the role of heteroatoms in the GPx activity of ebselen and related derivatives. In this paper, we also show that the low activity of ebselen and related derivatives is mainly due to the thiol exchange reactions and a novel approach to overcome these problems.

Results and Discussion

The GPx-like activity of ebselen was studied with H₂O₂ as a substrate and GSH, PhSH, and 4-MeC₆H₄SH as thiol cosubstrates. The catalytic activity of ebselen with GSH was studied by using the classical GSH–GSSG coupled assay,⁹ and with aromatic thiols it was studied by using reversed-phase HPLC methods. The initial rates (v_0) for the reduction of H₂O₂ by thiols in the presence and absence of ebselen were calculated from the first 5–10% of the reaction by choosing a linear fit (Table 1). The catalytic rates were corrected for the background reaction between H₂O₂ and thiols.

Interestingly, the rates for reduction of H₂O₂ in the presence of aromatic thiols were found to be much lower than those observed with GSH and also significantly lower than those of uncatalyzed reductions. In this regard, not only is ebselen an inactive catalyst in the PhSH and 4-MeC₆H₄SH thiol systems, but it also acts as an inhibitor of its own redox reactions. To probe this unexpected behavior, the active intermediates of ebselen in the GPx cycle (**2**–**4**) were studied using ⁷⁷Se NMR spectroscopy, HPLC methods, and quantum chemical calculations to identify the role of carbonyl oxygen during the catalytic cycle. Because the intramolecular Se \cdots O/Se \cdots N nonbonded interactions result in an apparent downfield shift of the ⁷⁷Se NMR chemical shift and an increase in the electropositive character of selenium,^{13,15} the calculated Se \cdots O/Se \cdots N distances and the theoretical and, in some cases, experimental ⁷⁷Se NMR

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Table 2. Theoretical Data for **1–4** Obtained by DFT Calculations at the B3LYP/6-31G(d)//B3LYP/6-311+G(d,p) Levels along with the GIAO ^{77}Se NMR Chemical Shifts

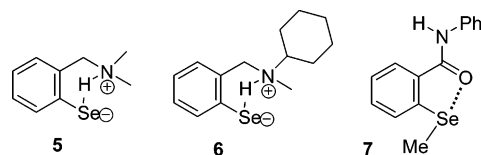
compd	$r_{\text{Se}\cdots\text{O-N}}$ (Å)	$\theta_{\text{O-Se}\cdots\text{X}}$ (deg)	q_{Se}	$\delta(^{77}\text{Se NMR})^a$ (calcd) (ppm)	$\delta(^{77}\text{Se NMR})$ (exptl) (ppm)	$E_{\text{Se}\cdots\text{O}}$ (kcal/mol)
1	1.893	86.6 ^b	0.622	941	959	
2	2.470	177.3	0.377	604	588	19.01
3	2.655	166.0	0.227	203	232	7.29
4	2.375	172.6	0.732	1110	1143	28.05

^a Referenced to the peak for Me_2Se . ^b N–Se–C (C atom of the benzene ring fused to the five-membered ring).

chemical shifts were used to probe the reactivity of various selenium species. The $\text{Se}\cdots\text{O}/\text{Se}\cdots\text{N}$ distances and the ^{77}Se NMR chemical shifts along with some important theoretical data for compounds **1–4** are summarized in Table 2.

The first step of the catalytic cycle of ebselen after the reduction of selenenyl sulfide **2** by thiols is believed to be the oxidation of selenol **3** by hydrogen peroxide to produce the corresponding selenenic acid **4**. It has been proposed that the introduction of amino/imino/ether groups in close proximity to selenium could enhance the GPx activity of organoselenium model compounds.^{13b} The heteroatoms present in these molecules have been proposed to (i) deprotonate the selenol moiety to increase its nucleophilic character and (ii) interact with selenium in the selenenic acid state to increase the electrophilic reactivity of selenium and hence to enhance the reaction with thiols before the possible conversion to “overoxidized” selenium species.^{9,10,13b,16} Therefore, theoretical investigations have been carried out to determine the role of the amide moiety in the catalysis. The B3LYP/6-31G(d)-level-optimized geometry and the calculated ^{77}Se NMR chemical shift for the selenol **3** show that the carbonyl oxygen or the $-\text{NH}-$ group does not facilitate the deprotonation of the selenol moiety. The experimental ^{77}Se NMR chemical shift of 232 ppm for this compound is in close agreement with the calculated value (203 ppm), but the signal is very downfield shifted compared with that of **5** having a basic amino group (6 ppm). Therefore, the GPx-like activity of **3** is expected to be lower than that of **5**, and this is indeed the case as shown in this study with aromatic thiols. Unexpectedly, the calculations on selenol **5** show that the basic amino group does not help in deprotonating the selenol, but it helps in stabilizing the selenol moiety by weak $\text{Se}\cdots\text{N}$ interaction with a stabilization energy ($E_{\text{Se}\cdots\text{N}}$) of 5.19 kcal/mol. Because the $\text{p}K_{\text{a}}$ of a quarternary trialkylammonium species is much higher (~ 10 – 11) than that of an aromatic selenol (~ 6), a selenol such as **5** having a tertiary amine side chain would be driven toward the zwitterion by about 4–5 $\text{p}K_{\text{a}}$ units. To understand the nature of the selenol moiety in aqueous solution, we included the solvent effects in the calculations by using the isodensity polarized continuum model (IPCM).¹⁷ The single-point energy calculations in water medium at the B3LYP/6-31G(d)-level-optimized gas-phase geometries show that the zwitterion form of **5** is at least 2.37 kcal/mol more stable in water than that with a weak $\text{Se}\cdots\text{N}$ interaction. On the other hand, the zwitterion form of **3** was found to be 5.45 kcal/mol less stable than that

with a weak $\text{Se}\cdots\text{O}$ interaction even after inclusion of the solvent effect. This suggests that selenol **3**, in contrast to **5**, may exist in the neutral form, favored by about 6–7 $\text{p}K_{\text{a}}$ units over the corresponding zwitterion. Therefore, the existence of **5** as the selenolate accounts for the higher reactivity of **5** as compared with the undissociated selenol **3**.



