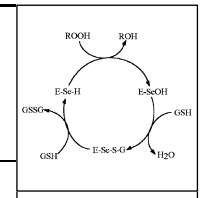
Synthetic organoselenium compounds as antioxidants: glutathione peroxidase activity



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Organoselenium compounds find applications in organic synthesis, materials synthesis, ligand chemistry and biologically relevant processes. This review deals with the use of various synthetic organoselenium compounds as mimics of glutathione peroxidase (GPx), a selenoenzyme which catalyses the reduction of a variety of hydroperoxides and protects the cell membranes from oxidative damage. The mechanism by which these compounds catalyse the reduction of peroxides is also reviewed. The cyclic selenenamides and diselenides with suitably positioned substituents exert their catalytic activity by a mechanism similar to that of the natural enzyme.

1 Organoselenium chemistry

The element selenium was discovered in 1817 by Berzelius[1] and the first organoselenium compound, diethyl selenide, was prepared by Löwig in 1836.[2] Although organoselenium compounds show formal and behavioural resemblances with their sulfur and tellurium counterparts in group 16 of the Periodic Table, there are many differences between organoselenium, -sulfur, and -tellurium compounds with respect to their stabilities, properties, and reactions. On the other hand, the differences between organoselenium and oxygen compounds are considerably more pronounced owing to certain properties of oxygen such as higher electronegativity, lower polarisability, stronger bonds with carbon, and lack of available d-orbitals for bonding. During the initial period, only simple compounds such as selenols (RSeH), selenides (RSeR), and diselenides (RSeSeR) were synthesized. These were mainly the simple aliphatic derivatives and they proved unpleasant to handle because of their highly malodorous nature. Difficulties in purification and the instability of certain derivatives also hampered the early development work. However, around 1970, the discovery of several useful new reactions and a variety of novel structures with unusual properties began to attract more general interest in the discipline. It was then found that the aryl-substituted derivatives and those containing selenium in higher oxidation states were less volatile and less offensive to handle than the original aliphatic compounds and that even the latter were relatively easy to manipulate by modern techniques.[3]

The selenols and their conjugate bases, selenolates, are most commonly prepared by the reaction of Grignard reagents or aryllithiums with elemental selenium, followed by acidification. Alternatively, selenols and selenolates can be obtained by the reduction of diselenides with reagents such as $NaBH_4$, alkali metals or NaH (Scheme 1a). The reduction or basic hydrolysis of selenocyanates (RSeCN) also affords selenols and

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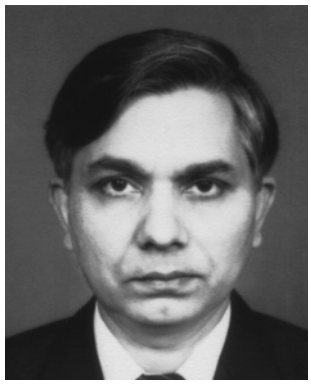

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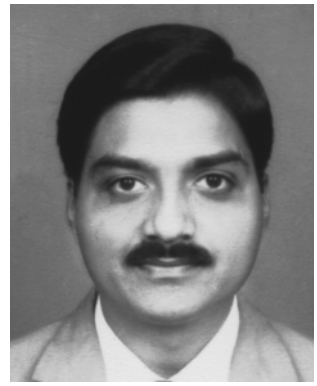

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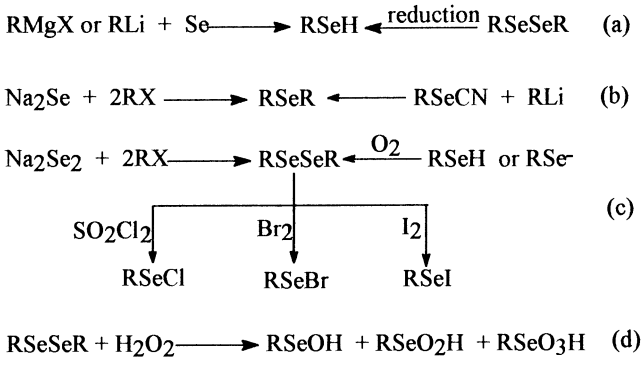


Scheme 1

selenolates. Dialkyl selenides are most conveniently prepared by the reaction of Na_2Se with alkyl halides (RX) or by the reaction of aryllithiums with aryl selenocyanates (Scheme 1b). Diaryl diselenides, which are central reagents of organoselenium chemistry, can be synthesized by the reaction of metal diselenides with aryl halides or by the oxidation of corresponding selenols or selenolates. Halogenation of diselenides with sulfuryl chloride, bromine and iodine affords selenenyl chloride, bromide and iodide, respectively (Scheme 1c). Iodination of diselenides with iodine also affords charge transfer complexes of the type $R_2Se_2.I_2$. The direct formation of selenenyl fluorides from the fluorination of diselenides has not yet been documented, but PhSeF, or an equivalent species, appears to be generated *in situ* from the sonication of PhSeBr and AgF in a suitable solvent.[3b] Oxidation of diselenides is known to give oxidized derivatives such as selenenic acid, seleninic acid and selenonic acid (Scheme 1d).

Recent advances in the area of organoselenium chemistry have been driven by the potential applications of selenium compounds in modern organic synthesis,[3] biochemistry,[4] photography,[5] as precursors for metal organic chemical vapour deposition (MOCVD) of semiconducting materials,[6] and in ligand chemistry.[7] The use of selenium-based synthetic methods is well established in organic synthesis. In contrast to oxygen and sulfur, the selenium-based methodologies offer several unique features in organic chemistry. In 1929 the first patent for the use of selenium dioxide as an oxidant in synthetic organic chemistry appeared.[8] Organoselenium moieties can be incorporated into a variety of substrates for functional group manipulations under mild conditions, either as nucleophiles or as electrophiles.[3] Asymmetric synthesis using organoselenium compounds is of current interest and presents a new trend in the field of organoselenium chemistry.[9] Asymmetric reactions such as selenoxide elimination and [2,3]sigmatropic rearrangement reactions using chiral selenoxides, transition metal mediated hydrosilylation of ketones using chiral diferrocenyl dichalcogenides, methoxyselenenylation of alkenes using chiral diselenides, selenocyclization for the synthesis of heterocyclic compounds such as lactones, cyclic ethers, lactams and nitrogen-heterocycles, and catalytic electrophilic addition reactions of organozinc reagents to aldehydes are some typical examples of asymmetric reactions using chiral organoselenium compounds. In addition to these reactions, the applications of chiral diselenides in asymmetric nucleophilic ring opening of meso-epoxides and the syntheses of natural products such as (+) samin, membrine and salsolidine have also been reported.[9]

From the material chemistry point of view, the importance of selenide semiconductors such as In_2Se_3, MnSe, PbSe, ZnSe, CdSe and HgSe has given rise to much current activity targeted at the synthesis of new organoselenide precursors for these substances.[6] Most of the selenide precursors synthesized earlier were thermally stable due to their polymeric nature and this led to an increase in defects in the metal selenide films. The use of complexes which contain both the metal and the selenium in the same molecule, the so-called "single-source" precursor concept, is one of the most promising strategies in this area. These complexes can deliver both the metal and the selenide element in a defined ratio, avoiding any stoichiometry problems. Moreover, these complexes are not prone to pre-reactions and are also significantly more stable, less toxic, non-pyrophoric and easier to handle.

