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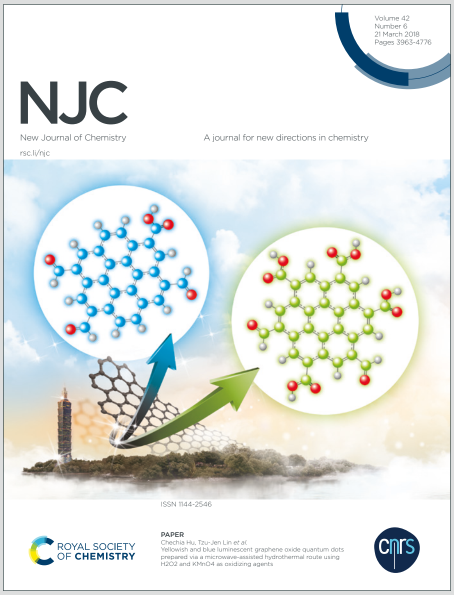

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ARTICLE

Free radical induced selenoxide formation in isomeric organoselenium compounds: Role of chemical structure on antioxidant activity

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With an aim to understand the role of structural modifications on antioxidant activty, two structurally isomeric selenium compounds, linear, bis(ethan-2-ol)selenide (SeEOH) and cyclic, DL-trans-3,4-dihydroxy-1-selenolane (DHS) were compared. The antioxidant activity, was assigned through their ability to scavenge reactive oxygen scavenging (ROS), where both compounds indicated formation of selenoxides. The ROS identified for the present study are peroxynitrite, hydroxyl ($^{\bullet}$OH) and carbonate ($CO_3^{\bullet-}$) radical. The rate constant for the scavenging of both peroxynitrite and $CO_3^{\bullet-}$ radical was higher for DHS as compared to that for SeEOH. The one-electron oxidation during these reactions indicated formation of selenium centred dimer radical cation $(>Se\therefore Se<)^+$, as the intermediate, which undergoes disproportionation reaction to form the corresponding selenoxide. Quantitative estimation of selenoxides indicated that cyclic compound DHS, produced higher yield of selenoxide than the linear compound SeEOH due to higher stability of $(>Se\therefore Se<)^+$. The selenoxide undergoes reduction by thiols, thereby regenerated back to the parent compound. The results thereby suggest that formation of selenoxide improves the antioxidant activity of organoselenium compounds and should be considered as an important step in the design of new selenium based antioxidants.

Introduction

Organoselenium compounds constitute a unique class of antioxidants due to their versatile redox properties. Such compounds exhibit antioxidant properties like free radical scavenging and glutathione peroxidase (GPx) like catalytic activity.[1,2] Studies from several synthetic molecules identified the role of important structural motifs such as aromaticity, non-bonding interactions, ring size, and nature of functional groups, on the GPx like activity.[3,4,5] Romano et al have found that the antioxidant activity of aromatic selenocynates is higher than the aliphatic selenocynates and Rafique et al have showed that 2-picolylamide-based diselenides having non-bonding interaction showed higher activity.[6,7] Engman et al, have reported that the ring size in cyclic seleno-tocopherols affects their radical trapping ability.[8] Similarly, Back et al reported that the GPx activity of a five member spirodioxaselenanonane was higher compared to those with six member ring.[9] Davies et al have reported that the rate constant for the reaction of various free radicals and molecular oxidants with biologically relevant organoselenium compounds is an important parameter.[10]

In search of water soluble and low molecular weight antioxidants/radioprotectors, our group has studied the hydroxyl ($^{\bullet}$OH) radical reaction with different classes of organoselenium compounds by pulse radiolysis technique.[11] The studies have shown that the initial attack of $^{\bullet}$OH radical takes place at selenium centre to form hydroxyselenouranyl radical adduct $(>Se\therefore OH)$ and the fate of this transient depends on the nature of functional group (hetero atom) attached to the selenium centre. Of the several compounds screened in our laboratory, trans-3,4-dihydroxyselenolane (DHS) a water-soluble organoselenium compound, has been found to reduce $^{\bullet}$OH radicals, hydroperoxides and peroxynitrite catalytically through the formation of a stable selenoxide, which is reverted back to DHS by thiols.[12] Further, this antioxidant activity was also verified under in vitro and in vivo models.[13] In contrast, its linear analogue bis(ethan-2-ol)selenide (SeEOH) showed low GPx like activity and was toxic to cells.[14] Therefore, to understand the contrasting behaviour, the two structural analogues have been investigated for their reactivity with ROS and the yields of products formed were estimated. To further compliment the observations, quantum chemical calculations have been carried out. The structures of DHS, SeEOH and their selenoxides are given in scheme 1.