The stability of selenenic acids against further oxidation and their fast reaction with thiols are the two major factors in the second step of the catalytic cycle. The secondary amino group in **4** may not play a crucial role in the activation of selenenic acid. Instead, the carbonyl oxygen is expected to interact with the selenium as shown by the crystal structure of **7** in which the oxygen atom interacts with selenium.¹⁸ In agreement with this, the B3LYP/6-31G(d)-level-optimized geometry of **4** shows the presence of strong $\text{Se}\cdots\text{O}$ interactions ($E_{\text{Se}\cdots\text{O}} = 28.05$ kcal/mol). The experimental ^{77}Se NMR chemical shift (1143 ppm) for this compound is in close agreement with the calculated value (1110 ppm), indicating the presence of a strong $\text{Se}\cdots\text{O}$ interaction in solution. The experimental ^{77}Se NMR chemical shift value is significantly downfield shifted compared with that of *o*-nitrobenzeneselenenic acid (1066 ppm), but this value is close to that reported for the selenenic acid derived from **6** (1173 ppm).^{10,19} In the natural GPx cycle, such interactions have been shown to facilitate the attack of nucleophilic sulfur at the electrophilic selenium atom, thereby producing the selenenyl sulfide. The crystal structure of human plasma GPx shows that the selenium atom is involved in weak interactions with Gln79 and Trp153 in the selenenic acid state ($\text{Se}\cdots\text{N}$ distances of 3.5 and 3.6 Å, respectively) in addition to the normal hydrogen-bonding interactions of other amino acid residues which are located in close proximity to selenium.²⁰

As selenenyl sulfides are the crucial intermediates in the GPx cycle, we next carried out a detailed theoretical investigation on some selenenyl sulfides derived from compounds having heteroatoms in close proximity to selenium. In general, the reduction of selenenic acids by thiols to the corresponding selenenyl sulfides increases the shielding of selenium nuclei. As expected, the B3LYP/6-31G(d)-level-optimized geometry of the selenenyl sulfide **2** shows a strong $\text{Se}\cdots\text{O}$ interaction ($r_{\text{Se}\cdots\text{O}} = 2.470$ Å; $E_{\text{Se}\cdots\text{O}} = 19.01$ kcal/mol), and the calculated ^{77}Se NMR chemical shift for this compound (604 ppm) is very downfield shifted compared to that of PhSeSPh (437 ppm), suggesting that the electron density around the selenium atom in **2** is considerably reduced by the coordinating group. It should be noted that the mesomeric effect of an aromatic ring having a delocalizing/electron-withdrawing carbonyl group in the ortho position may also reduce the electron density around selenium. However, the cross conjugation of the amide nitrogen with the π -electron system of the benzene ring may allow only a weak

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mesomeric effect. This can be rationalized by comparing the electron density on the sulfur atom in **2** with that of PhSSePh. In the case of the mesomeric effect, compound **2** should have an electron-deficient sulfur atom covalently bonded to the selenium. In contrast to this assumption, the calculations show that the sulfur atom in **2** carries less positive charge (0.029) than the sulfur in PhSSePh (0.089), indicating that the weak Se \cdots O interactions play a major role in decreasing the electron density around the selenium atom. Although Se \cdots O interactions favor the nucleophilic attack of thiol at selenium in the selenenic acid state, these interactions are detrimental to the reactivity of selenenyl sulfides as such interactions would lead to thiol exchange reactions rather than attack at sulfur to regenerate the selenol. The disadvantage of having a coordinating group near the selenium in the selenenyl sulfide state was also confirmed by experimental ^{77}Se NMR spectroscopy. The addition of an equimolar amount of PhSH to ebselen readily produced the expected selenenyl sulfide **2** (588 ppm), but the addition of an excess of PhSH did not produce the expected selenol **3**. Similarly, the addition of *N*-acetyl-L-cysteine to ebselen did not produce any signal for the selenol, but it produced the corresponding selenenyl sulfide **8** (565 ppm) as a stable product.

The results obtained by HPLC experiments are in agreement with those of ^{77}Se NMR experiments and theoretical calculations. Addition of an excess amount of PhSH to ebselen produced only the selenenyl sulfide as a stable product with a retention time of 6.9 min. Similarly, the reaction of ebselen with *N*-acetyl-L-cysteine also afforded the corresponding selenenyl sulfide as the only product (retention time 2.0 min) (see Figure S11 in the Supporting Information). These observations indicate that the selenenyl sulfides formed during the reactions are very stable and inactive toward further reaction with thiol or the excess thiol attacks at the selenium atom, leading to thiol exchange reactions. A crossover experiment was carried out using ebselen to confirm the possibility of a thiol exchange reaction. When ebselen was treated with *p*-thiocresol, it produced the expected selenenyl sulfide **9** (600 ppm); however, the addition of an excess amount of ethanethiol failed to produce the selenol even strictly under an inert atmosphere. On the other hand, it produced a signal at 532 ppm for a new selenenyl sulfide (**10**). These observations strongly suggest that the strong Se \cdots O interactions in the selenenyl sulfide state are detrimental to the biological activity of ebselen and related derivatives. The HPLC experiments also show that the addition of PhSH to selenenyl sulfide **8** produces compound **2** and the addition of *N*-acetyl-L-cysteine to **2** generates compound **8** by a thiol exchange reaction.