The ligand chemistry of selenium is also a subject of growing interest as a result of both their increasing accessibility and the realization that they may display significantly different properties from their sulfur analogues.[7] Metal complexes with selenols (RSeH), selenolates (RSe^-), selenoethers (RSeR) and diselenides (RSeSeR) have been extensively studied. Many alkali and alkaline earth metal selenolates, transition metal selenolates and main group selenolates have been synthesized and many of them structurally characterized.[10] Recently, the chemistry of macrocyclic ligands containing selenium has attracted much attention due to the fact that the lower electronegativity combined with the greater σ electron-donating properties of Se should yield complexes with interesting structures and redox behaviour.[11] Moreover, the incorporation of the NMR active nucleus (^{77}Se) would give valuable structural information about the macrocyclic ligands and their complexes. These ligands with "hard" and "soft" binding sites have the potential to coordinate to both "hard" and "soft" guest ions or molecules. The use of chiral selenium ligands in catalytic reactions has also been described. Nitrogen-containing diselenides have been used for asymmetric hydrosilylation, hydrogen transfer reactions and addition of organozinc reagents to aldehydes.[9]

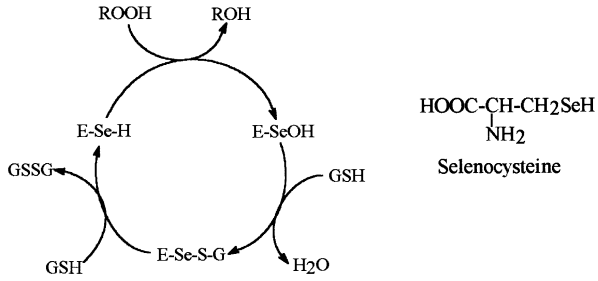
In addition to these applications, organoselenium compounds also show biological activities. Although selenium shares many chemical properties with its neighboring homologue sulfur, selenium differs from sulfur in a number of ways, the most significant being that selenol is a more powerful nucleophile than thiol. Hence, in several enzymes, the incorporation of a chemically more active selenol group into the active center instead of a thiol confers a dramatic catalytic advantage. Selenium in the selenocysteine residue acts as a strong nucleophile in the catalytic reaction. It is well known that organoselenium compounds are more reactive as nucleophiles than their sulfur counterparts. The lower redox potential of selenocysteine compared with cysteine is catalytically favorable for the reaction catalyzed by selenocysteine-containing enzymes. In contrast to thiols, selenols exist mostly in anionic form at neutral pH and represent good reducing groups under normal physiological conditions.[4] These properties combined with their facile reactions with thiols and peroxides have led to interest in their antioxidant properties. Much of the biological activity of selenium is associated with its incorporation into selenocysteine residues in enzymes such as glycine reductase, formate dehydrogenase, hydrogenase, glutathione peroxidase (GPx), tetraiodothyronine 5′-deiodinase, and plasma protein P.[12]

2 Glutathione peroxidases and their role in biological systems

Reactive oxygen species (ROS) such as superoxide anion or hydroxy radicals are known to destroy key biological components and cause damage to cell membranes. These reactive species are involved in the initiation, propagation and maintenance of both acute and chronic inflammatory processes.[13] There is a requirement for cellular defense against reactive oxygen species to protect cell membranes and other cellular component from oxidative damage and living organisms have evolved a number of defence mechanisms to cope with this oxidative stress. In this regard, intracellular and extracellular enzymes such as catalase, superoxide dismutase and the glutathione peroxidases play an important role in the detoxifica-

tion of these species. Among these enzymes, glutathione peroxidase (GPx) has been investigated extensively in recent years due to its link with hydroperoxide metabolism.[4,14]

The role of the enzyme glutathione peroxidase is to reduce hydrogen peroxide *via* its selenocysteine-containing active site, selenol (ESeH). The selenium atom in the enzyme catalytic site undergoes a redox cycle involving the selenolate anion as the active form which reduces hydrogen peroxides and organic peroxides. The selenolate, which is oxidized to selenenic acid, now reacts with reduced glutathione (GSH) to form a selenosulfide adduct (ESeSG). A second glutathione then regenerates the active form of the enzyme by attacking the selenosulfide to form oxidized glutathione (GSSG) (Scheme 2).[14] Thus, in the



Scheme 2 Proposed catalytic mechanism of GPx.

overall process, two equivalents of glutathione are oxidized to the disulfide and water, while the hydroperoxide is reduced to the corresponding alcohol.

Three different classes of selenium-dependent glutathione peroxidases (cytoplasmic, plasma, and phospholipid hydroperoxide) are known. Although they all catalyse the reduction of hydrogen peroxide and organic hydroperoxides by reduced glutathione, the cytoplasmic enzyme is the most thoroughly studied species. This enzyme comprises four identical subunits, each of 21000 Da and each containing a selenocysteine residue. In the catalytic center, the selenium of the selenocysteine is structurally stabilized and functionally activated in the form of dissociated selenol by hydrogen bridges to the amido group of the glutamine and the imino group of the tryptophan. Recently, the crystal structure of human plasma GPx has been determined and crystallographically refined at 2.9 Å resolution.[15] In contrast to the cytoplasmic enzyme where the selenium was present in the form of selenol, in the crystals of human plasma GPx the selenium was found to exist as a seleninic acid [E-Se(O)OH]. Although the overall active site architecture of the human plasma enzyme is similar to that of the cytoplasmic enzyme, the environment close to the selenocysteine residues is quite different in the two enzymes. Approximately only half of the residues close to the selenocysteine residue within a range of 10 Å are conserved in both enzymes. The residues conserved in the human plasma enzyme are Phe76, Gln79, Arg95, Trp153, Phe155, Asn154 and Arg173. Of these residues, Gln79 and Trp153 are located within hydrogen bonding distance of the selenium atom and have been suggested to play functional roles in catalysis. These two residues are in fact conserved in the whole GPx superfamily and probably account for the similarities in their catalytic mechanisms.

Unlike cytoplasmic enzyme, the biological function of the human plasma enzyme still remains unclear. Although the plasma enzyme can reduce hydrogen peroxide and organic hydroperoxides, its affinity to GSH is an order of magnitude lower than that of the cytoplasmic enzyme.[15] Since the concentration of GSH in plasma is far below the affinity of the enzyme it is questionable whether GSH is the natural substrate. The low reactivity of the plasma enzyme with GSH therefore suggests that this enzyme may use other thiols. The third class of GPx, phospholipid hydroperoxide glutathione peroxidase, also uses GSH as reductant, but acts only on phospholipid hydroperoxides.

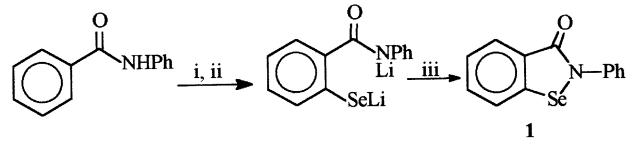
3 Synthetic organoselenium compounds as GPx mimics

Simple organoselenium compounds have been shown to mimic glutathione peroxidase activity *in vitro*. Based on structure, the GPx mimics reported so far can be classified into two major categories. (i) In the first type, the selenium atom is directly bonded to a heteroatom such as nitrogen. Cleavage of the Se–N bond in these compounds is expected to give the catalytically active species. (ii) In the second type, the heteroatom (N or O) is not directly bonded to selenium but it is located in close proximity to the selenium. In these cases, weak Se··N or Se··O intramolecular nonbonded interactions are expected.

3.1 Compounds with direct Se–N bonds

Various organoselenium compounds having a direct Se–N bond have been shown to mimic the active site of GPx. Among them the most promising drug is Ebselen[16] (PZ 51, 2-phenyl-1,2-benzoisoselenazol-3-(2*H*)-one) **1**, a heterocyclic compound exhibiting anti-inflammatory, anti-atherosclerotic and cytoprotective properties. Since the discovery that Ebselen mimics the hydroperoxide reducing ability of GPx, several groups have worked toward a better understanding of the pharmacology of Ebselen. It should be noted that the sulfur analog, 2-phenyl-1,2-benzisothiazol-3(2*H*)-one (**2**) does not exhibit GPx activity.[16] Ebselen contains a selenenic moiety stabilized by intramolecular cyclization in a cyclic *N*-aroyl selenenamide. Due to its strongly bound selenium moiety, demonstrated by ^{75}Se-labeling studies,[17] Ebselen does not release selenium from the molecule during the biotransformation, which probably accounts for its relative lack of toxicity. It is not surprising, therefore, that Ebselen is currently undergoing Phase III clinical trials in Japan as an antioxidant.

