Synthesis and Biological Evaluation of Ebselen and Its Acyclic Derivatives

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Five ebselen and three acyclic ebselen derivatives were synthesized. These compounds were screened for their glutathione peroxidase (GSH Px)-like activity and scavenging activity against 1,1-diphenyl-2-pycryl-hydrazyl (DPPH) and peroxynitrite radical. All tested compounds displayed similar significant GSH Px-like activity, which are slightly higher than that of ebselen. The peroxynitrite scavenging activity showed that the acyclic allylseleno 4c was five times more potent than ebselen.

Key words ebselen derivative; selenoorganic compound; antioxidant; peroxynitrite; glutathione peroxidase

Biological systems use enzymatic (superoxide dismutase, glutathione peroxidase, etc.) and nonenzymatic (uric acid, creatinine, polyamine, retinal, etc.) antioxidant systems to prevent oxidative stress. However, once the systems are disturbed the uncontrolled oxidative stresses initiate a series of harmful biochemical events, or generate them as a consequence of earlier tissue injury, thus aggravating the final damages. Such damages include brain dysfunction, cancer, and cardiovascular disease and inflammation.

Glutathione peroxidase (GSH Px, EC 1.11.1.9), a selenoenzyme, is one of the enzymes in the mammalian antioxidant systems, which perform the reduction of H₂O₂ and other hydroperoxides. It is well accepted that selenium (as selenocysteine), an essential component of the active sites of glutathione peroxidase, is responsible for the scavenging of reactive oxygen species and protecting biomembrane from oxidative stress. Although possessing potent antioxidative activity, like other protein drugs GSH Px is limited for the clinical use because of its instability, poor availability, and easy metabolism. Therefore many efforts have been directed to design and synthesize small selenium-containing organic molecules with GSH Px activity. Ebselen (2-phenyl-1,2-benzisoselenazole-3(2H)-one), a nontoxic low molecular weight selenium-containing heterocyclic compound, has been reported to mediate the reduction of hydroperoxides by glutathione, thereby mimicking the enzymic activity of GSH Px. Beside possessing antioxidative activity, ebselen also demonstrate a number of pharmacologic activities, such as antiinflammatory, antiatherosclerotic, and cytoprotective properties. Studies in our laboratory have recently focused on the design and synthesis of antioxidants as potential antiinflammatory agents. Based on the unique structure of ebselen, we speculated that simple modification of the chemical structure of ebselen might provide potent antioxidative activity. In this study, the ebselen molecule and its acyclic derivative were modified and their ability to scavenge free radicals and GSH Px-like effects were also investigated.

Chemistry Ebselen has been prepared previously by several methods. In the earliest approach 2,2′-diselenobis (benzoic acid) was converted to selenyl chloride benzoyl chloride, which was treated with aniline to give ebselen. More recent advance involves ortholithiation of benzanilide, subsequent insertion of selenium into benzanilide-derived diatomic and cyclization of selenium-containing diatomic to ebselen. Because of the short synthetic and easy handling steps, the latter method has been used in our synthetic work. Chart 1 summarizes our results and illustrates the structures of the new products obtained from benzoic acid. The N-substituted benzanilamides were conveniently synthesized by the treatment of benzoic acid with thionyl chloride, followed by substituted arylamine. Lithiation of N-substituted benzoyl-amine with two equivalents of n-butyl lithium at room temperature in dry tetrahydrofuran under nitrogen atmosphere gave the dilithiobenzoylamides. Addition of selenium to these intermediates resulted in the expected insertion of selenium into the carbon lithium bond giving diatomic intermediates. Cyclization of the diatomic intermediates with two equivalents of copper (II) bromide at −78°C provided compounds 3a–f, respectively. Treatment of 2a–c with two equivalents of n-butyl lithium, followed by selenium gave the diatomic intermediates. Without isolation, these intermediates reacted with either methyl iodide or allyl bromide to furnish compounds 4a–c. It is interesting that 4a and 4b were both N- and Se-methylated products while 4c was only a Se-allylic compound. No alloydilation was observed at the nitrogen atom of 4c.

Results and Discussion

The free radical-scavenging effects are expressed as the
scavenging concentration (SC_{50}) needed to reduce 50% of the initial amount of 1,1-diphenyl-2-pycryl-hydrazyl radical (DPPH).\(^9\) Ebselen and other tested compounds exhibited little radical-scavenging activity (SC_{50}>5\, \text{mM}) against DPPH radicals.

