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Evidence for a Common Selenolate Intermediate in the Glutathione Peroxidase-like Catalysis of α-(Phenylselenenyl) Ketones and Diphenyl Diselenide

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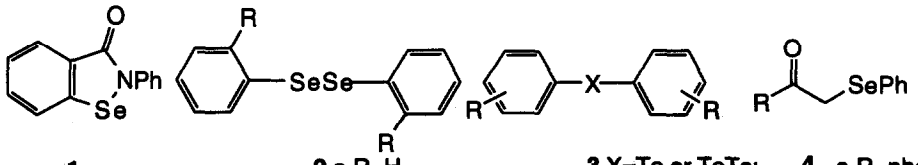
Abstract: The glutathione peroxidase-like catalysis of α-(phenylselenenyl) ketones was investigated. Degradation studies demonstrated the rapid cleavage of the aliphatic carbon-selenium bond of α-(phenylselenenyl) ketones by glutathione at pH 6.9 in a methanolic phosphate buffer under argon. On treatment with excess glutathione under aerobic conditions, α-(phenylselenenyl) ketones, S-(phenylselenenyl)glutathione and diphenyl diselenide were all shown to give benzeneselenolate. This material was found to be oxidized by hydrogen peroxide considerably faster than α-(phenylselenenyl) ketones, S-(phenylselenenyl)glutathione or diphenyl diselenide. A catalytic mechanism involving benzeneselenolate, benzeneselenenic acid and S-(phenylselenenyl)glutathione as cruical intermediates was proposed to account for the glutathione peroxidase-like catalysis of α-(phenylselenenyl) ketones and diphenyl diselenide.

INTRODUCTION

The essential micro-nutrient selenium plays an important role in the biological antioxidant defence network by serving in the active site of the selenium-containing glutathione peroxidases. These enzymes utilize thiols to reduce hydrogen peroxide to water and organic hydroperoxides to their corresponding alcohols, without the formation of potentially toxic free radical intermediates.[1,2] During recent years, several attempts have been made to synthesize simple organic compounds which mimic the properties of the natural enzymes. Such compounds might find clinical application in disease states where cell- or tissue-specific oxidative stress contribute to the pathophysiological mechanism. Ebselen (**1**, 2-phenyl-1,2-benzisoselenazol-3(2H)-one)[3] diaryl diselenides **2**,[4] diaryl ditellurides[5]/diaryl tellurides[6] **3**, α-(phenylselenenyl) ketones **4**[7] and the synthetic enzyme selenosubtilisin[8,9] are examples of glutathione peroxidase mimetics.

The catalytic action of the native enzymes and of the synthetic compounds has been attributed to the heteroatom, which can be readily cycled between different oxidation states. Despite this, the chemical structures of the intermediates involved in the respective mechanisms of catalysis are, in many cases, controversial. Recently, we demonstrated the formation of a selenolate during Ebselen-catalyzed reduction of hydrogen peroxide by glutathione (GSH).[10, 11] In the case of α-(phenylselenenyl) ketones, preliminary data suggested that the catalytic activity of the compounds was due to redox cycling of the intact α-



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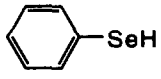
2 a R=H
b R=CH_2NMe_2
c R=CH_2N

3 X=Te or TeTe; R=H, OMe, NH_2, NMe_2, CH_2NMe_2

4 a R=phenyl
b R=4-nitrophenyl
c R=2-thienyl
d R=2-naphthyl
e R=4-methoxyphenyl
f R=t-butyl
g R=methyl

(phenylselenenyl) ketones. In this communication, we present chemical and UV-spectroscopic evidence that the catalysis is due to scission products formed from the parent molecules in the presence of GSH. The proposed catalytic cycle involves formation of benzeneselenol (**5**), an intermediate also indicated in the catalytic mechanism of diphenyl diselenide.