Since the first report on the synthesis of Ebselen in 1924 by Lesser *et al.*,[18] several further methods have been reported. The most useful method was reported by Engman *et al*. in 1989 and utilizes a one-pot procedure in which benzanilide is *ortho*-lithiated and treated with selenium powder followed by cupric bromide oxidative ring closure (Scheme 3).[19] Since the



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Scheme 3 *Reagents*: i, 2 n-BuLi; ii, Se powder, iii, CuBr.

discovery of this exciting compound, a number of attempts have been made to modify its basic structure, including substituent effects and isosteric replacement. In 1992, Renson *et al*. reported the Ebselen homologue (**3**) in which a supplementary tetrahedral carbon (–CH_2– group) is incorporated into the heterocycle.[20] Compound **3** preserves (i) a Se–C (aromatic) bond to avoid selenium release and maintain the low toxicity of Ebselen, (ii) a Se–N bond, which is responsible for the GPx activity, and (iii) a N–C=O bond to stabilize the selenenamide structure. Reich *et al*. reported the synthesis and redox chemistry of selenenamide **4**.[21] Similar to Ebselen, the selenazoline (**5**), is known to protect endothelial cells from the toxicity of hydroperoxides.[22] Regarding the substituent effect on the GPx activity, it appears that the substitution of an electron-withdrawing group in the *para*-position to the selenium decreases the GPx activity. For example, the GPx activity of the *para*-nitro derivative **6** is almost 2.5 times lower than that of **5**.[22] On the other hand, introduction of a –CH_2– group into the heterocycle has enhanced the catalytic activity of **5**. Compound **7** containing a six-membered heterocycle was, as

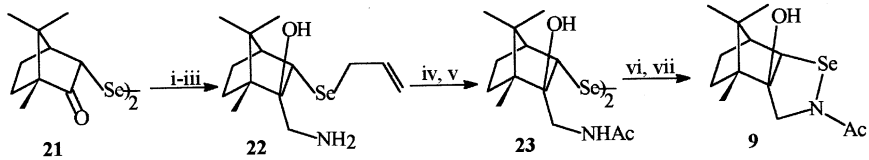
expected, found to be much more active than the parent compound. Substitution of an electron-donating group in compound **7** does not enhance the activity, as the GPx activity of compound **8** containing a *p*-methoxy substituent is identical to that of **7**. Recently, Back and Dyck have reported the camphor-derived cyclic selenenamide (**9**), which displays similar GPx mimetic behaviour as Ebselen.[23] The synthetic sequences for **9** are shown in Scheme 4. The camphor diselenide (**21**) was synthesized from commercially available (1*R*)-(+)-camphor and elemental selenium. The amino-substituted diselenide (**23**) was obtained from **22** by transformation of the amino alcohol moiety, followed by oxidation to the corresponding selenoxide and spontaneous [2,3]sigmatropic rearrangement to remove the protecting group and reductive workup with hydrazine to regenerate the diselenide linkage. The treatment of diselenide **23** with bromine and silver triflate resulted in its cyclization to the selenenamide **9** as a hydrolytically and thermally stable crystalline solid in high yield.[23]

All these mimics with Se–N bond have been developed with the assumption that the enzyme GPx may have a cyclic selenenamide structure in its oxidized form and the peptidic nitrogen atoms may function similarly in the formation of cyclic selenenamide species in the natural enzyme GPx. The importance of the Se–N bond for GPx activity was proved by Galet *et al*. The 1,3-benzoselenazolinones (**10**–**19**), which contain selenium and nitrogen atoms in the heterocycle but do not have any direct Se–N bonds were tested for GPx activity.[24] All 1,3-benzoselenazolinones were devoid of GPx activity under a variety of conditions. In contrast to Ebselen, which is readily opened by glutathione and other thiols, the 1,3-benzoselenazole ring in compounds **10**–**19** cannot be opened under nucleophilic attack by GSH and, therefore, cannot exert any catalytic activity. Moreover, partial or total reduction of aroyl substituents in the C-6 position did not affect the behaviour of these compounds. The work by Wilson *et al*., to design GPx mimics without direct Se–N bond, also met with only limited success.[25] The most active compound produced by this study, **20**, proved to be only 0.033 times as active as Ebselen.

3.2 Compounds with Se··N or Se··O nonbonded interactions

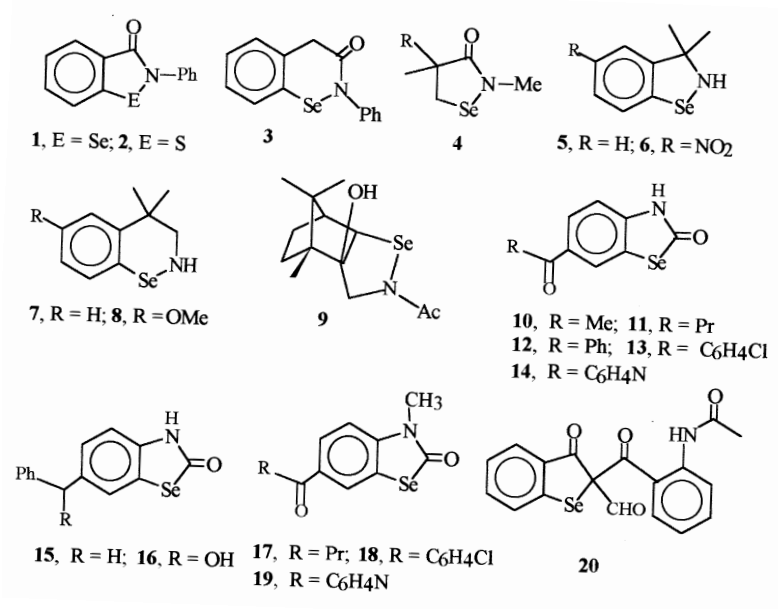
Studies on Ebselen and related derivatives revealed that the cyclic compounds are readily opened by thiol to afford selenenyl sulfide (RSeSR′) intermediates which disproportionate to the corresponding diselenide (RSeSeR) and disulfide (R′SSR′). Reich *et al*. reported that the selenenamide **4** equilibrates with seleninamide **24** and diselenide **25** under acid catalysis.[21] Moreover, the oxidation of selenol **26** with MCPBA afforded first diselenide **25** and then the seleninamide **24**. These observations led to the interest in more easily available diselenides that would function as effectively as the cyclic compounds, which are more difficult to synthesize. Moreover, the observation that diphenyl diselenide exhibits approximately 2 times the activity of Ebselen rules out the assumption that an Se–N bond is necessary for GPx activity.[25] However, the study by Wilson *et al*. stressed that the disubstituted selenium atom should have at least one selenium–heteroatom bond for high GPx activity. The diaryl selenides **27** and **28**, which do not have a Se–heteroatom bond, were found to be inactive catalysts.[25] In agreement with this, bis(4-aminophenyl)selenide (**29**)[26] and 2-phenylselenenyl-1-naphthol (**30**)[27] could not catalyse the reduction of H_2O_2 in the presence of thiols as stoichiometric reducing agents, although the tellurium analogs were found to be efficient catalysts. As a result of these observations, the diselenides, in which each selenium is bonded to a heteroatom (selenium), have been developed.