The increasing evidence for a role of peroxynitrite in biological processes prompted us to investigate the reaction of compounds with peroxynitrite. Indeed, it has been reported that ebselen rapidly reacts with peroxynitrite and protects against DNA damage caused by peroxynitrite.\(^9\) Peroxynitrite-scavenging activities were determined according to the method reported by Balavoine and Geletii\(^{10}\) and Bailly et al.\(^{11}\) Briefly, peroxynitrite induced the bleaching of Pyrogallol red (PR) dye, which was measured at 550 nm. Consumption of PR in the presence and absence of the test compounds was measured over a range of peroxynitrite concentrations (0—62.5 \, \text{mM}). Figure 1 shows the \(D/D_A\) data for different concentrations of antioxidant plotted against \([\text{antioxidant}]/[\text{PR}]_0\). \(D_A\) and \(D_A\) are the stoichiometries for the reaction of peroxynitrite with PR in the absence and presence of the tested antioxidant compounds, respectively. \(k_A\) and \(k_{PR}\) are the rate constants for the reaction of peroxynitrite with the tested antioxidants and PR, respectively. The ratio \(k_A/k_{PR}\), which represents the relative antioxidant activities, was calculated from the slope of the straight line plotting \(D_A/D_A\) against \([\text{antioxidant}]/[\text{PR}]_0\). The greater the ratio of \(k_A/k_{PR}\), the more potent the peroxynitrite-scavenging activity of the tested compounds. All tested compounds except for 3c, 4a, and 4b exhibited higher \(k_A/k_{PR}\) values than ebselen (Table 1). Compound 4c, containing the allylseleno moiety, was the most potent one with five-fold greater activity than ebselen. The scavenging potency was in the order of \(4c>3b>3d>3f>3e\,\text{ (ebselen)}\).

To evaluate the efficacy of the tested compounds as peroxide scavengers, the GSH Px-like activity was investigated. The GSH Px-like activity of ebselen results from two consecutive reactions: the oxidation of the thiols by peroxide and the reduction of the disulphides by glutathione. The consumption of NADPH\(^{11,12}\) upon the addition of \(\text{H}_2\text{O}_2\) in the absence of test compounds was 0.8 \, \text{\mu M} / \text{min}. There was no detectable activity with the solvent DMSO alone. Upon the addition of test compounds, catalytic NADPH consumption occurred, as summarized in Table 1. A typical reaction kinetic plot for compounds 3b—f is also shown in Fig. 2, with the compound 3a (ebselen) as standard control. The catalytic rates of other compounds are listed in Table 1. Our results indicate that all of the tested compounds have higher GSH Px-like activity than ebselen.

In conclusion, ebselen has been shown to be involved in a variety of effects such as antioxidant, free radical-scavenging, and antiinflammatory activities. Much of these activities can be attributed to its seleno-containing moiety with GSH Px-like activity. In the present study, we synthesized derivatives of ebselen and investigated their potential roles as antioxidants. All of the derivatives showed higher GSH Px-like activity than ebselen. Several of the derivatives possessed potent peroxynitrite-scavenging activity. In particular, 4c, con-

### Table 1. GSH Peroxidase-Like Activity and Inhibition of Test Compounds against Peroxynitrite-Mediated Oxidation

<table>
<thead>
<tr>
<th>Compound</th>
<th>GSH Px-like activity (relative to ebselen)(^a)</th>
<th>Inhibition of peroxynitrite-dependent oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(k_A/k_{PR})</td>
<td>Activity relative to ebselen</td>
</tr>
<tr>
<td>3a (Ebselen)</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>3b</td>
<td>1.36</td>
<td>2.48</td>
</tr>
<tr>
<td>3c</td>
<td>1.47</td>
<td>0.94</td>
</tr>
<tr>
<td>3d</td>
<td>1.17</td>
<td>2.19</td>
</tr>
<tr>
<td>3e</td>
<td>1.60</td>
<td>1.39</td>
</tr>
<tr>
<td>3f</td>
<td>1.60</td>
<td>1.81</td>
</tr>
<tr>
<td>4a</td>
<td>1.69</td>
<td>0.74</td>
</tr>
<tr>
<td>4b</td>
<td>1.33</td>
<td>0.87</td>
</tr>
<tr>
<td>4c</td>
<td>1.47</td>
<td>5.35</td>
</tr>
</tbody>
</table>

\(^a\) The consumption of NADPH upon addition of \(\text{H}_2\text{O}_2\) in the absence of test compounds was 0.8 \, \text{\mu M} / \text{min} and the consumption of NADPH for ebselen was 10.9 \, \text{\mu M} / \text{min}.