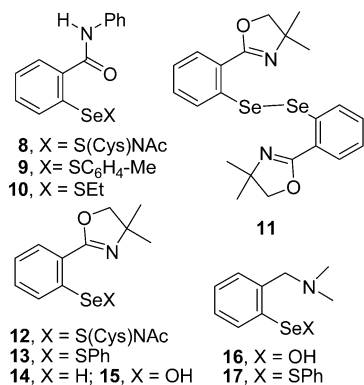


Table 3. A Comparison of the Calculated ^{77}Se NMR Chemical Shift Values of a Few Selenenyl Sulfides Having S \cdots N/O and Se \cdots N/O Interactions

compd	$r_{(\text{Se}\cdots\text{O})/(\text{S}/\text{Se}\cdots\text{N})}$ (Å)	$\delta(^{77}\text{Se NMR})^a$ (calcd) (ppm)	compd	$r_{(\text{Se}\cdots\text{O})/(\text{S}/\text{Se}\cdots\text{N})}$ (Å)	$\delta(^{77}\text{Se NMR})^a$ (calcd) (ppm)
2	2.470	604	19	2.751	405
13	2.608	547	20	2.630	500, 331
17	2.576	525	21	2.850	460
18	2.686	405			

^a Referenced to the peak for Me₂Se.

To support the observations that a strong Se \cdots O/N interaction increases the electrophilic reactivity of selenium, we carried out experiments on diselenide **11**, which has been shown to be an inactive catalyst in the reduction of H₂O₂ by PhSH.²¹ When diselenide **11** was treated with an excess amount of *N*-acetyl-L-cysteine in CDCl₃, the ^{77}Se NMR signal due to the diselenide disappeared completely to produce a new signal at 564 ppm for the corresponding selenenyl sulfide **12**. As in the case of ebselen, no signal was detected for the corresponding selenol even with a large excess of *N*-acetyl-L-cysteine. On the other hand, the addition of PhSH to the reaction mixture produced a signal at 574 ppm for another selenenyl sulfide (**13**) as a result of a thiol exchange reaction. The calculations on selenol **14** and selenenic acid **15** are also in close agreement with those of selenol **3** and selenenic acid **4** derived from ebselen (Table 4). Interestingly, the ^{77}Se NMR chemical shift (−48 ppm) calculated for selenolate **5** having a basic amino group is in close agreement with the experimental value (6 ppm),²¹ but it is very upfield shifted as compared with those of **3** and **14**. This indicates that selenol **5** can react with H₂O₂ much faster than **3** and **14**, which is consistent with the experimental observations. Tomoda et al. have shown, in a number of studies, that NBO second-order perturbation analysis can be conveniently used for studying hypervalent Se \cdots N/O/X (X = F, Cl, Br) interactions.^{10,13} Therefore, we carried out NBO analyses²² on a number of selenols, which show that the selenols can also be stabilized by hypervalent Se \cdots N/O interactions in the gas phase, although the stabilization energies ($E_{\text{Se}\cdots\text{N}}$) due to these interactions are relatively small (Tables 2 and 4). However, the calculations performed by including the effect of water show that selenol **5** exists in a completely dissociated form, whereas the selenols having amide and oxazoline substituents (**3** and **14**) exist as undissociated selenols. As can be seen from Table 3, the ^{77}Se NMR chemical shift for compound **17** (525 ppm) is very upfield shifted compared with that of **2** (602 ppm), suggesting that a nucleophilic attack of thiol at the selenium center in **17** is less favored as compared with the corresponding attack in **2**. This can also be rationalized by comparing the positive charge on the selenium atom in different selenenyl sulfides. According to these calculations, the selenenyl sulfides **2** (0.377) and **13** (0.352) carry a much higher positive charge on the selenium atom than the selenenyl sulfide **17** (0.289). This also suggests that a nucleophilic attack of thiol at the selenium center in **17** is less favored as compared to a similar attack at the selenium center in **2** or **13**. Therefore, the higher GPx activity of **5** as compared with that of **3** or **14** must be ascribed not only to the presence

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(22) (a) Reed, A. E.; Curtiss, L. A.; Weinhold, F. *Chem. Rev.* **1988**, *88*, 899. (b) Glendening, E. D.; Reed, J. E.; Carpenter, J. E.; Weinhold, F. *Natural Bond Orbital (NBO) Version 3.1*.

Table 4. Summary of Quantum Chemical Calculations, ^{77}Se NMR Chemical Shifts, and NBO Analysis on **5**, **7**, **13**–**17**, **19**, and **26**

compd	$r_{\text{Se}^{\text{S}}-\text{N}^{\text{O}}}$ (Å)	$\theta_{\text{N}^{\text{O}}-\text{Se}^{\text{S}}-\text{X}}$ (deg)	q_{Se}	$\delta(^{77}\text{Se NMR})^a$ (calcd) (ppm)	$\delta(^{77}\text{Se NMR})$ (exptl) (ppm)	$E_{\text{Se}^{\text{S}}-\text{N}^{\text{O}}}$ (kcal/mol)
5	2.829	165.9	0.144	81	6 ^b	5.19
7	2.725	171.8	0.446	253	253 ^c	6.13
13	2.608	177.5	0.352	547	574	13.45
14	2.759	167.0	0.219	197	270 ^b	6.35
15	2.471	172.3	0.694	1021	1206 ^b	23.01
16	2.485	172.7	0.617	1052	1164 ^b	21.64
17	2.576	175.2	0.289	525	562 ^b	15.49
19	2.751	176.9	0.215	405	455	5.46
26	2.569	177.1	0.374	632	622 ^d	14.35

^a Referenced to the peak for Me_2Se . ^b Reference 21. ^c Reference 27. ^d Reference 13g.

of **5** in its zwitterion form but also to the ability of selenenyl sulfide **17** to regenerate the selenol **5** in the catalytic cycle.

