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ARTICLE TYPE

Stable selones in glutathione peroxidase like catalytic cycle of selenonicotinamide derivative

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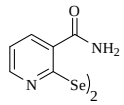
Selenonicotinamide, 2,2′-diselenobis[3-amidopyridine] (NictSeSeNict), exhibits glutathione peroxidase (GPx) like activity of catalyzing reduction of hydrogen peroxide (H_2O_2) by glutathione (GSH). Estimated reactivity parameters for the reaction of selenium species, according to Dalziel kinetic model, towards GSH (ϕ_{GSH}) and H_2O_2 (ϕ_{H2O2}), indicated that the rate constant for the reaction of NictSeSeNict with GSH is higher as compared to that with H_2O_2, indicating that the activity is initiated by reduction. ^{77}Se NMR spectroscopy, HPLC analysis, mass spectrometry (MS) and absorption spectroscopy were employed for understanding the nature of selenium intermediates responsible for the activity. The ^{77}Se NMR resonance at 525 ppm due to NictSeSeNict disappeared in the presence of GSH with initial appearance of signals at δ 364 and 600 ppm, assigned to selone (NictC=Se) and selenenylsulfide (NictSeSG), respectively. Reaction of H_2O_2 with NictSeSeNict produced a mixture of selenenic acid (NictSeOH) and seleninic acid ($NictSeO_2H$) with ^{77}Se NMR resonances appearing at 1069 and 1165 ppm, respectively. Addition of three equivalents of GSH to this mixture produced characteristic ^{77}Se NMR signal of NictSeSG. HPLC analysis of the product formed by the reaction of NictSeSeNict with GSH confirmed the formation of NictC=Se absorbing at 375 nm. Stopped-flow kinetic studies with global analysis revealed a bimolecular rate constant of $4.8 \pm 0.5 \times 10^3$ $M^{-1}s^{-1}$ and $1.7 \pm 0.6 \times 10^2$ $M^{-1}s^{-1}$ for the formation of NictC=Se produced in two consecutive reactions of NictSeSeNict and NictSeSG with GSH, respectively. Similarly the rate constant for the reaction of NictC=Se with H_2O_2 was estimated to be 18 ± 1.8 $M^{-1}s^{-1}$. These studies clearly indicated that the GPx activity of NictSeSeNict is initiated by reduction to form NictSeSG and a stable selone, which is responsible for its efficient GPx activity.

Introduction

Nicotinamide is an important moiety present in a wide range of biomolecules associated with processes such as energy production, synthesis of fatty acids, etc. It is a precursor for the coenzymes NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate),[1]and an important cofactor in numerous enzymatic redox reactions.[2] Nicotinamide exhibits anti-inflammatory[3] and antioxidant activities.[4] Antioxidants are required to minimize the oxidative stress in cellular systems. Oxidative stress is defined as a state in which an imbalance occurs in favour of reactive oxygen species (ROS) formation and as a result the biological system's ability to detoxify the reactive intermediates or to repair the resulting damage decreases.[5] ROS include molecular species like hydrogen peroxide (H_2O_2). Living organisms have developed several enzymatic systems such as catalases, superoxide dismutase and glutathione peroxidase (GPx) to detoxify these ROS.[6] GPx belongs to oxidoreductase family that catalyzes reduction of hydroperoxides by glutathione (GSH). The enzymatic activity of the GPx is due to the redox property of selenocysteine.[7]

Inspired by the biological function of selenocysteine in selenoenzymes like GPx as an antioxidant, attempts have been made during the past 10-15 years to synthesize a wide variety of organoselenium compounds that mimic the activity of GPx.[8] The first compound evaluated for GPx activity extensively was ebselen, an aromatic selenoamide.[9] Accordingly many new synthetic organoselenium compounds have been examined for GPx activity and a number of them showed promising results.[10] Further research in the development of selenium compounds indicated that weak non-bonding interactions between selenium atom and nearby heteroatoms either intramolecularly or intermolecularly play decisive role in the effectiveness of GPx like activity of ebselen and related compounds.[11] Since last one decade our group is involved in design and development of low molecular weight organoselenium compounds as antioxidants and radioprotectors.[12] With this background, nicotinoyl based organoselenium compounds were conceptulaized and synthesized. Preliminary investigations on the GPx like activity of NictSeSeNict (Scheme 1) revealed its superior activity as compared to ebselen and related derivatives.[13] Therefore to understand the factors responsible for its higher activity, detailed

catalytic mechanism was investigated by applying enzyme kinetics and the resultant intermediates were characterized by ^{77}Se NMR spectroscopy, HPLC, mass spectrometry (MS) and absorption spectroscopy.



