Development of Synthetic Compounds with Glutathione Peroxidase Activity

Stephen R. Wilson,[†] Paul A. Zucker,[†] Ruey-Ruey C. Huang,[‡] and Abraham Spector*[‡]

Contribution from the Department of Chemistry, New York University, Washington Square, New York, New York 10003, and the Biochemistry and Molecular Biology Laboratory, Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York, New York 10032. *Received July 28, 1988*

Abstract: Glutathione peroxidase is the major defense in the lens of the eye against hydrogen peroxide. The damage caused by hydrogen peroxide has been implicated in the formation of cataract. Organoselenium compounds which show catalytic activity in the enzyme assay for the selenoenzyme glutathione peroxidase have been prepared. These compounds were designed on the basis of the known chemistry of models for the active site of glutathione peroxidase. Diphenyl diselenide (**16**) has been shown to be approximately 2-fold more active in this assay then the most active compound previously known. Introduction of tertiary amine substituents onto the aromatic nucleus at a position ortho to the selenium atom in compounds **19** and **25** results in a further approximately 5-fold increase in activity. Diaryl selenides **26** and **27** are demonstrated to show no significant glutathione peroxidase activity.

Glutathione peroxidase is a selenoenzyme composed of four identical subunits of 21 000 Da which catalyzes the reduction of H_2O_2 and other hydroperoxides.[1,2] In many tissues, such as the lens of the eye, glutathione peroxidase is the major defense against hydroperoxides.[3] Glutathione [GSH; γGlu-Cys-Gly (**1**)] is utilized as a cofactor, supplying the electrons for the reductive reaction

$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG \qquad (1)$$

The X-ray crystal structure of GSH peroxidase has been determined with 0.2-nm resolution. The results from such analyses, as well as biochemical data, have been used to develop a reaction mechanism for the reaction.[2] The mechanism is shown in Scheme I. In this scheme, the selenium atom goes from a selenol (E–Se–H) to a selenenic acid (E–SeOH). [In the presence of high concentrations of peroxide, it can be further oxidized to a seleninic acid (E–SeOOH).] The scheme illustrates the two-step reduction of selenenic acid utilizing 2 mol of GSH/mol of enzyme.

Earlier work in this laboratory, directed toward the development of compounds having GSH peroxidase activity, met with only limited success.[4] This work was based on the design of small molecules which would mimic the structure of the active site of the enzyme containing the essential selenocystine residue. The most active compound, **2**, produced in this study proved to be only 0.033 as active as Ebselen (**3**), the most active compound previously known.[5]

Recently, a report by Reich and Jasperse[6] described the oxidation–reduction chemistry of compound **4** (Scheme II). The results of this study suggest that two mechanistic pathways (A and B) are possible for the catalysis of **4** of the reduction of hydroperoxides by thiols. Especially interesting from a mechanistic viewpoint were the following observations:

(1) Under acid catalysis, the selenenamide **4** equilibrates with seleninamide **8** and diselenide **7**. These compounds are produced by disproportionation of the selenenamide **4**.

(2) Oxidation of **6** with MCPBA gave first diselenide **7** and then the seleninamide **8**.

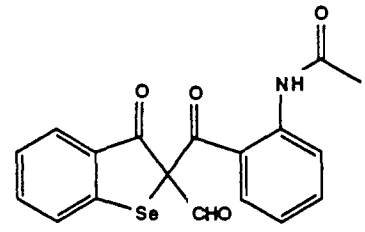
(3) Oxidation of **4** gave the seleninamide **8** and was faster than oxidation of the diselenide.

(4) Under weakly acidic conditions, treatment of **4** with thiols gave the selenosulfides **5** and disulfide.

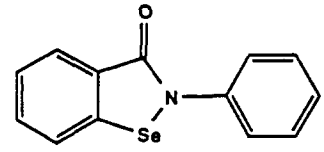
(5) The selenosulfide **5** and diselenide **7** did not react with thiol under neutral conditions. Upon addition of a strong amine base (DBU), however, both gave the selenolate and disulfide.

H_2N-γGLU-CYS-GLY-OH

1



2



3

These results suggested some guidelines for the construction of molecules showing GSH activity:

(1) More easily available diselenides would function as effectively as the more difficult to construct cyclic compounds in the production of the catalytically active species (observations 1–4 above).

(2) Inclusion in the molecule of a strongly basic group proximal to the active selenium atom is desirable as it would be expected to catalyze the reaction of thiols with the intermediate diselenide and selenosulfide. Presumably, the base functions to provide a source of nucleophilic thiolate anion (observation 5 above).

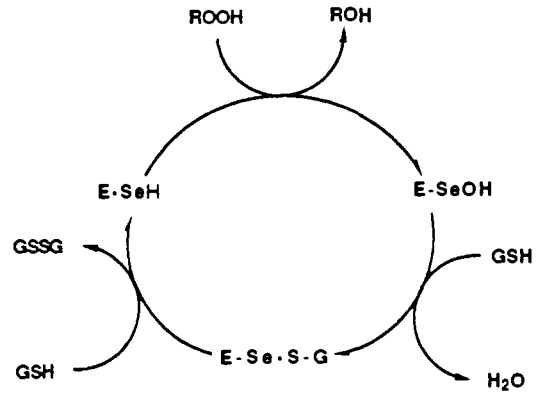
With these guidelines in mind, we choose as compounds for study tertiary amines of the type **13** (Scheme III). The use of tertiary amines seemed preferable to that of primary or secondary amines since the intermediates **10** and **14** are not stable compounds and are thus activated toward nucleophilic attack by thiol at the selenium atom.

