The Mechanism of Reaction of Ebselen with Superoxide in Aprotic Solvents as Examined by Cyclic Voltammetry and ESR

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The mechanism of the redox reaction of ebselen with superoxide was investigated using both ESR and electrochemical techniques. The reaction with superoxide in aprotic solvents was followed by means of cyclic voltammetry and ESR spin-trapping. A decrease in the oxidation current due to superoxide as a result of the addition of ebselen was clearly observed in the cyclic voltammograms. Ebselen reduced the ESR signal intensity of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO)–superoxide in a dose-dependent manner. The formation of an amidyl radical in this redox reaction was confirmed by rapid mixing continuous-flow ESR. The selenonate form and the seleninate form of ebselen were identified as the final products of the reaction of ebselen with superoxide. The following mechanism for this redox reaction can be proposed: First, ebselen reacts with superoxide and is converted to an ebselen anion radical; second, the ebselen anion radical reacts with superoxide and is converted to the amidyl radical. Hydrogen abstraction by the amidyl radical occurs and gives both a seleninate form and a selenonate form.

Key words ebselen; superoxide; antioxidant; cyclic voltammetry; ESR; spin trapping

Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one (Fig. 1) has been developed for use as an antioxidative agent. It is a lipophilic compound with varied antioxidant activities similar to those of glutathione (GSH) peroxidase.[1—3] These activities are thought to prevent against disease states by scavenging reactive free radicals, but the mechanisms of these redox reactions have not yet been clarified.

One of the most active oxygen species is the superoxide anion radical ($O_2\cdot^-$), which is produced by the one-electron reduction of molecular oxygen. This radical species is implicated in several harmful biological processes, such as lipid peroxidation and protein denaturation. The reactivity of $O_2\cdot^-$ in an aqueous media is quite different from that in aprotic media. In an aqueous system, $O_2\cdot^-$ spontaneously disproportionates into hydrogen peroxide and molecular oxygen, whereas in an aprotic media $O_2\cdot^-$ is stable and can have several reactivities, *e.g.* electrogenerated base, nucleophile, reductant or oxidant.

To evaluate the antioxidative mechanism of certain lipophilic compounds, it is important to understand the reactivity of these antioxidants with $O_2\cdot^-$ in aprotic media in order to mimic the lipophilic domain of the lipid bilayer. In this report we describe the mechanism of redox reactions of ebselen with $O_2\cdot^-$ in aprotic media using both electrochemical and ESR techniques. Electrochemical measurements provide useful information on the mechanisms of electron transfer and free radical reactions for redox active compounds. The voltammetric behavior of ebselen, primarily of its anodic oxidation,[4] has already been studied. The primary oxidation step has been identified as the formation of selenoxide. However, no cathodic reduction behavior of ebselen has been reported. Therefore, in the present study we used cyclic voltammetry to determine the interaction between superoxide anion radical and ebselen in both cathodic and anodic currents. As ESR spin-trapping is useful in discriminating trapped radical species and in estimating various short-lived radicals,[5,6] we employed this technique to evaluate the antioxidative activity of ebselen. Further, rapid mix, continuous-flow ESR was used to examine a short-lived free radical species which is generated during the reaction of ebselen with superoxide.

Experimental

Materials Ebselen was synthesized at Aventis Pharma AG (Frankfurt, Germany). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), potassium superoxide (KO_2) and 18-crown-6 ether were purchased from Sigma Co., Ltd., (St. Louis, MO, U.S.A). Tetraethylammonium perchlorate (TEAP) was purchased from Aldrich Chemical Co., Ltd., (Milwaukee, WI, U.S.A).

ESR Spin-Trapping ESR measurements were recorded on a JEOL JES-FE2XG spectrometer (JEOL, Tokyo, Japan) with 100 kHz field modulation operating at 9.44 GHz and at room temperature. The following instrument parameters were employed: modulation amplitude, 0.063 mT; microwave power, 8.0 mW; scan time, 2 min.

Five milliliters of a dry toluene–DMSO (3/2, v/v) solution containing 3.17 mg KO_2 (4.46×10^{-5} mol) and 20.8 mg 18-crown-6 ether (7.71×10^{-5} mol) was sonicated for 60 s. A 200 μl aliquot of toluene–DMSO (3/2, v/v) solution containing 40 μl DMPO (*ca.* 180 mM) and various concentrations of ebselen, DL-α-tocopherol or L-ascorbic acid was added. After stirring, the mixture was transferred to a flat quartz cell. ESR data collection began 60 s after addition of the DMPO solution. The signal intensity of the lowest field peak of DMPO–$O_2\cdot^-$ (S_2) was normalized against the signal intensity of MnO (S_1) in terms of relative height (S_2/S_1).