Scheme 1 Structure of 2,2′-diselenobis[3-amidopyridine] (NictSeSeNict)

Results and Discussion

a. GPx activity by NADPH assay

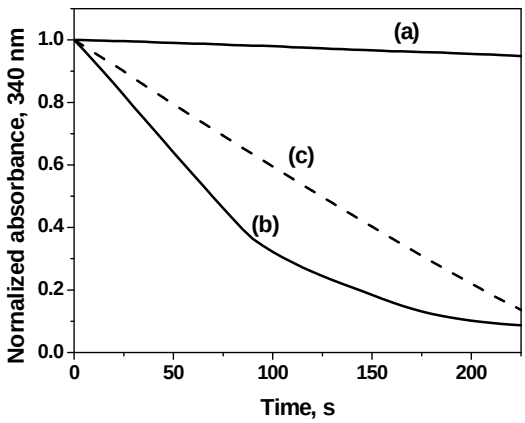


Fig. 1 Plots showing change in absorbance at 340 nm as a function of time due to NADPH oxidation. (a = blank; b = NictSeSeNict, c = ebselen). Concentration of NADPH, GSH, H_2O_2, glutathione reductase, and selenium catalyst were fixed at 0.34 mM, 1 mM, 1 mM, 5.0 Units/mL and 10 µM, respectively

GPx like catalytic activity of NictSeSeNict studied by using NADPH-GSSG reductase coupled assay indicated that the decay of NADPH at 340 nm (figure.1), increased significantly in the presence of NictSeSeNict (Fig. 1b). The initial decay (υ) under identical experimental conditions was compared with ebselen (Fig. 1c), and was used as a reference in the present study. The results indicated that, υ for NictSeSeNict (12.0±0.6 x 10^{-7} Ms^{-1}) is two-folds higher than that of ebselen (6.0 ± 0.3 x 10^{-7} Ms^{-1}). Considering that the former is a diselenide and the latter is a monoselenide the observed increase in the rate constant may indicate similar activity by the two compounds have similar GPx activity. To analyze this, a detailed enzyme kinetic study was performed where the following redox reactions may take place:

$$1/2\,\text{NictSeSeNict} + H_2O_2 \xrightarrow{k_1} 1/2\,\text{NictSeOH} + 1/2\,\text{NictSeO}_2\text{H} + 1/2\,H_2O \quad (1)$$

$$\text{NictSeSeNict} + \text{GSH} \xrightarrow{k_2} \text{NictSeH} + \text{NictSeSG} \quad (2)$$

$$\text{NictSeH} + H_2O_2 \xrightarrow{k_3} \text{NictSeOH} + H_2O \quad (3)$$

$$\text{NictSeOH} + \text{GSH} \xrightarrow{k_4} \text{NictSeSG} + H_2O \quad (4)$$

$$\text{NictSeOH} + H_2O_2 \xrightarrow{k_5} \text{NictSeO}_2\text{H} + H_2O \quad (5)$$

$$\text{NictSeSG} + \text{GSH} \xrightarrow{k_6} \text{NictSeH} + \text{GSSG} \quad (6)$$

$$\text{NictSeSG} + H_2O_2 \xrightarrow{k_7} \text{NictSeOH} + \text{GSOH} \quad (7)$$

$$\text{NictSeO}_2\text{H} + 3\text{GSH} \xrightarrow{k_8} \text{NictSeSG} + \text{GSSG} + 2H_2O \quad (8)$$

Depending upon its reactivity with the two substrates, i.e. GSH or H_2O_2, the GPx cycle is either initiated by the reaction of NictSeSeNict with H_2O_2 (eqn. 1) or GSH (eqn. 2).[14]In order to know the individual reactions with these substrates, the kinetic data was treated with Dalziel equation as applied in our earlier studies with selenocystine.[14b] The enzyme kinetic parameters were estimated using eqn. 9.

$$\frac{2\left[\text{NictSeSeNict}\right]}{\upsilon} = \phi_0 + \frac{\phi_{GSH}}{\left[\text{GSH}\right]} + \frac{\phi_{H_2O_2}}{\left[H_2O_2\right]} + \frac{\phi_{GH}}{\left[\text{GSH}\right]\left[H_2O_2\right]} \quad (9)$$