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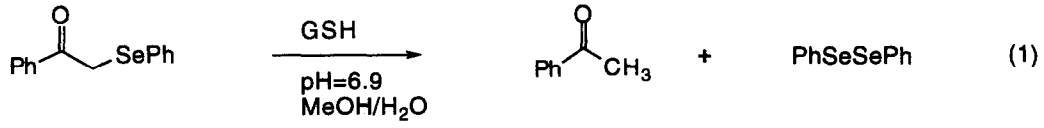
RESULTS

It is known that thiols show a dual reactivity towards certain α-substituted ketones[12] and benzylic halides,[13] giving reduction and/or substitution products under basic reaction conditions. When α-(phenylselenenyl)acetophenone (**4a**) in methanol was treated with three molar equivalents of GSH in an equal volume of water (pH≈2), no reaction was observed after 1 h as judged by TLC and ^1H NMR analysis (the latter carried out using deuterated solvents). However, when the same experiment was performed with the glutathione dissolved in a 0.1 M phosphate buffer (pH=6.9), the selenide was quantitatively consumed within a few minutes and diphenyl diselenide (99% yield) isolated together with the α-reduced carbonyl compound, acetophenone (64% yield) (eq. 1). The diselenide was isolated in the presence or absence of an atmosphere of



argon, which indicates that its formation does not involve air-oxidation of benzeneselenolate. A significantly longer reaction time (≈1 h) was required to complete the reaction when GSH was added in an acetate buffer (pH = 4.75). α-(Phenylselenenyl) ketones **4b** (99% diphenyl diselenide; 87% 4-nitroacetophenone), **4d** (99% diphenyl diselenide; 91% 2-acetonaphthone) and **4g** (92% diphenyl diselenide; acetone not isolated) were

similarly reduced within 10 min by GSH at pH=6.9. In order to study the relative reactivities of some of these compounds, α-(phenylselenenyl) ketones **4a** and **4g** (1:1 mixture) were allowed to compete for one equivalent of GSH. According to ^{1}H NMR analysis of the crude reaction product in comparison with an internal standard (4-bromoanisole), selenide **4a** was ≈ 6 times more reactive than selenide **4g**. Similarly, selenide **4b** was found to be ≈ 7 times more reactive than selenide **4a**.

As compared with the experiment using a three-fold excess of glutathione, a significantly lower yield (49%) of diphenyl diselenide was isolated when only one equivalent of GSH was added to α-(phenylselenenyl) ketone **4b** (the isolated yields of 4-nitroacetophenone were 87% and 100%, respectively). This seems to indicate that the phenylselenenyl group, to some extent, is lost into the aqueous phase during work-up, *e.g* as a water-soluble GSH conjugate, S-(phenylselenenyl)glutathione (**6**). An authentic sample of this material was prepared in 38% yield by treatment of GSH with phenylselenenyl bromide in dimethylformamide (eq. 2).

$$\text{GSH} + \text{PhSeBr} \xrightarrow[2)\ H_2O]{1)\ \text{DMF}} \underset{\mathbf{6}}{\text{GSSePh}} \qquad (2)$$

When treated with three equivalents of GSH for 15 min under our standard reaction conditions, selenosulfide **6** afforded diphenyl diselenide in 90% yield. However, under identical conditions, in the absence of GSH, only a 16% yield of the diselenide was isolated. Thus, the disproportionation of selenosulfide **6** (eq. 3) seems to be promoted by the presence of GSH, probably *via* formation of benzeneselenol and its equilibration with selenosulfide **6** (eq. 3).

$$\begin{aligned} &\text{GSSePh} + \text{GSH} \rightleftharpoons \text{GSSG} + \text{PhSeH} \\ &\text{PhSeH} + \text{GSSePh} \rightleftharpoons \text{PhSeSePh} + \text{GSH} \qquad (3) \\ &\hline \\ &2\ \text{GSSePh} \rightleftharpoons \text{GSSG} + \text{PhSeSePh} \end{aligned}$$