Cyclic Voltammetry The cyclic voltammetry measurements were performed on a dual potentio-galvanostat (DPGS-1: Nikko Keisoku Co., Ltd.), potential sweeper (NPS-2: Nikko Keisoku Co., Ltd.) and digital coulomb meter (NDCM-3: Nikko Keisoku Co., Ltd.) in dry acetonitrile containing the supporting electrolyte 0.1 M TEAP. A glassy carbon electrode for the working electrode, a platinum electrode as the counter electrode and a saturated calomel electrode (S.C.E.) was used as a reference electrode. The scan speed was 5 s/V. The voltammograms of ebselen alone were obtained by dissolving 50 mM of ebselen in dry, deoxygenated acetonitrile containing 0.1 M TEAP. The potential scan range was from −2.2 to 2.2 V *vs.* S.C.E. The voltammograms obtained with increasing concentrations of ebselen used acetonitrile which had been previously saturated with air for 15 min to ensure a constant oxygen concentration. The potential scan range was from −1.0 V to 0.0 V *vs.* S.C.E.

Structural Analysis of the Reaction Product of Ebselen with Superoxide Thirty milliliters of a methanol solution containing 100 mM of ebselen

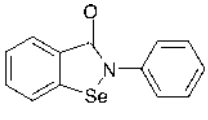

Fig. 1. Chemical Structure of Ebselen

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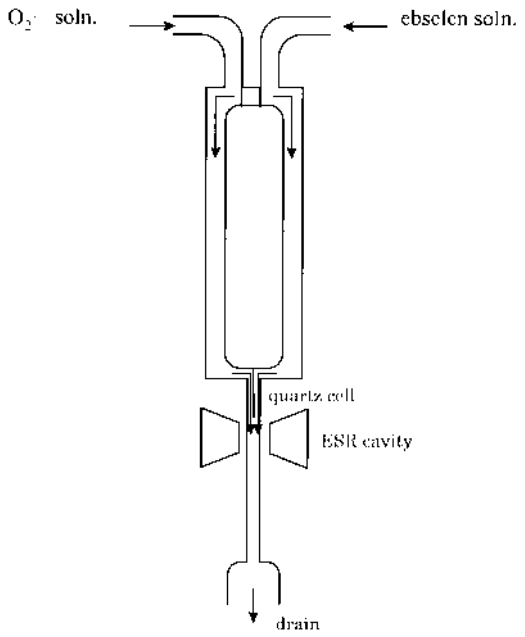

Fig. 2. Schematic Drawing of the Rapid Mix Continuous-Flow ESR Measurement

and 114 mM of KO_2 was stirred for one day and then concentrated under reduced pressure. A potassium salt of selenonate precipitated as a yellowish powder. IR spectrum of this powder was recorded on a HITACHI 270 IR spectrometer. ^{1}H-NMR spectrum was recorded on a JEOL EX-400 (400 MHz) spectrometer in D_2O. FAB-MS spectrum was recorded on a JEOL JMS HX-110 spectrometer. ^{1}H-NMR (D_2O) δ: 7.31 (1H, t), 7.51 (4H, m), 7.68 (1H, t), 7.82 (1H, t), 7.91 (1H, d), 8.19 (1H, d). MS (FAB) *m/z*: 364 (M+K+2H), 402 (M+2K+2H), 440 (M+3K+2H). IR (KBr) cm^{-1}: 904 (ν, Se=O), 920 (ν, O–Se–O). Structural analysis of the reaction product of ebselen with $O_2^{\cdot-}$ was also done in DMSO solution. Fifty milliliters of DMSO solution containing 16.3 mg ebselen (5.94×10^{-5} mol) and 40.3 mg 18-crown-6 ether (1.52×10^{-4} mol) were prepared. Approximately 50 mg of KO_2 was gradually added to this DMSO solution at room temperature, then the solution was stored at room temperature for 1 d, The reaction mixture was analyzed by liquid chromatography–mass spectrometry (LC-MS), which was performed using LCQ (Thermoquest) with a negative and a positive electrospray interface. HPLC conditions were as follows: column; TSKgel ODS-80TM (4.6 mm i.d. ×150 mm), eluent; 30% (v/v) acetonitrile, flow rate; 1.0 ml/min. Two peaks were observed at 2.2 and 5.7 min on the total ion chromatogram. The product eluted at 2.2 was the selenonate form which was the same product observed in MeOH [*m/z*: (pos.) 364 $(M+K+2H)^+$, (neg.) 324 $(M-H)^-$], and the product eluted at 5.7 min was the selenate form [*m/z*: (pos.) 310 $(M+H)^+$, (neg.) 308 $(M-H)^-$].