Here [*NictSeSeNict*] is the total enzyme concentration. NictSeSeNict being a diselenide produces two reactive species either during oxidation or reduction that can independently participate in the GPx-like cycle. Therefore, in this case the total enzyme concentration is twice the concentration of NictSeSeNict. In eqn. 9, ϕ_{GSH} is the reciprocal of the total reactivity of the enzyme and its intermediates with GSH, i.e. [$\phi_{GSH} = 1/k_G = 1/(k_2 + k_4 + k_6 + k_8)$], ϕ_{H2O2} is the reciprocal of the total reactivity of the enzyme and its intermediates with H_2O_2, i.e. [$\phi_{H2O2} = 1/k_H = 1/(k_1 + k_3 + k_5 + k_7)$]. Here, k_G and k_H are the apparent rate constants for the overall reactions involving GSH and H_2O_2, respectively. The term ϕ_{GH} is the parameter related to the rate of formation of the ternary complex. A non-zero value of ϕ_{GH} indicates the formation of a ternary complex between the enzyme and the two substrates. The term ϕ_0 is equal to the reciprocal of the turnover number which corresponds to the maximum catalytic rate at unit enzyme concentration ($\phi_0 = 1/k_{cat}$).[15] To estimate these parameters, a series of experiments were performed and the initial reduction rate (υ) of H_2O_2 which is directly related to the decay of NADPH at 340 nm, was measured in the presence of NictSeSeNict (10 µM) at a fixed initial concentration of GSH and different concentrations of H_2O_2 (0.1 mM –1.2 mM). A primary linear plot was obtained for the variation of [*2NictSeSeNict*]/υ as a function of reciprocal concentration of H_2O_2. The slope and intercept of the plot are represented by eqns. 10 and 11.

$$\text{Slope} = \phi_{H_2O_2} + \frac{\phi_{GH}}{\left[\text{GSH}\right]} \quad (10)$$

$$\text{Intercept} = \phi_0 + \frac{\phi_{GSH}}{\left[\text{GSH}\right]} \quad (11)$$

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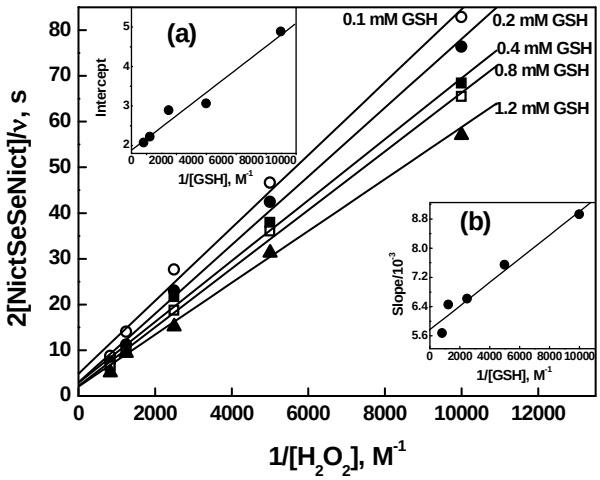


Fig. 2 Primary double-reciprocal plots according to Dalziel equation (eqn 9) for NictSeSeNict-catalyzed reduction of H_2O_2 in the presence of different concentrations of GSH. Insets (a) and (b) show secondary Dalziel plots in accordance with eqns 10 and 11, respectively

The above experiment was repeated at different concentrations of GSH (0.1 -1.2 mM), and from the primary linear plots as given in figure 2, different values for slope and intercept were obtained. The slope obtained from these values were plotted as a function of the reciprocal GSH concentration according to eqn. 10, and from the slope and intercept of this secondary plot, ϕ_{GH} and ϕ_{H2O2} values were estimated to be 2.0 ± 0.7 x 10^{-6} M^2s and 2.4 ± 0.1 x 10^{-2} Ms, respectively. Similarly, different intercept values obtained from the primary plots at different concentrations of GSH, when plotted against the reciprocal concentration of GSH, gave a secondary plot (Inset (b) of Figure 2). The slope and intercept of this secondary plot, according to eqn. 11, correspond to ϕ_{GSH} and ϕ_0, respectively, are 4.0 ± 0.2 x 10^{-3} Ms and 10.9 ± 0.7 s. A comparison of ϕ_{H2O2} with ϕ_{GSH} indicates that the overall reactivity of NictSeSeNict and the intermediates with GSH is nearly ten times higher than that of H_2O_2. To substantiate the results obtained from enzyme kinetic studies, intermediates formed during the catalytic cycle have also been characterized by ^{77}Se NMR, HPLC and MS data.

b. ^{77}Se NMR studies

NictSeSeNict exhibits a ^{77}Se NMR resonance at 525 ppm due to the diselenide moiety, which on treatment with three equivalents of GSH displayed new signals at 364 and 600 ppm (SI Figure1).To assign the nature of the species responsible for the ^{77}Se NMR signals, two independent experiments were performed as described below.