Since the chemical procedures detailed above were unable to demonstrate the formation of a selenolate intermediate in the reaction between α-(phenylselenenyl) ketones and GSH, spectrophotometric analysis was used to determine if a selenolate was transiently formed. The reported pK_a-value of 6.5 for benzeneselenol has been questioned.[14] As indicated by the insert of Fig. 1 , the compound is essentially present as a selenolate above pH 6. Fig.1 shows the spectrum of benzeneselenolate at pH 6.5 generated by $NaBH_4$ reduction of diphenyl diselenide(ε_{271} = 13.7 $mM^{-1}cm^{-1}$). On air oxidation, the peak at 271 nm gradually disappeared with the concurrent reformation of the spectrum of diphenyl diselenide (Fig. 1). The disappearance of the peak at 271 nm corresponds to an apparent spontaneous oxidation rate of 71.4 x 10^{-3} min^{-1}. When benzeneselenolate was added to a solution of 2,4-dinitrochlorobenzene (CDNB), a new peak, corresponding to the substitution product 2,4-dinitrophenyl phenyl selenide (**7**), rapidly appeared with an absorption maximum at 351 nm (Fig. 1; ε_{351} = 10.9 mM^{-1} cm^{-1}). Figure 2 shows the change in the UV spectrum on addition of excess GSH (1 mM) at pH 6.5 to a solution (100 mM) of α-(phenylselenenyl)acetophenone (**4a**) under aerobic conditions. A rapid increase of the 271 nm absorbance was noted. By time-resolved analysis at 280 nm, a wavelength chosen to minimize interference from the parent ketone, the apparent initial and maximal rates of selenolate formation

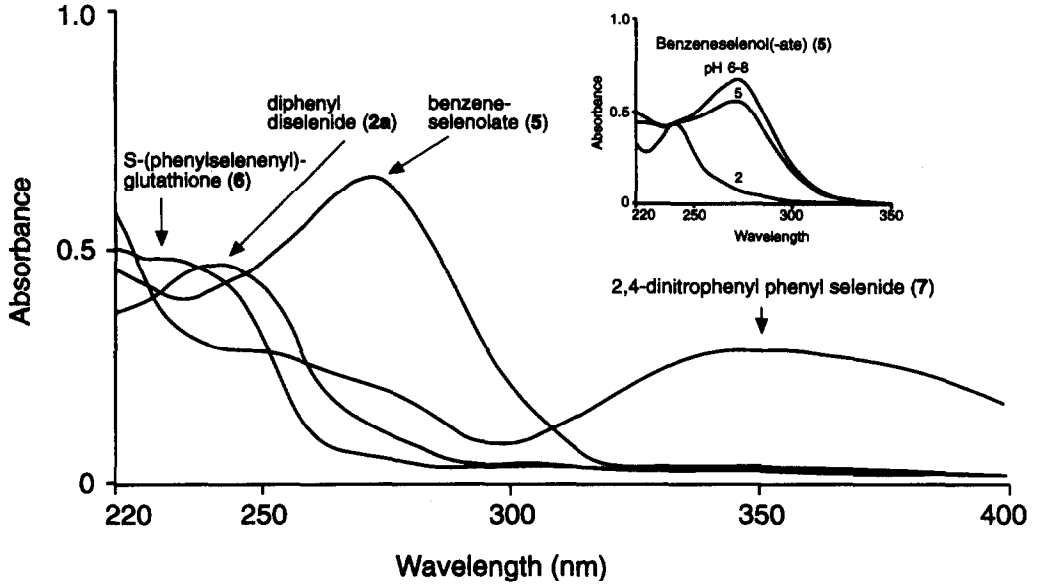
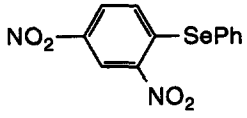


Figure 1. Absorption spectra of 25 μM S-(phenylselenenyl) glutathione (**6**), 25 μM diphenyl diselenide (**2a**), 50 μM benzene selenolate (**5**) and 25 μM 2,4-dinitrophenyl phenyl selenide (**7**) in 50 mM potassium phosphate, pH 6.5, 50% EtOH. Insert: Absorption spectra of benzene selenol(-ate) (**5**) in the same buffer at the pH values indicated.