It has been shown that the conversion of selenenyl sulfide to selenol could be achieved by increasing the thiol concentration.²¹ To gain further insight into the effect of thiol concentration in the GPx-like reaction of ebselen, a detailed kinetic study was undertaken by using an HPLC assay. The initial rates derived for various concentrations of PhSH were plotted against the concentration of thiol. In contrast to the GPx catalytic cycle, saturation kinetics was not observed even at a very high concentration of PhSH and also the expected linear increase in the rate was not observed at these concentrations. Alternatively, a polynomial increase in the rate was observed with an increase in the concentration of PhSH. The rates were calculated to be lower than the control rates at lower concentrations of thiol as shown in Table 1, but the rate of the reaction gradually increased over the control rates at higher concentrations (Figure 2). This

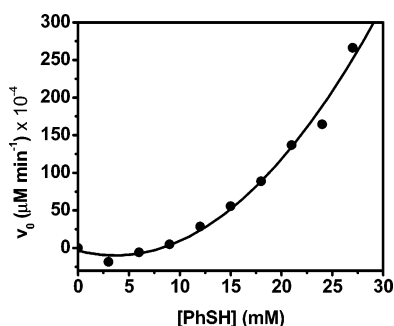


Figure 2. Initial rate (v_0) plotted against the substrate concentration. The initial rates were calculated from at least six HPLC experiments for each concentration of PhSH. The concentrations of ebselen and H_2O_2 were fixed at 0.47 and 9.8 mM, respectively.

is in agreement with the assumption that the thiol exchange reactions could be overcome, at least partially, by increasing the thiol concentration.

However, a closer look at the chromatograms of the reaction of ebselen with various concentrations of PhSH shows that the concentration of selenenyl sulfide **2** (retention time 6.3 min) with 9 mM PhSH (Figure 3A) is almost identical to that with 24 mM PhSH (Figure 3B). In fact, the selenenyl sulfide was found to be the major species during the entire concentration range, and the peak due to this compound was unaffected even after 300 min. These observations indicate that only a minor amount of selenenyl sulfide **2** could be converted to the corresponding selenol by increasing the concentration of thiol.

Although the poor catalytic activity of ebselen can be attributed both to the low reactivity of the selenol toward oxidation and to the thiol exchange reactions at selenium, further studies are required to give a firm conclusion regarding the rate-determining step. For this purpose, we prepared the selenol and selenenyl sulfide independently and compared their reactivity with peroxide and thiol to understand the origin of the poor catalytic activity. The reactions were followed by ^{77}Se NMR spectroscopy. The signal due to the selenenyl sulfide **2** was unaffected by the addition of PhSH, whereas the signal due to the selenol **3** completely disappeared upon the addition of peroxide to produce a new signal at 1143 ppm. This strongly suggests that the poor catalytic activity of ebselen is due to the thiol exchange reaction at selenium as opposed to sulfur in the selenenyl sulfide. Is there any other way to avoid the thiol exchange reactions and increase the GPx activity of organoselenium compounds? As already mentioned, the reduction of a selenenyl sulfide to selenol in the GPx cycle requires surmount-

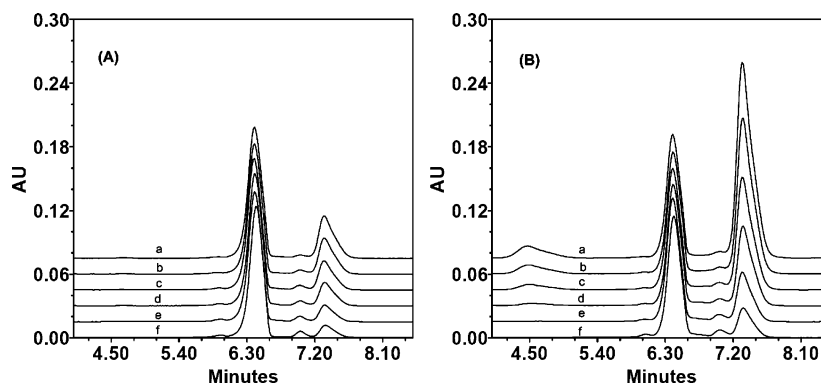
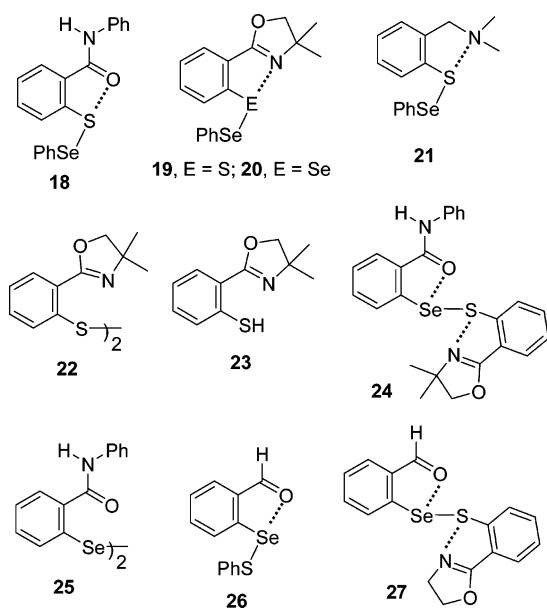