Generally, diselenides on reduction with thiols form selenol and selenenyl sulfide (eqn. 2).[16] Therefore in the first experiment, NictSeSeNict was reduced with sodium borohydride followed by addition of triflouro acetic acid. ^{77}Se NMR spectrum of this reaction mixture showed a signal at 364 ppm (SI Figure 2), which confirmed that the signal obtained in GSH reaction with NictSeSeNict is its reduced species, i.e. selenol or its equivalent. In general the ^{77}Se NMR resonance for a selenol would appear in the range -300 to 159 ppm,[17] however in compounds, where selenol is attached to a pyridine ring, it is reported to undergo tautomerization to form a stable selone (NictC=Se) as observed in the case of 2-pyridyl selone (δ ^{77}Se = 314.0 ppm) and 3-carboethoxy-N-hydroxypyridine-2-selone (δ ^{77}Se = 380.3 ppm).[18] Therefore in the present case the 364 ppm signal has been assigned to a stabilized selone. The down field shift of ~50 ppm with respect to that observed for simple 2-pyridyl selone would indicate further stabilization with the amino group of the amide substitution. The observed second peak at 600 ppm has been attributed to the selenenyl sulfide (NictSeSG), based on the literature reports[19]. To further confirm its nature, the following second experiment was performed. NictSeSeNict reacts completely with four equivalents of H_2O_2, to give two new signals at 1166 and 1069 ppm, (Figure 3), further over a period of time, the signal at 1069 ppm disappeared, only the 1166 ppm signal remained. Under these conditions addition of ~ 3 equivalents of GSH caused complete disappearance of the 1166 ppm signal with appearance of signal at 600 ppm. The 1166 ppm NMR signal is attributed to $NictSeO_2H$, in analogy with that reported for ebselen (eqn. 8) (Figure 3). The $NictSeO_2H$ peak is shifted downfield by 26 ppm as compared to the seleninic acid of ebselen (1143 ppm),[14a] probably due to non-bonding interactions. Assuming the 1166 ppm signal to be $NictSeO_2H$, addition of three equivalents of GSH would convert this to NictSeSG. This experiment although indirect, provided supportive conformation for the 600 ppm signal to be NictSeSG. The ^{77}Se NMR signal at 1069 ppm may correspond to NictSeOH, as reported in case of *o*-nitrobenzeneselenenic acid (δ 1066 ppm)[20].

NictSeSG formed during the GPx cycle can react with GSH to form NictC=Se (eqn. 6) or it can react with H_2O_2 to form NictSeOH (eqn. 7).The relative reactivity of NictSeSG with the two substrates i.e. GSH and H_2O_2 was studied by carrying out independent experiments. The ^{77}Se NMR spectra obtained on mixing NictSeSG with 3 equivalents of H_2O_2 showed complete disappearance of the 600 ppm signal with concomitant formation of two signals at 1069 ppm and 1166 ppm (SI Figure 3). In a separate experiment, NictSeSG reacts with excess GSH (3 equivalent) and shows signal at 364 ppm along with the 600 ppm. The ^{77}Se NMR signal of NictSeSG in the presence of H_2O_2 disappeared faster than that with GSH indicating that the reaction of NictSeSG is faster with H_2O_2 than that with GSH and the intermediate NictSeSG formed during the catalytic cycle would be consumed preferably by H_2O_2.

From the above ^{77}Se NMR studies, the GPx like catalytic activity of NictSeSeNict can be summarized as follows: The catalytic cycle is initiated by the reaction of NictSeSeNict with GSH to form NictC=Se and NictSeSG, both of them can react with H_2O_2 to form $NictSeO_2H$ via oxidation of another intermediate species, NictSeOH. Further, both $NictSeO_2H$ and NictSeOH can react with GSH to form NictSeSG which preferentially reacts with H_2O_2 to form $NictSeO_2H$, completing the catalytic cycle.

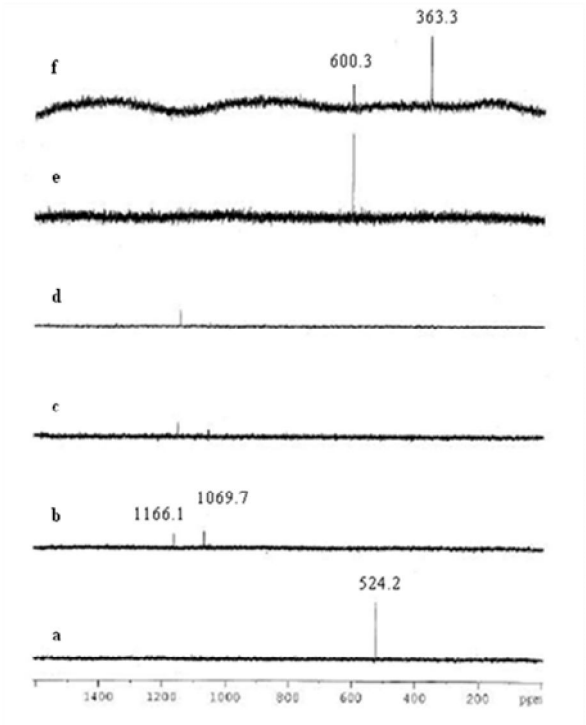


Fig. 3 ^{77}Se NMR spectra obtained in dmso(d_6) due to (a) NictSeSeNict; (b) reaction between NictSeSeNict and hydrogen peroxide (1:4); (c) reaction panel (b) after ~ 10 min; (d) reaction panel (c) after ~ 10 min; (e) reaction mixture from panel (d) treated with GSH (3 equivalent); (f) reaction mixture of panel (e) treated with another 3 equivalent of GSH.