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**Fig. 1.** Plot of \(D/D_A\) against \([\text{antioxidant}]/[\text{PR}]_0\)

Reactions were carried out at room temperature by adding peroxynitrite (12.5 \, \text{mM}) into tubes containing phosphate buffer (50 \, \text{mM}, pH 7.0) and 50 \, \text{\mu M} of [PR]_0. Results shown are the mean of two independent experiments.

**Fig. 2.** GSH Px-Like Activity of Tested Compounds and Ebselen (3a)

GSH Px-like activity was measured by the disappearance of NADPH. Reactions were initiated with the addition of 1 \, \text{mM} \, \text{H}_2\text{O}_2\) to the assay mixture containing 1.0 \, \text{mM} GSH, 0.28 \, \text{mM} NADPH, and 11 \, \text{uM} glutathione reductase. Ebselen 100 \, \text{\mu M} and its synthetic derivatives were added 2 \, \text{min} later (arrow). The reaction plot for compound 4a, 4b, and 4c are not shown here, but their activity is listed in Table 1. Results shown here represent the mean of at least two independent experiments.
taining the allylseleno moiety, displayed the highest activity and could serve as a lead compound to be further developed as an antiinflammation agent.

Experimental

General Melting points (mp) were taken on a BUCHI 530 apparatus and are uncorrected. Merck Art No 105554 plates precoated with Silica gel 60 containing a fluorescent indicator were used for thin-layer chromatography, and Silica gel 60 (Merck Art No 109385, 230—400 mesh) was employed for column chromatography. Evaporations were carried out at <50 °C using a rotary evaporator at reduced pressure (water aspirator). 1H and 13C-NMR spectra were obtained on a Varian 300 NMR spectrometer at 300 and 75 MHz, respectively. Where necessary, deuterium exchange experiments were used to obtain proton shift assignments. Mass spectra were recorded on a JEOL JMS-300 spectrophotometer. Analytical samples were dried under reduced pressure at 78 °C in the presence of P2O5 for at least 12 h unless otherwise specified. Elemental analyses were obtained using a Perkin-Elmer 2400 Elemental Analyzer.

N-Substituted-benzoilamide (2a—f), General Procedure Freshly distilled thionyl chloride (20 ml) was added to a solution of benzoic acid (5.0 g, 41.0 mmol), pyridine (2.0 ml), and dichloromethane (20 ml) and the reaction mixture was heated under reflux for 2 h. The mixture was concentrated under reduced pressure and the residue was coevaporated with toluene (5 ml) three times. Toluene (0.5 ml) and aniline (3.8 ml, 50 mmol) were added to the residue and the mixture was heated under reflux for 3 h. The solven was removed under reduced pressure, 10% sodium bicarbonate (20 ml) was added, and the mixture was extracted with dichloromethane (30 ml×3). The organic layer was concentrated and the residue was crystallized with ethanol to give pure 2a (6.4 g, 98%).

2b—f were obtained under the same conditions.

2-Phenyl-1,2-benzisoselenazol-3(2H-one (3a): mp 161—162 °C (EtOH). 1H-NMR (DMSO-d6): 7.95 (d, 2H, J=6.7 Hz, H-2, H-6), 7.36—7.30 (5, 3H, H-3, H-4, H-5, H-6), 7.35 (t, 2H, J=7.0 Hz, H-3, H-5), 1.74 (t, 1H, J=7.4 Hz, H-4). 13C-NMR (DMSO-d6): 131.9, 130.7, 129.6, 129.0, 125.0, 121.8, 107.4. Anal. Caled for C14H11NOSe: C, 79.59; H, 6.20; N, 6.96. Found: C, 79.26; H, 6.32; N, 6.96.


2-(2-Alkylseleno)benzoylamide (4a—c), General Procedure To a stirred solution of 2a (1.5 g, 7.6 mmol) in dry tetrahydrofuran (50 ml) under nitrogen at 0 °C was added n-butyl lithium (11 ml, 1.6 M). After 30 min, black selenium powder (0.4 g, 5.1 mmol) was added to the reaction mixture and the reaction was continued for another 4 h. To the reaction mixture methyl iodide (4.6 ml, 77 mmol) was added and the mixture was stirred for 30 min. The cooling bath was removed after 30 min, and the temperature was allowed to reach ambient temperature. After the reaction mixture was poured into water (100 ml) containing acetic acid (1 ml) and, after filtration, extracted with dichloromethane. The organic layer was concentrated under reduced pressure. Column chromatography on silica gel with 3:1 n-hexane/ethanol as eluent gave 4a (0.25 g, 14%) as a solid white product.

