Free Radical Research, April 2011; 45(4): 461–468

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Effect of functional groups on antioxidant properties of substituted selenoethers

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(Received date: 27 September 2010; Accepted date: 23 November 2010)

Abstract

Selenoethers attached to functional groups through propyl chain viz., bis(3-carboxypropyl)selenide (SeBA), bis(3-hydroxypropyl) selenide (SePOH) and bis(3-aminopropyl)selenide dihydrochloride (SePAm), have been examined for their ability to inhibit peroxyl radical mediated DNA damage, peroxyl radical scavenging ability and glutathione peroxidase (GPx) like activity. The DNA damage was monitored by gel electrophoresis, bimolecular rate constants for scavenging of model peroxyl radical were determined by pulse radiolysis and the GPx activity was followed by their ability to reduce hydrogen peroxide in the presence of glutathione utilizing NADPH decay and HPLC analysis. Among these compounds, SeBA showed maximum DNA protecting activity and it was also the most efficient in scavenging peroxyl radicals with the highest GPx mimicking activity. Quantum chemical calculations confirmed that SeBA with the highest energy level of HOMO (highest occupied molecular orbital) is the easiest to undergo oxidation and therefore exhibits better radical scavenging, GPx mimicking and DNA protecting activity than SePOH or SePAm.

Keywords: *Selenium, antioxidant, peroxyl radicals, GPx, HOMO level*

Introduction

Organoselenium compounds are important in biology as micronutrients, enzyme mimics, enzyme inhibitors, anti-tumour, anti-infective agents, cytokine inducers and immunomodulators [1–5]. Ebselen, an aromatic selenide, is one of the most extensively studied organoselenium compounds, which not only showed excellent glutathione peroxidase (GPx) mimicking activity but also exhibited a wide range of important pharmacological properties. However, its clinical applications are thwarted due to its insolubility in water [6,7]. This prompted synthetic chemists to develop new organoselenium compounds, which would mimic GPx-like activity. Accordingly, different types of selenium compounds such as those derived from ebselen and selenocystine, aromatic and aliphatic selenoethers and diselenides, having specific non-bonding interactions, have been synthesized and examined for GPx like antioxidant activity [8–15]. Recently our group has been designing and developing low molecular weight water-soluble selenium compounds as antioxidants and radioprotectors [16–19]. Preliminary screening of several aliphatic selenium compounds indicated that alkyl substituted selenoethers attached to the functional groups such as amine hydrochloride (–$NH_3^+Cl^-$), carboxylic acid (–COOH) and alcohol (–OH) at γ-position, through propyl chain, showed much better GPx activity as compared to similar compounds with smaller or larger alkyl chains. Therefore, selenoethers derived from the propyl group substituted with these functional groups at terminal position (Scheme 1) were studied in detail for free radical scavenging ability, GPx like activity and the ability to protect plasmid DNA from peroxyl radical-induced double strand breaks formation. To understand their differential activity, quantum chemical calculations were carried out. Finally, the antioxidant activity could be correlated with the energy level of the highest occupied molecular orbital (HOMO).

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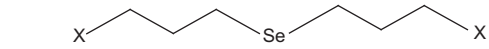
ISSN 1071-5762 print/ISSN 1029-2470 online © 2011 Informa UK, Ltd.
DOI: 10.3109/10715762.2010.543678



where X = -COOH,bis(3-carboxypropyl)selenide, SeBA
= -OH, bis(3-hydroxypropyl)selenide, SePOH
= $-NH_3^+Cl^-$, bis(3-aminopropyl)selenide dihydrochloride, SePAm

Scheme 1. Chemical structures of selenoethers.