c. HPLC and MS studies

The individual components formed during the reaction of NictSeSeNict with GSH were separated by HPLC. The chromatogram of NictSeSeNict, GSH and oxidized glutathione (GSSG) appeared at retention time of 19.3, 5.5 and 9.9 min, respectively. The products obtained on mixing NictSeSeNict with GSH (1.2 equivalent), showed peaks with retention time at 5.5, 8.3, 16.6 and 17.5 min, respectively, under this experimental condition, the peak due to NictSeSeNict was not observed (plot (B) of Figure 4). In analogy with the previous reports on selenoglutathione[21]as well as the analysis by photodiode array detection (SI. Figure 4), the peaks at 5.5 and 8.3 min have been attributed to NictC=Se and the peaks at 16.6 and 17.5 min to NictSeSG. The dual peaks may be due to the formation of different prototropic forms differing in the retention times as the pKa of nicotinamide is 3.4 and the eluent is at pH~2.0.[22]Another possibility for the dual peaks may be due to the possibility of two conformations with different orientation of the amido moiety. Increasing the concentration of GSH (240 equivalents) caused complete disappearance of NictSeSG with appearance of GSSG at 9.9 min and NictC=Se at 5.5 and 8.3 min (plot (D) of Figure 4). These results indicate that NictSeSeNict reacts with GSH (lower concentration) to form NictC=Se and NictSeSG (eqn. 2) and in the presence of excess GSH, the NictSeSG is further reduced to NictC=Se and GSSG (eqn. 6).

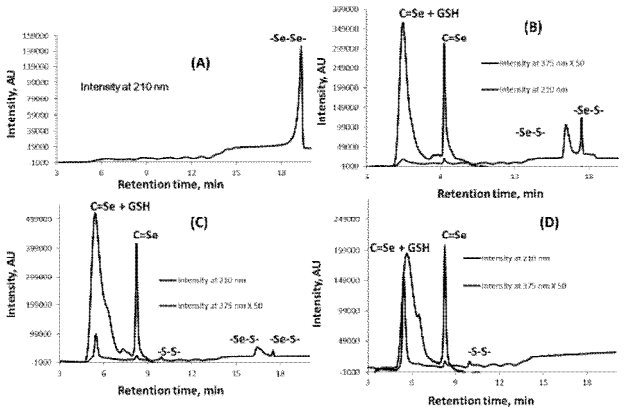


Fig. 4 HPLC chromatograms obtained on mixing NictSeSeNict with different concentration of GSH_{red}.Chromatogram (A) corresponds to NictSeSeNict. (B), (C) and (D) correspond to chromatogram obtained on mixing NictSeSeNict with 1.2, 40 and 240 equivalentsof GSH_{red}, respectively

The results from HPLC experiments were further supported by mass spectrometry (MS). In this study, NictSeSeNict was mixed with 1.2 equivalent of GSH and the reaction mixture was incubated for 30 minutes for completion and injected in MS in ESI(+) mode. The mass spectrum showed a molecular ion peak due to the formation of NictSeSG and NictSeSeNict at m/z value of 508.052 and 402.937, respectively (SI Figure 5A). The isotopic finger print of the spectrum as six lines confirmed selenium species. However under this condition, the signal due to selone was not observed. In the presence of higher GSH concentration (240 equivalents), the signal due to NictSeSeNict and NictSeSG disappeared and peak due to GSSG was observed at m/z value of 615.191 (SI Figure 5B). Even at this condition, the molecular peak due to NictC=Se was not observed, either in electron spray ionization (ESI (+/-)) or in Atmospheric pressure chemical ionization (APCI) mode. Similarly, the product formed on reacting NictSeSeNict with H_2O_2 could not be characterized by MS.

The spectrum of NictC=Se, separated from the HPLC eluent, showed a red shifted absorption maximum at 375 nm, which is distinctly different from that of NictSeSeNict and NictSeSG. In another separate experiment, we followed absorption spectral changes on mixing NictSeSeNict with GSH. The absorption spectrum due to NictSeSeNict showed absorption maximum at 290 nm (Figure 5a*)*, which onaddition of equimolar GSH resulted in distinct peak at 375 nm, due to NictC=Se. Therefore, stopped-flow spectrometry using absorption detection was utilised to determine the rate constants for the individual reaction between NictSeSeNict and GSH and NictC=Se and H_2O_2.

