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Antioxidant Activity of Amine-Based Organoselenium Compounds having Intramolecular Se–Se Bonds

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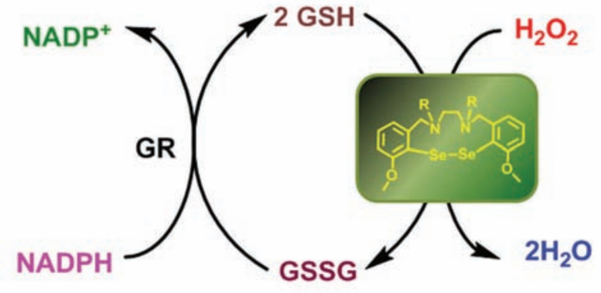
(Dedicated to Prof. S.P. Singh on his 80th Birthday)

ABSTRACT Amine-based diselenides have been studied as synthetic mimics of the antioxidant enzyme glutathione peroxidase (GPx). In this study, a series of intramolecular diaryl diselenides were synthesized and studied for their antioxidant activity. A structure-activity correlation study reveals that these intramolecular diselenides are more active than the standard GPx mimetic ebselen, but the activity is significantly lower than that of the corresponding diselenides having intermolecular diselenide bonds. The crystal structure of a representative compound suggests that the poor activity is due to the absence of any strong Se–N interactions, which has been shown to play important roles in the catalytic activity. As the diselenide bonds are not activated for cleavage by thiols, the reduction of peroxides involves an initial reaction of the selenium center with peroxide during the catalytic cycle.

Graphical Abstract



KEY WORDS Antioxidant, Glutathione, Selenium, Enzyme mimics, Intramolecular diselenide.

INTRODUCTION

Design and synthesis of efficient functional mimics of the mammalian selenoenzyme glutathione peroxidase (GPx) have been a major challenge. The enzyme plays important roles in protecting the human body from oxidative damages by maintaining the cellular hydroperoxide concentration using glutathione (GSH) as the cofactor.[1,2] Several research groups have developed different kinds of synthetic mimics based on the enzyme structure or mechanism.[3,4] Diaryl diselenides constitute an important class of organoselenium compounds that were studied extensively as the GPx mimics.[3,4] The catalytic cycle of native GPx consists of three intermediates, i.e., selenol, selenenic acid, and selenenyl sulfide in the active site. The selenol is the active intermediate, which reduces peroxides in the catalytic cycle to water or alcohol.[5,6] Therefore, design and synthesis of GPx mimics that can generate reactive selenol intermediate in the catalytic cycle are a major goal of our synthetic approach. The synthetic mimics were designed based on the proposed catalytic triad of native enzyme featuring a heteroatom at the close proximity to

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the selenium center.[7,8] It is known that the cyclic selenenyl amide ebselen **1 [Figure 1]**, which is reported as the first synthetic GPx mimic, exhibits moderate catalytic activity using GSH as the cofactor.[9,10] Interestingly, the activity is reduced significantly in the presence of aromatic thiols such as benzyl thiol or thiophenol. Further, experimental and theoretical studies confirmed that ebselen does not generate any selenol intermediate during its catalytic mechanism.[11-13] Ebselen and related cyclic selenenyl amides behave as poor catalysts due to the strong Se…O non-covalent interaction, which leads to an extensive thiol exchange reaction in the selenenyl sulfide intermediate, preventing the regeneration of active selenol species.[11] In contrast, Wilson *et al*. reported amine-based diaryl diselenide **2**, which displayed very high activity as compared to that of ebselen in the presence of both GSH and aromatic thiols **[Figure 1]**.[14] Detailed mechanistic studies by Iwaoka and Tomoda using compound **3** suggest that the weak Se…N non-covalent interaction plays crucial roles in the catalytic cycle to enhance the catalytic activity of amine-based diaryl diselenides significantly.[15] Subsequently, a series of diaryl diselenides containing either oxygen (**4, 5**) or nitrogen atom (**6-13**) were synthesized and investigated for their antioxidant activities **[Figure 1]**.[16-21]

