Isolation of Unusual *N*-Thiophenyl Ebselenamines and other Intermediates during the ^{77}Se NMR Mechanistic Study of Azo-Bis-Ebselen: Their Antioxidant Behaviour against Oxidative Stress

MANISH KUMAR
PANJAB UNIVERSITY CHANDIGARH https://orcid.org/0000-0002-9859-3793
VIJAY P. SINGH (vijay@pu.ac.in)
Panjab University Chandigarh

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Abstract

Based on the traditional ^{77}Se NMR spectroscopy investigation, a catalytic cycle for the formation of *N*-thiophenyl ebselenamine **12** involving diselenide **9**, selenenyl sulfide **10** and ebselenamine **7a** was reported by the reaction of azo-bis-ebselen **8** with PhSH and H_2O_2. The signals detected in the ^{77}Se NMR spectrum corresponding to **7a**, **10** and **12** were directly isolated from the NMR mixture. Mechanistic investigation for the formation of *N*-thiophenyl ebselenamine **12** was confirmed from an independent reaction of diselenide **9** and PhSSPh in the presence of H_2O_2. This was further supported by another diselenide **19** containing *p*-tolyl group with equimolar amount of H_2O_2 and PhSH in an independent experiment followed by the ^{77}Se NMR spectroscopy, yielding similar observations. These results, which illustrated diselenide has been observed as the main precursor in the formation of all intermediates. The new novel selenium antioxidants quenched lipidperoxyl radicals much more efficiently than α-tocopherol and were regenerable by the aqueous ascorbic acid in a two-phase (chlorobenzene/water) azo-initiated peroxidation system. The notable benefit of the organoselenium biology, the novel ebselenamine analogues and their corresponding selenenyl sulfides were found to mimic the activity of the glutathione peroxidase enzymes better than ebselen in the coupled reductase assay.

Introduction

Normally, cells have their antioxidant defence against reactive oxygen species (ROS) such as lipid peroxyl radicals (LOO•) and organic peroxides, produced during the aerobic metabolism thus causing oxidative damages in living organism.[1] Some natural enzymes, antioxidants such as most abundant molecular weight tripeptide reduced glutathione (GSH) maintain the homeostasis defence against ROS level.[2] In an early research, higher or moderate level of GSH has very crucial role in the treatment and prevention of COVID-19.[3] The redox state of the GSH is controlled by the glutathione reductase (GR), a flavoenzyme that uses *β*-nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor to reduce the disulphide bond (S–S) bond in oxidized glutathione (GSSG).[4] In addition to antioxidant functions and scavenging effects of ROS[5], GSH acts as substrate for the selenoenzymes for the reduction of harmful peroxides.[6]

The great excitement was elicited in mammalian selenoenzymes containing the 21st amino acid such as selenocysteine (SeCy) at the active site have shown a crucial role to detoxify the organic peroxides in living organisms and therefore, protect them from oxidative damages.[7] Among the 25 selenoenzymes in animals, the glutathione peroxidase (GPx) enzymes are the most important hydroperoxide-decomposing enzymes in humans served as an antioxidant.[6] The GPx enzymes destroy the harmful hydrogen peroxide (H_2O_2) using GSH as the stoichiometric reducing agent.[8] In the catalytic cycle of the GPx enzymes, selenocysteine selenenic acid (SeCy-SeOH), selenocysteine selenol (SeCy-SeH) and the corresponding selenenyl sulphide (SeCy-SeSG) have been proposed as the key intermediates.[9] The presence of selenenic acid in the mechanism of the GPx is very crucial.

In a very recent finding, Flohè et al. observed a bypass mechanism where the selenenic acid underwent intramolecular cyclization to the corresponding Se–N heterocycle.[10] Intramolecular cyclization of unstable selenenic acids produced from their corresponding selenols has been proposed as stable heterocyclic compounds.[11] Many examples of highly reactive selenenic acids and selenols were implicated during the investigation of catalytic mechanisms of several organoselenium compounds *in situ* using the ^{77}Se NMR kinetic experiments.[12] In few reports, diselenides formation was predicted from selenenic acid, presumably due to the presence of unreacted selenol in the mixture.[12e,13] However, isolations of few stable selenenic acids and selenols have been reported.[14,15]

Initial work done by Sies[16] and Wendel[17] showed that ebselen (**1**, PZ 51, 2-phenyl-1,2-benzisoselenzol-3-(*2H*)-one), a cyclic Se–N compound exhibited both anti-inflammatory activity *in vivo* and GPx-like activity *in vitro* with low toxicity (Figure 1). It catalytically reduced the harmful peroxides by GSH or other thiols mimicking the activity of the GPx-enzymes and protects the lipid membranes and other cellular components against oxidative damage.[18,19]

Mechanistic studies of ebselen followed by the ^{77}Se NMR spectroscopy using thiols such as PhSH or GSH and H_2O_2 indicated that Se–N bond is reductively cleaved to form the corresponding selenenyl sulfide intermediate, which further reacts with H_2O_2 to produce selenol and selenenic acid intermediates.[12f,13] Furthermore, it has been demonstrated in phase II for the safety of noise-induced hearing loss and phase III for cerebal ischemia bipolar disorder diseases.[20] Recently, it exhibited promising antiviral activity as inhibitor of the main coronavirus protease in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in COVID-19.[21] Inspired with ebselen as the most redox active molecule, several other important biologically active organoselenium compounds have been reported including ebselen analogues and other derivatives.[22-24]

Ebselen analogues with different substituents at the *N*-phenyl ring by introducing amino acid functions and electron-donating groups were prepared with a capacity to improve the catalytic activity.[22b-d] For example, the GPx-like activity of **2** was found several fold greater than ebselen.[22d] The presence of *ortho*-coordinating groups in a close proximity to the Se atom in organoselenium compounds have been reviewed towards their synthesis and antioxidant activities.[25] The nitro-derivative **3a** of ebselen possessing weak secondary Se•••O interactions exhibited better GPx activity.[11b,26] Recently, we have done further modification in the *N*-phenyl ring of **3a** that resulted **3b-c** with enhanced activity as compared to ebselen.[27] In contrast to ebselen, another class of selenenamides such as camphor-derived cyclic selenenamide **4**[12b], isoselenazoles **5**[28] and **6**[12g] has been discovered as very good GPx mimics.

During the last two decades, in search for better antioxidants, other than organoselenium compounds, aromatic amines have been considered as potent inhibitors of lipid LOO• radicals.[29] Aminic radical-trapping antioxidants (RTAs) inhibited the spontaneous autoxidation process with high impact than phenols.[30] Among the phenols, α-tocopherol (α-TOH) has been optimized by Nature and considered as

the best lipophilic RTA.[31] It inhibits the lipid peroxidation *via* the formal H-atom transfer (HAT) from the phenolic OH group to the chain-carrying LOO• forming hydroperoxide (LOOH) as non-radical products.

Over the years, we and Engman focused to improve the radical-trapping activity of both phenolic and aminic antioxidants by incorporating chalcogenide (Se, Te) atom in a two-phase lipid/aqueous biological membrane-like model system.[32] These compounds multi-functionally inhibited the lipid peroxidation and reduced LOO• directly into alcohols (LOH) using water-soluble co-antioxidants acted as more reactive than α-TOH.[33To account for the high reactivity, a rather unconventional mechanism was proposed involving the initial O-atom transfer from LOO• to the Se/Te atom, followed by HAT reaction, in a solvent cage, from the nearby H-atom donor group to the resulting alkoxyl radical.[34] Interestingly, these were found to be highly regenerable by the presence of water-soluble co-antioxidants. Recently, we reported ebselenamines **7** and *N*-methylated ebselenamines as multifunctional antioxidants with higher capacity to inhibit the lipid peroxidation as well as good GPx-like activity.[35-36]

Herein, as a consequence, in view of the above and our longstanding interest with pleasant results of organochalcogen chemistry, we are highly encouraged to examine the intermediates involved in the catalytic mechanism followed by the ^{77}Se NMR spectroscopy. During our recent findings on ebselenamines, we observed that the thiolysis of azo-bis-ebselen followed by oxidation with H_2O_2 afforded several intermediates during the ^{77}Se NMR mechanistic studies. Among the other isolated products, new *N*-thiophenyl ebselenamines and the corresponding selenenyl sulfides were obtained. We present results of our investigations from the direct NMR spectroscopic evidences for stable intermediates and their isolations. We also report their antioxidant behaviour with a capacity to scavenge the lipid peroxyl radicals and the GPx-like activities.