Materials and methods

Glutathione (GSH), hydrogen peroxide (H_2O_2), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^{2-}$) from (Sigma/Aldrich, Steinheim, Germany) have been procured from the local agents. The concentration of aqueous H_2O_2 (30%) was estimated by iodometric titration. Solutions were prepared in nanopure water. The ^1H, $^{13}C\{^1H\}$ and $^{77}Se\{^1H\}$ NMR spectra were recorded on a Bruker Avance-II spectrometer operating at 300, 75.47 and 57.24 MHz, respectively. Chemical shifts are relative to $CHCl_3$ for ^1H (δ = 7.26 ppm) and ^{13}C (δ = 77.0 ppm) peaks and external diphenyl diselenide in C_6D_6 for ^{77}Se (δ = 463 ppm). Selenoetheres were synthesized according to literature methods [20,21] and were characterized by NMR spectroscopy. The supporting figure (Figure S1; online version only) shows the chromatograms of the compounds.

Characterization of SeBA

White solid, mp 88–91°C, IR (cm^{-1}): 1697 (CO), 646 (CH). ^1H NMR (D_2O) δ: 2.37 (t, J = 7.4 Hz, 2 H, Se $CH_2CH_2CH_2$); 2.01 (t, J = 5.1 Hz, 2 H, $SeCH_2$), 1.66 (m, J = 7.5 Hz, 2H, $SeCH_2CH_2$). ^{13}C NMR (D_2O) δ: 182.2 (O=*C*−), 37.0 ($SeCH_2CH_2CH_2$), 26.2 ($SeCH_2CH_2$), 22.5 ($SeCH_2$, $J_{C\text{-}Se}$ = 59 Hz); ^{77}Se NMR (D_2O) δ: 139.6 ppm.

Characterization of SePOH

Pale yellow liquid, IR (cm^{-1}): 3397 (OH), 673(CH). ^1H NMR (D_2O) δ: 3.54 (t, J = 6.8 Hz, 2H, $SeCH_2CH_2CH_2$), 2.53 (t, J = 7.2 Hz, 2H, $SeCH_2$), 1.78 (m, J = 6.9 Hz, 2H, $SeCH_2CH_2$). ^{13}C NMR (D_2O) δ: 61.2 ($SeCH_2CH_2CH_2$), 32.1 ($SeCH_2CH_2$), 19.6 ($SeCH_2$, $J_{C\text{-}Se}$ = 58 Hz); ^{77}Se NMR (D_2O) δ: 138.5 ppm.

Characterization of SePAm

Pale yellow solid, mp: 186–189°C IR (cm^{-1}): 3421 (NH_3^+), 631(CH). ^1H NMR (D_2O) δ: 3.00 (t, J = 6.6 Hz 2H, $SeCH_2CH_2CH_2$), 2.58 (t, J = 7.2 Hz, 2H, $SeCH_2$), 1.93 (m, J = 6.6 Hz, 2H, $SeCH_2CH_2$). ^{13}C NMR (D_2O) δ: 39.2 ($SeCH_2CH_2CH_2$), 27.3 ($SeCH_2CH_2$), 19.2 ($SeCH_2$, $J_{C\text{-}Se}$ = 60 Hz); ^{77}Se NMR (D_2O) δ: 146.3 ppm.

DNA nicking assay

DNA nicking assay [22,23] was carried out in 0.5 ml polypropylene tubes in a final reaction volume of 20 µl, containing pBR322 DNA (200 ng), 5 mM AAPH and a selenoether (0.1–2.0 mM) in 10 mM phosphate buffer at pH 7.4. The reaction was initiated by adding AAPH and incubated at 37°C for 30 min and 5 µl of loading buffer (0.25% bromophenol blue, 10% glycerol) was added. The treated DNA samples were loaded on agarose gel (1.2%) containing 1.3 µM ethidium bromide. The gel was run for ~ 3 h (constant voltage at 50 V) submerging in 1X TAE buffer. Photographs were taken on a Syngene Gel documentation system and from this the DNA band intensity was determined.