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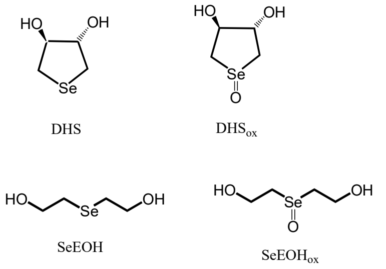

Scheme 1: Structure of 3,4-dihydroxy selenolane (DHS), bis(ethan-2-ol)selenide (SeEOH) and their corresponding selenoxide DHS$_{ox}$ and SeEOH$_{ox}$.

Results

Kinetics of reaction of DHS/SeEOH with different ROS: As reported earlier, SeEOH and DHS reacted with $^{\bullet}$OH radical with a bimolecular rate constants of (1.0 ± 0.1) x 10^{10} $M^{-1}s^{-1}$ and (9.1 ± 0.1) x 10^{9} $M^{-1}s^{-1}$, respectively.[12,14] To further understand the antioxidant activity under different conditions, rate constants for the reaction of these compounds with peroxynitrite, carbonate ($CO_3^{\bullet-}$) radical and nitrogen dioxide ($NO_2^{\bullet}$) radical, were studied. Under physiological condition peroxynitrite readily reacts with CO_2 to form nitroso-peroxocarbonate which decomposes to form $CO_3^{\bullet-}$ radical and nitrogen dioxide $NO_2^{\bullet}$ radical, which are important for peroxynitrite induced oxidative stress.[18]

The reaction between DHS/SeEOH with peroxynitrite, was studied by employing competition kinetics using Dihydrorhodamine 123 (DHR123) as reference solute.[17]

$$DHR123 + Peroxynitrite \xrightarrow{k_1} R123 \qquad (1)$$

$$DHS / SeEOH + Peroxynitrite \xrightarrow{k_2} Products \qquad (2)$$

The bimolecular rate constant (k_2) was estimated by plotting (F_0/F)-1 as a function of ((SeEOH or DHS)/DHR123) according to equation (3):

$$\frac{F_0}{F} = \frac{k_2}{k_1} X \frac{[SeEOH / DHS]}{[DHR123]} \qquad (3)$$

Where, F_0 and F are the intensities of fluorescence of R123 in absence and in presence of SeEOH/DHS respectively, k_1 (8.2 x 10^{3} $M^{-1}s^{-1}$) is the bimolecular rate constant for the reaction of DHR123 with peroxynitrite.[12d] The estimated values for k_2 were (9.2 ± 0.1) x10^{2} $M^{-1}s^{-1}$ and (2.2 ± 0.2) x10^{3} $M^{-1}s^{-1}$ for SeEOH and DHS respectively (Figure S1). The results indicate that DHS showed higher rate constant in neutralizing peroxynitrite as compared to SeEOH.

Rate constant for the reaction of DHS with $CO_3^{\bullet-}$ radical was reported to be (1.2 ± 0.2) x 10^{9} $M^{-1}s^{-1}$.[12] In the present study, SeEOH was tested for its ability to scavenge $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ radicals using pulse radiolysis technique. The kinetics between SeEOH and $CO_3^{\bullet-}$ radical was estimated by following the characteristic decay of $CO_3^{\bullet-}$ at ~600 nm ($\varepsilon_{600\ nm}$=1680 $M^{-1}cm^{-1}$) in presence of varying concentration of SeEOH. Figure S2 shows the absorption-time plot of $CO_3^{\bullet-}$ radical in absence and in presence of 10 µM SeEOH. In the absence of SeEOH, $CO_3^{\bullet-}$ radical decayed by second order kinetics with 2k/εl value of (5.7 ± 0.1) x 10^{7} s^{-1} at pH 7.4. On addition of 10 µM SeEOH, $CO_3^{\bullet-}$ radical decayed very fast and the reaction was found to follow pseudo-first order kinetics (k_{obs}). The bimolecular rate constant was estimated from the slope of the linear plot of k_{obs} at different concentration of SeEOH (10 -100 µM), which was found to be (6.5 ± 0.3) x 10^{8} $M^{-1}s^{-1}$. Both DHS, SeEOH did not showed any reaction with $NO_2^{\bullet}$ radical, which could be due to the unfavourable redox potential of these compounds.