d. Spectrophotometric and stopped flow studies

The bimolecular rate constant for the reaction between NictSeSeNict and GSH was estimated by following the built-up kinetics at 375 nm by using stopped flow spectrometer in a single mixing mode. The reaction was initiated by mixing 50 μM of NictSeSeNict with different concentration of GSH (3.3 – 5.6 mM) and was monitored as a function of time. The absorption-time plot was fitted to first order kinetics according to the eqn. 12:

$$Y(t) = at + b + \sum_{i=1}^{N} C_i \exp(-k_{obs} t) \quad (12)$$

where Y(t) is the time dependent absorbance at 375 nm, a and b correspond to the absorbance at infinite time. The parameters C_i and k_{obs} are respectively the amplitude and observed rate constant. Formation of NictC=Se during the reaction of GSH with NictSeSeNict may proceed via two reaction routes, i.e. eqn. 2 and 6. The final absorbance as given in the absorption-time plots (inset of figure 5) at two different GSH concentrations do not show any change, which indicates that the formation of NictC=Se is complete in both the cases. However, in the presence of excess GSH thefirst step becomes much faster while the second step is the rate determining. Under these conditions, we can assume that k_{obs} is mainly dueto the reaction shown in eqn.6. The plot of k_{obs} as a function of GSH concentration, is linear and the slope corresponds to the bimolecular rate constant for the reaction given in eqn. 6, which was found to be $1.5 \pm 0.1 \times 10^2$ $M^{-1}s^{-1}$. This was further confirmed by performing Global kinetic trace analysis of absorption-time plots with singular value decomposition and non-linear regression modelling by means of the Levenberg-Marquardt method.[23] From this, the individual bimolecular rate constant for eqn. 2 and 6 was estimated to be $4.8 \pm 0.5 \times 10^3$ $M^{-1}s^{-1}$ (k_2) and $1.7 \pm 0.6 \times 10^2$ $M^{-1}s^{-1}$ (k_6), respectively (SI Figure 6). The value for k_6, obtained with the two different analyses is the same within experimental errors. The initial reaction of NictSeSeNict with GSH is therefore ten times higher than that for NictSeSG with GSH

Similarly, the bimolecular rate constant for the reaction of NictC=Se with H_2O_2 (eqn. 3) was estimated. For this study, NictC=Se was formed by reducing 50 μM NictSeSeNict with 50 μM dithiothreitol (DTT_{red}) as this reaction is free from contamination of selenenyl sulfide.[10k] After five minutes, the mixture was treated with different concentration of H_2O_2 (3 – 10 mM) and the time dependent decrease in the absorption at 375 nm after addition of H_2O_2 was immediately monitored at each concentration of H_2O_2 (SI Figure 7). By fitting the data to first order kinetics equation (eqn. 13), the k_{obs} was estimated. From the linear plot of the k_{obs} against the H_2O_2 concentration, the bimolecular rate constant between NictC=Se and H_2O_2 was estimated to be 18 ± 1.3 $M^{-1}s^{-1}$. This reaction is definitely slower than the other reactions but compared to the rate constant for similar reactions with compound like ebselen[28] with H_2O_2, can be considered as competing. The bimolecular rate constant (k_7) for the reaction between NictSeSG and H_2O_2 could not be estimated due to experimental limitations.

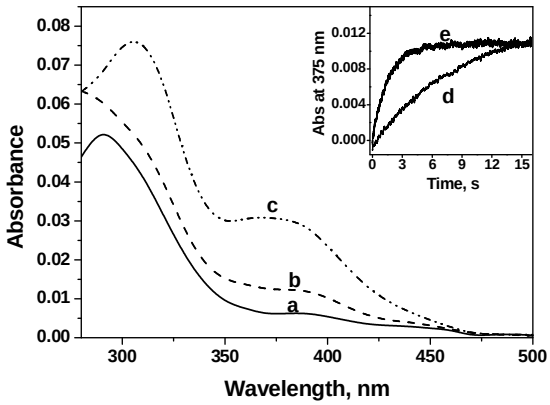


Fig. 5 Time dependent absorption spectra obtained on mixing 50 μM NictSeSeNict with 2 mM GSH in 1% DMSO-aqueous solution (a = 0 sec, b = 1 sec, c = 7.5 sec).Inset shows representative absorption – time plot obtained at 375 nm on mixing 50 μM NictSeSeNict with (d) = 0.3 mM and (e) = 4 mM GSH

In a control experiment, when 20 μM NictSeSeNict was treated with 1 mM H_2O_2, no change in absorption spectrum was observed initially. However, after few hours, the absorbance at 315 nm started decreasing with appearance of a new absorbance band at 325 nm. As the reaction was very slow (few hours), its kinetics could not be followed. This control experiment is performed to rule out contribution of the direct reaction in the estimation of rate constant for eqn. (1).