Results And Discussion

To ascertain the kinetic study, the required azo-bis-ebselen **8** was prepared using modified procedure from previous report.[27] We employed a mixture of compound **8** and PhSH in $CDCl_3$ by subsequent addition of H_2O_2 to the mixture at room temperature monitored by the ^{77}Se NMR resonance which revealed full consumption of compound **8** (Scheme 1). Various signals were observed in the ^{77}Se NMR spectrum at 362, 450, 533, 868, and 926 ppm and assigned corresponding to diselenide **9**, selenenyl sulfide **10**, diselenide **11**, *N*-thiophenyl ebselenamine **12** and ebselenamine **7a**, respectively (See Figure S13-S23 in the Supporting Information). The identities of diselenide **9** and ebselenamine **7a** were in agreement with the reported ^{77}Se NMR chemical shifts.[35, 36]

Initially, the unknown unstable signal was observed at 533 ppm together with **12** by addition of PhSH (8 equiv) to a solution of compound **8**. During the kinetic experiments, the signal 533 ppm was assumed to be diselenide **11** (*vide infra*, Scheme 2 and 4). Again, excess of PhSH (24 equiv) to the above mixture produced new signals at 450 and 868 ppm. The signal at 533 ppm was very unstable and disappeared completely after the addition of H_2O_2 (6 equiv) to the NMR mixture. Considering the successful outcome

of the reaction, addition of H_2O_2 (excess) to the above mixture, the most stable signals at 450, 868 and 926 ppm were observed only. During the isolation of stable intermediates corresponding to their signals as above, a signal for diselenide **9** (δ 362 ppm) was also detected together with other intermediates. Selenenyl sulfide **10**, *N*-thiophenyl ebselenamine **12** and ebselenamine **7a** were isolated from the NMR mixture after purification in 28%, 25% and 16% yields, respectively. Here, *N*-thiophenyl ebselenamine **12** recognition has not been reported previously. This is our new finding of ebselen analogue carrying *N*-thiophenyl group at *ortho*-position to the Se atom in the heterocyclic ring.

It was hypothesized that compound **8** was reduced by PhSH, leading to diselenide **9** containing free $-NH_2$ group at *ortho*-position to the Se atom. Alternatively, we already established the protocol for the preparation of **9** by treating with *in situ* generated PhTeNa in ethanol.[27] Surprisingly, the azo (−N=N−) group was reduced fully to $-NH_2$ group and the Se−N heterocyclic bond also cleaved reductively to produce ultimately **9** as the only one major product. Studies of azo-benzene derivatives reduction with GSH were reported by Moroder et al., where the −N=N− group was reduced into hydrazo (−NH−NH−) group.[37] This mechanism was based on acid and base catalysis. The pK_a values of PhSH (6.5) and GSH (9.2) might be able to alter the reactivity at the −N=N− group.[38, 39] The acidic nature of the thiolate (PhS^-) is more prone to reduce −N=N− into $-NH_2$ group, cleanly. Further, diselenide **9** reacted with PhSH again in the NMR mixture produced the corresponding **10** (*vide infra*, Scheme 3). It has been reported that diselenides have been found to produce selenenyl sulphides by treating with thiols (GSH/ PhSH).[12h,36] Additionally, The attack of the selenol upon selenenyl sulphide afforded the generation of diselenide and thiol.

In order to identify all possible intermediates observed in the mechanism, the structure of unstable diselenide **11** was optimized using DFT calculations (please see the Supporting Information for optimized geometry and coordinates). The calculated ^{77}Se NMR chemical shift 548 ppm was close to that of experimental value 533 ppm (Table S1 in the Supporting Information). This might be due to the involvement of two *ortho*-coordinating groups that further cyclized into **12**. The precise mechanism for the formation of **12** from diselenide **9** *via* the intermediate **13** has been proposed (Scheme 2). In the mechanism, it has been observed that $-NH_2$ group in diselenide **9** acted as nucleophile towards PhSSPh. The formation of intermediate **13** with two *ortho*-coordinating groups was possible *via* the S_N2 reaction at both the free $-NH_2$ group. Further, the S_N2 reaction in intermediate **11** could not take place due to involvement of d-orbitals of the Se atom with the nearby lone pair of electrons on the N-atom in -NHPhS group. Then, PhS anion abstracted the H-atom from the N-centre, resulting **11**. This hindered diselenide **11** destabilized and cyclized due to the unexpected disproportionation into arylselenolate ($ArSe^-$) anion and **15** through the intermediate **14**. This is in good agreement with the recently reported organoselenium compounds bearing two coordinating groups at *ortho*-position to the Se atom underwent disproportionation reaction due to ring strain in the molecule.[11,12g,28,40,41] Since diselenide species are dimeric molecules, and therefore, each equivalent should lead to compound **12** (2 equiv). It has been observed that the compound **12** formed *via* the returning of intermediate selenol **16** to the cycle.

In order to confirm the formation of selenenyl sulfide **10**, we performed another ^{77}Se NMR experiment by treating diselenide **9** with PhSH followed by subsequent addition of H_2O_2 in Scheme 3 (see Figure S24-S30 in the Supporting Information for spectra). With one equiv of PhSH, a signal for selenenyl sulfide **10** was appeared immediately. While in excess of PhSH, signals corresponding to selenol **17** (δ 104 ppm) were observed along with selenenyl sulfide **10** and unreacted diselenide **9**. The oxidation of selenol **17** in the presence of H_2O_2 produced ebselenamine **7a** *via* the selenenic acid **18**.[36]

Again, addition of PhSH to the above mixture, ebselenamine **7a** was reductively cleaved to form selenenyl sulfide **10**. Ebselen and its analogues carrying the Se–N heterocyclic bond were found to react directly with thiol and produced first their corresponding selenenyl sulphides. Further, addition of thiol would generate selenol intermediates and disulphide.[11b,12b,f,g,42]

Diselenides containing basic groups at *ortho*-position to the Se atom have attracted much attention as the GPx mimics since the Se•••N/O intramolecular secondary interactions:[19, 43] a) to activate the Se–Se bond towards the oxidative cleavage; b) to stabilize the resulting selenenic acid intermediate against further oxidation; and c) to enhance the nucleophilic attack of the PhSH at the S rather than Se atom in the selenenyl sulfide to produce the highly reactive selenol and PhSSPh.[44] We observed that ebselenamine **12** has been implicated by the disproportionation of selenenyl sulfide **10** into diselenide **9** and PhSSPh in the presence of H_2O_2. Similar observations were reported where selenenyl sulphide intermediates disproportionated to the corresponding diselenides and disulphide.[12f,13] The rate of disproportionation depends on the strength of the Se•••O interactions in the selenenyl sulphide. The strong interactions were found to enhance the stability of selenenyl sulphide in solution (like ebselen selenenyl sulfide), which is the rate determining step.[12e–f]

As a result, compound **10** was relatively unstable in solution, and therefore disproportionated into diselenide **9** and PhSSPh. A detailed ^{77}Se NMR kinetic study of diselenide **9** was carried out by treating PhSSPh in the presence of H_2O_2 to confirm the formation of **12** as shown in Scheme 4 (see Figure S31-S34 in the Supporting Information). As anticipated, compound **12** was formed only *via* unstable diselenide **11** as implicated in the ^{77}Se NMR spectrum during the kinetic studies. In a control experiment, diselenide **9** and PhSSPh (3 equiv) did not make any change in the ^{77}Se NMR spectra.

Interestingly, addition of H_2O_2 (1 equiv) to the mixture produced immediately a signal corresponding to **7a**. Whereas excess of H_2O_2 (3 equiv) was found crucial to generate the signals for **7a**, **10** and **12** along with unreacted **9**. In the next step, the formation of **7a** was observed mainly due the oxidation of diselenide **9** with H_2O_2 as in Scheme 3. Diselenide **11** underwent a facile intramolecular cyclization of ebselenamine **12** was observed only from diselenide **11** containing two bulky *ortho*-coordinating groups (*vide supra*). This is in good agreement that diselenides bearing two *ortho*-coordinating or hindered groups led to stable cyclic compounds.[11, 28, 41]

To confirm the formation of the products formed in Scheme 3 and 4, the diselenide **19** was prepared and used for further kinetic experiments.[27, 36] Diselenide **19** was treated with equimolar amounts of H_2O_2 and PhSH in DMSO-d_6. The progress of the reaction monitored using the ^{77}Se NMR spectroscopy (Scheme 5). In the ^{77}Se NMR, three signals at 409, 842 and 929 ppm were observed (See Figure S35 in the Supporting Information). These signals were assigned to the corresponding selenenyl sulfide **20**, *N*-thiophenyl ebselenamine **21** and ebselenamine **7b** that were obtained in 27%, 30% and 7% yields, respectively.