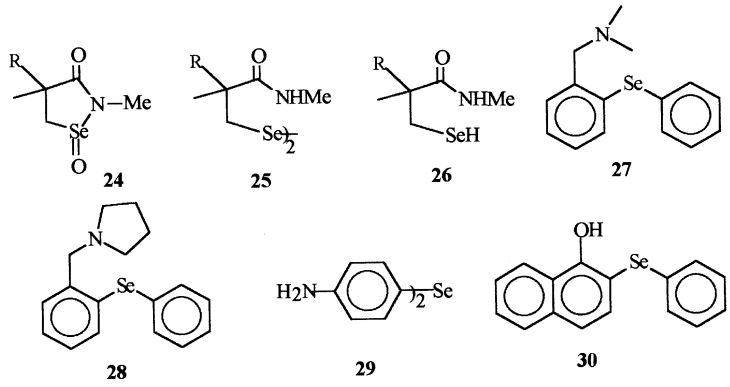
Further developments in the area have started in accordance with the finding that the active site of GPx may involve in some interactions with other amino acid residues that would alter the enzyme's activity. According to Wendel *et al*.,[14] the catalytically active selenocysteine residue in GPx is located at the N-terminal end of helix α_1. It was proposed that the amino acid residues near the selenium atom might interact weakly with the selenium, which would certainly stabilize the active site selenolate and enhance its nucleophilic reactivity.[14] Hilvert *et al*. reported that selenosubtilisin, a selenoenzyme synthesized



21 **22** **23** **9**

Scheme 4 *Reagents*: i, $NaBH_4$, allylic iodide; ii, Me_3SiCN; iii, $LiALH_4$, iv, Ac_2O, pyridine; v, MCPBA, NH_2NH_2; vi, Br_2; vii, AgOTf.



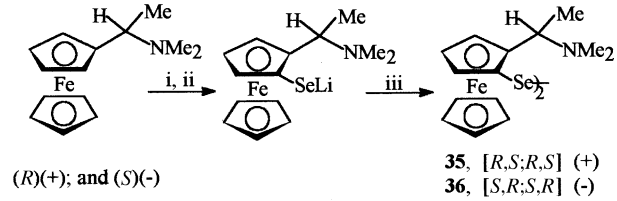


by chemical modification of the serineprotease subtilisin, can also mimic the catalytic behaviour of GPx.[28] While this study was primarily undertaken to demonstrate how a single atom change —from oxygen to selenium— at the serine active site of subtilisin can radically alter the enzyme's pattern of reactivity, this study also proved the importance of a basic histidine residue at the active center of this enzyme. In 1997, Flohé *et al*. studied the simulation of the catalytic cycle of GPx by computer-assisted molecular modeling.[29]Starting from the established X-ray structure of bovin GPx, all kinetically defined intermediates and enzyme substrate complexes were subjected to force field calculations and molecular dynamics. According to this model, the selenium in the active site interacts weakly with the imino group of Trp 165 and the amido group of Gln 87 (Se··N distances: 3.31 and 3.37 Å, respectively). The crystal structure of seleno GPx from human plasma also shows interactions between selenium and Gln79 and Trp153 residues (Se··N distances: 3.5 and 3.6 Å, respectively) in the active site.[15]

Based on these observations, various organoselenium compounds containing heteroatoms in close proximity to the selenium have been synthesized in order to study their GPx activity. Wilson *et al*. first reported the interesting result that the protonated derivatives of diselenides **31** and **32**, each of which possesses a basic amino nitrogen near the selenium, exhibit strong GPx antioxidant activity.[25] Tomoda *et al*. also studied the effect of an amino group on the antioxidant activity of GPx by using various model compounds (**31**–**33**).[30] The initial reduction rates of H_2O_2 were studied by using PhSH as a glutathione alternative. The results of these studies were in agreement with the report of Wilson *et al*.[25] and suggested two important conclusions as to the effects of an intramolecular amino moiety in a selenium catalyst: (i) The effects of selenium and the basic amino nitrogen on the GPx activity are remarkably cooperative for H_2O_2 reduction. (ii) The selenium as an active center catalyses the reduction of H_2O_2 by interacting with the proximate basic amino nitrogen.

The detailed mechanistic studies on GPx mimics reveal that the Se··N intramolecular nonbonded interactions, (i) activate the Se–Se bond towards an oxidative cleavage, (ii) stabilise the selenenic acid form of the catalyst against further oxidation, (iii) enhance the nucleophilic attack of thiol at sulfur rather than selenium, and (iv) activate the selenol intermediate toward oxidation through conversion into its conjugate base, selenolate. In addition, the amine may serve to deprotonate the thiol sulfhydryl group and thus provide a high local concentration of nucleophilic thiolate anion. These observations are also in agreement with the report of Reich *et al*. that the selenosulfide (**34**) and diselenide (**25**) derived from selenenamide **4** reacted with thiol only in the presence of a strong base.[21]

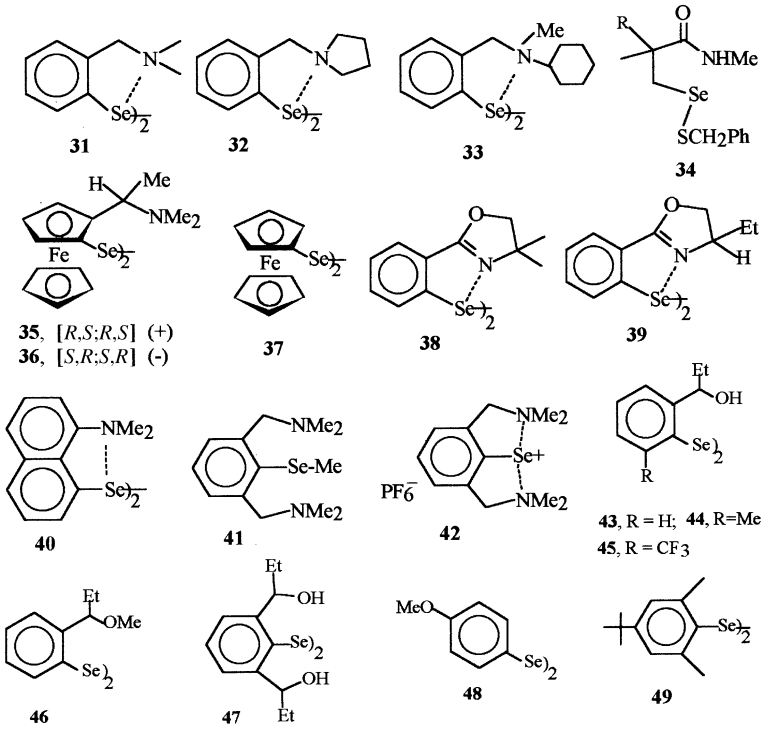
Singh *et al*. recently reported a comparison of GPx activities between various amino substituted diaryl diselenides. The redox-active diferrocenyl diselenides (**35** and **36**) containing basic amino groups near the selenium exhibited excellent GPx activity.[31] The chiral diselenides **35** and **36** could be synthesized by diastereoselective lithiation of commercial (*R*)- and (*S*)-[1-(dimethylamino)ethyl]ferrocene with s-BuLi followed by addition of elemental selenium and air oxidation (Scheme 5).[32]



Scheme 5 *Reagents*: i, s-BuLi; ii, Se powder; iii, H_2O, air oxidation.

Interestingly, the X-ray crystallographic data of **35** and **36** indicate that these compounds do not have any Se··N interactions in the solid state as the observed Se··N distances of 3.697 and 4.296 Å for **35** and 3.98 and 4.12 Å for **36** are greater than the sum of their van der Waals radii (3.54 Å).[31,32] On the other hand, compound **37**, which does not have basic amino groups showed a very low activity. Compounds **38**, **39** and **40** with strong Se··N interactions did not show any noticeable activity under similar experimental conditions. These results are in agreement with the reports of Ladenstein *et al*,[15] and Flohé *et al*.[29] which clearly demonstrate that the presence of a hetero-atom near the selenium is very important as long as the Se··N interactions are not very strong. A detailed mechanistic study on the catalytic activity of **35** and **36** shows that the Se··N interactions, though they are weak, contribute to the enhancement of GPx activity.[33] While the Se··N interactions were absent in compounds **35** and **36**, the ^{77}Se NMR chemical shifts indicate the presence of strong Se··N interactions in the selenenic acid (RSeOH) state. This certainly increases the possibility of a nucleophilic attack of thiol at selenium. It should be noted that the presence of basic amino groups in the *ortho*-position of monoselenides (RSeR) does not improve their catalytic activity as compounds **27** and **28** were found to be inactive.[25] However, aryl alkyl selenides may become the selenocysteine model system when the selenide is converted into cationic species. Se-dealkylation of selenide **41** with t-BuCl afforded a selenenium cation (**42**) which is stabilized by two neighbouring amino groups.[34] While the X-ray data reveal no interaction at all between the counteranion and countercation, there are short intramolecular Se··N contacts of 2.154 and 2.180 Å which are remarkably shorter than the sum of the van der Waals radii of the two atoms. Compound **42** oxidizes two equivalents of benzenethiol to the corresponding disulfide quantitatively and the deprotonation of the thiol by two amino groups facilitates the reaction.