Further to understand the mechanism of electron transfer in these compounds, the nature of the transients produced during these ROS reactions was investigated as discussed below.

Transient studies:

The reaction of SeEOH and DHS with $^{\bullet}$OH radical formed two centered three electron bonded dimer radical cation (>Se∴Se<)$^{+}$, which decayed by following second order kinetics with 2k/εl of (2.7 ± 0.2) x 10^{5} s^{-1} and (3.9 ± 0.3) x 10^{5} s^{-1} for DHS and SeEOH, respectively. Using the reported ε value for (>Se∴Se<)$^{+}$ radical of DHS, and presuming similar value for SeEOH, the 2k value for DHS and SeEOH was estimated to be (1.6 ± 0.1) x 10^{9} and (2.3 ± 0.2) x 10^{9} $M^{-1}s^{-1}$, respectively. This indicates that the (>Se∴Se<)$^{+}$ radical of DHS is more stable compared to that from SeEOH. In case of SeEOH along with the (>Se∴Se<)$^{+}$ radical, a species absorbing at 320 nm was attributed to a carbon centred radical of the type α-(hydroxyl ethyl) seleno methyl radical ($HOCH_2CH_2SeCH_2^{\bullet}$) or α-reducing radical (-SeC$^{\bullet}$). Such radicals are formed by rearrangement of (>Se∴OH) radical at α-position to the selenium centre followed by elimination of water and formaldehyde (HCHO) molecules via Barton reaction.[19] In case of DHS, no indication for α-reducing radical formation was observed.

The relative yield of the radical cations, estimated by $ABTS^{\bullet-}$ reaction was found to be 1.8 times higher for DHS than SeEOH. As reported earlier, the (>Se∴Se<)$^{+}$ radical undergoes radical-radical disproportionation to form selenoxides, as estimated below.

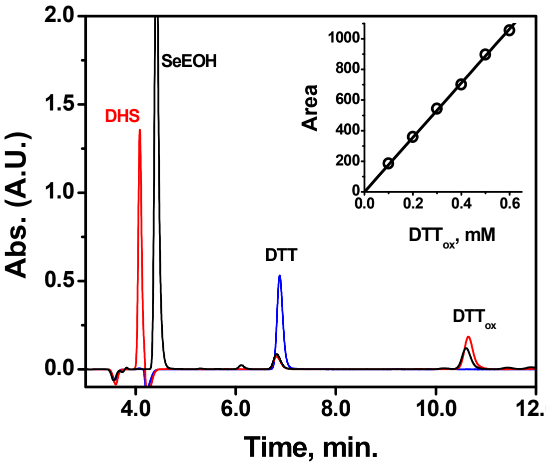

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Figure 1: HPLC chromatogram generated on treating irradiated aqueous solution (N_2O saturated) of 5 mM SeEOH/DHS with 1 mM DTT at pH 7. Inset shows the calibration curve for DHS_{ox}

Product analysis by HPLC :

Formation of selenoxide and formaldehyde products formed during the reaction of $^{\bullet}$OH radicals with SeEOH/DHS were identified and quantified by HPLC measurements.

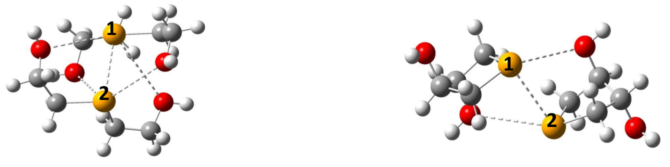
Selenoxide estimation: The HPLC chromatogram for SeEOH and DHS showed peaks at 4.2 min and 4.0 min, while their corresponding selenoxides being more polar, were fast eluted and therefore could not be detected on HPLC directly. Therefore the selenoxides formation was quantified by employing their reaction with DTT and monitoring the amount of DTT_{ox} formed thereof at 240 nm as described.[14b] From this analysis, it was found that DHS showed higher yield of selenoxide (0.45 ± 0.04) µmol/J) than SeEOH (0.23 ± 0.02) µmol/J) (Figure 1). The relative yield of selenoxides is in the same order as estimated yield of their radical cations within experimental limits.

Formaldehyde (HCHO) estimation: Formation of HCHO was confirmed by treating with (2,4-dinitrophenyl)hydrazine (DNPH) followed by HPLC analysis as described in reference.[22] Using the calibration curves plotted for the known concentration of HCHO treated with DNPH, the amount of HCHO formed during γ-radiolysis of SeEOH in N_2O saturated condition was (0.11 ± 0.01) µmoles/J which corresponding to ~18 % of the total $^{\bullet}$OH radical yield (Figure S3). However no peak corresponding to HCHO-DNPH was observed in case of DNPH derivatized DHS samples (irr) indicating absence of any HCHO.