were determined (v_{init} = 1.2 μM ·min^{-1}; v_{max} = 2.2 μM· min^{-1}). It was calculated that the maximal selenolate concentration under the conditions of the experiment, c_{max}, was 22 μM. Experiments performed as above, but in the presence of CDNB, showed build-up of absorbance at 351 nm, corresponding to selenide **7**. The UV spectra of S-(phenylselenenyl)glutathione (**6**) and diphenyl diselenide (**2a**) are shown in Fig. 1. When these compounds, 100 μM and 50 μM, respectively, were treated with GSH as described above, benzeneselenolate was formed as indicated by the rapid build-up of absorbance at 271 nm. The apparent initial and maximal rates of selenolate formation and maximal selenolate concentrations under the conditions of the experiments were as follows: compound **6** $v_{init} = v_{max}$=22μM·min^{-1}, c_{max}=40μM; compound **2a** $v_{init}=v_{max}$=7.2μM·min^{-1}, c_{max} =48μM. As determined from the build-up of absorbance at 271 nm, benzeneselenolate is formed 2-3 times more rapidly from ketone **4b** than from ketone **4a**. On the other hand, the formation of benzeneselenolate from



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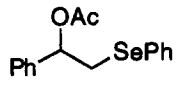

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compound **4f** was considerably faster(Fig. 3).

As determined by UV spectroscopy, benzeneselenolate was quantitatively oxidized by hydrogen peroxide to give diphenyl diselenide (k_{app} = 6.6 x 10^{-3} μM^{-1} · min^{-1}). Other organoselenium compounds investigated (compounds **2a**, **4a**, **4b**, **6**) did not react with hydrogen peroxide to any significant extent during the period of assay as determined either by UV-spectroscopy or hydrogen peroxide removal.[7] By using the

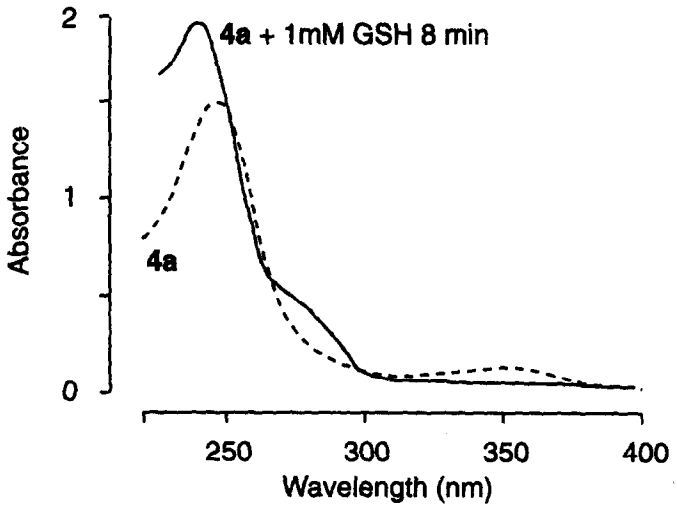


Figure 2. Absorption spectra of a solution of 100 μM α-(phenylselenenyl) acetophenone (**4a**) before and 8 minutes after the addition of 1 mM GSH in 50 mM potassium phosphate, pH 6.5, 50% EtOH.