Peroxyl radical scavenging assay

Reaction of model peroxyl radicals, trichloromethyl peroxyl radical ($CCl_3O_2^{\bullet}$) with selenoethers was carried out by pulse radiolysis using high-energy electron pulses (7 MeV, 500 ns) generated from a linear electron accelerator [24]. Aerated aqueous solution of 0.01 M KSCN was used for determining the dose delivered per pulse, which is close to 10–12 Gy [25]. The bimolecular rate constant for the reaction of the compound with this radical was estimated by employing competition kinetics using $ABTS^{2-}$ as a reference solute [26]. The $CCl_3O_2^{\bullet}$ radicals were generated by radiolysis of aerated aqueous solution containing 48% isopropanol and 4% carbon tetrachloride [17,26].

GPx like catalytic activity

This was performed by two methods, viz. (i) Glutathione disulphide (GSSG) reductase assay [27] and (ii) HPLC method [28].

i) In the GSSG-reductase assay, the test mixture contained NADPH, GSH and glutathione reductase in 0.1 M phosphate buffer (pH 7.4). Selenoether was added to the mixture and the reaction was initiated by the addition of H_2O_2. The concentrations of NADPH, GSH, glutathione reductase, selenoether and H_2O_2 were 0.3 mM, 4 mM, 5.0 mU/mL, 0.1 mM and 1 mM, respectively. The initial rate (v_o) of NADPH oxidation was determined by monitoring the decrease in absorbance due to NADPH at 340 nm over a period of 300–600 s.

ii) In the HPLC method, the GPx like activity was monitored by estimating the ratio of the concentrations of oxidized and reduced glutathione (GSH and GSSG). In this method, reaction was initiated by adding H_2O_2 (320 µM) to a mixture of GSH (160 µM) and

16 μM selenoether and leaving it for 5 min. GSH and GSSG were separated by HPLC on a reverse phase C18 column using a mobile phase of aqueous solution containing 10% methanol in 0.1% trifluoroacetic acid (TFA) and detected at 215 nm. Forty microlitres of the reaction mixture was injected into the column at regular intervals (45 min to 300 min). HPLC analysis was performed on a JASCO PU-280 plus unit, equipped with a binary gradient pump system and a variable-wavelength detector. Separate calibration plots for GSH and GSSG were made by plotting percentage peak area against the concentration.

Quantum chemical calculation

Quantum chemical calculations were performed by using a Gaussian 03 software package (revision B.04) [29]. *Ab initio* calculations were performed by Hartree Fock theory with a 6-31G(d) basis set for both geometry optimizations and molecular energy calculations. The energies were not corrected with zero-point energies. Effects of solvent on these structures were followed by using a conductor-like solvation model (CPCM) [30,31], which is a modified form of the polarizable continuum model (PCM). In PCM, the solvent is modelled as a continuous static medium characterized by a dielectric constant (ε), which is modified as a scaled conductor boundary in CPCM.

Results

Protection against AAPH-induced damage of pBR322 DNA

Aqueous solutions of AAPH on thermal degradation decompose to generate alkyl radical (R'$^{\bullet}$), which in the presence of oxygen is converted into the corresponding peroxyl radicals (R'OO$^{\bullet}$). The half-life of AAPH at 37°C in neutral water is ~ 175 h. The radicals are generated at a rate of 1.3×10^{-6} mol/s [32]. These peroxyl radicals induce oxidative damage to DNA by oxidation of sugar-phosphate diester bond and nitrogenous bases. Consequently, DNA undergoes quick damage and loses its integrity, leading to the formation of double strand breaks [33]. In the presence of antioxidants that can scavenge peroxyl radicals, the oxidative damage to DNA may be inhibited. During this process, the antioxidant would convert the peroxyl radical to non-reactive species [34]. In the present study, the extent of damage to DNA was monitored by agarose gel electrophoresis [22,23]. As seen in Figure 1, pBR322 DNA on incubation with AAPH induced extensive double strand breaks. This would result in the increase in the intensity of the open circular (OC) form with subsequent decrease in the intensity of super coiled (SC) form of plasmid