Figure 3. (A) Reversed-phase HPLC chromatogram (305 nm) of ebselen (0.5 mM) with PhSH (9.0 mM) and H_2O_2 (9.8 mM). The chromatographic runs were carried out at (a) 300 min, (b) 240 min, (c) 200 min, (d) 160 min, (e) 120 min, and (f) 0 min. (B) Reversed-phase HPLC chromatogram of ebselen (0.5 mM) with PhSH (24.0 mM) and H_2O_2 (9.8 mM). The chromatographic runs were carried out at (a) 0 min, (b) 120 min, (c) 160 min, (d) 200 min, (e) 240 min, and (f) 300 min.

ing a substantial barrier (~ 50 kcal/mol).¹¹ A careful analysis of the active site features of GPx reveals that the sulfur atom in the selenenyl sulfide intermediate is involved in a weak interaction with the amido nitrogen of the Thr54 residue, lowering the energy barrier to increase the possibility of a nucleophilic attack of negatively charged thiolate at the sulfur atom in the $-\text{Se}-\text{S}-$ bridge.^{23,24} Another selenoenzyme, thioredoxin reductase (TrxR), having proximal histidines²⁵ may also use such a strategy to overcome the thiol exchange reactions. To probe the role of Thr54 in the GPx cycle, we carried out experimental as well as theoretical investigations on some model compounds which have internal chelating groups near the sulfur. We carried out investigations on three different types of selenenyl sulfides having (i) only a $\text{Se}\cdots\text{O}/\text{N}$ interaction (selenenyl sulfide **2**), (ii) only a $\text{S}\cdots\text{O}/\text{N}$ (selenenyl sulfide **19**), and (iii) both $\text{Se}\cdots\text{O}/\text{N}$ and $\text{S}\cdots\text{O}/\text{N}$ interactions (selenenyl sulfide **24**).



As previously shown, the $\text{Se}\cdots\text{O}$ interaction in **2** does not favor a nucleophilic attack at sulfur, but it favors a nucleophilic attack at selenium. The reaction of diphenyl diselenide with thiol **23** having a heteroatom in close proximity to sulfur produced selenenyl sulfide **19**. Interestingly, addition of an excess amount of thiol completely reduced the selenenyl sulfide to the corresponding selenol, which was unstable and oxidized in solution to produce diphenyl diselenide. Similarly, when pure selenenyl sulfide **19** was treated with 1 equiv of thiol **23**, it produced a signal at 460 ppm for diphenyl diselenide. To confirm that the formation of diphenyl diselenide is not due to the disproportionation reactions, which are normally observed with selenenyl sulfides, the reaction of **19** with thiol **23** was monitored by ^{77}Se NMR spectroscopy and reversed-phase HPLC. When the reaction of **19** with **23** was carried out strictly under an inert atmosphere, it produced the expected selenol, which was trapped by treating the reaction mixture with iodoacetic acid. The ^{77}Se NMR spectrum recorded in $\text{CDCl}_3/\text{MeOH}$ showed a sharp signal at 203 ppm, indicating the

formation of the expected selenide. The HPLC experiments also show that the selenenyl sulfide **19** is stable enough to react with **23**, producing the disulfide and PhSeSePh . These studies clearly show that the thiol readily attacks at the sulfur center in **19** to produce the corresponding disulfide and benzeneselenol and the instability of the resulting selenol leads to the oxidation of this compound to the corresponding diselenide.

The experimental ^{77}Se NMR chemical shift (455 ppm) for selenenyl sulfide **19** is very downfield shifted compared to those for **13** (575 ppm) and PhSeSPh (526 ppm). This clearly indicates a possibility that the $\text{S}\cdots\text{N}$ interaction decreases the electro-positive character of the selenium atom in **19**. The calculations show that the nitrogen atom in **19** interacts with the sulfur ($\text{S}\cdots\text{N} = 2.751 \text{ \AA}$), leading to an elongation of the $-\text{S}-\text{Se}-$ bond ($\text{Se}-\text{S} = 2.238 \text{ \AA}$). The NBO second-order perturbation energy for the $\text{S}\cdots\text{N}$ interaction ($E_{\text{S}\cdots\text{N}}$) in **19** is calculated to be 5.46 kcal/mol. The calculated ^{77}Se chemical shift for **19** (405 ppm) also shows a large upfield shift as compared with that of **13** (547.0 ppm), confirming the expected decrease in the electrophilic character of selenium. The experimental ^{77}Se NMR results are in agreement with the theoretical results, and the upfield signal observed for an authentic sample of **19** (455 ppm) also suggests that the selenium nucleus in **19** is considerably shielded due to the $\text{S}\cdots\text{N}$ interactions even in solution. The NBO analysis shows a decrease in the positive charge on selenium and an increase in the positive charge on sulfur on moving from **13** [0.352 (Se), 0.033 (S)] to **19** [0.215 (Se), 0.152 (S)]. This indicates that the nonbonded interactions with sulfur in the selenenyl sulfide intermediate not only increase the electropositive character of sulfur but also decrease the electrophilic character of selenium. In other words, the $\text{S}\cdots\text{N}$ interactions would certainly enhance the possibility of the thiol attack at sulfur rather than at selenium. The increase in the electron density around selenium or the decrease in the electron density around sulfur in **19** by $\text{S}\cdots\text{N}$ interactions was further verified by replacing the sulfur atom in **19** with selenium (compound **20**). In this case, the calculated ^{77}Se chemical shifts show that the selenium atom located near the amino group is more deshielded (500 ppm) as compared with the other selenium atom (331 ppm).