Wirth *et al.* have reported a new class of diselenide GPx mimics (**43**–**48**) containing an oxygen atom in close proximity to selenium.[35] In these cases, the Se··O non-bonded interactions, though they are expected to be weaker than the Se··N interactions, were shown to increase the catalytic activity. Compound **48** containing an electron-donating substituent (methoxy group) in the *para*-position showed the highest



activity of the series. However, diselenide **49** containing other electron-donating substituents such as t-butyl group did not show any noticeable activity.[33] The inactivity of this compound may be ascribed to steric hindrance due to the presence of 2,6-substituents near the selenium.

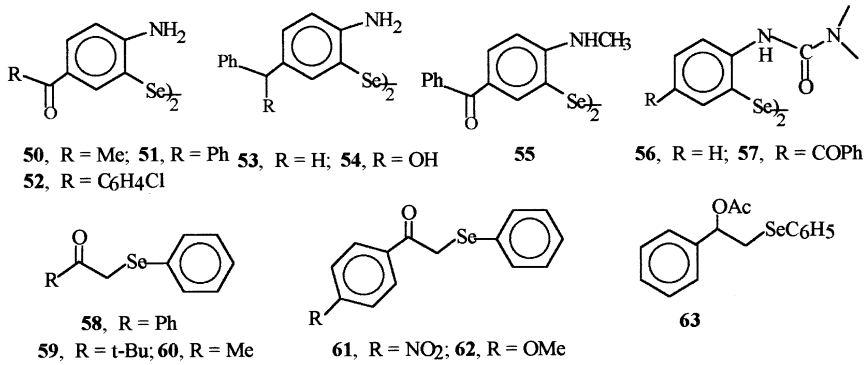
In order to compare the GPx activity of cyclic compounds with the diselenides, Galet *et al*. have synthesized two series of compounds, substituted benzoselenazolinones (**10**–**19**) and their opened analogs (diselenides, **50**–**57**) and tested these compounds for GPx activity.[24] The diselenides were found to be very potent (up to 3 times more active than Ebselen), whereas the benzoselenazolinones were inactive. As already described, this is due to the fact that these cyclic derivatives cannot be opened *in vitro* by thiol, whereas the opened derivatives (including Ebselen which is opened by the nucleophilic attack of glutathione) can react with peroxides. Among compounds **50**–**57**, diselenides **51**, **52** and **55** were the most active compounds. As in the case of benzoselazolinones, partial or total reduction of the aroyl substituents in the C-6 position in the diselenides does not increase the GPx activity.

In addition to these two classes of compounds, some α-(phenylselenenyl) ketones (**58**–**62**) also exhibit GPx activity.[36] In their case, the catalytic activity of the compounds is due to scission products formed from the parent molecules in the presence of GSH. An electron-withdrawing substituent in the acetophenone moiety (**61**) was shown to potentiate the catalysis whereas a mesomerically electron-donating substituent (**62**) decreased the catalytic activity of the parent compound. Similarly, substitution of the acetophenone aryl group for alkyls (**59**, **60**) or reduction/acetylation of the carbonyl group (**63**) caused a decrease in the catalytic activity of the compounds.

4 Cytotoxic effect of GPx mimics

The major factor in the toxicity of the organoselenium compounds is the ability of either alkyl- and arylselenolates or arylselenenyl sulfides to behave as catalysts for the reductive activation of dioxygen. Various model studies of diselenides and cyclic selenenamides have shown that in the presence of thiol, the reactive oxygen species can be produced by the reduction of oxygen in a one-electron transfer process. The glutathione oxidase (GOx) activities of organoselenium compounds are, therefore, a measure of the cytotoxicity of these compounds. Unfortunately, reliable predictions of cytotoxic effects are often missing in the design of GPx mimics.

A few years ago, Chaudiere *et al.*[37] demonstrated that some alkyl diselenides, *e.g.* selenocystamine, behave as GOx catalysts in the presence of excess GSH and ambient oxygen. The glutathione reductase-coupled assay studies indicate that the selenocystamine catalyses the fast production of GSSG even in the absence of exogenous hydroperoxide. At neutral pH, an excess of GSH reduces the diselenide to the selenolate RSe^-, with RSeSG as a transient intermediate. This rate-limiting process is followed by a three-step reduction of dioxygen to water. In the first step, oxygen is reduced by the RSe^- in a one-



electron transfer leading to the production of superoxide $O_2^{\bullet -}$ and the selenenyl radical $RSe^{\bullet}$. Hydrogen peroxide is produced in the second step by another one-electron transfer from RSe^- to superoxide. The third step is a two-electron transfer from RSe^- to H_2O_2. Catalytic recycling of R_2Se_2 or RSeSG is ensured by the very fast recombination of $RSe^{\bullet}$ and by nucleophilic scavenging of RSeOH. Since the GPx and GOx cycles occur simultaneously, this results in the consumption of GSH, accumulation of GSSG and the continuous production of toxic peroxides which, may hit other targets in a biological medium. It is still unclear whether the enzyme GPx contains any special features at the selenocysteine active site to avoid one-electron transfers.

Recent studies have shown that aromatic selenium compounds are less toxic than aliphatic compounds. However, many aromatic selenium GPx mimics, whose Se is not bioavailable, also exhibit a one-electron transfer process. Design and synthesis of organoselenium compounds with high GPx activity and low GOx activity has been a difficult task. For example, although the GOx activity and toxicity of compound **5** are very weak, this compound exhibits only moderate GPx activity.[22] The selenolate RSe^- derived from this compound was found to be a poor monovalent reductant of oxygen. This observation led to the assumption that the oxidase activity of RSe^- could be reduced by decreasing the electron density on selenium. Although the substitution of a nitro group in the *para*-position (**6**) decreased the GOx activity, the GPx activity of this compound was also found to be much lower than that of **5**.[22] Due to the electron withdrawing effect of the NO_2 group, ring opening by GSH yields a dead-end product RSe^-. On the other hand, the GOx activity and toxicity of the *ortho*-nitro analog **64** is found to be very high at neutral pH. It is interesting to note that in the presence of NADPH and glutathione reductase, compound **64** significantly increased the rate of NADPH oxidation which is indicative of GOx activity.[22] To establish the involvement of selenolate in this cycle, iodoacetamide was added to trap the selenolate. However, since the rate of oxidation was not affected by iodoacetamide, the formation of selenolate derivative RSe^- is unlikely. Although heterolytic cleavage of the Se–N bond by GSH yields the RSeSG intermediate, the NO_2–Se interaction possibly prevents bimolecular displacement of the selenium.

Wirth *et al*. studied the diselenides **43**–**48** and found that the GOx activities of these compounds are very low and some are less toxic than Ebselen.[35] The oxidase activity of the compounds decreases with the electron density on selenium, as evidenced by the undetectable GOx activities of **6** and **45**. On the other hand, electron-donating substituents such as the methoxy group increase the GOx activity. Compounds **8**, **43** and **48** are more cytotoxic than the other compounds. From the data obtained, it seems that compounds **5**, **44** and **47** might be good candidates for GPx as well as GOx activities. It is, therefore, not possible to design selenoaromatic GPx mimics with electron-withdrawing substituents since these substituents decrease both GPx and GOx activities.