Although the Se…N interaction is much weaker in the amine-based diselenides, this interaction is sufficient to cause significant thiol exchange reactions. The ^{77}Se…nuclear magnetic resonance (NMR) experiments showed that the selenenyl sulfide derived from the diselenide **2** requires a large excess of thiol to convert entirely to the respective selenol.[20] As regeneration of selenol from the selenenyl sulfide by thiol is a crucial step in the catalytic cycle, weakening of the Se…N non-covalent interaction in the selenenyl sulfide intermediate is highly important for the better catalytic activities of the amine-based diselenides. Mugesh *et al.* previously observed that substitution of the aromatic ring with ferrocenyl moiety weakens the Se…N interaction considerably.[17] Similarly, our group showed that substitution of the aromatic hydrogen in the ortho-position of diselenide **2** by methoxy group (diselenide **12**) enhances the activity dramatically.[20] A detailed studies showed that the steric influence of the methoxy group in the ortho-position weakens the Se…N non-covalent interaction significantly in the selenenyl sulfide intermediate preventing the undesired thiol exchange reaction.[20,21] These observations suggest that further reduction of the Se…N interaction in the diselenide molecule would provide a better mimic for the GPx enzyme. The amine-based diaryl diselenides (**2-13)** reported till now are intermolecular in nature.[14-21] Reductive cleavage of the diselenide bond by thiol produces two separate selenenyl sulfide moieties in which each selenium center interacts with one nitrogen atom. In this study, we have synthesized a series of amine-based intramolecular diselenides **[Figure 2**, **14-17]** and studied their catalytic activities in the presence of different peroxide substrates and thiol cofactors. Intramolecular selenium containing metallomacrocycle are reported as metal chelator[22], however, the diselenides in this study are the first examples of intramolecular diselenides as the GPx mimics.

RESULTS AND DISCUSSION

Synthesis of compounds 14-17

Compounds **14-17** were synthesized from the diselenide **18**,[23,24] which reacts with one equivalent of ethylenediamine

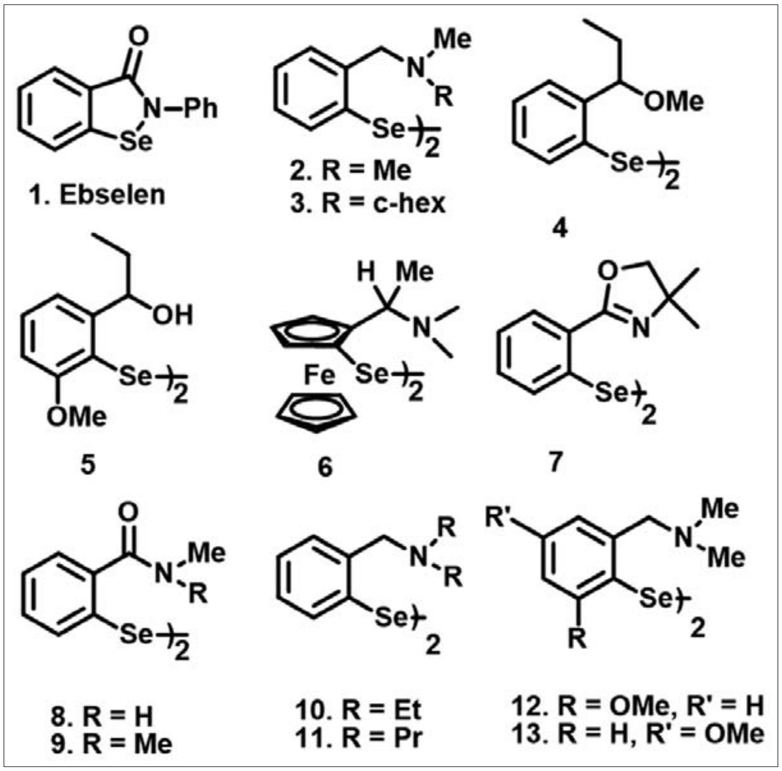


Figure 1: Chemical structures of the ebselen (1) and intermolecular diselenides 2-13

to produce the corresponding Schiff base **19**. Compound **19** was reduced with sodium borohydride in methanol to generate the diselenol **20**, which after oxidation produced the diselenide **14 [Scheme 1]**. The *sec*-amino moiety in compound **14** was alkylated with the corresponding alkyl halide in the presence of a base to obtain the corresponding diselenides **15-17**. All the intramolecular diselenides were characterized by ^{1}H,^{13}C,^{77}Se NMR spectroscopy, mass spectrometry (MS), and one of them by single crystal X-ray diffraction. The Se…N distances of 3.395Å and 3.440Å in compound **15** indicate that the nitrogen atoms do not interact with the selenium atoms.