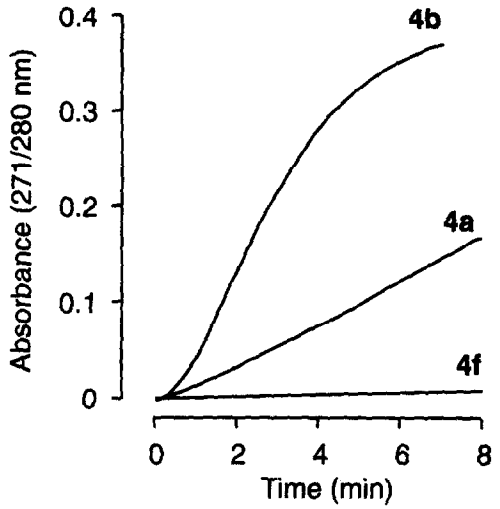


Figure 3. Increase in absorbance at 280*/271 nm for α-(phenylselenenyl) acetophenone* (**4a**), α-(phenylselenenyl)-4-nitroacetophenone (**4b**) and α-(phenylselenenyl) pinacolone (**4f**) (all 100 μM) on treatment with excess GSH (1 mM) in 50 mM potassium phosphate, pH 6.5, 50% EtOH.

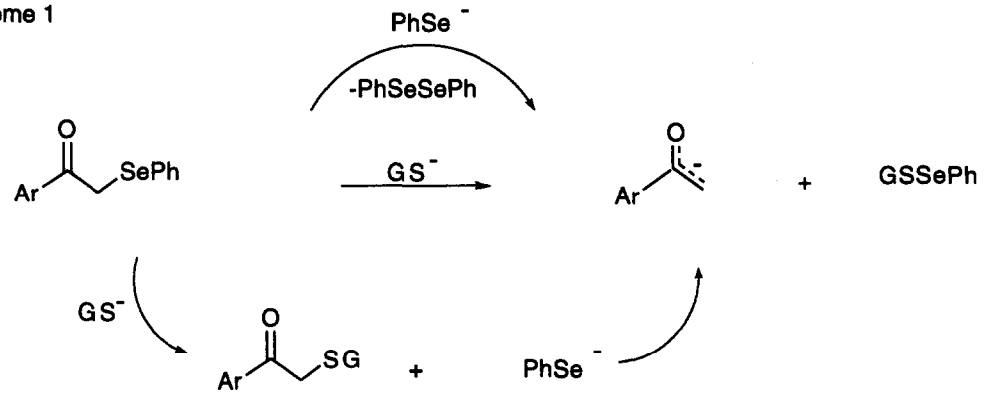
apparent rate constants for air- and H_2O_2-stimulated oxidation of benzeneselenolate, it can be calculated that the latter reaction at 100 μM H_2O_2 is approximately 100 times faster than the former .

DISCUSSION

By using the coupled GSSG reductase assay, we have previously demonstrated the catalysis by α-(phenylselenenyl) ketones of the reduction of hydrogen peroxide and other organic peroxides using GSH as reductant.[7] An electron withdrawing substituent in the acetophenone moiety (compound **4b**) was shown to potentiate the catalysis whereas a mesomerically electron donating substituent (compound **4e**) decreased the catalytic activity of the parent compound. Similarly, substitution of the acetophenone aryl group for alkyls (compounds **4f** and **4g**) or reduction/acetylation of the carbonyl group (compound **8**) caused a decrease in the catalytic activity of the compounds. Several mechanisms were proposed to account for the observed catalysis. The data presented here provide evidence for the involvement of a selenolate in the glutathione peroxidase-mimetic activity of α-(phenylselenenyl) ketones.

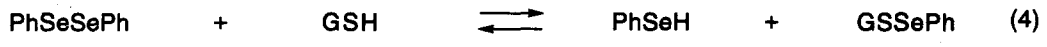
The facile reduction of selenide **4b** under mild reaction conditions in the presence of only one equiv. of GSH at pH 6.9 seems to indicate that the nucleophilic thiolate ion of the tripeptide attacks the α-(phenylselenenyl) ketone on selenium, with the ketone enolate serving as a leaving group (Scheme 1). This

Scheme 1



mechanistic view is also in accord with the relative reactivities of compounds **4a**, **4b** and **4g** (the reactivity increases with increasing enolate stability). However, an alternative mechanism,[15] involving thiolate attack on carbon, followed by rapid selenolate attack on sulfur can not be excluded. Also, benzeneselenolate, generated as indicated in eq. 3, may contribute to reduce the α-(phenylselenenyl) ketone (Scheme 1).