Another type of selenium toxicity arises from the one-electron reduction of certain enzymes by active selenium species. It has been demonstrated that certain selenium-containing compounds (selenocysteine, GPx) catalyse the thiol mediated one-electron reduction of ferric cytochrome c.[38] Engman *et al*. reported the catalytic effect of some GPx mimics on the thiol reduction of cytochrome c.[38b] The reaction sequences are summarized in eqns. 1–4. In the presence of excess thiol, the diselenide (RSeSeR) is reduced to the selenolate RSe^- with the formation of R′SSeR. Nucleophilic attack of thiol on R′SSeR produces the disulfide and the selenolate RSe^-. The arylselenolate ion thus formed acts as a one-electron reductant towards cytochrome c as shown in eqn. 4. The resulting selenium centered radical could then dimerize to regenerate the catalyst in the diselenide form. The spontaneous reduction of cytochrome c by thiols which probably involves electron transfer from thiolate ions to iron could be ruled out since selenolates are stronger reductants than thiolates and selenols are more acidic than thiols.

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + HO^{o} + HO^- \quad (1)$$

$$R'SH + RSeSeR \rightleftharpoons R'SSeR + RSe^- + H^+ \quad (2)$$

$$R'SSeR + R'SH \rightleftharpoons R'SSR' + RSe^- + H^+ \quad (3)$$

$$\text{cyt-}Fe^{III} + RSe^- \rightarrow \text{cyt-}Fe^{II} + RSe^{o} \quad (4)$$
$$\downarrow$$
$$RSeSeR$$

The alkyl aryl selenosulfides **65** and **66**, selenenamide **67** and α-(phenylselenenyl) acetophenone **68**, which all could be expected to readily generate benzeneselenolate ion in the presence of thiol, showed catalytic activity similar to diphenyl diselenide.[38b] The high oxidation potential of Ebselen (1.59 V *versus* Ag/AgCl) suggests that the compound does not exert its catalytic effect by direct one-electron transfer from selenium to iron. From this study it is clear that the potentially toxic thiol-mediated reduction of ferric cytochrome c catalysed by Ebselen is slower than the reaction catalysed by other GPx mimics. This effect may contribute to the observed toxicity of certain organoselenium compounds and must be considered in the design and synthesis of new GPx mimics.

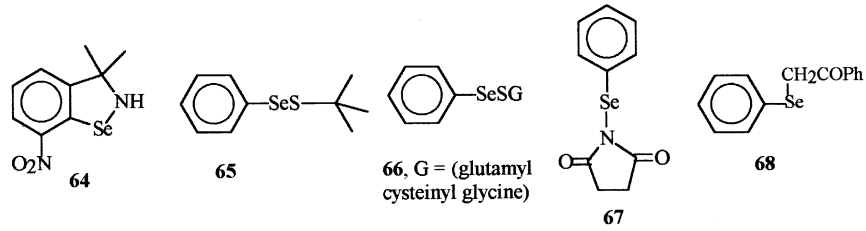
5 Methods for the assessment of GPx activity

There are several methods available to determine the GPx activity of selenium model compounds.

5.1 Enzymatic method

This method was first used for diaryl diselenides by Wilson *et al*.[25] The GPx activity of the compounds was determined using H_2O_2 as the substrate in the presence of GSH (eqns. 5–7). Glutathione reductase was used to reduce the oxidized GSH with NADPH as a cofactor.

$$2GSH + H_2O_2 \xrightarrow[\text{Peroxidase}]{\text{GSH}} GSSG + 2H_2O \quad (5)$$



$$GSSG + NADPH + H^+ \xrightarrow[\text{Reductase}]{\text{GSH}} 2GSH + NADP^+ \quad (6)$$

$$H^+ + NADPH + H_2O_2 \rightarrow NADP^+ + 2H_2O \quad (7)$$

The initial decrease in NADPH monitored spectrometrically at 340 nm was a measure of GPx activity. The relative activities of the compounds prepared were compared with that of glutathione peroxidase. The assay mixture was prepared by dissolving potassium phosphate buffer, EDTA, sodium azide, GSH, NADPH, GSSG reductase, and an appropriate amount of test compound. Reaction was initiated by the subsequent addition of H_2O_2.

5.2 NMR method

Engman *et al.*[39] proposed this direct method to determine the thiol peroxidase activity of diaryl diselenides and diaryl ditellurides. In this assay, thiols were oxidized to the corresponding disulfides in the presence of H_2O_2 and the catalyst to be evaluated. The time required to reduce the thiol concentration by 50%, t_{50}, was determined as a measure of the thiol peroxidase activity of the catalyst. For example, *N*-acetylcysteine was dissolved in a mixture of D_2O and CD_3OD in an NMR tube, and hydrogen peroxide was added by syringe. After some time, the basal oxidation was checked by ^1H NMR, and the catalyst to be evaluated was added. The ^1H NMR spectrum of the solution was then recorded at various time intervals, and the conversion of thiol into disulfide was plotted against time to give a linear correlation. The time required to reduce the thiol concentration by 50% in the presence of different catalysts was determined, usually by extrapolation, from the equation of the line.

5.3 UV method using aromatic thiols as GSH alternative

Hilvert *et al.* used 3-carboxy-4-nitrobenzenethiol as an alternative for GSH for the reduction of H_2O_2 and t-BuOOH in the presence of selenosubtilisin.[28] The enzyme-catalysed reduction of peroxide by thiol was studied by following the disappearance of the thiolate absorption at 410 nm. This method was also employed by Tomoda *et al*. using benzenethiol (PhSH) as a glutathione alternative (eqn. 8).[30]

$$2PhSH + H_2O_2 \xrightarrow{\text{Catalyst}} PhSSPh + H_2O_2 \quad (8)$$

The initial reduction rates of H_2O_2 (v_0) were obtained by monitoring the UV absorption increases at 305 nm due to the formation of diphenyl disulfide (PhSSPh). In this assay, the catalytic GPx model reaction was initiated by the addition of an excess amount of H_2O_2 to a methanol solution of PhSH (C_0) containing a selenium catalyst and was monitored by UV spectroscopy at 305 nm. The molar extinction coefficient of PhSSPh ($\varepsilon_1 = 1.24 \times 10^3\ M^{-1}cm^{-1}$) at the wavelength was much larger than that of PhSH ($\varepsilon_1 = 9\ M^{-1}cm^{-1}$). The concentration of PhSH (C) was therefore calculated from the absorbance (a) according to the following equation: $C = (\varepsilon_1 C_0 - 2a)/(\varepsilon_1 - 2\varepsilon_2) \cong C_0 - 2a/\varepsilon_1$. The initial reduction rate of H_2O_2 (v_o) was then determined by $1/v$ *vs* $1/C$ plots.

5.4 HPLC method

This method was recently employed by Back and Dyck using phenylmethanethiol and *tert*-butyl hydroperoxide (eqn. 9).[23]

$$BnSH + t\text{-}BuOOH \xrightarrow{\text{Catalyst}} BnSSBn + t\text{-}BuOH \quad (9)$$

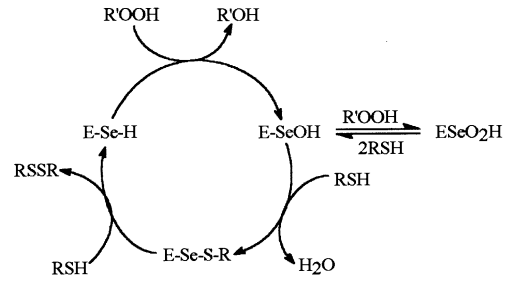
The formation of dibenzyl disulfide (BnSSBn) was determined by HPLC using naphthalene as an internal standard. For example, t-BuOOH was added to a solution containing phenylmethanethiol and naphthalene in dichloromethane. Selenenamide **9** was added, and the progress of the reaction was monitored by HPLC.