GPx-like activities of compounds 14-17

The GPx-like catalytic activities of the diselenides **14-17** were studied using PhSH or GSH as the thiol cofactor and three different peroxides (H_2O_2, Cum-OOH, or *t*-BuOOH) as substrates. In the presence of PhSH, the formation of PhSSPh from PhSH was followed by a reverse-phase high-performance liquid chromatography (HPLC) method and the time required for 50% conversion of PhSH to PhSSPh ($t_{1/2}$) was calculated.[19-21,25] The $t_{1/2}$ values, as summarized in **Table 1**, indicate that all the intramolecular diselenides **14-17** are better GPx mimics than the standard compound ebselen (**1**) in all three peroxide systems. The $t_{1/2}$ values of compounds **14-17** are found to be much lower than that of the ebselen (300.5 min) for the reduction of H_2O_2. Interestingly, in contrast to our expectation, the $t_{1/2}$ values in **Table 1** suggest that the intramolecular diselenides **14-17** are much lower active than the corresponding intermolecular diselenides **12** and related diselenides,[19-21] which exert very high activities even at 5.0–10.0 µM concentrations.

The poor catalytic activities of the intramolecular diselenides can be understood from the X-ray crystal structure of diselenide **15**. A close look at the crystal structures of compound **15 [Scheme 1]** shows that the Se…N distances (3.395Å and 3.440Å) are only slightly shorter than the sum of the Van der Waals radii of selenium and nitrogen atoms (3.54 Å), indicating the absence of any strong Se…N non-covalent interactions. The extremely weak Se…N interactions are probably not sufficient to increase the catalytic activity of intramolecular diselenides **15-17** significantly. This is in contrast to the observations of Tomoda *et al.* that weak Se…N interaction is essential for the catalytic activity of amine-based diselenides.[15] Compound **14** exhibited the highest activity among all the intramolecular diselenides in the presence of PhSH as the cosubstrate. Similar to the intermolecular diselenides, catalytic activities of intramolecular diselenides are also dependent on the nature of the peroxides used in the studies.

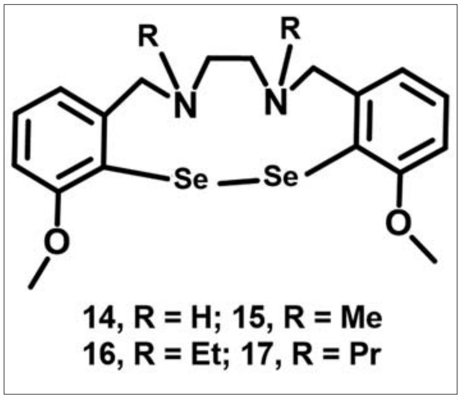


Figure 2: Chemical structures of the intramolecular diselenides 14-17

As GSH is the preferred cofactor for native GPx enzyme, the catalytic activities of compounds **14-17** were also studied using GSH. The initial rate (v_0) of the reaction was determined by following the oxidation of NADPH at 340 nm using GSH-GSSG coupled assay in the presence of enzyme GSH reductase in phosphate buffer.[3,11,12,14] The initial rate values, as summarized in **Table 2**, indicate that the diselenides **15-17** exhibit higher activities than that of ebselen (**1**) for the reduction of H_2O_2. However, the activity is decreased considerably when other two peroxides (Cum-OOH and *t*-BuOOH) are used as the substrate. Furthermore, compound **14**, which exhibited the highest activity in the PhSH assay, showed poor catalytic activity with GSH. The size of the substituent on the nitrogen atom appears alter catalytic activity of the amine-based diselenide, as the larger substituent experiences a severe steric interaction with the GSH molecule, which is also larger in size than PhSH. Therefore, the intramolecular diselenides having less accessible selenium centers exhibit poor activities than that of the corresponding intermolecular diselenides in the presence of GSH.

Reactivity of compounds 14-17 toward thiol

The reactivity of intermolecular amine-based diselenides toward thiols is well established. In the presence of one