The reduced and O-acetylated derivative **8** of α-(phenylselenenyl)acetophenone, which does not contain an enolate leaving group, was unchanged after treatment with GSH for 1h under our standard conditions. The compound showed essentially no glutathione peroxidase-like activity by using the coupled reductase assay.[7] The formation of benzeneselenolate in the reactions of selenides **4a**, **4b** and selenosulfide **6** with glutathione was shown by UV-spectroscopy, a technique previously used to show the transient formation of a selenolate in the glutathione peroxidase-like catalysis of Ebselen.[10-11] Also, diphenyl diselenide was found to readily form benzeneselenolate in the presence of GSH. This shows that the equilibrium reaction depicted in eq 4 is readily

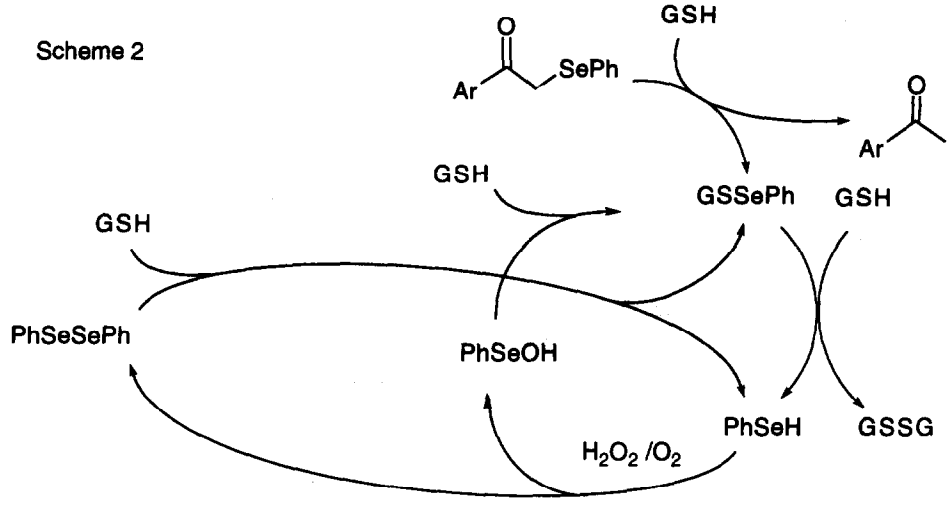


established. According to eq. 4, benzeneselenolate is also suggested as a crucial intermediate in the glutathione peroxidase-like catalysis of diphenyl diselenide.[4] Indeed, it can be proposed that the mechanisms of catalysis of α-(phenylselenenyl) ketones and diphenyl diselenide are identical, once the aliphatic carbon-selenium bonds of the former are cleaved.

Of the investigated compounds(**2a**, **4a**, **5** and **6**), only benzeneselenolate showed an appreciable reactivity towards hydrogen peroxide. This is in contrast to the situation with Ebselen where the corresponding selenol and diselenide intermediates were both reactive towards hydrogen peroxide.

Based on the above observations, the catalytic cycle shown in Scheme 2 was formulated. As indicated, the α-(phenylselenenyl) ketones serve as "procatalysts". Once they are decomposed, a catalytic cycle

Scheme 2



involving S-(phenylselenenyl)glutathione, benzeneselenolate and benzeneselenenic acid is responsible for the reduction of hydrogen peroxide with GSH serving as the stoichiometric reducing agent. For diphenyl diselenide, the cycle is entered via equilibrium reaction 4. This protocol is similar to those proposed to account for the glutathione peroxidase-like activities of Ebselen,[11] glutathione peroxidase,[16] selenosubtilisin[9] and diaryl ditellurides.[5] There are probably additional reactions which, to some extent, serve to interconvert the compounds shown in Scheme 2. For example, benzeneselenolate could be expected to react with α-(phenylselenenyl) ketones or S-(phenylselenenyl)glutathione to give diphenyl diselenide.