6 Mechanism of peroxidase reactions catalysed by GPx mimics

The mechanism by which the GPx mimics exert their catalytic activity has been the subject of numerous investigations. While the catalytic cycle of the semisynthetic enzyme selenosubtilisin and a few other GPx mimics is found to be identical to that of GPx, most of the cyclic selenenamides and diselenides show their GPx activity *via* different mechanisms depending upon the concentration of thiol and hydroperoxide.

6.1 Selenosubtilisin

The kinetic and NMR studies on the peroxidase activity of selenosubtilisin show that the catalytic cycle proceeds *via* selenenic acid, selenenyl sulfide and selenol forms of selenosubtilisin as shown in Scheme 6.[28] While the seleninic acid



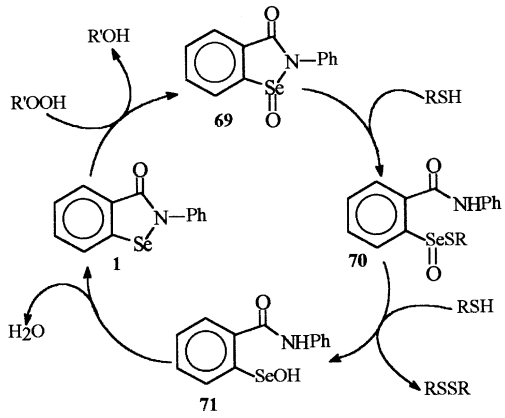
Scheme 6

($ESeO_2H$), the oxidized form of selenosubtilisin, lies off the main catalytic cycle, it may become important at very high concentrations of hydroperoxides. However, under conditions of excess thiol, which represents a more realistic scenario *in vivo*, $ESeO_2H$ reacts with thiol to afford the selenenyl sulfide (ESeSAr). Investigations into the reduction of $ESeO_2H$ by 3-carboxy-4-nitrobenzenethiol revealed saturation kinetics and were consistent with a significant lowering of the pK_a of the seleninic acid at the enzyme active site. The conversion of (ESeSAr + ArS^-) to (ESe^- + ArSSAr) is found to be the rate-determining step, and the histidine residue near the active site (His64) is implicated as a general acid or electrostatic catalyst, facilitating the departure of the selenolate group. Studies on the enzyme's thiol specificity for the reduction of *tert*-butyl hydroperoxide indicate that, while 3-carboxy-4-nitrobenzenethiol is turned over by the enzyme at least 2000-fold faster than by relevant model systems, most aliphatic thiols are poor substrates for selenosubtilisin. By analogy with the natural peroxidase, a variety of hydroperoxides are accepted as substrates for selenosubtilisin and the k_{max} is dependent upon the nature of hydroperoxide, indicating that peroxide-mediated oxidation of the enzyme selenolate is at least partially rate limiting.[28]

6.2 Cyclic compounds.

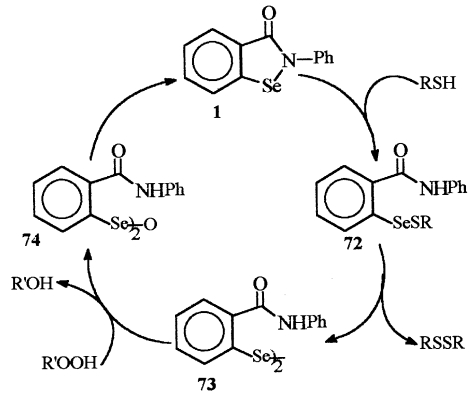
Although Ebselen (**1**) is known to be a major GPx mimic, its peroxide decomposition pathway is entirely different from that

of the enzyme GPx. Mechanistic studies by several research groups reveal that Ebselen behaves differently with different concentrations of peroxide and thiol. Ebselen is readily oxidized to the corresponding seleninamide (**69**) by hydrogen peroxide. The rapid reaction of seleninamide with thiol to form the corresponding thioseleninate (**70**), further reaction with a second equivalent of thiol to give selenenic acid (**71**) and disulfide, and the elimination of water to give Ebselen are known to follow the pathway shown in Scheme 7.[23]



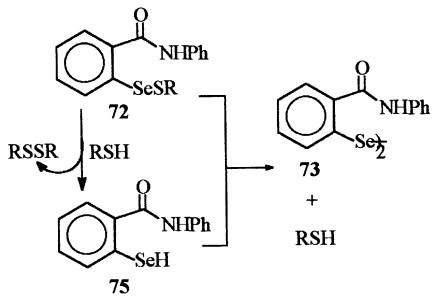
Scheme 7

In the presence of excess thiol, **1** reacts with the thiol to afford a selenenyl sulfide (**72**) intermediate. Compound **72** disproportionates slowly to the corresponding diselenide (**73**) with the elimination of disulfide. Further oxidation of the diselenide then regenerates the original seleninamide *via* a selenenic anhydride (**74**) (Scheme 8). A slight modification to Scheme 4 has been



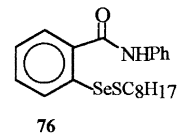
Scheme 8

reported where the selenenyl sulfide reacts directly with a second equivalent of the thiol to produce a selenol (**75**) and disulfide, followed by attack of the selenol upon more selenenyl sulfide to afford the diselenide (**73**) and thiol (Scheme 9).[23]



Scheme 9

Studies by Engman *et al.*[39] with selenenyl sulfide **76** indicate that a third mechanism has also to be considered, involving slow



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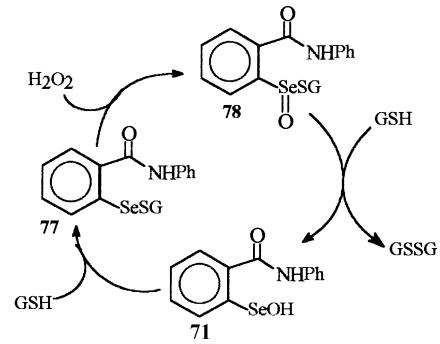
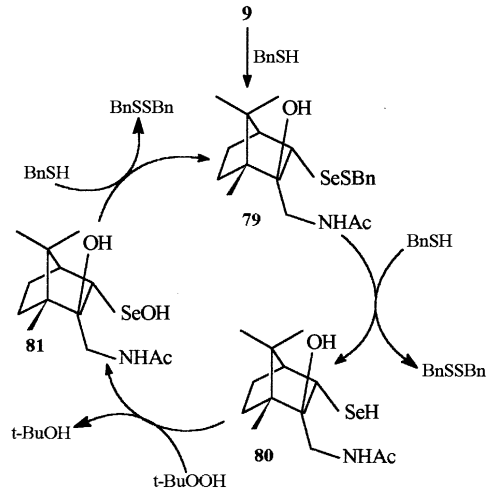
oxidation of selenenyl sulfide (**77**) followed by rapid attack by GSH to give disulfide and a selenenic acid (**71**) which could reform the selenenyl sulfide (**77**) in the presence of thiol (Scheme 10). The GPx activity and mechanistic studies on



Scheme 10

isoselenazolidin-3-one **4** show that the selenenamide produces seleninamide (**24**), selenenyl sulfide (**34**), thioseleninate, selenol (**26**) and diselenide (**25**), the precise mechanism depending upon whether acid or base catalysed.[21] Reduction of **4** with phenylmethanethiol under weakly acidic conditions gave the selenenyl sulfide (**34**). Although thiolysis of the selenenyl sulfide in the presence of base produced the corresponding selenol, the oxidation of the latter by *m*-chloroperbenzoic acid or *tert*-butyl hydroperoxide leads to the diselenide, rather than directly to the original selenenamide **4**. The diselenide **25** and selenenyl sulfide **34** did not react with phenylmethanethiol under neutral conditions, but with an excess base each gave the selenolate **26** and disulfide. The selenolate was quantitatively trapped *in situ* by benzyl bromide to give the benzyl selenide.