Table 1: Reduction of peroxides by PhSH in the presence of compounds 1, 14-17

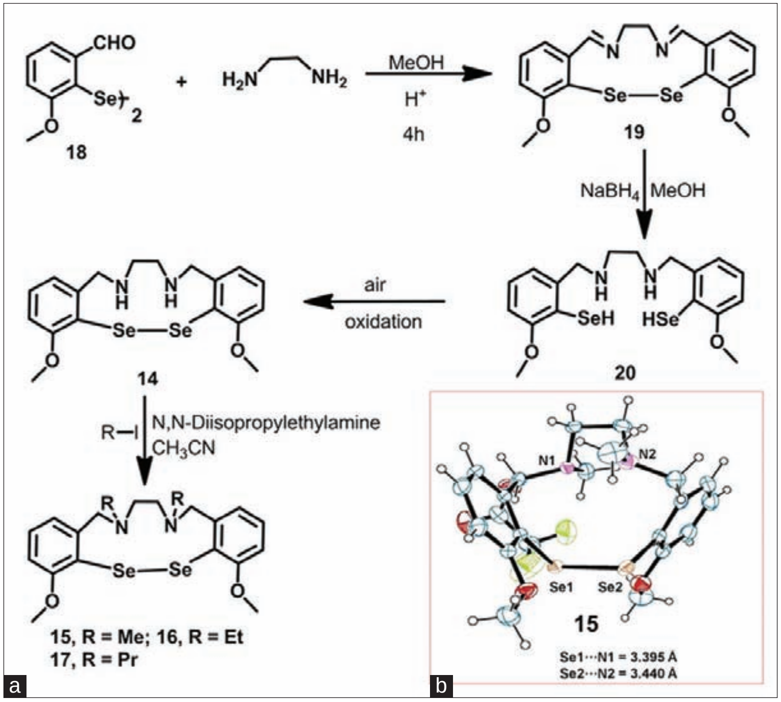
Compound	$^{t1/2}$ values (min)[a]		
	H_2O_2	CuOOH	*t*-BuOOH
Ebselen (1)	300.5±10.2	153.9±8.5	427.3±12.5
14	20.8±2.2	30.0±2.5	71.4±7.2
15	77.6±5.6	100.3±9.2	158.6±5.3
16	79.3±6.5	98.7±6.4	177.9±4.0
17	57.6±3.4	58.8±3.8	114.0±5.6

[a] Assay condition: The reactions were carried out in MeOH at 22°C. Catalyst: 20.0 µM; PhSH: 1.0 mM; peroxides: 2.0 mM

Table 2: Reduction of peroxides by GSH in the presence of compounds 1, 14-17

Compound	Initial rate (v_o) [μMmin^{-1}][a]		
	H_2O_2	CuOOH	*t*-BuOOH
Ebselen (1)	100.0±6.2	40.5±3.9	16.1±1.5
14	95.5±7.6	34.2±2.8	2.5±0.2
15	121.4±3.8	28.5±5.3	20.7±1.6
16	111.7±10.6	25.3±3.2	12.3±2.0
17	109.3±10.8	23.4±2.1	5.4±1.6

[a] Assay condition: The reactions were carried out in phosphate buffer (0.1 M, pH 7.5) at 22°C. Catalyst: 80.0 µM; GSH: 2.0 mM; NADPH: 0.4 mM; EDTA: 1.0 mM; GSH disulfide reducatase: 1.7 units/mL; peroxides: 1.6 mM. GSH: Glutathione



Scheme 1: (a) Synthetic routes to the intramolecular diselenides 14-17. (b) Single crystal X-ray structure of compound 15. Oak Ridge Thermal Ellipsoid Plot diagram with the ellipsoids representing 50% probability. Significant bond lengths (Å) and bond angles (°). 15: rSe1…N1 = 3.395; rSe2…N2 = 3.440; rSe1…Se2 = 2.342; θN1…Se1-Se2 = 88.9; θN2…Se2-Se1 = 94.4.

equivalent of PhSH, intermolecular diselenides produce a mixture of the corresponding selenenyl sulfides and selenols.[19-21] However, the complete conversion of the selenenyl sulfides to the corresponding selenols by PhSH depends on the extend of thiol exchange reaction that takes place in the selenenyl sulfide intermediates. Thiol exchange reaction, on the other hand, is controlled by the strength of the Se…N non-covalent interaction between the selenium center and the amino moiety.[19-21] Selenenyl sulfides with very less or negligible Se…N non-covalent interactions are readily converted to the respective selenols in the presence of one equivalent of PhSH due to less thiol exchange reaction,[20] whereas the selenenyl sulfides having considerable Se…N interactions require a large excess (30–40 equivalent) of PhSH for their complete conversion to the selenols.