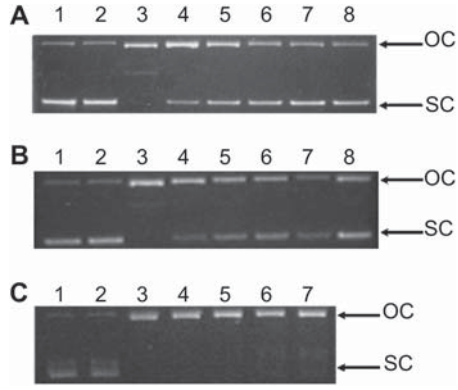


Figure 1. Protection against AAPH-induced DNA damage. (A) SeBA shows a concentration-dependent inhibition in DSB formation when compared with AAPH (5 mM) treated DNA till 1 mM. Control DNA (lane 1), DNA + 1 mM SeBA (lane 2), DNA + 5 mM AAPH (lane 3), DNA + 5 mM AAPH + 0.1 mM SeBA (lane 4), DNA + 5 mM AAPH + 0.25 mM SeBA (lane 5), DNA + 5 mM AAPH + 0.5 mM SeBA (lane 6), DNA + 5 mM AAPH + 0.75 mM SeBA (lane 7) and DNA + 5 mM AAPH + 1.0 mM SeBA (lane 8). (B) SePOH also showed a similar trend like SeBA, but the degree of inhibition was lesser compared to SeBA (lanes were similar like SeBA). (C) SePAm did not show any protection at all till a concentration up to 2 mM. Control DNA (lane 1), DNA + 2 mM SePAm (lane 2), DNA + 5 mM AAPH (lane 3), DNA + 5 mM AAPH + 0.25 mM SePAm (lane 4), DNA + 5 mM AAPH + 0.5 mM SePAm (lane 5), DNA + 5 mM AAPH + 1.0 mM SePAm (lane 6), DNA + 5 mM AAPH + 2.0 mM SePAm (lane 7).

DNA. On incubating the plasmid DNA with AAPH in the presence of selenoethers, reduction in the double strand break formation was noticed. As the concentration of selenoether is increased, a gradual decrease in the OC form is observed in a concentration-dependent manner. The relative DNA protecting ability of the compounds was compared at a fixed concentration. Thus, in the presence of 1.0 mM of selenoether, the percentage DNA protecting ability for SeBA, SePOH and SePAm was found to be 66.5, 59.0 and 14.6, respectively. The IC_{50} value, that is the concentration of the selenoethers capable of inhibiting 50% double strand break formation in plasmid DNA, was estimated by following the protection at different concentrations of selenothers (0.1–2.0 mM). It is evident from Table I that, among all the three selenoethers, SeBA showed highest protection to the plasmid DNA from the peroxyl radical-induced oxidative damage.

Peroxyl radical scavenging ability of selenoethers

The above results on protection of DNA from the oxidative damage could be due to the peroxyl radical scavenging ability of the selenoethers and the differential protection exhibited by them may be due to their difference in reactivity. This can be compared by estimation of rate constants for the scavenging of a peroxyl radical. $CCl_3O_2^{\bullet}$ is a model peroxyl radical that is conveniently employed for studying the reactions of antioxidants using a pulse radiolysis technique [16,17,26].

Table I. Antioxidant activity and HOMO values of selenoethers.