e. Quantum chemical calculation

To confirm the nature and relative stability of seleone and selenol, quantum chemical calculation was performed at B3LYP/6-31++G(d,p) in the polarizable continuum model (PCM) of water. The obtained structures are shown in Figure 6. In both the cases, two stable conformers are possible due to internal rotation of the amide group. In one of the selenol conformers, the amide group is roughly perpendicular to the benzene ring and there is no significant interaction between the selenol and amide groups, while the Se–H···O hydrogen bond is formed in the other. These selenol conformers are, however, less stable than the selone conformers by more than 10 kcal/mol in water. The stability of the selone conformers is further enhanced in water compared to vacuo. The results support that only a selone form would be present in polar solvents. The one selone conformer, which is a global energy minimum structure, has a planar structure stabilized with an intramolecular N–H···Se hydrogen bond. For this stabilised selone, ^{77}SeNMR chemical shift was estimated to be 244.0 ppm in water and 394.0 ppm in vacuo. These values are almost consistent with the experimental observations, indicating that the species having absorption at 364 ppm should not be a selenol but a selone. On the other hand, the other selone conformer with a perpendicular amide group is slightly unstable (ΔE = +1.15 kcal/mol) in water. To further characterize the formation of the selone form (NictC=Se), we measured the ^{1}H NMR spectrum of the reaction mixture of NictSeSeNict and DTT_{red}, which showed a signal at 14.4 ppm corresponding to the tautomeric hydrogen present on the N atom of the nicotinamide group (SI. Figure 8). Further presence of non-

bonding interaction between Se and hydrogen atom in the selone was confirmed by recording the ^{77}Se NMR spectrum of NictC=Se by keeping the proton decoupler turned off, where the 364 ppm signal showed a doublet with coupling constant value of 10.7 Hz, which corresponds to the Se-H coupling (SI. Figure 9).[29]

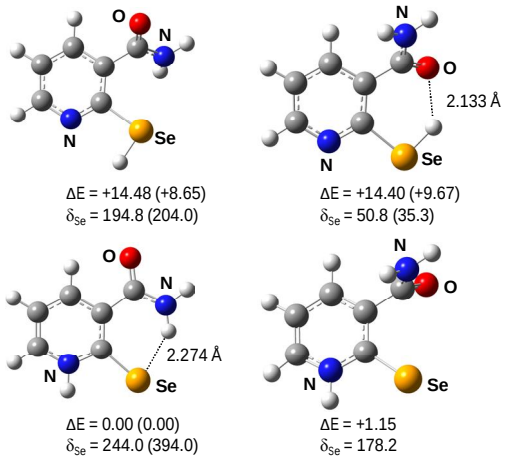
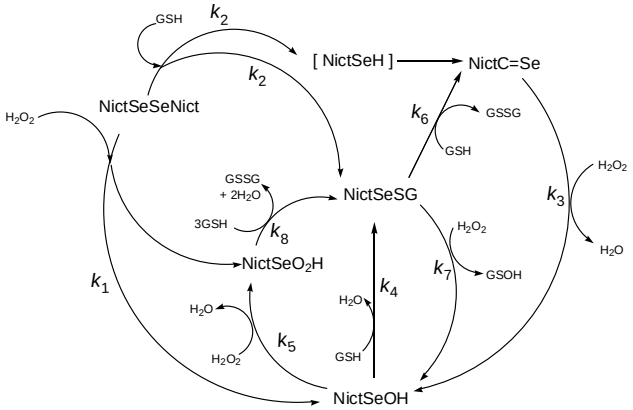


Fig. 6 The stable structures obtained by quantum chemical calculation at B3LYP/6-31++G(d,p) in the polarizable continuum model (PCM) using water as solvent. The relative energies are shown in kcal/mol. The calculated Se NMR chemical shifts with respect to Me_2Se in vacuo are shown in ppm as δ_{Se}. The values in parentheses are those obtained in vacuo at the same calculation level

Summary and Conclusion

In the present investigation, NictSeSeNict was synthesized and examined for GPx like catalytic activity. The compound is as active GPx mimic as ebselen. Enzyme kinetic studies applying Dalziel kinetic model, ^{77}Se NMR and stopped flow kinetics suggested that the GPx catalytic cycle is initiated predominantly by reduction of NictSeSeNict with GSH. ^{77}Se NMR studies on the intermediate species formed in the GPx cycle confirmed that during reduction by GSH, NictSeSeNict is converted to a selone (NictC=Se) and NictSeSG. Formation of NictC=Se has been confirmed by HPLC and that of NictSeSG by MS studies. Quantum chemical studies further supported that the reduced species is a selone (NictC=Se), which is stabilized by ~10 kcal/mol as compared to the selenol form. Bimolecular rate constants for the reaction between NictSeSeNict and GSH and also for NictC=Se and H_2O_2 indicated that the reduction of the former is faster than that of the oxidation of the latter. The possible reactions involved in the GPx activity of NictSeSeNict are summarized in scheme 2. Thus our studies for the first time identified formation of stable selone in the GPx activity of diselenides. The studies performed on the GPx like activity of ebselen did not show the formation of selenol even at high concentration of GSH, because of the fast sulfur exchange reactions.[14a, 30] Thus from the present results, we propose that introduction of the pyridine ring in the selenoamide moiety provides extra stabilization to the reduced species, selone. The results of this study inspire us to design new water-soluble pyridine based GPx mimics by substituting suitable functional groups. Recently, formation of such selone on reduction of selenoneine, a diselenide and an antioxidant naturally found in ocean fish like tuna is reported,[31] which makes selone moiety a viable candidate to be explored for its free radical scavenging and antioxidant properties.