In contrast, the reactivity of diselenides **15-17** toward thiol was found to be different from that of the corresponding intermolecular diselenides. The treatment of diselenides **15-17** with two equivalents of PhSH did not generate any peak in the ^{77}Se NMR spectra for the corresponding selenenyl sulfides **21-23** and selenols **24-26 [Figure 3]**. Addition of an excess of PhSH (30 equivalent) leads to no change in the spectra, which strongly suggests that compounds **15-17** do not react with the PhSH. However, compounds **15-17** can be reduced by strong reducing agents such as sodium borohydride ($NaBH_4$) to the corresponding selenolate anions **27-29**, respectively, **[Figure 3]**, which could trapped with iodoacetic acid (ICH_2CO_2H) to obtain the corresponding monoselenides **30-32 [Figure 3]**. In contrast, the *sec*-amine-based diselenide **14** readily reacts with one equivalent of PhSH to produce a mixture of selenenyl sulfide **33** and selenol **34 [Figure 3]** and a second equivalent of PhSH was sufficient to convert the selenenyl sulfide **33** completely to the selenol **34**. However, no peak was detected in the ^{77}Se NMR spectra for the selenol **34** due to a rapid proton exchange reaction between the selenol moiety and the *sec*-amino group. The selenol was trapped with iodoacetic acid to obtain the corresponding monoselenide at 174 ppm. An immediate formation of the selenol **34** after reductive cleavage of the Se–Se bond by PhSH is mainly responsible for higher GPx-like activity of compound **14** compared to that of the other intramolecular diselenides in the presence of PhSH. Although the X-ray crystal structure of compound **15** shows larger Se…N distances, compound **14** undergoes a rapid reaction with PhSH to cleave the Se–Se bond. This indicates that the orientation is probably different for compounds **14** and **15** in the solution phase, bringing the nitrogen atom near to the selenium centers, which favors reasonably strong Se…N non-covalent interaction in compound **14**. Thus, compound **14** exhibits higher activities in the presence of PhSH as the cofactor.

Reactivity of compounds 14-17 toward peroxides

As most of the intramolecular diselenides exhibited very poor or no reactivity toward thiol, these compounds were treated with H_2O_2 to understand their reactions with the peroxides. In the presence of peroxides, the selenium centers in compounds **15-17** underwent oxidation. It is shown earlier that selenenic acids derived from the intermolecular diselenide **12** do not undergo overoxidation due to the presence of the methoxy group in the *ortho*-position.[20] In contrast, all the intramolecular diselenides **15-17** react with excess H_2O_2 to generate the overoxidized products, despite having the methoxy group. Reactions of

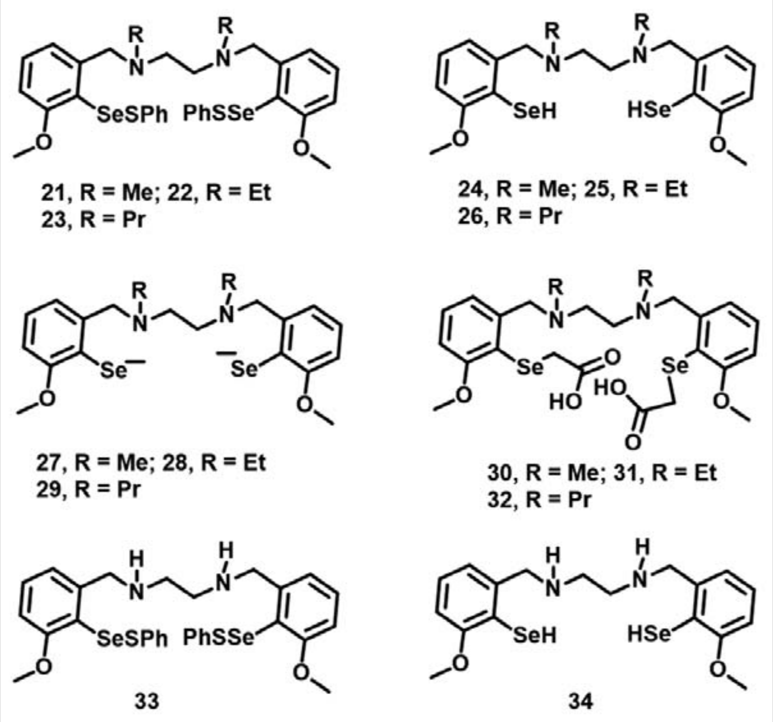


Figure 3: Reactions of compounds 15-17 with PhSH to produce the selenenyl sulfides 21-23 and selenols 24-26