Finally, we focused our attention on selenenyl sulfide **24**, which has both $\text{Se}\cdots\text{O}/\text{N}$ and $\text{S}\cdots\text{O}/\text{N}$ interactions. As expected, the reaction of ebselen with thiol **23** produced the corresponding selenenyl sulfide **24**. This compound exhibited a ^{77}Se NMR signal at 547 ppm, which is significantly upfield shifted compared with that of **2** (588 ppm). Crucially, the addition of an excess amount of **23** to selenenyl sulfide **24** readily produced the corresponding selenol, which is unstable and upon oxidation afforded the diselenide **25** (440 ppm) in nearly quantitative yield. These observations strongly suggest that the introduction of coordinating amino or other groups in the thiols would enhance the GPx-like catalytic activity of ebselen and other related organoselenium compounds (Figure 4). Although ebselen effectively reduces H_2O_2 in the presence of thiol **23**, all attempts to obtain the reaction rate were unsuccessful. The reaction of **24** with thiol **23** produced the desired selenol **3** and disulfide **22**, but unfortunately, the disulfide, in contrast to PhSSPh , reacted with H_2O_2 to produce the corresponding sulfenic acid or other oxidized products. The cleavage of the $-\text{S}-\text{S}-$ bond in **22** by H_2O_2 may be due to the presence of strong $\text{S}\cdots\text{N}$ interactions ($\text{S}\cdots\text{N} = 2.815 \text{ \AA}$, $\text{S}\cdots\text{N} = 2.787 \text{ \AA}$),²⁶ which

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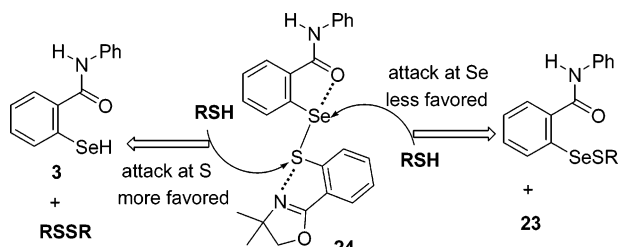


Figure 4. $S\cdots N$ interactions modulate the attack of an incoming thiol at the sulfur center in ebselen selenenyl sulfide **24**.

activates the $-S-S-$ bond. Because of this unexpected behavior of **22**, the initial rate could not be determined by HPLC or UV spectroscopic methods.

Having established the effect of novel $S\cdots N/O$ interactions on the reactivity of selenenyl sulfides, we next set out to investigate the correlation between $S\cdots N/O$ distances and the strength of $Se\cdots N/O$ interactions. For this purpose, we carried out DFT calculations on **26** and **27**, which are simplified models for **2** and **24**, respectively. The $Se\cdots O$ distance in **27** (2.631 Å) is found to be longer than that in **26** (2.569 Å). NBO analysis shows that the orbital interaction energy $E_{Se\cdots O}$ in **27** (11.43 kcal/mol) is lower than that in **26** (14.35 kcal/mol) due to the presence of $S\cdots N$ interactions ($E_{S\cdots N} = 4.78$ kcal/mol). As the decrease in interaction energy would lead to a decrease in the electrophilic character of selenium, the calculated ^{77}Se NMR chemical shift for **27** is very upfield shifted (586 ppm) as compared with that of **26** (632 ppm). A comparison of the experimental ^{77}Se NMR chemical shift between **2** (588 ppm) and **24** (547 ppm) also supports that $S\cdots N$ interactions are indeed present in **24**, which increases the possibility of a nucleophilic attack at the sulfur center of the $-Se-S-$ bridge. The energy profile obtained for **27** by varying the dihedral angle $\gamma(1,2,3,4)$ from -180° to $+180^\circ$ stepwise by 10° is shown in Figure 5. In the rotation profile, the energy minima were found at -160° (A), 0° (C), and $+160^\circ$ (E) and the maxima were found at -90° (B) and $+80^\circ$ (D). The $S\cdots N$ distance was found to be the shortest (2.786 Å) at the point where the most stable minimum (C) occurs, and this distance is in good agreement with the optimized structure (Figure 5). The other two minima at -160° (A) and $+160^\circ$ (E) correspond to conformations where the nitrogen atom of the oxazoline ring is completely away from the sulfur atom. At this stage, the structures are stabilized by interaction between sulfur and the oxygen atom present in the five-membered ring. The $S\cdots O$ distances (2.623 Å at $\gamma = +160^\circ$ and 2.623 Å at $\gamma = -160^\circ$) indicate that the $S\cdots O$ interactions also lead to some stabilization of the molecule although these conformations are less stable than those obtained by $S\cdots N$ interactions (relative energy for **27**, 0.0, 2.77, and 2.58 kcal/mol at $\gamma = 0^\circ$, $+160^\circ$, and -160° , respectively). The conformations become unstable when the oxazoline ring lies perpendicular to the benzene ring. In these conformations, there is no $S\cdots N$ or $S\cdots O$ interaction as the nitrogen and oxygen atoms lie either above or below the plane of the benzene ring.

The theoretical data²⁸ obtained under the present study are in good agreement with the experimental results. For example, bond lengths and angles obtained by theoretical calculations for **13** are consistent with the data obtained from single-crystal

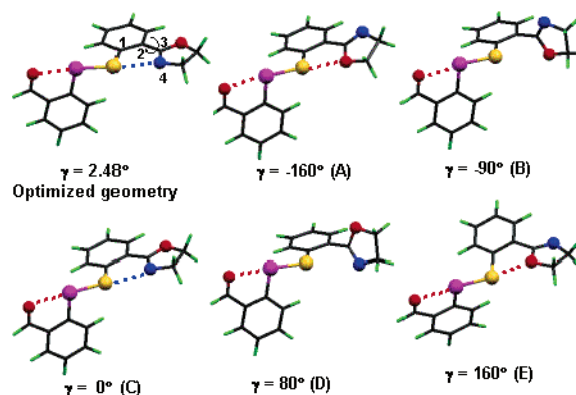
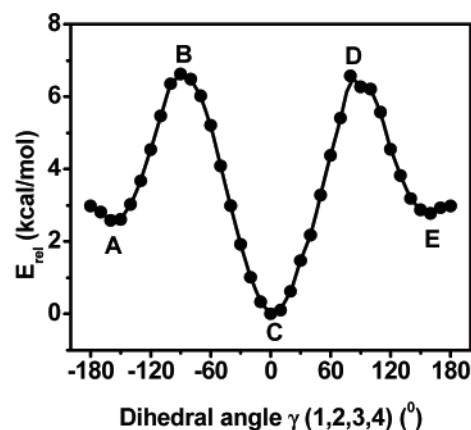