Rapid Mix Continuous-Flow ESR Spectroscopy on the Reaction of Ebselen with Superoxide KO_2 (10 mM) and 18-crown-6 ether (20 mM) were dissolved in toluene–DMSO (3 : 2 v/v). This solution was introduced into the ESR cavity through a twin-type flow cell. The ebselen solution (100 mM/100 ml toluene–DMSO (3 : 2 v/v) was also introduced into the ESR cavity from a second inlet (Fig. 2). ESR spectra were recorded following sufficient mixing. The following instrument parameters were employed: modulation amplitude, 0.125 mT; microwave power, 10.0 mW; scan time, 2 min.

Results and Discussion

Superoxide Scavenging Activity of Ebselen Estimated by ESR Spin-Trapping We employed DMPO as a spin-trapping agent of $O_2^{\cdot-}$ generated by KO_2– 18-crown-6 ether in toluene–DMSO. Figure 3 shows a typical ESR spectrum of the DMPO–$O_2^{\cdot-}$ adduct using this system. The obtained hyperfine coupling constants for a_N and a_H^β were 1.43 and 1.15 mT, respectively. The splitting due to the γ-hydrogen which is observed in an aqueous solution system[5] was not observed here using the toluene–DMSO system. However, a considerable amount of DMPO–$\cdot CH_3$ was detected as indicated in Fig. 3.

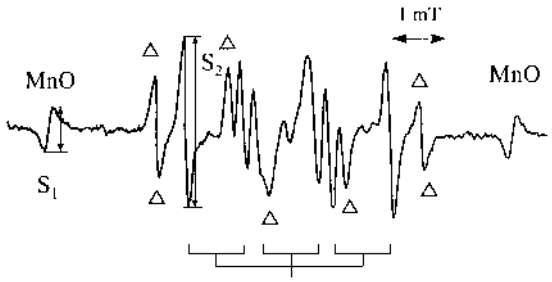

Fig. 3. ESR Spectra of DMPO–$O_2^{\cdot-}$ Formed in KO_2–18-crown-6 System
DMPO–$\cdot CH_3$ (△) was found in the spectra.

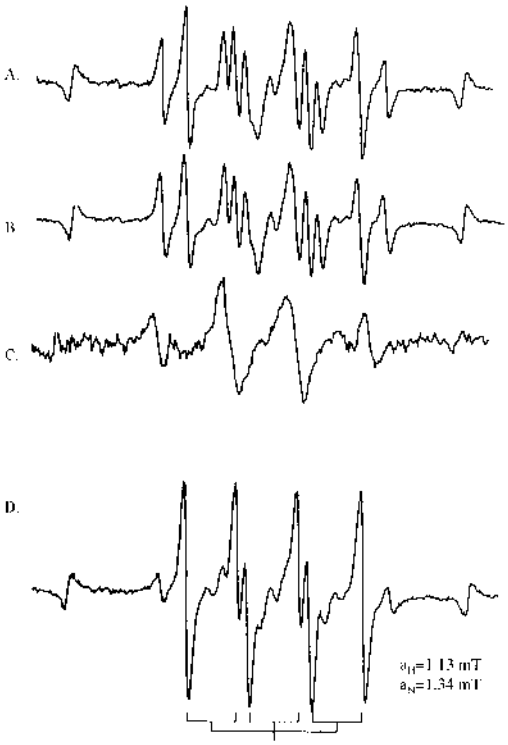

Fig. 4. ESR Spectra of DMPO–$O_2^{\cdot-}$ in Toluene–DMSO in the Presence of Various Concentrations of Ebselen
A: 100 μM, B: 1 mM, C: 10 mM, D: 100 mM.