intramolecular diselenides with one equivalent of H_2O_2 were very slow at room temperature, and therefore, excess of peroxides was added to them. Unfortunately, all the oxidized intermediates could not be detected due to the rapid reaction with excess of H_2O_2. Thus, detailed mechanistic studies with H_2O_2 were performed for compound **14**, which exhibited higher catalytic activities in the presence of PhSH. Treatment of compound **14** with one equivalent of H_2O_2 generated a peak at 933 ppm in the ^{77}Se NMR spectra, which corresponds to compound **36** produced by the cyclization of the intermediate selenenic acid **35** after removal of two equivalents of water molecules. The peak for the selenenic acid **35** could not be detected in the ^{77}Se NMR spectra due to the rapid cyclization. Compound **36** undergoes oxidation in the presence of excess H_2O_2 (2 equivalent) to generate the selenoxide **37** (1162 ppm) **[Scheme 2]**, which undergoes spontaneous hydrolysis to the corresponding seleninic acid **35**, which showed a signal at 1172 ppm. Compound **38** may also be produced by the reaction of compound **35** with excess of peroxides when the thiol concentration is low in the reaction medium. The seleninic acid **38** thus produced undergoes further oxidation (10 equivalent) to produce the selenonic acid **39** (1140 ppm) **[Scheme 2]**. The overoxidation of the selenenic acid to the corresponding selenonic acids appears to reduce the catalytic activities of intramolecular diselenides **14-17**, although all the overoxidized products are reduced back to the corresponding selenenyl sulfides by excess amount of thiol.

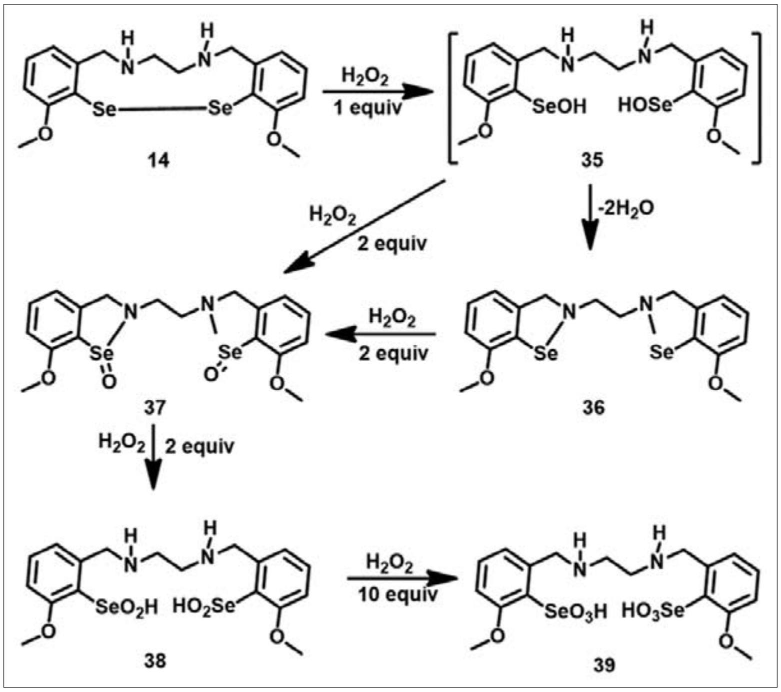
CONCLUSIONS

In this study, we have synthesized a series of intramolecular diselenides and studied their antioxidant activities as the GPx mimics. Although they exhibit much higher activities than that of the standard GPx mimics ebselen, the activity is considerably less than that of the corresponding intermolecular diselenides. The crystal structure of one of the diselenides suggests that the lower activity is probably due to extremely weak or absence of Se…N non-covalent interaction in the molecule. Steric hindrance was also found to be an important factor for the lower activities of intramolecular diselenides in the presence of GSH as the thiol cosubstrate. Most of these diselenides do not react with the thiol to cleave the Se–Se bonds. The poor reactivity toward thiol is mainly due to the weak Se…N interaction, which is not sufficient to activate the diselenide bonds toward the facile cleavage by the nucleophilic thiol. Therefore, oxidation of the selenium centers by the peroxides appears to be the first step in the catalytic cycle. In the presence of excess peroxides, they undergo facile overoxidation, which may reduce the catalytic activity.

Experimental section

General procedure

n-Butyllithium was purchased from Acros Chemical Co. (Belgium). Methanol was obtained from Merck and dried before use. All other chemicals were of the highest purity available. All the reactions were carried out under nitrogen with the use of standard vacuum-line techniques. Due to the unpleasant odors and toxic nature of several of the reaction mixtures involved, most manipulations were carried out in a well-ventilated fume hood. Diethyl ether was dried over sodium metal with benzophenone. Thin-layer chromatography (TLC) analyses were carried out on pre-coated silica gel plates (Merck), and spots were visualized