According to the proposed mechanism, the extent of carbon selenium bond-cleavage in the α-(phenylselenenyl) ketone moiety would be limiting for the glutathione peroxidase-like activity of the catalyst. The observed reactivities of compounds **4a**, **4b** and **4g** towards GSH in an inert atmosphere seem to parallel the glutathione peroxidase-like activity as determined by the coupled reducatase assay (**4b** > **4a** > **4g**). Also, by monitoring selenol formation spectrophotometrically under aerobic conditions, a similar order of reactivity was observed (**4b** > **4a** > **4f**). Concerning the enzymatic assay, a word of caution must be added. Since this procedure involves premixing of NADPH, GSH, catalyst and GSSG reductase before the peroxide is added,[7]

erratic results may be obtained if the time of incubation of the catalyst with GSH is not kept constant from one experiment to another.

In conclusion, the data presented above indicate that the glutathione peroxidase-like activity of α-(phenylselenenyl) ketones is due to cleavage of the aliphatic carbon-selenium bond of the catalyst. The catalytic cycle responsible for reduction of hydrogen peroxide is likely to involve benzeneselenolate, S-(phenylselenenyl)glutathione and benzeneselenenic acid as crucial intermediates. The catalytic mechanisms are proposed to be identical for α-(phenylselenenyl) ketones and diphenyl diselenide.

EXPERIMENTAL SECTION

Melting points (uncorrected) were determined by using a Büchi 510 melting point apparatus. ^{1}H NMR spectra were obtained with a Bruker AC-F250 instrument and recorded in $CDCl_3$ or D_2O solutions. Reduced glutathione, diphenyl diselenide and phenylselenenyl bromide were obtained from Aldrich. α-(Phenylselenenyl) ketones **4**,[7] and selenide **8**[7] were prepared as previously described. 2,4-Dinitrophenyl phenyl selenide, mp 130-31 °C(lit.[17] 129-30°C) was prepared from 2,4-dinitrochlorobenzene and benzeneselenolate in analogy with a literature procedure.[18]

*Reaction of α-(Phenylselenenyl)acetophenone (**4a**) with Glutathione. Typical Procedure:* To a stirred solution of α-(phenylselenenyl)acetophenone (0.025 g, 0.091 mmol) in methanol (20 mL) under argon was added a solution of glutathione (0.084 g, 0.27 mmol) in a 0.1 M phosphate buffer (20 mL). The reaction mixture soon turned light yellow and then heterogeneous as diphenyl diselenide started to precipitate. TLC analysis after 4 min showed that all starting material was consumed. After dilution with water (50 mL), extraction with CH_2Cl_2 (2 x 50 mL), drying ($CaCl_2$), evaporation and flash chromatography (SiO_2; CH_2Cl_2/hexanes = 1/1) 0.014 g diphenyl diselenide (99%) and 0.007 g acetophenone (64%) were isolated. Both compounds were compared with authentic samples.

α-Phenylselenenyl ketone **4b** similarly afforded diphenyl diselenide (99%) and 4-nitroacetophenone (87%), mp 80-1 °C (lit.[19] 80-1 °C).

α-Phenylselenenyl ketone **4d** similarly afforded diphenyl diselenide (99%) and 2-acetonaphthone (91%), mp 54-5 °C (lit.[20] 54 °C).

α-Phenylselenenyl ketone **4g** similarly afforded diphenyl diselenide (92%). No attempt was made to isolate acetone in this reaction.

Selenide **8** was unreactive towards glutathione after 1 h under the conditions of the typical procedure.

When only one equivalent (0.028 g) of glutathione was allowed to react with selenide **4b** for 15 min under the conditions of the typical procedure, diphenyl diselenide (49%) and 4-nitroacetophenone (100%) were isolated.