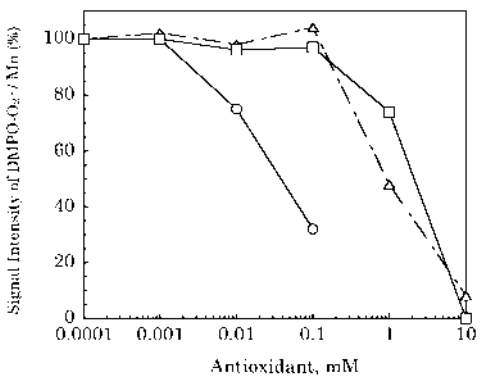

Fig. 5. Inhibitory Effect of Antioxidants on the Formation of DMPO–$O_2^{\cdot-}$

When ebselen was added to the system, the signal intensity of DMPO–$O_2^{\cdot-}$ decreased in proportion to the concen-

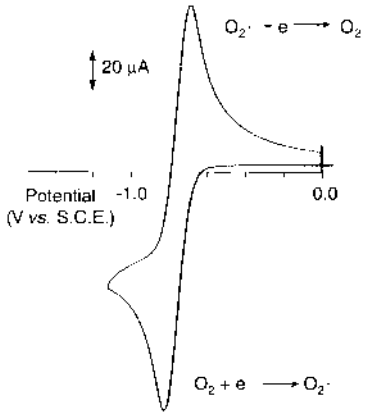

Fig. 6. Cyclic Voltammograms of $O_2/O_2 \cdot^-$ in Acetonitrile

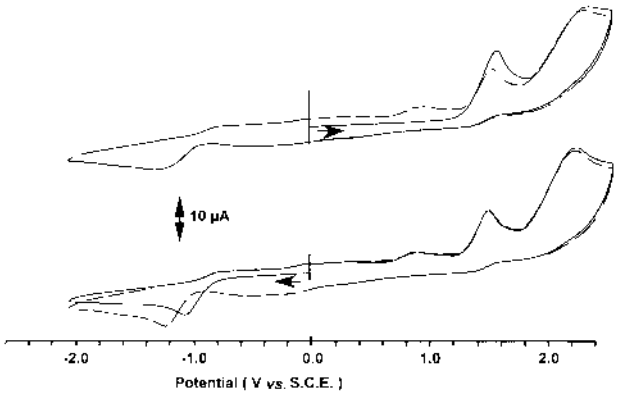

Fig. 7. Cyclic Voltammograms of Ebselen in Acetonitrile

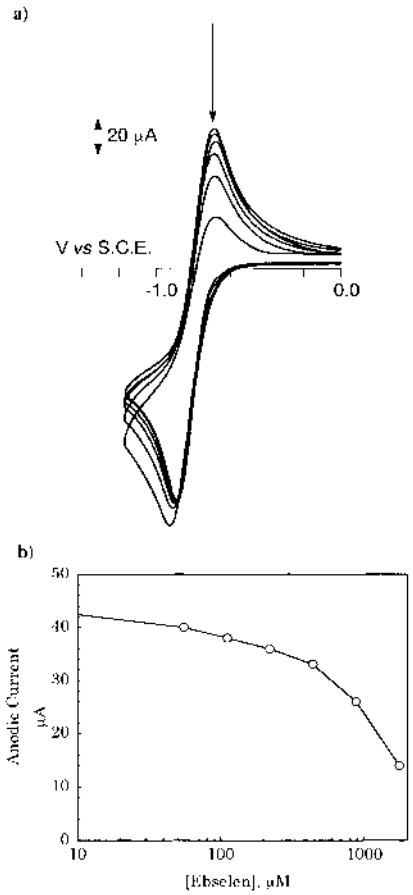

Fig. 8. Cyclic Voltammograms of $O_2/O_2 \cdot^-$ in Acetonitrile in the Presence of Increasing Concentrations of Ebselen (a) and the Decrease of Anodic Current (Oxygen Regeneration) by Adding Various Concentrations of Ebselen to Acetonitrile (b)

tration of added ebselen (Fig. 4), suggesting that ebselen scavenged $O_2 \cdot^-$. The scavenging ability of ebselen was compared with the other antioxidants, L-ascorbic acid and DL-α-tocopherol (Fig. 5). Relative $O_2 \cdot^-$ scavenging activity for these compounds was in the following order: DL-α-tocopherol>ebselen≅L-ascorbic acid. The scavenging activity of ebselen was almost equal to L-ascorbic acid.

At the highest dose of ebselen examined (100 mM), a signal due to an unknown radical adduct appeared in the ESR spectrum (a_N=1.34 mT, a_H^β=1.13 mT) (Fig. 4, trace D). The structure of this unknown radical adduct is discussed below.