The ^{77}Se NMR chemical shifts of *N*-thiophenyl ebselenamines **12** and **21** were δ 833 and 856, respectively. The signals shifted more upfield as compared to ebselen (δ 960)[12f] and ebselenamines **7a-d** (δ 926-930).[36] The ^{77}Se NMR chemical shifts of selenenyl sulfides **10** (δ 452) and **20** (δ 453) were also upfield shift 135 ppm in comparison to ebselen selenenyl sulfide (δ 588). This downfield shift in ebselen selenenyl sulfide is due to the presence of strong secondary Se•••O interaction.[44] Earlier, we have observed the similar upfield ^{77}Se NMR chemical shifts in the catalytic cycle of ebselenamine-based selenenyl sulphides (δ 407).[35, 36] The calculated ^{77}Se NMR chemical shifts of *N*-thiophenyl ebselenamines **12** and **21**, selenenyl sulfides **10** and **20** were found to be comparable with the experimental values (Table S5-S6 in the Supporting Information).

Based on our experimental data obtained from ^{77}Se NMR mechanistic studies of compound **8**, we thus propose a mechanism for the formation of *N*-thiophenyl ebselenamine **12** and others intermediate species such as diselenide **9**, selenenyl sulfide **10**, and ebselenamine **7a** (Scheme 6). According to the cycle, compound **8** reacts with PhSH and gives diselenide **9**, which further produces **10** in the presence of PhSH (Step A). The formation of selenenyl sulfide **10** was confirmed by the reaction of diselenide **9** with 1 equiv of PhSH (Scheme 3). A signal corresponding to selenol **17** could not be detected this time. Ebselenamine **7a** produced in the reaction mixture can also generate selenenyl sulfide **10** with equimolar PhSH as in Scheme 3. Selenenyl sulfide **10** could produce compound **12** (Step B). It undergoes disproportionation reaction in the presence of H_2O_2 to produce diselenide **9** and PhSSPh (Step C). The rapid reaction of diselenide **9** with the formed PhSSPh in the NMR mixture produces hindered diselenide **11** detected in the ^{77}Se NMR spectrum (Step D). This is highly unstable and rapidly cyclizes into a stable cyclic ebselenamine **12** in solution (Step E). Already this step has been confirmed by an independent experiment carried out using diselenide **9** with PhSSPh (Scheme 4). A signal corresponding to compound **12** was observed immediately in the ^{77}Se NMR spectrum. Another possibility for the formation of compound **12** is from the reaction between **7a** and the PhSSPh formed in the reaction mixture. Since the presence of **7a** has been deducted in the ^{77}Se NMR and therefore, it is possible to generate compound **12** during the kinetic studies (data not shown here). Ebselenamine **7a** was produced in the reaction from diselenide **9** and H_2O_2.[36] This was further confirmed as shown in Scheme 3.

The selenenyl sulfide **10**, ebselenamine **7a** and *N*-thiophenyl-ebselenamine **12** are the main dominant products formed in the reaction. First of all in the mechanism, diselenide **9** was probably formed from **8**

and then selenenyl sulfide **10** *via* the reduction and disproportionation reaction, respectively. Since, thiol is very important for the cleavage of the Se–N in ebselen and related heterocycles reductively, which is the main basis for the biological properties.[1b,12g] Overall, it seems that all intermediates **7a**, **10** and **11** that were produced by diselenide **9** were responsible for the formation of **12**.

The radical quenching capacity and regenerability of compounds **12** and **21** were evaluated in a two-phase (water phase/lipid phase) system in which an azo-initiated peroxidation of linoleic acid was carried out.[45] Autoxidation of linoleic acid in open air was initiated by radicals formation using thermal decomposition of 22′-azobis(2,4-dimethylvaleronitrile; AMVN) at 42 ^{o}C in lipid phase (chlorobenzene). The water phase containing ascorbic acid (AscOH) as a co-antioxidant could regenerate the lipid-soluble antioxidants and thus inhibited the peroxidation for longer time. The rate of quenching efficiency of LOO• i.e. rate of inhibition (R_{inh}) was determined as the slope of the line during the inhibited phase (Figure 2).

The rate of peroxidation increases for the uninhibited peroxidation reaction (R_{uninh}). The inhibition time (T_{inh}) i.e. regenerability was determined as the cross-point between R_{inh} and R_{uninh}. α-TOH, the best lipid soluble radical-trapping antioxidant was used as a benchmark which could inhibit the lipid peroxidation with R_{inh} = 25 μM/h; T_{inh} = 95 min in the presence and absence of aqueous AscOH (Table 1).

Table 1
Inhibited rates (R_{inh}) and inhibition times (T_{inh}) of conjugated diene formation with and without AscOH for antioxidants **12** and **21** (40 μM) tested in the two-phase model system. The GPx-like activities of ebselen, **10**, **12**, **20** and **21** as determined by the initial rate of NADPH-consumption (v_0) in the presence of H_2O_2, GSH and glutathione reductase (GR)

Catalysts	With AscOH				Without AscOH		GPx activity
	(0.5 mM)		(1.0 mM)				
	R_{inh}[a] (μM/h)	T_{inh}[b] (min)	R_{inh}[a] (μM/h)	T_{inh}[b] (min)	R_{inh}[a] (μM/h)	T_{inh}[b] (min)	v_0[c] (μM/min)
α-TOH	25 ± 4	97 ± 8	23 ± 2	96 ± 6	28 ± 2	109 ± 2	—
Ebselen	514 ± 9	0	510 ± 6	0	540 ± 4	0	60.3 ± 2.2
12	13 ± 4	30 ± 5	14 ± 3	43 ± 6	26± 4	30 ± 4	58.4 ± 3.5
21	12 ± 3	43 ± 4	9 ± 2	88 ± 4	22 ± 3	56 ± 3	112.1 ± 4.6
10	—	—	—	—	—	—	88.9 ± 2.2
20	—	—	—	—	—	—	164.8 ± 3.6

[a] Rate of peroxidation during the inhibited phase (uninhibited rate ca. R_{uninh} = 544 μM/h). [b] Duration of the inhibited phase of peroxidation. Reactions were monitored for 150 min. Errors correspond to ± SD for triplicates. [c] Assay conditions: Phosphate buffer (100 mM), pH 7.5 with EDTA (1 mM), GSH (2 mM), NADPH (0.4 mM), GR (1.65 unit/mL), H_2O_2 (1.60 mM) and catalysts (80 μM). Initial rates (v_0) were corrected for the spontaneous oxidation of GSH (6.7 ± 0.30 μM/min). Errors correspond to ± SD for triplicates.

The low value of R_{inh} Indicates that the antioxidants could inhibit the lipid peroxidation more efficiently. The radical quenching efficiency of **12** (26 ± 4 μM/h) and **21** (22 ± 3 μM/h) was found almost comparable with α-TOH in the absence of AscOH but for lesser T_{inh} (30 ± 4 and 56 ± 6 min). This is only due to formal HAT reaction. The formed selenoxide species due to the presence of linoleic LOOH present in the sample could be the reason as very poor retarder not inhibitors. Whereas in the presence of AscOH (0.5 and 1.0 mM), compounds **12** and **21** showed remarkable radical quenching efficiency (Table 1). Here, AscOH as two-electron reducing agent is able to reduce selenoxides (Se^{4+}) into lipid-soluble selenides (Se^{2+}) and maintains the lower value of R_{inh}. The R_{inh} values for both compounds **12** and **21** are notably better than α-TOH. The compoud **12** (R_{inh} = 12 ± 2 μM/h; T_{inh} = 88 ± 4 min) inhibited the lipid peroxidation 2 times better than α-TOH with almost silimar T_{inh}. On the other hand, ebselen only retarded the lipid peroxidation, but could not show any inhibition. This suggests that *N*-thiophenyl substituted

ebselenamines **12** and **21** could be even more effective peroxyl radical-trapping antioxidants than ebselen.

The most notable advancement is based on the recent findings which involving the introduction of *ortho*-substituent to the Se atom. This may cause to a dramatic increase in the rate constant for radical quenching. These quench the peroxyl radicals *via* initial O-atom transfer from LOO• to the Se in *N*-thiophenyl ebselenamne **12**, followed by HAT in a solvent cage from the nearby NH group resulting alkoxyl radical (LO•) in Scheme 7.

In a solvent cage, LO• would then abstract an H-atom from the –NHPhS group leading to an aminyl radical selenoxide (**22**). The regeneration of antioxidant **12** occurs by AscOH at the liquid-aqueous interphase. Selenoxide **22** is reduced back to the active form of antioxidant **12** with AscOH and is accompanied by the dihydroascorbate (DHA) formation.