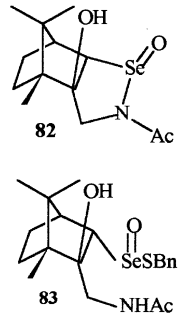
Recently Back and Dyck reported the mechanism of the camphor-derived selenenamide (**9**) using phenylmethanethiol and *tert*-butyl hydroperoxide.[23] The catalytic cycle is different from that of Ebselen and related heterocycles, but closely resembles that of GPx (Scheme 11). Compound **9** functions by reaction with thiol to afford selenenyl sulfide **79**, which



Scheme 11

undergoes further attack by the thiol to produce dibenzyl disulfide and selenol **80**. The latter compound is oxidized by the

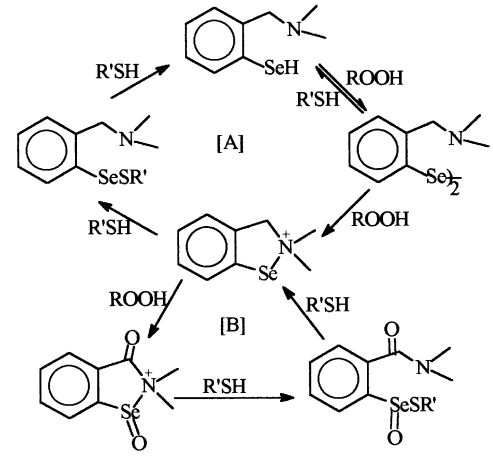
hydroperoxide to the selenenic acid **81**, which in turn reacts with additional thiol, thus regenerating the selenenyl sulfide and forming water as the by-product. The original selenenamide, therefore, acts as a procatalyst in this process and is not regenerated, whereas the selenenyl sulfide is the true catalyst. In this catalytic cycle, neither seleninamide (**82**) nor thioseleninate



(**83**) is produced. Interestingly, the diselenide (**23**) is not generated during the catalytic process although the synthesis of **9** is carried out through cyclization of **23** with bromine and silver triflate.

6.3 Diaryl Diselenides

The mechanism by which the diaryl diselenides catalyse the GPx reaction is less complicated than that of the cyclic seleninamides. Wilson *et al.*[25] have proposed a mechanism for the diselenides possessing a basic amino nitrogen near the selenium, which involves six active intermediates and two reaction cycles (Scheme 12), and is significantly different from that of GPx. A modified catalytic mechanism was proposed by Engman *et al.*[39] for the diaryl diselenides in which the diselenides (RSeSeR) first react with thiol to afford the selenenyl sulfide (RSeSR′). Further reaction of this selenenyl sulfide with hydrogen peroxides affords a seleninic acid thiol ester (RSe(=O)SR′). Nucleophilic attack by thiol on sulfur would then give disulfide and a selenenic acid (RSeOH), which would react with thiol to regenerate the selenenyl sulfide. In accordance with the report of Back *et al.*,[23] in this particular case the selenenyl sulfide acts as a true catalyst. Tomoda *et al.*[30] have proposed a mechanism for the amino-substituted diselenides which is similar to that of the actual enzyme, involving both selenolate and selenenyl sulfide as intermediates (Scheme 13). The initial step is the reaction of diselenide with RSH to produce selenenyl sulfide and selenolate. Because of hypervalent Se···N interaction, compound **84** undergoes facile

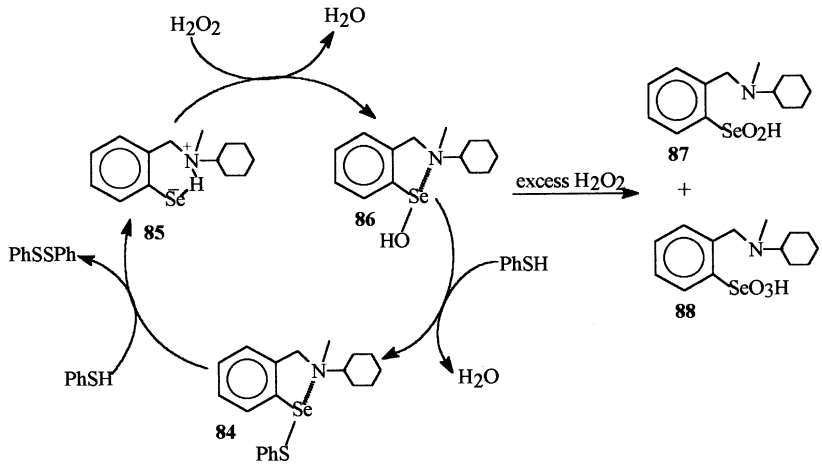


Scheme 12

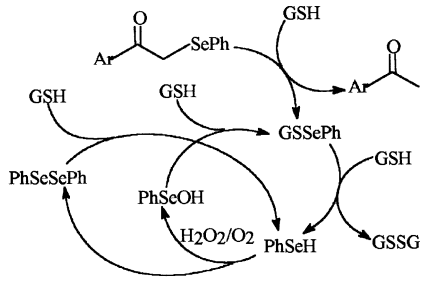
bimolecular displacement preferentially at the sulfur atom with RSH to give selenolate (**85**). Both theory (MO calculations) and experiments (^{77}Se NMR) suggest that the proximate nitrogen base activates the selenol intermediate into corresponding selenolate anion, which should play a key role in accelerating the catalytic cycle. Oxidation of **85** with H_2O_2 produces selenenic acid **86**, which then rapidly undergoes bimolecular displacement at the selenium atom with RSH to regenerate **84**. With excess of H_2O_2 (4 equivalents), the formation of other oxidized products such as seleninic acid (**87**) and selenonic acid (**88**) was also observed. The formation of other oxidized products such as seleninic acid and selenonic acid may depend upon the various degree of Se···N interactions. Diselenides **35** and **36** did not produce any overoxidized products even when a large excess of H_2O_2 was used.[33]

Similarly to diselenides, selenenium cation (**42**) also behaves catalytically when reducing thiol equivalents are used.[34] The thiol attacks the selenium atom of **42** to form the intermediate selenosulfide, which is converted into the selenolate anion by reaction with a second thiol. The selenolate anion formed is oxidized to the selenenium cation. Although phenylselenenyl halides and pseudohalides are known to give addition products, **42** does not react with olefins (norbornene, stilbene) and phenylacetylene.

Several mechanisms have been proposed to account for the observed catalytic acitivity of α-(phenylselenenyl) ketones (**58**–**62**). A catalytic mechanism involving benzeneselenolate as a crucial intermediate was proposed by Engman *et al.*[36] According to this mechanism (Scheme 14), the α-(phenylselenenyl) ketones serve as "procatalysts". Once they are decomposed, a catalytic cycle involving *S*-(phenylselenenyl)-glutathione, benzeneselenolate and benzeneselenenic acid is



Scheme 13



Scheme 14

responsible for the reduction of hydrogen peroxide with GSH. While the benzeneselenolate produced in the cycle is expected to react with α-(phenylselenenyl) ketones or *S*-(phenylselenenyl)glutathione to give diphenyl diselenide, the mechanism is proposed to be identical with that of diphenyl diselenide. It was also shown by these studies that the selenenyl sulfide is the main catalytically active species.

7 Conclusion

In this article, we have attempted to highlight the developments in the area of glutathione peroxidase activity of organoselenium compounds during past decade. Although it has long been known that many organoselenium compounds exhibit antibacterial, antiviral, antifungal, antiparastic and antiradiation properties, the application of organoselenium compounds as enzyme mimics has only recently attracted much attention. After the discovery of Ebselen as a glutathione peroxidase mimic, there has been a major development in the area of design and synthesis of organoselenium compounds that mimic the action of the natural enzyme GPx. Despite the fact that some aspects of the biological role of GPx can no longer be questioned, several other functions such as the involvement of GPx in the inhibition of lipoxygenase or cyclooxygenase and reduction of other reactive oxygen species such as peroxynitrite are still not clear. We envisage that the progress and prospectives described in this review—both structure–activity correlations and mechanistic aspects—will stimulate further efforts from researchers all across the organoselenium community.

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