Assessment of Relative Reactivity of α-(Phenylselenenyl)Ketones towards Glutathione. To a stirred solution of selenides **4a** (0.025 g, 0.091 mmol), **4g** (0.019 g, 0.091 mmol) and 4-bromoanisol (0.018 g, 0.096 mmol) in methanol (20 mL) under argon was added glutathione (0.028 g, 0.091 mmol) in a 0.1 M phosphate buffer (20 mL). Work-up (15 min later as described above in the typical procedure) and ^{1}H NMR analysis of the crude

product showed that 85% and 15%, respectively, of compounds **4a** and **4g** were reduced in the reaction with glutathione.

In a similar competitive experiment, selenide **4b** was found to react ≈7 times faster than selenide **4a**.

S-(Phenylselenenyl)glutathione (**6**):To a stirred solution of phenylselenenyl bromide (0.38 g, 1.6 mmol) in dry dimethylformamide (2 mL) at 0 °C was added glutathione (0.50 g, 1.6 mmol). The ice-bath was then removed and stirring continued for 30 min to give a light-yellow solution. Evaporation of the solvent (ambient temperature, 10^{-2} mm Hg), afforded a semi-solid which was first triturated with methylene chloride (5 mL) and then with water (5 mL) to give light-yellow crystals of the title compound. Yield 0.28 g (38%). The analytical sample, m.p. 189-91 °C, was obtained by recrystallization from water/ethanol. Anal. Calcd for $C_{16}H_{21}N_3O_6SSe$: C, 41.56; H, 4.58. Found: C, 41.56; H, 4.44. ^1H NMR (D_2O) δ 1.93-1.99 (several peaks, 2H), 2.23-2.32 (several peaks, 2H), 3.07 (dd, 1H J = 10.0 and 14.4 Hz), 3.33 (dd, 1H J = 4.3 and 14.4 Hz), 3.64 (t, 1H), 3.78 (s, 2H), 4.45 (m, 1H), 7.30-7.33 (several peaks, 3H), 7.64-7.68 (m, 2H).

When compound **6** was treated with glutathione for 15 min as described in the typical procedure, diphenyl diselenide (90%) was isolated. If glutathione was omitted in the reaction, a considerably lower yield (16%) of diphenyl diselenide was obtained.

Spectrophotometric characterization of benzeneselenolate. Benzeneselenolate was prepared from diphenyl diselenide by a method previously described.[10,11] Thus, diphenyl diselenide was reduced with excess sodium borohydride in ethanol to give a solution of benzeneselenolate. The excess borohydride was destroyed with HCl(0.1 M; added dropwise). Aliquots were then added to an ethanol/ phosphate buffer, pH 6.5 =1/1 and the UV spectrum recorded. Upon addition of increasing amounts of benzeneselenolate to 2,4-dinitrochlorobenzene(200 μM in the same buffer), the characteristic absorbtion spectrum of 2,4-dinitrophenyl phenyl selenide appeared. The apparent rate of air-oxidation of benzeneselenolate was determined by following the decrease in absoption with time at 271 nm.

*Benzeneselenolate from compounds **2a**, **4a**, **4b**, **4f**, and **6**.* The rates of benzeneselenolate formation from diphenyl diselenide (25 μM), ketones **4a** (50 μM), **4b** (50 μM), **4f** (50μM) and S-(phenylselenenyl) glutathione (50 μM) on treatment with GSH (1 mM) were followed spectrophotometrically at ambient temperature at 271, 280, 271, 271 and 271 nm, respectively, in ethanol/100 mM phosphate buffer, pH 6.5 = 1/1.

*Reactivity of H_2O_2 towards compounds **2a**, **4a**, **5** and **6**.* The reactions of compounds **2a**, **4a**, **5** (generated *in situ* from diphenyl diselenide as described above) and **6** (10–100 μM in EtOH/100 mM phosphate buffer pH 6.5 = 1/1) at ambient temperature with hydrogen peroxide (1 mM) were monitored spectrophotometrically as well as by a method for peroxide consumption.[7]

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