We have calculated the BDE_{N-H} of **12** and **21** was found very weak ~ 70 kcal/mol to generate the aminyl radicals. But our two phase model system contradicts this study in which the T_{inh} of compounds are not much desirable as what we have hypothesized. To justify this result, we have calculated the spin density of radical cation formation upon 1-electron oxidation of **12** and **21** suggested that there is delocalized electron density on the phenyl ring and benzamide *N*-phenyl only (see also Figure S56 in the Supporting Information). The spin density is mainly localized to the N–H bond and nearby which alter the reactivity of compounds. Further calculations indicated that the stability of the aminyl radical cation formed. Indeed, the considerable effect of the bridging heteroatom is assigned to a stabilizing π-electron contribution to the aminyl radical, followed by HAT, and σ-electron withdrawal destabilizing the aminyl radical cation, followed by one-electron oxidation.[46]

Additionally, the presence of the Se atom would also contribute with a capacity to serve as the GPx mimetics. Since, it is easily two electron redox-cycled. This may be the reason that Nature has selected this element for this valuable task.[47] Organic peroxides and H_2O_2 cause oxidation and mild reducing agents such as thiols regenerate the catalysts. The catalytic GPx-like activity of compounds **10**, **12**, **20**, and **21** was evaluated in the coupled reductase assay, using H_2O_2 as a substrate, GSH as a thiol reducing agent, GR and NADPH as a cofactor (Table 1).[48] The values were corrected for the spontaneous oxidation of GSH by H_2O_2. Ebselen (60.3 ± 2.2 μM/min) was included as benchmark in the study. Ebselenamine **12** was found to be nearly as active as ebselen. Interestingly, ebselenamine **21** showed an activity (112.1 ± 4.6 μM/min) almost two-fold larger than recorded for ebselen. The enhanced activity of **21** could probably be ascribed due to the presence of electron donating group at the *N*-phenyl ring. It was also observed that selenenyl sulfide **10** (88.9 ± 2.2 μM/min) and **20** (164.8 ± 3.6 μM/min) showed better GPx-activity than ebselen. Selenenyl sulfide **20** was the most active catalyst and nearly three-fold more active than ebselen (Figure 3).

In conclusion, we have observed various intermediates and isolated novel *N*-thiphenyl-ebselenamines during the ^{77}Se NMR mechanistic experiments of azo-bis-ebselen with PhSH and H_2O_2. Particularly, the

mechanism in Scheme 6 provides the rationale for the formation of selenenyl sulfide, ebselenamine and *N*-thiophenyl-ebselenamine from their corresponding diselenide. The mechanism is consistant with all our observations as observed by the ^{77}Se NMR spectroscopy. In the mechanism, azo-bis-ebselen is fully reduced in the first step by PhSH to diselenide. In the presence of AscOH, *N*-thiophenyl-ebselenamines quenched 5 peroxyl radicals with a stoichiometric number *n* = 5. The stoichiometric *n* for α-TOH is 2. Antioxidants **12** and **21** are likely to quench the lipidperoxyl radicals as much as for α-TOH where the reaction takes place via the formal HAT. *N*-Thiophenyl ebselenamines and their corresponding selenenyl sulfides catalyzed the reduction of H_2O_2 more efficiently than ebselen to mimic the action of the GPx-enzymes.

Experimental Section

^{1}H, ^{13}C and ^{77}Se NMR spectra for all isolated compounds were recorded on 400 and 500 MHz (^{1}H: 399.97 MHz; ^{13}C: 100.6 MHz and ^{1}H: 500 MHz; ^{13}C: 125 MHz) and spectrometers, using the residual solvent peaks of $CDCl_3$ (^{1}H: δ 7.26; ^{13}C: δ 77.2), as an indirect reference to TMS (δ = 0 ppm). ^{77}Se NMR spectra were recorded on 400 MHz and 500 MHz (^{77}Se: 76 MHz and 100 MHz) spectrometers with Ph_2Se_2 (δ = 460 ppm) as an indirect reference to Me_2Se (δ = 0 ppm). Column chromatography was performed using silica gel (0.14 - 0.25 mm). Melting points are uncorrected for all solid compounds with digital melting point apparatus. The high resolution mass spectra (HRMS) were obtained using a time of flight (TOF) instrument equipped with electron ionization (EI) operating in the positive ion mode. Solvents were distilled first and then used for the column chromatography.

Preparation of Bis-[2-phenyl-1,2-benzisoselenazol-3-(2H **)-one-7-yl]diazene (8)**: To a brown suspension of *in situ* prepared Na_2Se_2 (12.46 mmol) in dry THF (20 mL) under an inert atmosphere was slowly added a solution of 2-bromo-3-nitro-*N*-phenylbenzamide (1 g, 3.113 mmol) in THF (10 mL) at room temperature. After heating at reflux for 5 h, the reaction was allowed to stir at room temperature. Water was added to quench the reaction, and the reaction mixture was further stirred at room temperature for additionally 30 min. Following extraction with $CHCl_3$, the combined organic layers were dried over anhydrous Na_2SO_4. Removal of the solvent and purification of the residue by silica gel column chromatography, eluting first with hexane/ethyl acetate (1:1), then with $CHCl_3$, and finally with $CHCl_3$/MeOH (98:2), afforded compound **8** as dark black solid. Yield: (550 mg, 32%); mp > 300 ℃; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.36 (d, *J* = 7.5 Hz, 2H), 7.46 (t, *J* = 7.5 Hz, 4H,), 7.62 (d, *J* = 7.5 Hz, 4H), 7.73 (t, *J* = 7.5 Hz, 2H), 8.32 (d, *J* = 7 Hz, 2H), 8.53 (d, *J* = 7.5 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) : *δ* = 119.9, 125.1, 126.5, 128.1, 128.8, 129.4, 131.4, 131.9, 139.1, 144.2, 163.8 ppm. ^{77}Se NMR (100 MHz, $CDCl_3$): *δ* = 928 ppm.

All data correspond to literature.[27]

Typical Procedure for the Preparation of Diselenides:

Bis[3-Amino-*N*-phenylbenzamide-2-yl] Diselenide (9): This was prepared according to the procedure reported using azo-bis-ebselen **8** (500 mg, 0.870 mmol) and *in situ* prepared colourless solution of NaTePh prepared from Ph_2Te_2 (712 mg, 1.74 mmol) and $NaBH_4$ (132 mg, 3.48 mmol) in ethanol (20 mL) at 0 ^oC. The mixture was then heated at 80 ^oC for 4 h and allowed to cool at room temperature. After adding water to the above mixture, the extraction was carried out with ethyl acetate and separated organic layers were dried over anhydrous $MgSO_4$. Evaporation of the solvent and purification by column chromatography, using 80% ethyl acetate/n-pentane mixture as an eluent afforded the title compound as orange crystalline solid. Yield = (280 mg, 55%); mp 220-223 ^oC (lit. 222-224 ^oC). ^1H NMR (500 MHz, $CDCl_3$): δ = 4.46 (s, broad, 4H), 6.61 (d, *J* = 8 Hz, 2H), 6.72 (d, *J* = 7 Hz, 2H,), 7.01 (t, *J* = 7.5 Hz, 2H), 7.10 (t, *J* = 7 Hz, 3H), 7.23 (d, *J* = 8 Hz, 3H), 7.29 (d, *J* = 6 Hz, 6H), 8.40 (s, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) : δ = 110.6, 115.8, 116.7, 120.7, 124.5, 128.6, 131.2, 137.5, 144.2, 149.6, 168.4 ppm. ^{77}Se NMR (100 MHz, $CDCl_3$): δ = 360 ppm. All data correspond to literature.[35-36]

Bis[3-Amino-*N*-(*p*-tolyl)benzamide-2-yl] Diselenide (19): Yield = (750 mg, 80%); mp 220-222 ^oC (lit. 219-221 ^oC). ^1H NMR (500 MHz, $CDCl_3$): δ = 2.29 (s, 6H), 4.43 (s, broad, 4H), 6.60 (d, *J* = 8 Hz, 2H), 6.68 (d, *J* = 7.5 Hz, 2H), 7.01 (m, 6H), 7.13 (d, *J* = 8 Hz, 6H), 8.22 (s, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) : *δ* = 20.9, 110.7, 115.7, 116.7, 120.7, 129.1, 131.2, 134.1, 134.9, 144.4, 149.6, 168.2 ppm. ^{77}Se NMR (100 MHz, $CDCl_3$): δ = 360 ppm. All data correspond to literature.[27]

Reaction of compound 8 with H_2O_2 and PhSH: To the NMR tube containing compound **8** (50 mg, 0.087 mmol) in 1.0 mL of $CDCl_3$ was added 8 equiv of PhSH (72 µL). The progress of the reaction was monitored by the ^{77}Se NMR spectroscopy. Further, more total of 16 equiv of PhSH were added subsequently to the above mixture. A solution of 6 equiv of H_2O_2 (40 µL) was added to the above mixture. Then another more 6 equiv of H_2O_2 was added and the ^{77}Se NMR spectrum recorded again. More 12 equiv of H_2O_2 was added subsequently to the NMR mixture. All the ^{77}Se NMR spectra were recorded after every addition of the PhSH and H_2O_2 to the NMR tube. Water was added to the above mixture and the extraction was carried out with $CHCl_3$. The separated organic layers were combined and dried over anhydrous $MgSO_4$. Evaporation of the solvent and purification by column chromatography on silica gel, using 40% ethyl acetate/n-pentane mixture as an eluent afforded compounds **10**, **12** and **7a**.