4b—e were obtained under the same conditions.

2-Phenyl-1,2-benzisoselenazol-3(2H-one (3a): mp 161—162 °C (EtOH). 1H-NMR (DMSO-d6): 7.95 (d, 2H, J=6.7 Hz, H-2, H-6), 7.36—7.30 (5, 3H, H-3, H-4, H-5, H-6), 7.35 (t, 2H, J=7.0 Hz, H-3, H-5), 1.74 (t, 1H, J=7.4 Hz, H-4). 13C-NMR (DMSO-d6): 131.9, 130.7, 129.6, 129.0, 125.0, 121.8, 107.4. Anal. Caled for C14H11NOSe: C, 79.59; H, 6.20; N, 6.96. Found: C, 79.26; H, 6.32; N, 6.96.

2-(4-Methylphenyl)-1,2-benzisoselenazol-3(2H-one (3c): mp 154—155 °C. 1H-NMR (DMSO-d6): 7.95 (d, 2H, J=6.7 Hz, H-2, H-6), 7.36—7.30 (5, 3H, H-3, H-4, H-5, H-6), 7.33 (t, 2H, J=6.7 Hz, H-3, H-5), 1.71 (t, 1H, J=7.4 Hz, H-4). 13C-NMR (DMSO-d6): 140.0, 133.5, 130.4, 129.3, 128.7, 127.6, 126.6, 123.2, 22.4. IR (KBr) cm⁻¹: 3065, 1647. UV λmax (MeOH) nm (log e): 327 (3.82). FAB-MS m/z: 289 (M+H)+. Anal. Caled for C14H11NOSe: C, 58.34; H, 3.85; N, 4.86. Found: C, 58.30; H, 3.70; N, 4.66.

2-(4-Methylphenyl)-1,2-benzisoselenazol-3(2H-one (3d): mp 154—155 °C. 1H-NMR (DMSO-d6): 7.95 (d, 2H, J=6.7 Hz, H-2, H-6), 7.36—7.30 (5, 3H, H-3, H-4, H-5, H-6), 7.33 (t, 2H, J=6.7 Hz, H-3, H-5), 1.71 (t, 1H, J=7.4 Hz, H-4). 13C-NMR (DMSO-d6): 139.0, 135.6, 131.1, 129.3, 128.4, 127.7, 127.6, 123.2, 22.4. IR (KBr) cm⁻¹: 3065, 1647. UV λmax (MeOH) nm (log e): 327 (3.82). FAB-MS m/z: 289 (M+H)+. Anal. Caled for C14H11NOSe: C, 58.34; H, 3.85; N, 4.86. Found: C, 58.30; H, 3.70; N, 4.66.
(KB) cm$^{-1}$: 3052, 1638. UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$): 318 (3.79). FAB-MS m/z: 305 (M$^+$).

\textit{Anal.} Cacld for C$_{16}$H$_{15}$NOSe: C, 60.76; H, 4.78; N, 4.43. Found: C, 60.97; H, 4.74; N, 4.52.

-N-Toluidyln-methyl(2-methylseleno)benzoylamide (4b): $R_f$: 0.28 (n-hexane/ethyl acetate=3:1). mp 77—78°C. $^1$H-NMR (CDCl$_3$) $\delta$: 7.32 (d, 1H, J=7.59 Hz, H-6), 7.14—7.00 (m, 4H, H-2, H-3, H-5, H-6'), 3.45 (s, 3H, N-CH$_3$), 2.31 (s, 3H, SeCH$_3$), 2.24 (s, 3H, CH$_3$), 1.3°C-NMR (CDCl$_3$) $\delta$: 138.1, 132.4, 131.1, 130.7, 129.5, 128.0, 127.1, 39.3, 25.2, 9.3. IR (KBr) cm$^{-1}$: 3023, 1633. UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$): 318 (3.79). FAB-MS m/z: 319 (M$^+$). 

\textit{Anal.} Cacld for C$_{16}$H$_{15}$NOSe: C, 60.38; H, 5.38; N, 4.40. Found: C, 60.49; H, 5.19; N, 4.34.