Superoxide Scavenging Activity of Ebselen Estimated by Cyclic Voltammetry The reactivity of ebselen with $O_2 \cdot^-$ was studied by cyclic voltammetry in order to determine the scavenging mechanism. The cyclic voltammogram for oxygen in dried acetonitrile (Fig. 6) shows that the one-electron reduction of oxygen in a cathodic scan ($O_2 \cdot^-$ formation) and the oxidation of the superoxide anion radical in a cathodic scan (oxygen regeneration) are reversible as shown in reaction 1.

$$O_2 + e \rightleftharpoons O_2 \cdot^- \quad (1)$$

The reproducibility of the cyclic voltammogram for $O_2/O_2 \cdot^-$ is high since $O_2 \cdot^-$ is stable in an aprotic solvent.[7]

The cyclic voltammograms of ebselen alone were obtained in deoxygenated acetonitrile containing 0.1 M TEAP and both anodic and cathodic scan are shown in Fig. 7. No cathodic peak was observed from 0 to −1.0 V *vs*. S.C.E., but an irreversible peak at *ca*. −1.2 V *vs*. S.C.E. was seen.

Figure 8a shows the cyclic voltammograms of various concentrations of ebselen in oxygenated acetonitrile containing 0.1 M TEAP. With the addition of ebselen, the oxidation peak current of $O_2 \cdot^-$(anodic current, oxygen regeneration) decreases (Fig. 8b), while the reduction current (cathodic current, $O_2 \cdot^-$ formation) remains unchanged. These data suggest that ebselen reacts with $O_2 \cdot^-$, that is, it scavenges $O_2 \cdot^-$ in acetonitrile, in a concentration-dependent way.

Mechanism of the Redox Reactions of Superoxide with Ebselen in Aprotic Solvents ^{1}H-NMR, IR and MS spectral analyses of the structure of the reaction product of ebselen with $O_2 \cdot^-$ determined the product was a selenonate form and a seleninate form (Fig. 9).

Ebselen is easily oxidized by hydrogen peroxide, *tert*-butyl-hydroperoxide and *m*-chloroperbenzoic acid,[8] and the main oxidation product is a selenoxide of ebselen (Fig. 10).[7] Further, the selenoxide of ebselen was irreversibly produced by the electrochemical oxidation of ebselen in acetonitrile at +1.35 V *vs*. S.C.E.

On the other hand, as shown in Fig. 4, trace D, in the spin-trapping ESR spectrum of $O_2 \cdot^-$ with DMPO in the presence of 100 mM ebselen, the unknown-sextet signal appeared. It has been reported that DMPO can be oxidized to produce DMPOX, 5,5-dimethyl-2-oxo-1-pyrrolidinyloxyl,[9] however, the observed unknown sextet signal in this ESR spectrum is

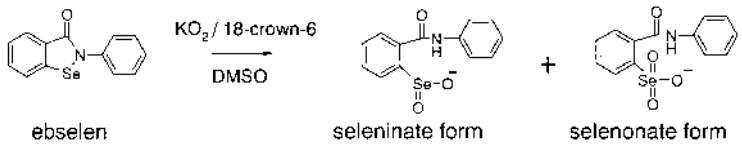

Fig. 9. The Reaction of Ebselen with $O_2 \cdot^-$

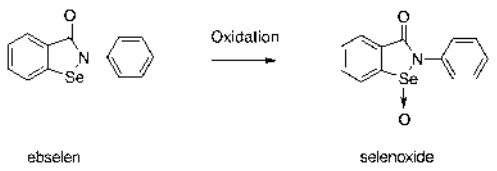

Fig. 10. Anodic Oxidation of Ebselen

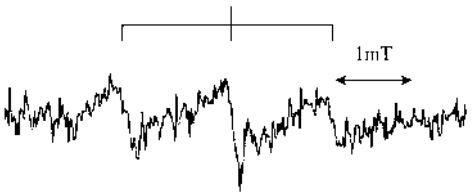

Fig. 11. ESR Spectrum of an Intermediate in the Reaction of Ebselen with $O_2 \cdot^-$ by Means of Rapid Mix Continuous Flow Method