3-Amino-*N*-phenyl-2-((phenylthio)selanyl)benzamide (10):

Yield: 18 mg (28%); mp 197-201 ^oC. ^1H NMR (500 MHz, $CDCl_3$): δ = 6.84 (t, *J* = 7 Hz, 1H), 7.10 – 7.21 (m, 4H), 7.34 (t, *J* = 7.5 Hz, 2H), 7.39 – 7.46 (m, 3H), 7.49 – 7.74 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) : δ = 116.6, 120.2, 120.6, 121.2, 124.6, 125.0, 127.3, 128.3, 128.9, 129.0, 129.2, 131.2, 132.4, 149.4, 167.4 ppm. ^{77}Se NMR (76 MHz, $CDCl_3$): δ = 452 ppm. HRMS (TOF MS ESI) *m/z* calcd for $C_{19}H_{16}N_2OSSe$ [*M* + H]$^+$: 401.0227; found: 401.0226.

2-Phenyl-7-((phenylthio)amino)benzoselenazol-3(*2H*)-one (12):

Yield: 17 mg (25%); mp 211-215 ^{o}C. ^{1}H NMR (500 MHz, CD_3OD): δ = 6.51 (s, 1H), 6.65 (dd, *J* = 1, 7.5 Hz, 1H,), 6.67 (dd, *J* = 1, 8 Hz, 1H), 6.88 – 6.99 (m, 2H), 7.14 (t, *J* = 7.5 Hz, 1H), 7.21 (dd, *J* = 1, 8.5 Hz, 1H), 7.26 (dd, *J* = 0.5, 7.5 Hz, 3H), 7.38 (t, *J* = 7.5 Hz, 1H), 7.47 – 7.50 (m, 2H), 7.98 (t, *J* = 8 Hz, 1H). ^{13}C NMR (125 MHz, CD_3OD) : δ = 115.1, 115.4, 115.5, 120.9, 121.2, 124.1, 125.9, 127.1, 128.1, 128.4, 130.6, 137.8, 144.1, 150.7, 168.8 ppm. ^{77}Se NMR (76 MHz, $CDCl_3$): δ = 833 ppm. HRMS (TOF MS ESI) *m/z* calcd for $C_{19}H_{14}N_2OSSe$ [*M* + H]$^{+}$: 399.0070; found: 399.067.

7-Amino-2-phenyl-1,2-benzisoselenazol-3(*2H*)-one (7a): Yield: 8 mg (16%); mp 180-183 ^{o}C (lit. 182-186 ^{o}C); ^{1}H NMR (500 MHz, DMSO-d_6): δ = 5.87 (s, 2H), 6.91 (d, *J* = 7.5 Hz, 1H,), 7.18 (d, *J* = 7.5 Hz, 1H), 7.24 (t, *J* = 7.5 Hz, 1H), 7.30 (t, *J* = 7.5 Hz, 1H), 7.47 (d, *J* = 7.5 Hz, 2H), 7.62 (d, *J* = 7.5 Hz, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) : δ = 115.8, 116.1, 121.1, 125.0, 126.3, 127.8, 128.1, 129.3, 139.4, 144.4, 165.8 ppm. ^{77}Se NMR (100 MHz, DMSO-d6): *δ* = 929 ppm. All data correspond to literature.[36]

Reaction of diselenide 19 with H_2O_2 and PhSH: To an NMR tube containing diselenide **19** (50 mg, 0.082 mmol) in 0.50 mL of DMSO-d_6 was added 2 equiv of H_2O_2 (19 µL, 0.16 mmol) and mixed together. The oxidation reaction of was followed by the ^{77}Se NMR spectroscopy. Thereafter, observing the signal corresponding to selenoxide[35], an equimolar of PhSH (16 µL, 0.16 mmol) was added to the above mixture (data not shown here). Immediately, three signals were observed at δ 409, 842 and 929 ppm in the ^{77}Se NMR spectrum (Figure S35 in the Supporting Information). The signal corresponding to diselenide **19** at δ 368 was fully disappeared. The mixture was extracted with ethyl acetate and purified by column chromatography. Purification was performed using 40% ethyl acetate/*n*-pentane as eluent produced products **20, 21**, and **7b**.

3-Amino-2-((phenylthio)selanyl)-*N*-(*p*-tolyl)benzamide (20): Yield: 18 mg (27%). mp 182-185 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$): *δ* = 2.34 (s, 3H), 6.81 (dd, *J* = 4, 8.4 Hz, 2H,), 7.05 (s, 1H), 7.09 – 7.20 (m, 6H), 7.34 (d, *J* = 8 Hz, 2H), 7.43 ppm (d, *J* = 6.8 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): *δ* = 21.1, 116.6, 116.7, 120.4, 127.6, 128.4, 129.1, 129.2, 129.5, 131.3, 132.6, 134.4, 135.2, 144.3, 149.5, 167.4 ppm. ^{77}Se NMR (76 MHz, $CDCl_3$): *δ* = 453 ppm. HRMS (TOF MS ESI) *m/z* calcd for $C_{20}H_{18}N_2OSSe$ [*M* + Na]$^{+}$: 437.0198; found: 437.0191.

7-((Phenylthio)amino)-2-(*p*-tolyl)benzoselenazol-3(*2H*)-one (21): Yield: 20 mg (30%); mp 155-158 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$): *δ* = 2.33 (s, 3H), 5.77 (s, 1H), 7.04 (d, J = 8 Hz, 1H), 7.19 (d, *J* = 8.4 Hz, 2H), 7.31 (m, 4H), 7.48 (dd, *J* = 1.2, 8 Hz, 2H), 7.72 ppm (d, *J* = 7.6 Hz 1H) (2 proton merge in the solvent). ^{13}C NMR (100 MHz, $CDCl_3$) : *δ* = 21.2, 117.6, 121.5, 124.5, 125.3, 127.7, 128.2, 129.5, 129.8, 130.0, 131.5, 136.2, 136.8, 140.7, 141.6 ppm (1 peak missing). ^{77}Se NMR (76 MHz, $CDCl_3$): *δ* = 856 ppm. HRMS (TOF MS ESI) *m/z* calcd for $C_{20}H_{16}N_2OSSe$ [*M* + H]$^{+}$: 413.0223; found: 413.0228.

7-Amino-2-[(4-tolyl)-1,2-benzisoselenazol-3(*2H*)-one (7b): Yield: 5 mg (7%). mp 157-160 ^{o}C (lit. 159-162 ^{o}C). The ^{1}H NMR spectrum corresponds to literature.[36]

Mechanistic study of compound 8 with H_2O_2 and PhSH: To the NMR tube containing compound **8** (50 mg, 0.082 mmol) in 1.0 mL of $CDCl_3$ was added 8 equiv of PhSH (72 µL). The progress of the reaction was monitored by the ^{77}Se NMR spectroscopy. Further, more total of 16 equiv of PhSH were added subsequently to the above mixture. The ^{77}Se NMR spectra were recorded after each addition to the NMR tube. A solution of 6 equiv of H_2O_2 (40 µL) was added to the above mixture. The NMR spectra were recorded followed by ^{77}Se NMR spectroscopy. Then another more 6 equiv of H_2O_2 was added and the ^{77}Se NMR spectrum recorded again. More 12 equiv of H_2O_2 was added subsequently to the NMR mixture. All the ^{77}Se NMR spectra were recorded after every addition of the PhSH and H_2O_2 to the NMR tube. See the Supporting Information for the NMR spectra of the mixture.

Mechanistic study of diselenide 9 with PhSH and H_2O_2: To the NMR tube containing diselenide **9** (30 mg, 0.05 mmol) in 0.5 mL of $CDCl_3$ was added 1 equiv of PhSH (5 µL, 0.05 mmol). The progress of the reaction was followed by the ^{77}Se NMR spectroscopy. The signal corresponding to selenenyl sulfide **10** was observed. Further addition of PhSH in excess (2-4 equiv), selenol peak was appeared in the spectrum. Thereafter, the signal corresponding to ebselenamine **7a** was observed by adding 2 equiv of H_2O_2 (12 µL, 0.10 mmol) to the above mixture. Again, addition of extra PhSH (2 equiv) to the above mixture produced selenenyl sulfide **10** and diselenide **9**. All the ^{77}Se NMR spectra were recorded from time to time with subsequent addition of H_2O_2 and PhSH. See the Supporting Information for the ^{77}Se NMR spectra.