Figure 5. Variation of the relative stabilization energy of **27** for the rotation of the oxazoline ring around the C–C bond attached to the benzene ring. The calculations were performed at the B3LYP/6-31G* level of theory.

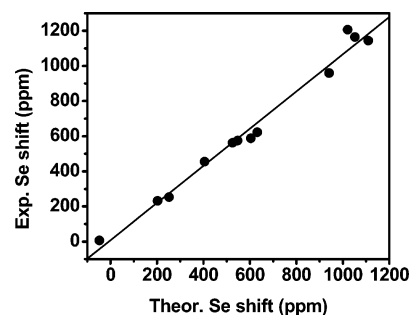


Figure 6. Theoretical versus experimental ^{77}Se NMR chemical shifts (ppm). The data were taken from Tables 2 and 4.

X-ray analysis.²⁹ The calculated GIAO ^{77}Se NMR chemical shifts for most of the compounds in the present study also correlate well with the experimental data. A plot of theoretical ^{77}Se NMR chemical shifts versus the experimental shifts shows a linear correlation as shown by Bayse,³⁰ demonstrating the accuracy of the calculations (Figure 6).

Conclusions

The experimental and theoretical observations on ebselen and related derivatives suggest that strong $Se\cdots O/N$ interactions in the selenenyl sulfide states are detrimental to the biological activity of synthetic selenium compounds due to thiol exchange

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reactions. The relatively poor GPx-like catalytic activity of ebselen is, therefore, due to the undesired thiol exchange reactions that take place at the selenium center in the selenenyl sulfide intermediate. This study provides the first experimental evidence that any substituent that is capable of enhancing the nucleophilic attack of thiol at sulfur in the selenenyl sulfide state would enhance the antioxidant potency of organoselenium compounds. These results lead to an assumption that some of the glutathione peroxidases utilize non-GSH cosubstrates probably to overcome thiol exchange reactions.

Experimental Section

All reactions were carried out under nitrogen or argon using standard vacuum-line techniques. Solvents were purified by standard procedures and were freshly distilled prior to use. ^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. Mass spectral studies were carried out on a Q-TOF micro mass spectrometer with ESI MS mode analysis. In the case of isotopic patterns, the value given is for the most intense peak. Ebselen was synthesized by following the literature method.³¹

Synthesis of 2. To a stirred solution of ebselen (**1**) (21 mg, 0.076 mmol) in CH_2Cl_2 was added benzenethiol (8 μL , 0.076 mmol). After the resulting solution was stirred for 1 h at room temperature, the solvent was evaporated under reduced pressure, and the product obtained was washed with petroleum ether to remove any unreacted thiol or disulfide formed during the reaction. The selenenyl sulfide **2** was obtained as a light yellow oil in quantitative yield: ^1H NMR (CDCl_3) δ 8.23 (d, 1H), 7.94 (s, 1H), 7.71 (d, 1H) 7.62 (d, 2H) 7.51 (d, 2H) 7.32–7.41 (m, 3H) 7.13–7.28 (m, 5H); ^{13}C (CDCl_3) δ 166.1, 137.7, 137.2, 136.5, 132.5, 131.2, 129.4, 129.3, 129.1, 128.9, 126.5, 126.2, 125.7, 125.2, 120.7; ^{77}Se (CDCl_3) δ 588; HRMS (TOF MS ES^+) m/z 407.9937 ($\text{M} + \text{Na}$) $^+$.

Synthesis of 9. To a stirred solution of **1** (21 mg, 0.076 mmol) in CH_2Cl_2 was added 4-methylbenzenethiol (9.42 mg, 0.076 mmol). After the resulting solution was stirred for 1 h at room temperature, the solvent was evaporated under reduced pressure, and the product obtained was washed with petroleum ether to remove any unreacted thiol or disulfide formed during the reaction. The selenenyl sulfide **9** was obtained as a light yellow oil in quantitative yield: ^1H NMR (CDCl_3) δ 8.27 (d, 1H), 7.93 (s, 1H), 7.69 (d, 1H) 7.61 (d, 2H) 7.50 (t, 1H) 7.30–7.42 (m, 5H) 7.18 (t, 1H) 7.04 (d, 2H) 2.29 (s, 3H); ^{13}C (CDCl_3) δ 166.1, 137.8, 137.2, 136.8, 133.1, 132.4, 131.3, 129.7, 129.5, 129.3, 129.0, 126.6, 126.2, 125.2, 120.6, 20.2; ^{77}Se (CDCl_3) δ 600; HRMS (TOF MS ES^+) m/z 422.0094 ($\text{M} + \text{Na}$) $^+$.