Compound	(v_0) (μmol/min)	t_{50}(min)	k_{CCl3O2}(M^{-1} s^{-1})	IC_{50} (mM)	HOMO(eV)
SeBA	0.0372	60	4.2×10^8	0.4	−8.426
SePOH	0.0246	90	3.5×10^8	0.5	−8.644
SePAm	0.015	115	1.0×10^8	No inhibition, even at 1 mM	−9.086

v_0 = initial velocity for the decay of NADPH absorption at 340 nm in presence of selenoether; t_{50} = time required for the conversion of 50% GSH to GSSG in presence of selenoether and H_2O_2; k_{CCl3O2} = bimolecular rate constant for the reaction of $CCl_3O_2^{\bullet}$ radicals with selenoethers; IC_{50} = concentration of selenoether required to inhibit AAPH-induced DNA damage by 50%. HOMO is the calculated energy level of the highest occupied molecular orbital in water.

Therefore, the bimolecular rate constant for the reaction of selenoethers with $CCl_3O_2^{\bullet}$ was determined using a competition kinetics method employing $ABTS^{2-}$ as a reference solute. The competing reactions under such conditions are given in equations (1) and (2).

$$CCl_3O_2^{\bullet} + ABTS^{2-} \xrightarrow{k_1} ABTS^{\bullet -} \quad (1)$$

$$CCl_3O_2^{\bullet} + RSeR \xrightarrow{k_2} Products \quad (2)$$

The above reactions can be related by equation (3)

$$\frac{A_0}{A} - 1 = \frac{k_2}{k_1} X \frac{[RSeR]}{[ABTS^{2-}]} \quad (3)$$

Here A_0 and A are the absorbance at 645 nm due to $ABTS^{\bullet -}$ in the absence and presence of different concentrations of selenoether, respectively. The bimolecular rate constant (k_2) for the reaction between the selenoether and $CCl_3O_2^{\bullet}$ was estimated by using the bimolecular rate constant for the reaction between $CCl_3O_2^{\bullet}$ and $ABTS^{2-}$ (k_1) as 1.2×10^9 M^{-1} s^{-1} [35].

Thus, the absorbance at 645 nm decreased with increasing concentration of selenoether. Figure 2 shows linear plots for the variation of A_0/A as a function of concentration of the selenoether in the presence of 500 μM $ABTS^{2-}$. According to equation (3), the values of k_2 for SeBA, SePOH and SePAm were estimated (Table I). These rate constants indicate that all the three selenoethers have the ability to scavenge the peroxyl radicals, with SeBA being the most active.

GPx-like activity

An important aspect of organoselenium compounds, which is not seen in other antioxidants, is their ability to show GPx-like activity. Therefore, the observed protection to the plasmid DNA by the selenoethers could also be due to their ability to act as GPx mimics. To compare GPx-like catalytic activity of these selenoethers, we utilised two methods, viz., GSSG-reductase coupled assay [27] and HPLC assay [28]. In the absence of selenium catalyst (Figure 3A), NADPH was consumed completely in 650 s, but in the presence of 0.1 mM selenoether, its rate increased. Figures 3B–D show change in the rate of loss of NADPH absorbance at 340 nm in the absence and presence of the three selenoethers. The estimated v_o (Table I), which is a measure of the catalytic activity, was in the order SeBA > SePOH > SePAm.

HPLC method by estimating the ratio of concentrations of a thiol and dithiol is an alternate approach,

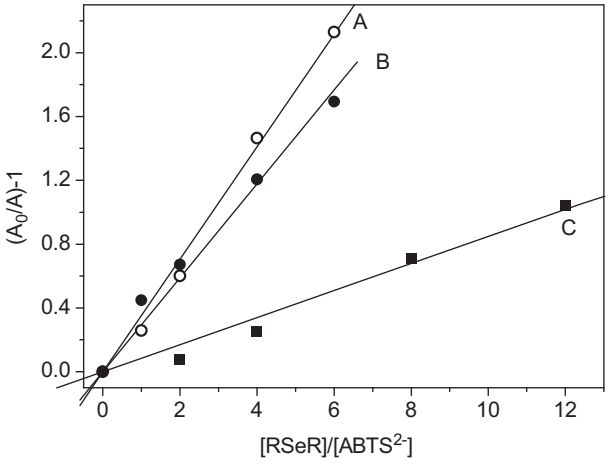


Figure 2. The absorbance changes at 645 nm due to $ABTS^{\bullet -}$ in the absence and presence of selenoethers at pH 7, fitted to equation (3). SeBA (A), SePOH (B) and SePAm (C).