To explain the differential yield of selenoxides obtained during reaction with $^{\bullet}$OH radicals and their ROS scavenging activity, quantum chemical calculations were performed and the results are discussed below.

Quantum chemical calculations: For these studies, ground state geometry of DHS/SeEOH and different transients observed during electron transfer process were optimized at B3LYP/6-31+G(d,p) level in water followed by HOMO and LUMO level calculations.[23] Figure S4 represents the optimized structures and corresponding HOMO level of SeEOH and DHS. The HOMO values indicate that the cyclization in DHS structure leads to introduction of ring strain, causing increase in ground state energy and its HOMO energy levels. Due to this, it becomes easier to remove outermost electron compared to its linear (relaxed) form where HOMO level is well stabilized.

To explain the nature and stability of the resultant $(>Se\therefore Se<)^+$ radicals of SeEOH and DHS, their geometries were optimized followed by correction for BSSE (basis-set superposition error) using counter-poise method. Figure 2 represents the optimized ground state structure of $(>Se\therefore Se<)^+$ radical at B3LYP/6.31+G(d,p) level. The bond distance between two selenium atoms is less (3.035 Å) in $(>Se\therefore Se<)^+$ radical of SeEOH which enhances the inter electronic repulsion and destabilizes the bond. On the other hand in $(>Se\therefore Se<)^+$ radical of DHS, Se-Se bond distance (3.071 Å) is slightly more leading to decrease in inter electronic repulsion and stabilisation of the two centred three electron bond in $(>Se\therefore Se<)^+$ radical. Additionally, $(>Se\therefore Se<)^+$ of DHS is stabilized by two non-bonded Se···O interactions from the hydroxyl functional group, which is not seen in SeEOH. Therefore, $(>Se\therefore Se<)^+$ radical of DHS is more stable than that of SeEOH.



Se_1—Se_2=3.035 Å

Se_1—Se_2= 3.071 Å

Figure 2: Optimized geometries of $(>Se\therefore Se<)^{\bullet +}$ radicals of a) SeEOH and b) DHS using DFT at B3LYP-6/31+G(d,p) level in water.

Further the energetics for the initial reaction of $^{\bullet}$OH radical with these compounds and consequent formation of $(>Se\therefore Se<)^+$ radical was calculated. The results showed that initial attack of $^{\bullet}$OH radical at Se-centre to form $(>Se\therefore OH)$ radical is more preferred in DHS (ΔE=-17.29 kcal/mol) than SeEOH (ΔE=+3.49 kcal/mol). This $(>Se\therefore OH)$ generally decays by proton catalyzed dehydration to form selenium centered radical cation $(>Se^{\bullet +})$ which on reaction with another parent molecule forms $(>Se\therefore Se<)^+$. The dimerization process was found to be more exothermic in DHS (-19.3 kcal/mol) as compared to SeEOH (-14.9 kcal/mol). These results suggest that formation of $(>Se\therefore Se<)^+$ radical is more favourable in case of DHS than SeEOH and support the experimental observations. The preferential formation of $(>Se\therefore Se<)^+$ radical can also be explained on the basis of HOMO energy values of different transients involved in its formation. Table 1 lists the HOMO, LUMO and HOMO-LUMO energy gap in different transients formed for SeEOH and DHS. The HOMO energy values of DHS and $DHS^{\bullet +}$ radical are comparable and therefore an efficient overlap is expected.

Compounds	HOMO (eV)	LUMO (eV)	HOMO-LUMO Energy gap (eV)
SeEOH	-5.7822	-0.2829	-5.4992
$(SeEOH)^{\bullet +}$	-7.8693	-5.2380	-2.6312
$(SeEOH)_2^{\bullet +}$	-5.7414	-3.5918	-2.1496
DHS	-5.6625	-0.1006	-5.5618
$(DHS)^{\bullet +}$	-6.8081	-1.3496	-5.4584
$(DHS)_2^{\bullet +}$	-6.0489	-0.9088	-5.1401

Table 1: HOMO values of SeEOH/DHS and their proposed intermediates formed during electron transfer reactions.