-N-Phenyln-(2-allylseleno)benzoylamide (4c): $R_f$: 0.46 (n-hexane/ethyl acetate=3:1). mp 106—107°C. $^1$H-NMR (CDCl$_3$) $\delta$: 8.15 (s, 1H, H-6), 7.73—7.58 (m, 3H, H-3, H-4, H-5), 7.41—7.27 (m, 4H, H-2, H-3, H-5, H-6'), 7.19 (t, J=7.40 Hz, H-4'), 6.00—5.86 (m, 1H, CH=CH$_2$), 5.10—4.98 (m, 2H, CH=CH$_2$), 3.55 (d, 2H, J=7.14 Hz, SeCH$_2$). IR (KBr) cm$^{-1}$: 3282, 3052, 1638. UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$): 321 (3.82). FAB-MS m/z: 317 (M$^+$).

\textit{Anal.} Cacld for C$_{16}$H$_{17}$NOSe: C, 60.38; H, 5.38; N, 4.40. Found: C, 60.54; H, 4.57; N, 4.51.

**Determination of the Scavenging Effect on 1,1-Diphenyl-2-pycryl-hydrazyl radical (DPPH·)**

The experimental procedure was adapted from Ohnishi et al.$^{14}$ The ethanolic solution of DPPH· was added to 2 ml of the test compounds at different concentrations in ethanol (12.5, 25, 37.5, 50 μM). Each mixture was then shaken vigorously and kept for 30 min at room temperature in the dark. The decrease in absorbance of DPPH· at 517 nm was measured. Ethanol was used as a blank solution and DPPH· solution in ethanol served as the control. The percentage of remaining DPPH· was then calculated, and the radical-scavenging effects of the tested compounds were compared in terms of IC$_{50}$ (the concentration needed to reduce 50% of the initial amount of DPPH· and expressed as the molar ratio of each compound to the radical). All tests were performed in triplicate.

**Determination of the Scavenging Effect on Peroxynitrite**

Peroxynitrite synthesis was carried out as described by Radd et al.$^{15}$ Briefly, acidified hydrogen peroxide (1 M in 0.7 M HCl, 20 ml) and sodium nitrite (0.2 M, 20 ml) solution were drawn into two separate syringes. The contents of both syringes were simultaneously injected into an ice-cooled beaker containing 20 ml solution to remove excess hydrogen peroxide. The solution was filtered and the concentration of the resulting stock was determined spectrophotometrically at 302 nm ($\varepsilon$=1670 M$^{-1}$ cm$^{-1}$). The typical yield of freshly prepared peroxynitrite was 30 mM. The peroxynitrite was diluted in 0.1 M NaOH. Experiments were conducted at 25°C in 50 mM phosphate-buffered saline containing 0.1 mM diethylenetriaminepentaacetic acid, 90 mM NaCl, and 5 mM KC1, pH 7.4. Blanks using DMSO alone in the absence of test compounds and peroxynitrite allowed to degrade for 5 min in phosphate-buffered saline, pH 7.4, were also examined. There was no interference by DMSO and degraded peroxynitrite on the PR. Peroxynitrite induced the bleaching of PR dye, which was measured at 542 nm ($\varepsilon$=24000 M$^{-1}$ cm$^{-1}$). Consumption of PR (50 μM) in the presence and absence of test compounds was measured over a range of peroxynitrite concentrations (0—62.5 μM). Antioxidative activities were determined according to the methods reported by Balavoine et al.$^{10}$

The ratios of rate constants $k_3$/$k_{9p}$, which represent the relative antioxidant activities, were determined by plotting $D_0/D_0$ against [antioxidant]/[PR]. $k_3$ and $k_{9p}$ are the rate constants for reaction of peroxynitrite with the antioxidants and PR, respectively. $D_0$ and $D_0$ are the stoichiometries for the reaction of peroxynitrite with PR in the absence and presence of the antioxidant compounds, respectively. $N'$-Acetyl cysteine was used as a control compound and our results showed that it has a very low level of peroxynitrite-scavenging activity.

**Determination of GSH Px-Like Activity**

GSH Px-like activity was determined by the reduction of GSSG formed via the NADPH-glutathione reductase system as a continuous indicator system.$^{16}$ Loss of NADPH was monitored continuously at 340 nm using a spectrophotometer (Perkin-Elmer, Shelton, CT, U.S.A.) and a molar absorption coefficient of 6220 M$^{-1}$ cm$^{-1}$. Experiments were conducted at room temperature in 50 mM phosphate-buffered saline, pH 7.4, containing 1.0 mM EDTA and 1.0 mM Na$_2$S, 1.0 mM GSH, 0.25 mM NADPH, 1 U of GSH reductase, and 10—100 μM of test compounds. The reaction was initiated by addition of 0.5 mM tert-butylhydroperoxide, and ebselen or synthetic compounds were added later. GSH Px-like activity was proportional to the decrease in NADPH absorption at 340 nm.

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**References**