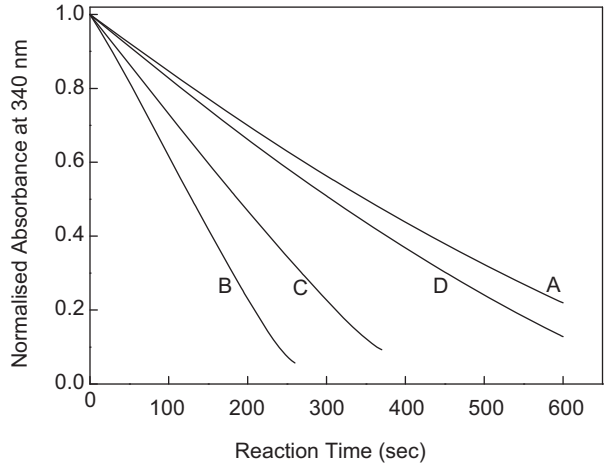


Figure 3. Plots showing the variation in the absorbance of NADPH at 340 nm as a function of the reaction time in the absence (A) and in presence of 0.1 mM of SeBA (B), SePOH (C) and SePAm (D).

adopted by many research groups [28]. However, most of them employed thiols that are not biologically relevant. Therefore, in the present study, we followed the conversion of GSH to GSSG in the presence of hydrogen peroxide (equation 4).

$$2GSH + H_2O_2 \xrightarrow{\text{selenoethers}} GSSG + 2H_2O \quad (4)$$

Both GSH and GSSG can be separated by HPLC and detected by absorption method. At a fixed concentration of one of the selenoethers, the ratio of GSH and GSSG was monitored as shown in Figure 4. From this, t_{50} value, i.e. the time required to convert 50% GSH to GSSG, was evaluated. The results indicated that the GPx activity of the compounds is in the same order as observed by NADPH-reductase assay, i.e. SeBA > SePOH > SePAm. The results thus confirmed that among the selenoethers, SeBA showed the highest GPx-like catalytic activity. The supporting figure (Figure S2; online version only) shows the HPLC chromatograms indicating change in GSH and GSSG in the presence of SeBA as a function of time from 5–230 min.

Quantum chemical calculations

The geometry of the selenoethers was optimized by the *ab initio* calculations. The stable structures of SeBA, SePOH and SePAm were sought in vacuum by exhaustive conformer search, in which all possible dihedral angles were systematically changed and the geometry of each structure was optimized. However, as these compounds bear ionizable functional groups, a similar exhaustive conformer search for the ionic form was employed in the *ab initio* calculations at HF/6-31G(d) in water with the CPCM model. The global

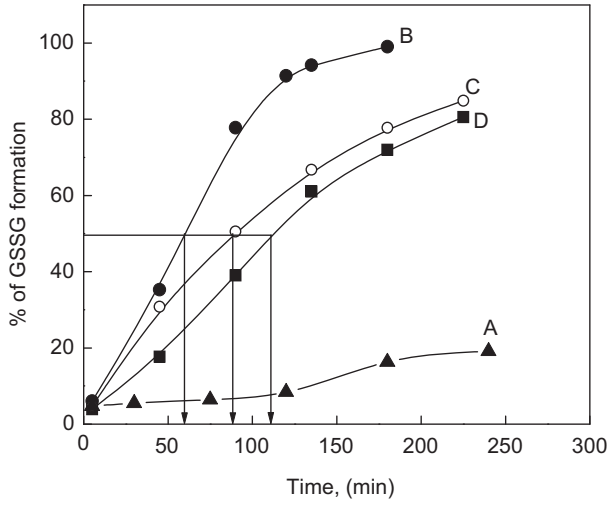


Figure 4. Plot showing the percentage of disulphide formed as a function of time on incubating 160 μM H_2O_2, 320 μM GSH in the absence (A) and presence of 16 μM of (B) SeBA, (C) SePOH and (D) SePAm.