The monomer radical cation of organoselenium compound tends to undergo irreversible deprotonation to form a carbon

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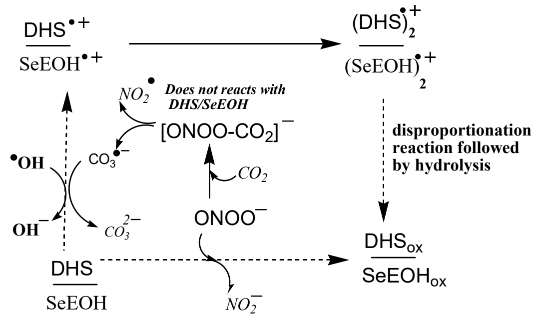
centred radical (α-reducing radical) on the carbon adjacent to the chalcogen centre. In our earlier work, we have shown that the deprotonation is favoured by the effective overlap between the σ* of the selenium centre with the σ-C-H bond at the α-position. The energy of such non-bonding interactions can theoretically be calculated by employing natural bond orbital (NBO) analysis.[24] The non-bonding interaction energy (E_{nb}) indicates the extent of the interactions taking between two orbitals. The optimized structure of ($>Se^{\bullet+}$) derived from DHS/SeEOH were calculated and the E_{nb} value of DHS and SeEOH was calculated to be 0.36 kcal/mol and 1.61 kcal/mol, respectively. From this it can be inferred that the ($>Se^{\bullet+}$) derived from DHS is less prone to undergo irreversible deprotonation as compared to SeEOH. Further, the energetics for conversion of ($>Se\therefore Se<)^+$ to form selenoxides indicated that this radical in DHS disproportionates in an energetically favored path (ΔE=+31.1 kcal/mol), as compared to SeEOH (ΔE= +32.5 kcal/mol).

Discussion

In the present study two isomeric compounds, DHS and SeEOH were investigated to understand the effect of small structural alteration on their ROS scavenging activity. DHS/SeEOH were examined for their ability to scavenge ROS like $^{\bullet}OH$, $CO_3^{\bullet-}$ radical and peroxynitrite. The rate constants for $^{\bullet}OH$ radical reaction for both SeEOH/DHS were comparable. However teh rate constant with peroxynitrite, was two times more for DHS as compared to SeEOH. To explain this significantly different activity by minor change in the structure, detailed mechanism of the radical reaction with DHS/SeEOH was studied. The overall reaction of SeEOH and DHS is depicted in scheme 2.



Scheme 2: Reaction of DHS and SeEOH with different oxidants

On reaction with $^{\bullet}OH$, both the compounds were converted to their corresponding selenoxide, where DHS produced significantly higher amount of selenoxide than SeEOH

The experimental results were complimented by calculating the energetics determined by quantum chemical calculations. The HOMO energy of DHS is higher than that of SeEOH indicating the easier oxidation of DHS. The reaction of initial addition of $^{\bullet}OH$ radical on selenium atom to form ($>Se\therefore OH$) adduct is endothermic in SeEOH (ΔE=+3.48 kcal/mol) but exothermic in DHS (ΔE=-17.29 kcal/mol). This ($>Se\therefore OH$) adduct generally decays to form $(>Se\therefore Se<)^+$. The dimerization process was found to be more exothermic in DHS as compared to SeEOH. Additionally, the stability of dimer radical cation of DHS is due to the direct interaction between selenium and oxygen of DHS and this prevents deprotonation to form carbon centred radical. Thus higher HOMO value coupled with higher stability of the intermediate $(>Se\therefore Se<)^+$ results in higher yield of selenoxide formation and thereby better antioxidant activity of DHS as compared to SeEOH. The selenoxide being reversible in presence of thiols, imparts higher antioxidant activity in DHS than in SeEOH against ROS.

Experimental

The organoselenium compounds studied were synthesized as per the reported method.[14a,c] 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid-sodium salt) ($ABTS^{2-}$), DHR123, Diphenylpicrylhydrazine (DNPH), dithiothretol reduced (DTT) and dithiothretol oxidized (DTT_{ox})were purchased from Sigma Aldrich and were of >95% purity. All the other chemicals and reagents were of "Analar" grade and used as such. The solutions were freshly prepared for each experiment in nanopure water with a conductivity of 0.1 μS cm^{-1}, obtained from a milipore water purification system. The pH of the solutions was adjusted using monosodium phosphate (NaH_2PO_4), disodium phosphate ($Na_2HPO_4.2H_2O$) and perchloric acid ($HClO_4$).