Scheme 2: Reaction of compound 14 with H_2O_2 produces the selenenic acid 35. In the presence of excess H_2O_2, compound 35 undergoes further oxidation to produce the seleninic acid 38 and selenonic acid 39. Elimination of water molecule from compound 35 afforded compound 36, which is oxidized to the selenoxide 37 by H_2O_2

with ultraviolet (UV) radiation. Column chromatography was performed on glass columns loaded with silica gel or on automated flash chromatography systems (Biotage) with the use of preloaded silica cartridges.^{1}H (400 MHz),^{13}C (100.56 MHz), and ^{77}Se (76.29 MHz) NMR spectra were obtained on a Bruker 400 MHz NMR spectrometer. Chemical shifts are cited with respect to $SiMe_4$ as internal (^{1}H and^{13}C) and Me_2Se as external (^{77}Se) standard. A Perkin–Elmer Lambda 5 UV/visible spectrophotometer was used to measure the GPx-like activities. Mass spectral studies were carried out on a Bruker Daltonics esquire 6000 plus mass spectrometer with electrospray ionization MS (ESI-MS) mode analysis. Ebselen (**1**) was synthesized following the reported procedure.[9,10]

Synthetic procedures

Synthesis of 14

Compound **18 (**0.5 g, 1.16 mmol) was dissolved in dry acetonitrile (20 mL) along with 2 mL of dry dichloromethane to dissolve the compound. 50 µL of acetic acid was added to it and stirred for 15 min. Ethylenediamine (77 µL, 1.16 mmol) was added to it and stirred at room temperature. After 4 h, the solvent was evaporated and then dissolved in methanol (15 mL). Sodium borohydride (0.171 g, 4.64 mmol) was added to it portion wise with stirring at 0°C. It was stirred for 6 h at room temperature and the solvent was evaporated. The crude product was dissolved in dichloromethane (20 mL) and washed with water (20 mL) 3 times. The organic layer was dried with Na_2SO_4, filtered, and evaporated to obtain yellow-colored oil. It was purified by column chromatography using dichloromethane and methanol as the eluents. It was characterized by NMR and MS. Yield: 0.22 g (40%),^{1}H NMR ($CDCl_3$), δ (ppm): 3.34 (s, 4H), 3.91 (s, 6H), 3.97 (s, 4H), 6.88–6.90 (d, *J* = 6.8 Hz, 2H), 7.13–7.15 (d, *J* = 6.8 Hz, 2H), 7.33–7.37 (t, *J* = 8.0 Hz, 2H); ^{13}C NMR ($CDCl_3$), δ (ppm): 45.7, 52.5, 57.0, 112.4, 112.8, 124.1, 131.9, 139.1, 160.4; ^{77}Se NMR ($CDCl_3$), δ (ppm): 369. ESI-MS: m/z: Calcd for $C_{18}H_{22}N_2O_2Se_2[M+H]^+$: 456.2995; found: 455.8619.

General procedure for the synthesis of compounds 15-17

Compound **14 (**0.30 g, 0.66 mmol) was dissolved in dry acetonitrile (20 mL). Diisopropylethylamine (0.66 µL, 1.97 mmol) was added to it and heated at 60°C for 30 min. Then, corresponding alkyl iodide (0.146 mmol) in 2 mL of acetonitrile was added to the mixture dropwise under N_2-atmosphere with a proper cold-water circulation to avoid the evaporation of alkyl iodide. The reaction was monitored by TLC, and after completion of the reaction, the solvent was evaporated under reduced pressure. Then, it was dissolved in dichloromethane and washed 3 times with water (20 mL). It was dried with Na_2SO_4, filtered and evaporated to obtain yellow oil. It was purified by column chromatography using dichloromethane and methanol as eluents. The product was characterized by NMR and MS.

Compound 15: The compound was crystallized in chloroform and *n*-hexane with small amount of trifluoroacetic acid. Yield: 0.124 g (39%), ^{1}H NMR ($CDCl_3$), δ (ppm): 1.93 (s, 6H), 2.96 (s, 4H), 3.99 (s, 6H), 4.16 (s, 4H), 6.99–7.04 (m, 4H), 7.39–7.43 (t, *J* = 8.0 Hz, 2H); ^{13}C NMR ($CDCl_3$), δ (ppm): 40.7, 49.1, 57.2, 61.6, 112.5, 121.4, 124.4, 132.5, 138.9, 161.8; ^{77}Se NMR ($CDCl_3$), δ (ppm): 364. ESI-MS: m/z: Calcd for $C_{20}H_{26}N_2O_2Se_2[M]^+$: 486.0325; found: 486.9369.