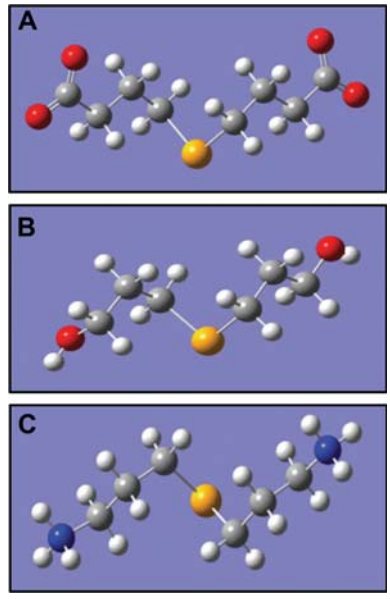


Figure 5. Global energy minimum structures of (A) SeBA, (B) SePOH and (C) SePAm obtained in water at HF/CPCM/6-31G(d).

energy minimum structures of SeBA, SePOH and SePAm obtained in water are shown in Figure 5. The HOMO energy levels for the compounds, as listed in Table I, are in the order SeBA > SePOH > SePAm.

Discussion

With an aim to evaluate water-soluble organoselenium compounds as antioxidants, we have earlier synthesized a number of selenoethers, containing different functional groups, like −COOH, $-NH_3^+$ and −OH, in the alkyl chains of varying length. Our preliminary studies indicated that, among the selenoethers, those attached to the functional group through the propyl chain showed the highest GPx-like activity. Activities of such selenium compounds were previously studied extensively in organic solvent by using a benzyl thiol as a reducing substrate, and it was demonstrated that the cyclized selenurane structures play roles in the high activities [36–38]. This prompted us to test selenoethers, SeBA, SePOH and SePAm, having functional groups −COOH, −OH and $-NH_3^+$, respectively, at the terminal position of propyl group, for *in vitro* antioxidant activity.

The selenium compounds were first evaluated for their ability to protect, cellular DNA against peroxyl radical-induced strand break formation. The results indicated that in the range of concentrations employed (< 2 mM), only the −COOH and −OH functionalized selenoethers showed protection to DNA, but not the $-NH_3^+$ functionalized ether. An attempt was made to understand the factors contributing to such differential activity.

This antioxidant activity could be either due to their ability to scavenge peroxyl radicals or to reduce the

hydroperoxides. The pulse radiolysis studies on the reaction of these compounds with model peroxyl radicals $CCl_3O_2^{\bullet}$ indicated that all the compounds have the ability to scavenge peroxyl radicals (rate constants ~ 10^8 M^{-1} s^{-1}) and they were in the order SeBA > SePOH > SePAm. SeBA exhibited maximum rate constant.

To evaluate their ability to reduce hydroperoxides, we studied GPx-like catalytic activity by NADPH-reductase assay and by monitoring the oxidative conversion of GSH to GSSG in the presence of H_2O_2. The catalytic ability of SeBA is nearly 2-times more than that of SePAm, while that of SePOH falls between the two.

Electron transfer generally mediates the above reactions. One of the factors controlling such an electron transfer process is the energy of the frontier molecular orbital, e.g. HOMO [15,39] of the donor molecule. The quantum chemical calculations for these compounds in water indicated that the HOMO energy levels of the three compounds are in the order SeBA > SePOH > SePAm. This suggests that SeBA having the highest HOMO level is the easiest to undergo oxidation. Accordingly, it shows highest free radical scavenging ability, leading to efficient antioxidant capacity.