Mechanistic Study of diselenide 9 with PhSSPh: The 1.5 equiv of PhSSPh (29 mg, 0.13) was added to the NMR tube containing diselenide **9** (50 mg, 0.086 mmol) in 0.5 mL of $CDCl_3$. The ^{77}Se NMR recorded next day. Only the diselenide **9** peak was appeared at 361 ppm. Further, 1.5 equiv of PhSSPh was added to the above mixture. The ^{77}Se NMR spectra were recorded after each addition to the NMR tube. Now, added one more equiv (6 µL) of H_2O_2 in the same reaction mixture. The NMR spectra were recorded followed by ^{77}Se NMR spectroscopy. Then another three more equiv of PhSSPh was added and the ^{77}Se NMR spectrum recorded again. For the spectra related to kinetic experiment, please see the Supporting Information.

Mechanistic study of diselenide 19 with H_2O_2 and PhSH: To the NMR tube containing diselenide **16** (50 mg, 0.082 mmol) in 0.50 mL of DMSO-d_6 was added 2 equiv of H_2O_2 (19 µL, 0.16 mmol) and mixed together. The oxidation reaction of was followed by the ^{77}Se NMR spectroscopy. Thereafter, observing the signal corresponding to selenoxide[36], an equimolar of PhSH (16 µL, 0.16 mmol) was added to the above mixture. Immediately, three new signals corresponding to **20**, **21** and **7b** were observed in the ^{77}Se NMR spectrum. See the Supporting Information for the NMR spectrum of the mixture.

HPLC Peroxidation Assay: The experimental setup for recording inhibition times (T_{inh}) and inhibited rates of peroxidation (R_{inh}) during azo-initiated peroxidation of linoleic acid using AMVN at 42 °C in a two-phase chlorobenzene/water system has been recently described with slight modifications.[45] Fresh linoleic acid was used as purchased. The values of R_{inh} and T_{inh} reported for reactions performed in the presence and absence of AscOH in Table 1 are means ± SD based on triplicates. As R_{inh}- and T_{inh}- values indicated slight variations depending on the amount of linoleic acid hydroperoxide which is always present in commercial samples as an impurity, and increases upon storage. In a fresh sample of linoleic acid was added small amounts of peroxidized linoleic acid from an older bottle until the concentration, as assessed by UV spectroscopy of conjugated diene formation at 234 nm was about 170-180 μM.

Coupled Reductase Assay: The GPx-like activities of compounds prepared were determined using UV-spectroscopy by following the reported protocol with slight modifications.[48] The test mixture contained GSH (2 mM), ethylene diamine tetra acetate (EDTA, 1 mM), GR (1.65 unit×mL^{-1}), and NADPH (0.4 mM) in potassium phosphate buffer (100 mM), pH 7.5. Catalysts (80 μM) were added to the test mixture at 21 °C and the reaction was initiated by addition of H_2O_2 (1.6 mM). Initial reaction rates were based on the consumption of NADPH as assessed by UV-spectroscopy at 340 nm. The initial reduction rates were triplicated and calculated from the first 10 seconds of reaction by using 6.22 $mM^{-1}cm^{-1}$ as the extinction coefficient for NADPH. GPx-data reported in Table 1 are means ± SD.

Computational Details: Computational calculations for compounds **10**,**12** **20**, and **21** were executed by using the Gaussian 09 suite of quantum chemical programs.[49] The hybrid Becke 3-Lee-Yang-Parr (B3LYP) exchange correlation functional was implemented for density functional theory (DFT) calculations.[50] The geometry optimizations and frequencies were carried out at the B3LYP/6-311+G(d) basis sets. The quantifications of orbital interaction were done by natural bond analysis at B3LYP/6–311+G(d,p) level.[51] The ^{77}Se NMR calculations were performed at B3LYP/6-311+G (d,p) level on B3LYP/6–311+G(d)-level-optimized geometries by using the gauge-including atomic orbital (GIAO) method (referenced with respect to the peak of Me_2Se).[52]

Declarations

ASSOCIATED CONTENT

Supporting Information

^{1}H, ^{13}C, ^{77}Se NMR and HRMS spectra for all new compounds prepared. Results from ^{77}Se NMR kinetic studies of compounds prepared and coordinates of optimized geometries. This material is available free of charge *via* the Internet.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: vijay@pu.ac.in

Notes

The authors declare no competing financial interest

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Supplementary Figures And Table

Table S1 and Figures S1-S23 are not available with this version.

Figures

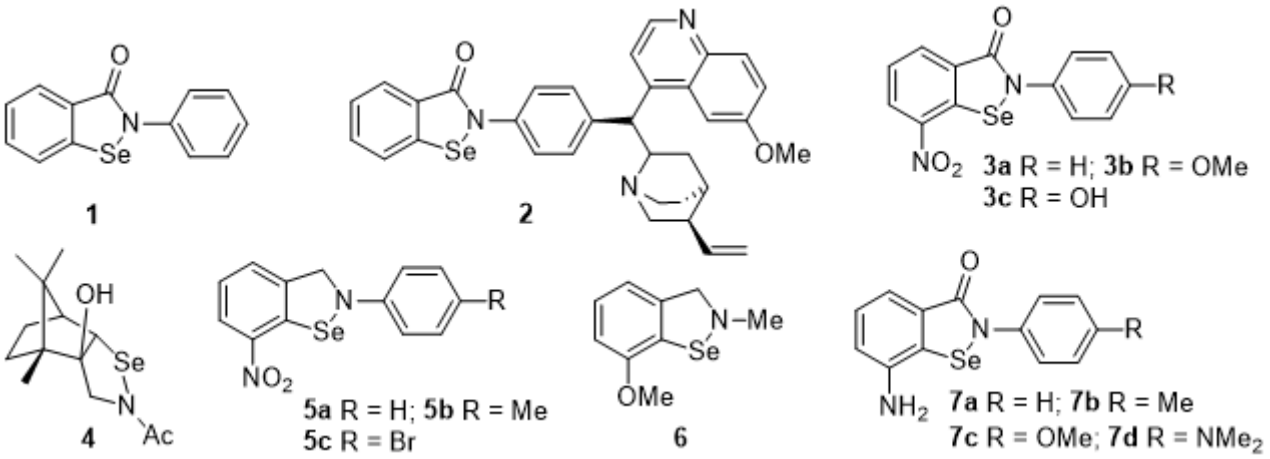

Figure 1

Some representative organoselenium GPx mimetics (**1**-**6**) and radical-trapping antioxidants (**7a**-**d**)

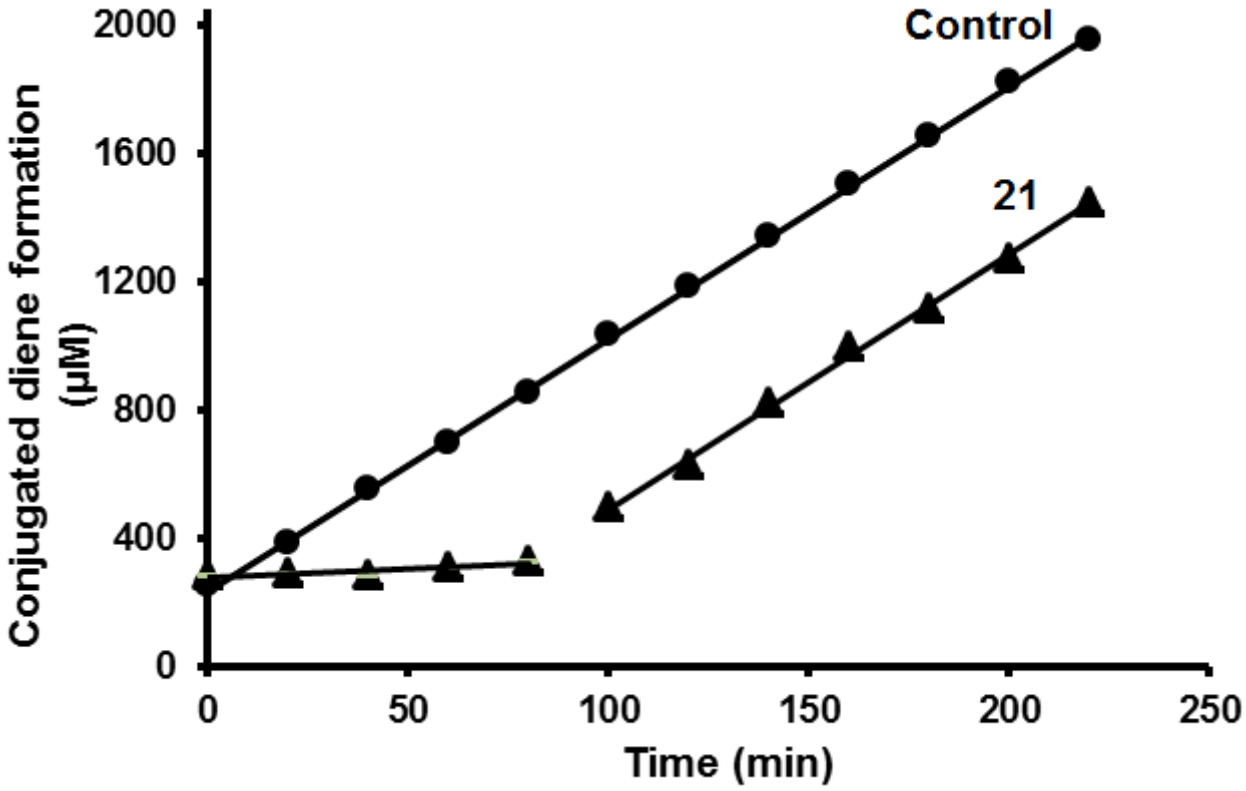

Figure 2

Peroxidation traces (conjugated diene formation *versus* time) recorded in the absence i.e. control and presence of antioxidant **21** in the lipid layer and AscOH in the aqueous phase.