Compound 16: Yield: 0.128 g (39%), ^{1}H NMR ($CDCl_3$), δ (ppm): 0.83–0.86 (t, *J* = 6.8 Hz, 6H), 2.30–2.31 (br, 4H), 2.79 (s, 4H), 3.96 (s, 6H), 4.14 (s, 4H), 6.96–6.98 (d, *J* = 8.4 Hz, 2H), 7.08–7.10 (d, *J* = 7.2 Hz, 2H), 7.37–7.41 (t, *J* = 8.0 Hz, 2H); ^{13}C NMR ($CDCl_3$), δ (ppm): 30.1, 46.3, 47.6, 57.2, 57.7, 112.4, 121.0, 125.1, 132.4, 139.1, 161.7; ^{77}Se NMR ($CDCl_3$), δ (ppm): 358. ESI-MS: m/z: calcd for $C_{22}H_{30}N_2O_2Se_2[M]^+$: 512.4058; found: 512.9707.

Compound 17: Yield: 0.120 g (34%),^{1}H NMR ($CDCl_3$), δ (ppm): 0.63–0.67 (t, *J* = 7.6 Hz, 6H), 1.71 (br, 4H), 1.99 (br, 2H), 2.13–2.16 (br, 2H), 2.84-2.88 (br, 2H), 3.22–3.24 (br, 2H), 3.96 (s, 6H), 4.12–4.15 (br, 2H), 4.23–4.26 (br, 2H), 6.97–6.99 (d, *J* = 8.0 Hz, 2H), 7.18–7.19 (d, *J* = 6.4 Hz, 2H), 7.39–7.43 (t, *J* = 8.0 Hz, 2H);^{13}C NMR ($CDCl_3$), δ (ppm): 11.7, 17.0, 47.2, 55.1, 57.3, 58.3, 112.3, 120.9, 125.2, 132.3, 139.3, 161.9;^{77}Se NMR ($CDCl_3$), δ (ppm): 357. ESI-MS: m/z: Calcd for $C_{24}H_{34}N_2O_2Se_2[M]^+$: 540.4590; found: 540.9491.

GPx-like activity HPLC assay

GPx-like activity was carried out by HPLC with use of a 2695 separation module and a 2996 photodiode array detector and a fraction collector. The assays were performed in 1.8 mL sample vials, and a built-in autosampler was used for sample injection. In this assay, we used mixtures containing a 1:2 molar ratio of PhSH and peroxide in methanol at room temperature (22°C) as our model system. Runs with and without catalyst were carried out under the same conditions. Periodically, aliquots were injected onto the reverse-phase column (Princeton C18 column, 4.6 mm × 150 mm, 5 µm) and eluted with methanol and water (85:15), and the concentrations of the diphenyl disulfide (PhSSPh) produced in the reaction were determined at 254 nm with the aid of pure PhSSPh as an external standard. The amount of disulfide formed during the course of the reaction was calculated from the calibration plot for the standard (PhSSPh). The plots for kinetic parameters were obtained by use either of linear or of sigmoidal curve fitting.

GPx-like activity: GSH-GSSG coupled assay

The GPx activity was followed spectrophotometrically. The test mixture contained GSH (2.0 mM), EDTA (1 mM), GSH disulfide reductase (1.7 units/ml), and NADPH (0.4 mM) in 0.1 M potassium phosphate buffer of pH 7.5. GPx samples (80 µM) were added to the test mixture at 23°C temperature and the reaction was started by the addition of peroxide (1.6 mM). The initial reduction rates were calculated from the rate of NADPH oxidation at 340 nm in GSH assay. Each initial rate was measured at least 3 times and calculated from the first 5–10% of the reaction using 6.22/mM/cm as the molar extinction coefficient for NADPH. For the peroxidase activity, the rates were corrected for background reaction between peroxide and thiol.

Single-crystal X-ray structure determination

X-ray crystallographic studies were carried out using a Bruker CCD diffractometer with graphite-monochromatized $Mo_{K\alpha}$ radiation (λ = 0.71073 Å) controlled by a Pentium-based PC running the SMART software package.[26] Single crystals were mounted at room temperature on the ends of glass fibers, and data were collected at room temperature. The structures were solved by direct methods and refined using the SHELXTL software package.[27,28] All non-hydrogen atoms were refined anisotropically and hydrogen atoms were assigned idealized locations. Empirical absorption corrections were applied to all structures using SADABS.[29] The structures were solved by direct method (SIR-92) and refined by full-matrix least-squares procedure on F^2 for all reflections (SHELXL-97).[30]

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