The above results can be fitted to a structure–activity correlation by using Taft's sigma constant of substituent (σ^*), which is related to the electronic effect of the substituent in an aliphatic system [40]. In a series of compounds, the effect of polar substituent (−X) on the rate constant for any reaction with respect to no substituent (-H) can be studied by using equation (5)

$$\log k(-X) = \log k(-H) + \rho\sigma^* \quad (5)$$

For this correlation, we followed the rate constants $k(-X)$ for $CCl_3O_2^{\bullet}$ radical as a function of σ^* (−1.06 for COO^-, 1.34 for OH and 3.76 for NH_3^+). The plot for variation of [log $k(-X)$] as a function of σ^* showed a linear dependence with slope (−0.13) equating to substituent reaction constant (ρ), which is also a measure of the total polar effect exerted by a substituent X (relative to no substituent) on the reaction centre. The negative value of ρ indicates that the transition state of the rate-determining step is stabilized by electron-donating groups and destabilized by electron-withdrawing groups through σ bonds or space. This further supports the hypothesis that the oxidation of selenium is the most crucial in deciding the antioxidant activity of the selenoethers. However, because the plots shown in Figure 6 have large deviations from a linear correlation, it is possible that other factors can also participate in the observed trends of the activities. For example, formation of cyclized selenurane intermediates, as suggested by Back [36–38], may be plausible for SeBA and SePOH. Our attempts to characterize such intermediates, however, failed, probably due to the rapid hydrolysis in aqueous medium. Future studies will be performed with many more compounds having different functional groups so that a better evaluation of the role of electron transfer in the antioxidant activity will be made.

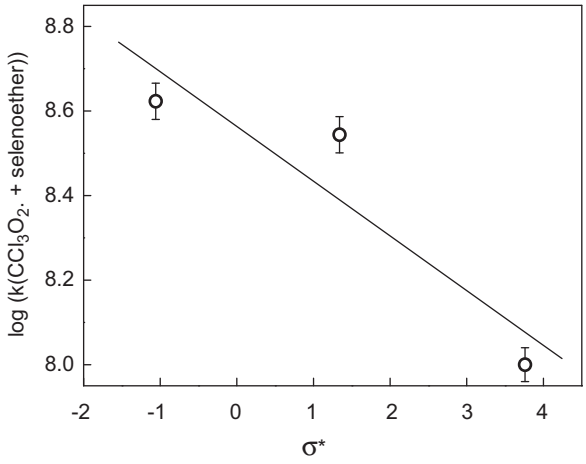


Figure 6. Linear plot showing the variation in logarithmic value of bimolecular rate constant for the reaction between $CCl_3O_2^{\bullet}$ radical and selenoethers as a function of Taft's sigma constant of the substituents (σ^*).

Conclusion

Three functionalized, water-soluble aliphatic selenoethers, SeBA, SePOH and SePAm, have been examined for antioxidant activity, free radical scavenging ability and GPx-like activity. Among the three, SeBA exhibited highest antioxidant activity, peroxyl radical reducing ability and GPx-like catalytic property, compared to its functional analogues, viz. SePOH and SePAm. The higher antioxidant activity can be ascribed to the electron donating nature of the carboxylate functional group, which elevates the HOMO energy level and makes the selenium more nucleophilic. These studies on the structure–activity correlation of the selenoethers would be useful in the future design of new water-soluble, simple organoselenium compounds with high antioxidant and GPx mimicking ability

Acknowledgements

We thank Dr T. Mukherjee, Dr S. K. Sarkar, Dr P. Phadnis and Dr D. Das for encouragement of this work.

Declaration of interest

We are grateful to BRNS for the research grant under the Prospective Research Fund (PRF) Scheme (Grant No. BRNS/2007/38/5) as well as India-Japan Science Promotion Scheme (Grant No. DST/INT/JAP/P-45/08).

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This paper was first published online on Early Online on 18 January 2011.

Supplementary material available online

Figures S1 and S2