Synthesis of 19. To a stirred solution of diphenyl diselenide (150.72 mg, 0.48 mmol) in CH_2Cl_2 was added the thiol **23** (100 mg, 0.48 mmol). After the resulting solution was stirred for 1 day at room temperature, the solvent was evaporated under reduced pressure to give a yellow residue. This was purified by column chromatography using petroleum ether/ethyl acetate (5:1) as eluent to give compound **19** as a yellow oil in 30% yield: ^1H NMR (CDCl_3) δ 7.84 (d, 1H), 7.74 (d, 1H), 7.56 (d, 2H) 7.34 (t, 1H) 7.20–7.29 (m, 4H), 4.11 (s, 2H), 1.44 (s, 6H); ^{13}C (CDCl_3) δ 160.9, 137.8, 132.0, 131.1, 129.8, 129.5, 129.3, 129.2, 127.4, 126.7, 125.8, 78.9, 68.7, 28.6; ^{77}Se (CDCl_3) δ 455; HRMS (TOF MS ES^+) m/z 364.0274 ($\text{M} + \text{H}$) $^+$.

Synthesis of 22. The disulfide **22** was synthesized by following the literature method with some modifications.²⁶ To a solution of 4,4-dimethyl-2-phenyloxazoline (1.75 mL, 1.79 g, 10 mmol) in dry hexane (50 mL) was added a 1.6 M solution of *n*-BuLi in hexane (6.8 mL, 11 mmol). After the resulting solution was stirred for 1 h at room temperature, a white precipitate of lithiated compound was obtained. The supernatant solvent was removed with a syringe. The white

precipitate was dissolved in dry ether (30 mL), the solution was cooled to 0 $^\circ\text{C}$, and sulfur powder (0.32 g, 10 mmol) was added. After 1 h all sulfur was consumed to give a yellow solution. This solution was poured into a beaker containing $\text{K}_3\text{Fe}(\text{CN})_6$ and stirred for 2 days by adding CH_2Cl_2 from time to time. The resulting solution was extracted with CH_2Cl_2 , dried over anhydrous sodium sulfate, and concentrated to give a yellow oil. Recrystallization of the product in chloroform/methanol (1:1) afforded the disulfide as white needles: yield 0.8 g, (50%); ^1H NMR (CDCl_3) δ 7.78 (d, 1H), 7.72 (d, 1H), 7.32 (t, 1H), 7.20 (t, 1H), 4.12 (s, 2H), 1.44 (s, 6H); ^{13}C NMR (CDCl_3) δ 160.7, 138.6, 131.2, 129.8, 126.2, 125.9, 125.4, 78.9, 68.8, 28.6; MS (TOF MS ES^+) m/z 435 [$\text{M} + \text{Na}$] $^+$.

Synthesis of 23. The thiol **23** was prepared by reducing the corresponding disulfide with NaBH_4 at 0 $^\circ\text{C}$ under a N_2 atmosphere. To a cooled solution (0 $^\circ\text{C}$) of the disulfide **22** in MeOH was added NaBH_4 (10 equiv). After the resulting solution was stirred for 1 h at 0 $^\circ\text{C}$, the organic layer was extracted with CH_2Cl_2 , dried over anhydrous sodium sulfate, and concentrated to give a green-yellow oil. This compound was essentially pure and was used without further purification. This compound is stable under an inert atmosphere, but oxidizes slowly under air to produce the corresponding disulfide.

Synthesis of 24. To a stirred solution of **1** (21 mg, 0.076 mmol) in CH_2Cl_2 was added the thiol **23** (15.75 mg, 0.076 mmol). After resulting solution was stirred for 1 h at room temperature, the solvent was evaporated under reduced pressure, and the product obtained was washed with petroleum ether to remove any unreacted thiol or disulfide formed during the reaction. The yellow oil of **24** was obtained in good yield (90%): ^1H NMR (CDCl_3) δ 8.05 (d, 1H), 7.99 (s, 1H), 7.72 (d, 2H), 7.65 (t, 4H), 7.41 (q, 4H), 7.16–7.24 (m, 2H), 4.16 (s, 2H), 1.47 (s, 6H); ^{13}C (CDCl_3) δ 166.2, 161.4, 137.4, 137.3, 137.1, 132.5, 131.7, 131.1, 129.9, 129.4, 129.2, 127.0, 126.9, 126.7, 126.2, 125.5, 125.1, 120.7, 78.9, 68.7, 28.6; ^{77}Se (CDCl_3) δ 547; MS (TOF MS ES^+) m/z 477 [$\text{M} - 5$] $^+$.

HPLC Assay. In this assay, we employed a mixture containing a 2:1 molar ratio of PhSH or 4-MeC₆H₄SH and H₂O₂ in dichloromethane/methanol (95:5) at room temperature as our model system. Runs with and without 10 mol % added ebselen were carried out under the same conditions. Periodically, aliquots were removed, and the concentrations of the product diphenyl disulfide (PhSSPh) were determined by reversed-phase HPLC, using pure PhSSPh as an external standard. The catalytic activity of ebselen with 4-MeC₆H₄SH was studied by following a similar method using the corresponding disulfide as an external standard. The amount of disulfide formed during the course of the reaction was calculated from the calibration plot for each standard.

Computational Methods

All calculations were performed using the Gaussian98 suite of quantum chemical programs.³² The hybrid Becke 3–Lee–Yang–Parr (B3LYP) exchange correlation functional was applied for DFT calculations.³³ Geometries were fully optimized at the B3LYP level of theory using the 6-31G(d) basis sets. All stationary points were characterized as minima by corresponding Hessian indices. The NMR calculations were done at the B3LYP/6-311+G(d,p) level on B3LYP/6-31G(d)-level-optimized geometries using the GIAO method.³⁴ Orbital interactions were analyzed using the natural bond orbital (NBO) method at the B3LYP/6-31G(d) level, and charges were calculated from natural population analysis (NPA).²²

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Supporting Information Available: Experimental data (^{77}Se NMR spectra) and archive entries for the optimized geometries

and calculated GIAO chemical shifts (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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