Scheme 2 The GPx like catalytic activity by NictSeSeNict

Experimental Section

NictSeSeNict was synthesized according to the method reported[13] and characterized by ^{1}H, ^{13}C, and ^{77}Se NMR. NADPH, GSH, DTT_{red}, H_2O_2, glutathione reductase, trifluoro acetic acid (TFA), acetonitrile (ACN) from Sigma/Aldrich were procured from the local suppliers. Concentration of aqueous H_2O_2 (30% H_2O_2), was estimated by iodometric titration. Solutions were prepared using water from nanopure system. UV-visible absorption studies were carried on JASCO V-630 spectrophotometer. NMR spectra were recorded on a Bruker Avance-II 300 MHz spectrometer operating at 300.13 (^{1}H) and 57.25 MHz (^{77}Se{^{1}H}). ^{1}H NMR chemical shifts were relative to internal dmso peak (δ = 2.49 ppm). The ^{77}Se{^{1}H} NMR chemical shifts were relative to external diphenyl diselenide (Ph_2Se_2) in $CDCl_3$ (δ 463.0 ppm relative to Me_2Se (0 ppm). For all the NMR studies, to ensure complete solubility of the selenium compound, DMSO (d_6) was used as solvent.

HPLC analysis was performed on a Shimadzu VP series highperformanceliquid chromatograph system, equipped with a binary gradient pump system and a photodiode array detector. The individual components formed during the GPx like catalytic cycle were separated on a TOSOH TSKgel ODS-100V reverse phase column which was equilibrated with 0.1 % aqueous solution of TFA (eluent A) at a flow rate of 1.0 mL/min. A solvent gradient using 0.1 % TFA in ACN (eluent B) was applied by increasing the ratio of eluent B from 0 to 5% in 0-10 min, then 5 to 20% in 10-18 min, from 20 to 60% in 18-30 min, and finally 60 to 100% in 30-31 min. The flow rate was kept constant at 1.0 mL/min throughout the experiment. The mass spectra were recorded on JMS-T100LP mass spectrometer. In general to characterize the selenol, the aqueous solution containing mixture of NictSeSeNict and varying GSH (3 equiv) was incubated in air saturated condition for 30 min, and the reaction products were analyzed by ESI(+)-mass spectrometer. Due to the incompatibility of the HPLC and MS instruments with DMSO solvent, the reagents for these studies were dissolved in 70 % ACN – 30 % water mixture.

Kinetics for the reaction between NictSeSeNict and GSH was studied by employing BioLogic SFM-300 stopped-flow spectrometer (from BioLogic Scientific Instruments, France) equipped with a xenon lamp as a light source, in the single mixing mode. The flow cell has a path length of 1.5 mm. The two mixing syringes contained the reactants separately and the two solutions were mixed in the stopped-flow cell. The absorption changes at any suitable wavelength after the mixing were monitored as a function of time. The time-resolved difference spectra were analyzed by singular value decomposition (SVD) and global exponential fitting using the built in S-Fit software with the stopped flow spectrometer.[23c]

GPx activity of NictSeSeNict was monitored spectrophotometrically by using NADPH-GSSG reductase coupled assay.[24] In brief, the test mixture contained NADPH, GSH, hydrogen peroxide and glutathione reductase in 0.1 M potassium salts of phosphate buffer (pH 7.4) and the reaction was initiated by addition of NictSeSeNict. The concentrations of NADPH, glutathione reductase, and NictSeSeNict were fixed at 0.34 mM, 5.0 Units/mL and 10 μM, respectively, while the concentration of the two substrates i.e. GSH and H_2O_2 were varied. Other details related to the experiments are given in the results section. The initial reduction rate of the hydroperoxide (υ) was calculated from the rate of NADPH oxidation by following the decay of absorbance due to NADPH at 340 nm.[15a]

Quantum chemical calculation: Calculation was performed for both selenol and selone forms of NictSeH in the polarizable continuum model (PCM) of water[25] at B3LYP/6-31++G(d,p). For possible conformations, the structure was fully optimized in vacuo and then in water to obtain two stable conformers for each form. All structures were characterized as an energy minimum by vibrational analysis. The NMR chemical shifts were subsequently estimated by the Gauge-Independent Atomic Orbital (GIAO) method[26]at the same calculation level. Dimethyl selenide (Me_2Se) was employed as a reference compound, which showed the Se isotropic values of 1925.77 ppm in vacuo and 1971.1245 ppm in water at the same calculation level. All calculations were performed by using a Gaussian 03 software package.[27]

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