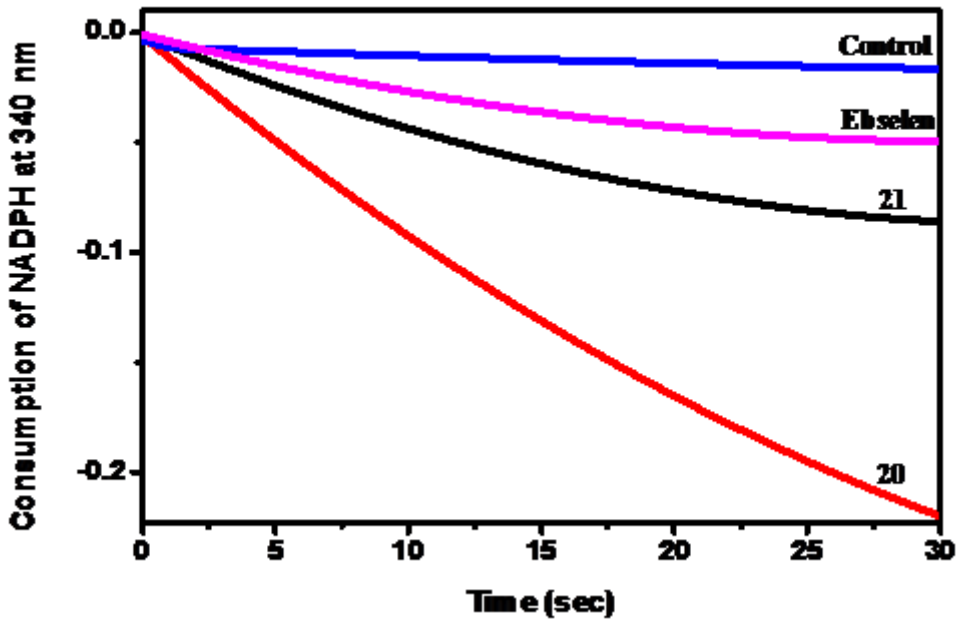

Figure 3

The GPx-like activities of measured by plotting the absorbance of NADPH-consumption *versus* time

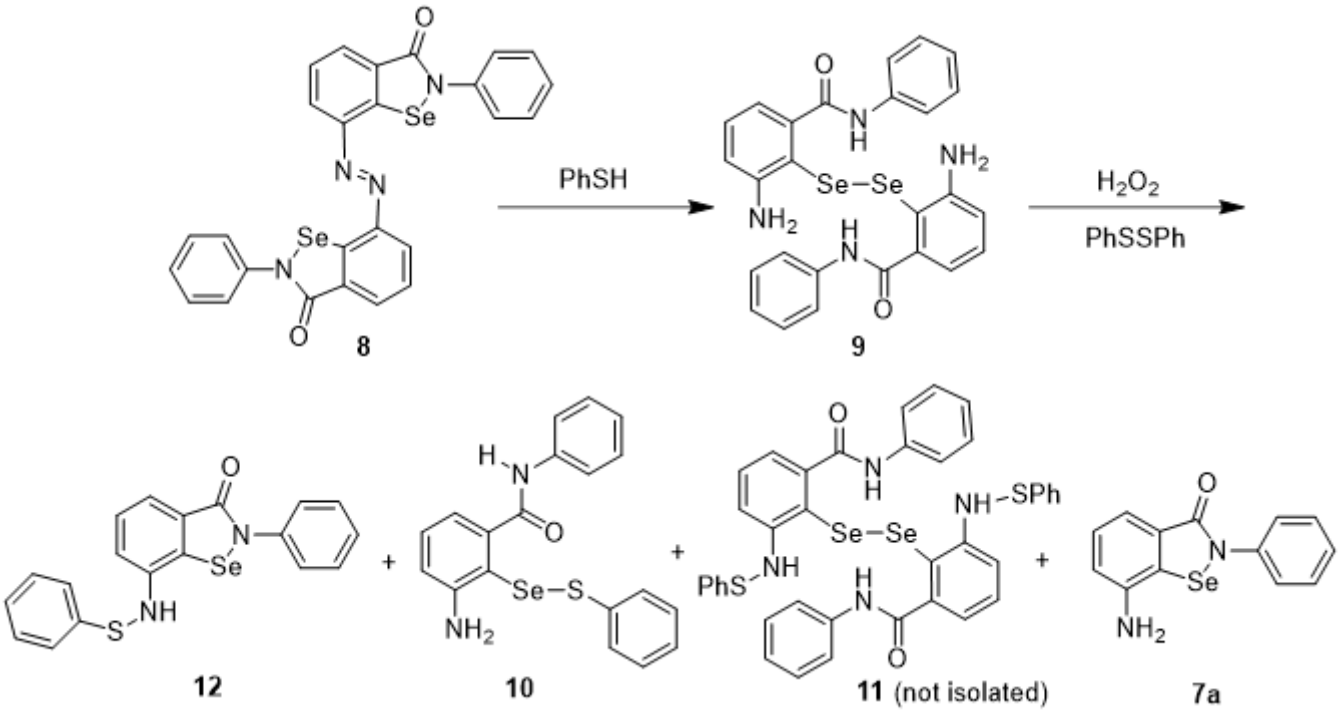

Figure 4

Scheme 1. Reaction of **8** with PhSH and H_2O_2 followed by the ^{77}Se NMR spectroscopy

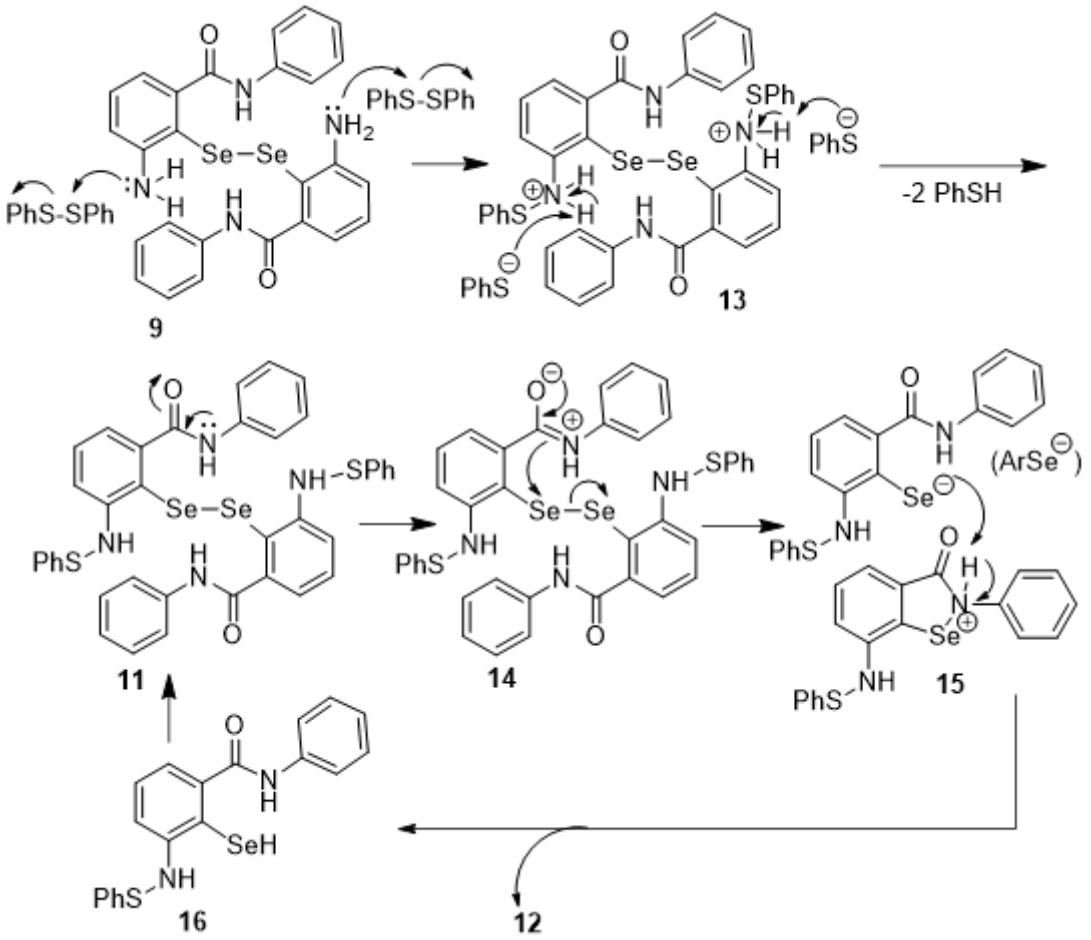

Figure 5

Scheme 2. Proposed mechanism for compound **12** from diselenide **9** *via* intermediate **11**