Peroxynitrite was prepared by the ozonolysis of 0.1 M sodium azide solution containing 0.1 M NaOH.[25] For all experiments stock peroxynitrite solutions were diluted using 10 mM NaOH and during the experiments, the pH was maintained at 7.4 by using 70 mM phosphate buffer. The concentration of peroxynitrite was estimated by measuring the absorbance at 302 nm ($\varepsilon_{302\ nm}$=1705 M^{-1} cm^{-1}) on UV-Visible spectrophotometer (Jasco V-639).

Pulse radiolysis studies were carried using 7 MeV electron beam with 100 ns pulse width and the details of the instrument is reported. An average dose of 9 Gy as estimated by using thiocyanate dosimeter (aerated aqueous solution of 10 mM KSCN, $G\varepsilon^{475\ nm}$ =2.59 x 10-4 m^2/J) was used for all experiments.[26] Solutions were saturated with N_2O to monitor the $^{\bullet}OH$ radical reaction ($G_{\bullet OH}$=0.6 µmole/J). Carbonate ($CO_3^{\bullet-}$) radical was generated on pulse radiolyzing N_2O saturated solution containing sodium carbonate (0.1M) at pH 7. The product formed on reaction of $^{\bullet}OH$ radical with the organoselenium compound was quantified by HPLC technique. For this, N_2O saturated aqueous solutions of 10 mM SeEOH were radiolysed using ^{60}Co γ-source with a dose rate of 40 Gy/min. The absorbed dose was set in a way that not more than 10% of the compound undergoes radiolysis. Selenoxide estimation was performed as per the reported method. In brief, 180 µl of radiolysed samples were treated with 20 µl DTT (10 mM) and incubated for 5 minutes. 20ul of the mixture was injected to C-18 reverse phase column. The different compounds were eluted using acetonitrile:water (5:95) mixture as mobile phase and absorption peaks were detected at 240 nm. The yield of formaldehyde formed during radiolysis of the organoselenium compound was estimated by derivatizing the aldehyde with DNPH. The radiated solutions were mixed with 1 mM DNPH dissolved in 10 mM HCl and stirred for 5 minutes. 20 µl of resulted solutions were injected to C-18 reverse phase column. Acetonitrile:water (60:40) was used as mobile phase and detector was set at 345 nm.

Reaction of the organoselenium compounds with peroxynitrite was studied by competition kinetics using dihydrorhodamine (DHR)123 as standard. The fluorescence measurements were done on Hitachi F-4500 fluorescence spectrophotometer with excitation and emission wavelength at 510 nm and 536 nm respectively. Peroxynitrite solution (5 µM) was added to DHR123 (10 µM) containing 0.1 mM DTPA in 70 mM phosphate buffer (pH 7.5) in absence and presence of the organoselenium compound (10-100 µM).

The structures of transients were optimized in vacuo by regressive variation in starting geometry at B3LYP/6-31++G(d,p) level (Becke non local model and Lee–Yang–Parr nonlocal correlation functionals). The geometries obtained were checked by frequency calculations. The global minima structures were then further optimized in water at B3LYP/6-31+G(d,p) level in water using PCM-SMD model. Geometry optimization and frequency calculations were performed by adopting the GAMESS suite of programs on a PC-based LINUX cluster platform.[27] The absorption maxima wavelength were calculated in water by using UCIS model at B3LYP; 6-31-G(d,p) level using Gaussian 09. The transient's geometries and molecular orbitals were visualized using chemissian V4.38 software.

Conclusions

Two isomeric organoselenium compounds, cyclic compound, DHS and a linear compound, SeEOH were studied for their antioxidant ROS scavenging activity, where DHS, a cyclic compound was found to be much better than its linear isomer SeEOH. The enhanced activities in DHS have been attributed to the strain induced in the cyclic structure that elevated the HOMO energy level and easy oxidation. Further, the cyclic structure assists in stabilizing the selenium centred dimer radical cations which favour formation of stable selenoxides, that can be reversed by the thiols. Thus the cyclic structural feature of DHS can be used as a molecular design for developing new organoselenium compounds with enhanced antioxidant activity.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Table of contents entry

Free radical induced selenoxide formation in isomeric organoselenium compounds: Role of chemical structure in antioxidant activity *Beena G. Singh*, Pavitra Kumar, P. Phadnis, Michio Iwaoka, and K. Indira Priyadarsini* Formation of selenoxide improves the antioxidant activity of organoselenium compounds and should be considered as an important marker in the design of new selenium based antioxidants **.**