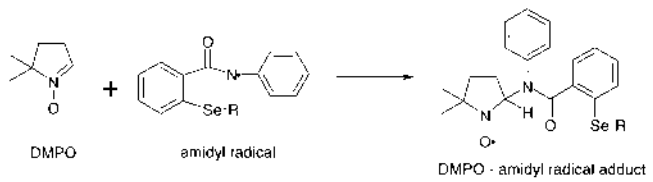

Fig. 12. The Reaction of Amidyl Radical with DMPO

clearly different from that of DMPOX. The color of the solution is brilliant pink immediately after mixing the KO_2 and ebselen solutions in toluene–DMSO and the color disappears within a few seconds. No signal was observed in the ESR spectrum of this reaction mixture using normal measurement conditions. This phenomenon suggested that an intermediate, short-lived, active species was generated during the reaction of ebselen with $O_2 \cdot^-$. As a result, we attempted to directly detect this intermediate free radical by employing rapid mix, continuous-flow ESR spectroscopy for the KO_2–ebselen reaction. Figure 11 shows the ESR spectrum obtained with the continuous-flow method. A triplet signal was observed. As the final structure of the oxidative reaction product for ebselen with $O_2 \cdot^-$ was the selenonate form, we concluded that this signal was due to an amidyl radical. A new signal observed in the spin-trapping ESR spectrum of $O_2 \cdot^-$ with DMPO (Fig. 4D) might be the DMPO–amidyl radical adduct (Fig. 12). Hence, we propose the following mechanism of reactions of ebselen with $O_2 \cdot^-$ (Chart 1): Firstly, ebselen reacts with superoxide and is converted to ebselen anion radical; secondly, the ebselen anion radical reacts with superoxide and is converted to the amidyl radical. Hydrogen abstraction by amidyl radical occurs and gives the seleninate form and the selenonate form. The amidyl radical may abstract hydrogen radical from a trace of water in DMSO. Therefore, we propose that this reaction proceeds *via* an amidyl radical and may generate intermediates in the form of peroxyselenenate,

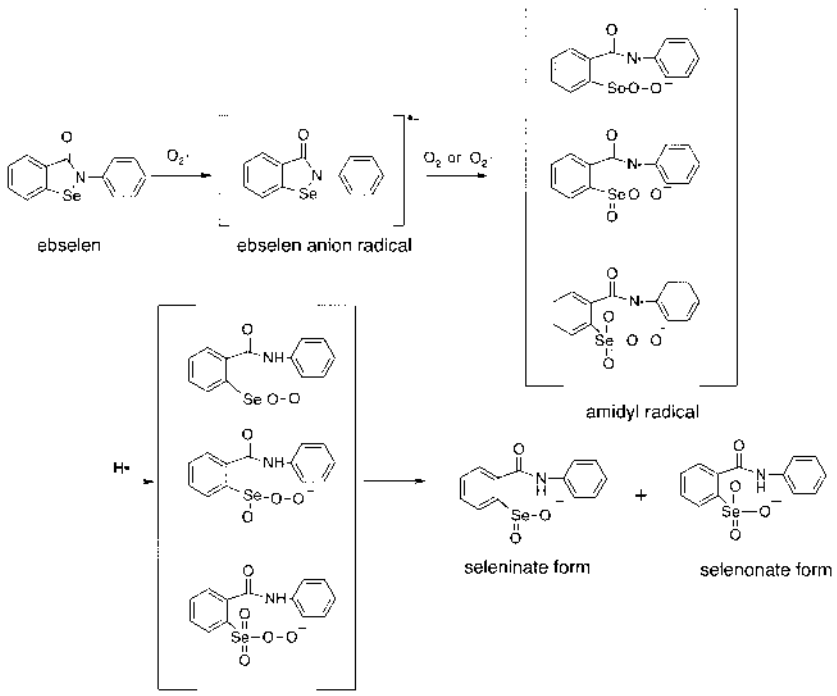

Chart 1. Proposed Reaction Mechanism of $O_2 \cdot^-$ with Ebselen in Aprotic Media

peroxyseleninate and peroxyselenonate. This proposed reaction sequence is consistent with reactions of a variety of sulfur organic compounds (homologous series of selenium compounds) with $O_2\cdot^-$,[10] in which peroxysulfenate, peroxysulfinate and peroxysulfonate are formed.

In conclusion, one of the antioxidative properties of ebselen in an aprotic system similar to the lipophilic domain of the liposomal membrane is explained by the formation of an amidyl radical which was formed by the attack of free radicals. The ability of ebselen to participate in redox reactions such as these probably plays a role in its pharmacological benefit.

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