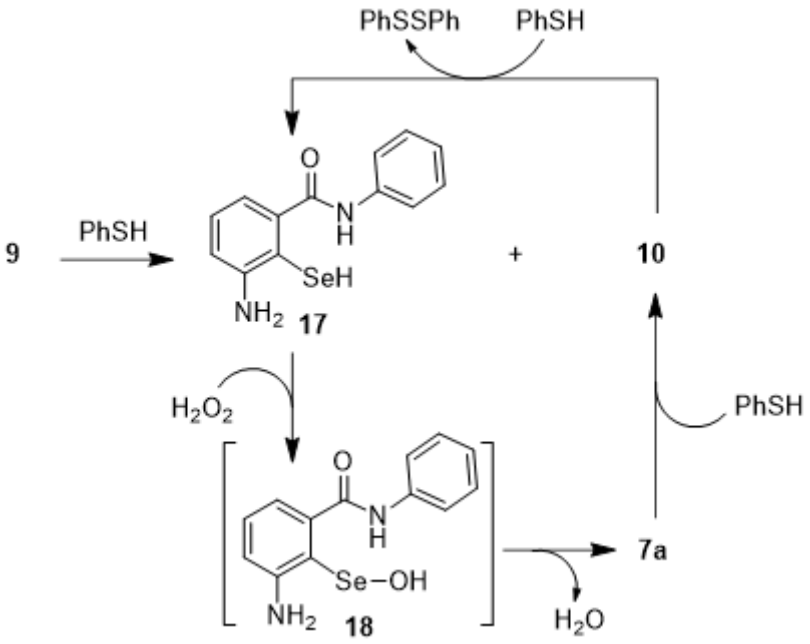

Figure 6

Scheme 3. Mechanistic study of diselenide **9** with PhSH and H_2O_2

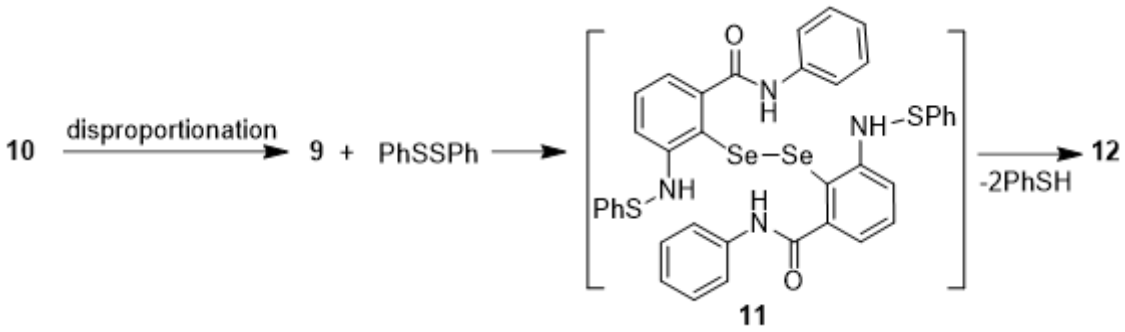

Figure 7

Scheme 4. Disproportionation of **10** into diselenide **9** and PhSSPh, and further reaction produced **12** *via* the unstable intermediate **11**

Figure 8

Scheme 5. Reaction of diselenide **19** with H_2O_2 and PhSH

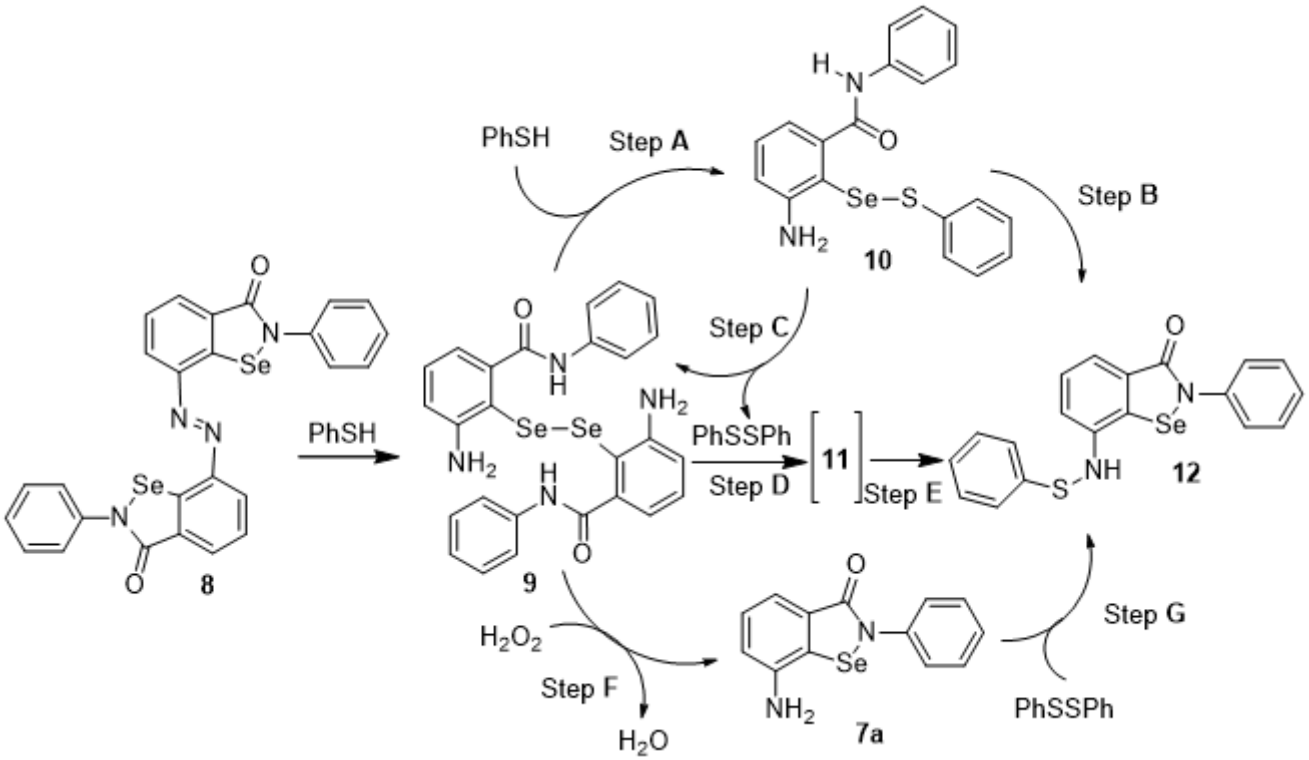

Figure 9

Scheme 6. The ^{77}Se NMR mechanistic study of compound **8** with PhSH and H_2O_2

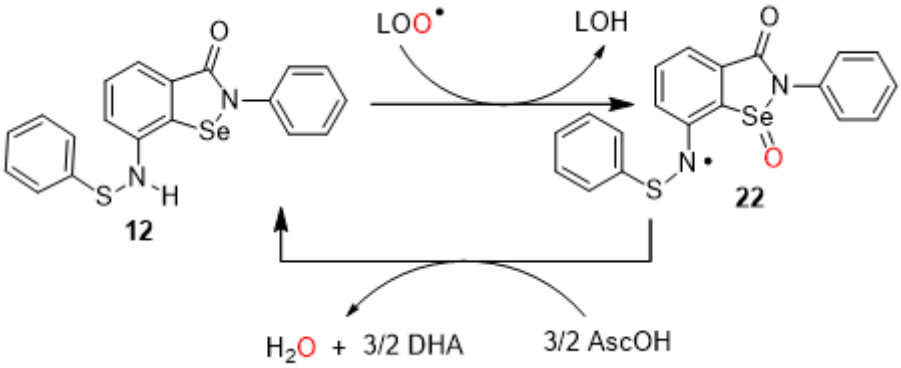

Figure 10

Scheme 7. Proposed mechanism for the radical-trapping antioxidant activity of **12**

Supplementary Files

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