[†] New York University.
[‡] Columbia University.
* Address correspondence to this author.

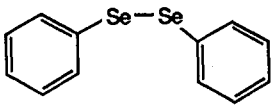
(1) Chance, B.; Boveris, A.; Nakase, Y.; Sies, H. *Functions of Glutathione in Liver and Kidney*; Springer-Verlag: Berlin, 1978; pp 95–106.
(2) Epp, O.; Ladenstein, R.; Wendel, A. *Eur. J. Biochem.* **1983**, *133*, 51–69.
(3) Spector, A. *Invest. Ophthal. Vis. Sci.* **1984**, *25*, 130–146.
(4) Unpublished observations.
(5) Wendel, A. European Patent 0,165,534, 1985.
(6) Reich, N. J.; Jasperse, C. P. *J. Am. Chem. Soc.* **1987**, *109*, 5549–5551.

0002-7863/89/1511-5936$01.50/0

Scheme I

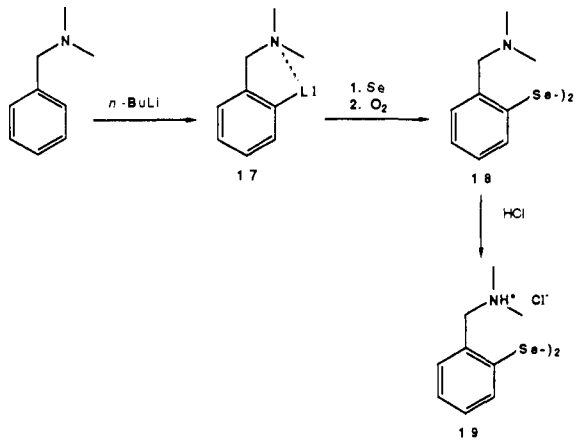


Synthesis of Amines. Diphenyl diselenide (**16**) was obtained from Aldrich and used without further purification.



16

Diaminodiselenide **18** was synthesized in two steps from commercially available materials (eq 1). Ortho-metalation of *N,N*-dimethylbenzylamine was readily accomplished according to the procedure of Klein and Hauser.[7] Thus, *N,N*-dimethylbenzylamine was treated with a slight excess of *n*-butyllithium in ether–hexane at room temperature for 24 h and the resulting solution of aryllithium **17** treated with metallic selenium. The resulting arylselenol was oxidized with air to the corresponding diselenide.[8] The oily product was most easily handled as its bis(hydrochloride salt) **19**.

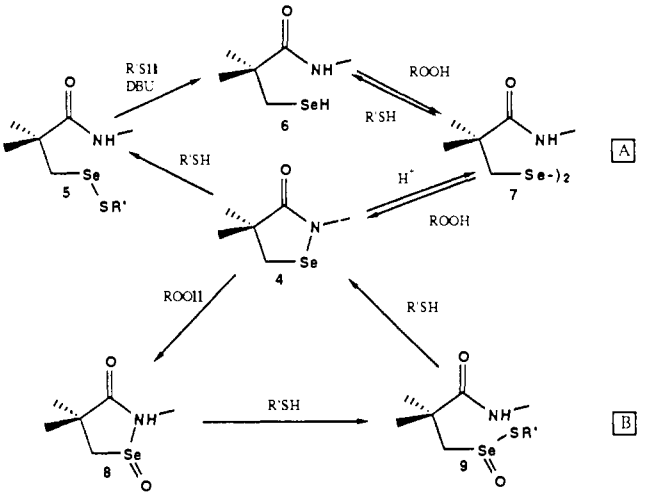


The amine **22**, required for the synthesis of **24**, was readily prepared by alkylation of pyrrolidone (**20**) with 2-bromobenzyl bromide (**21**). The corresponding Grignard reagent failed to form upon treatment of **22** with magnesium. Halogen–metal exchange with 2 equiv of *n*-butyllithium at −78 °C in THF proceeded smoothly to give the desired aryllithium **23**. This reagent readily reacted with metallic selenium in the presence of anhydrous $MgCl_2$ to give the desired diselenide **24**. As in the case of **18**, the diselenide was converted for ease of handling into the bis(hydrochloride salt) **25**.

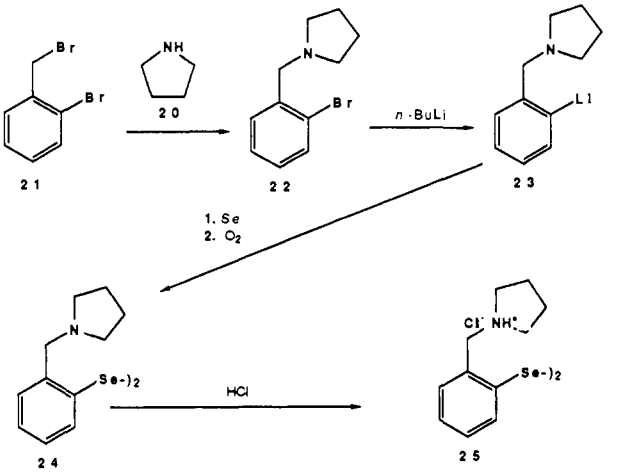
The synthesis of the diaryl selenide **26** was accomplished in a straightforward manner by the reaction of aryllithium **17** with diphenyl diselenide. Diaryl selenide **26** proved to be much easier

(7) Klein, K. P.; Hauser, C. R. *J. Org. Chem.* **1967**, *32*, 1479–1483.
(8) Gould, E. S.; McCullough, J. D. *J. Am. Chem. Soc.* **1951**, *73*, 1109–1112.

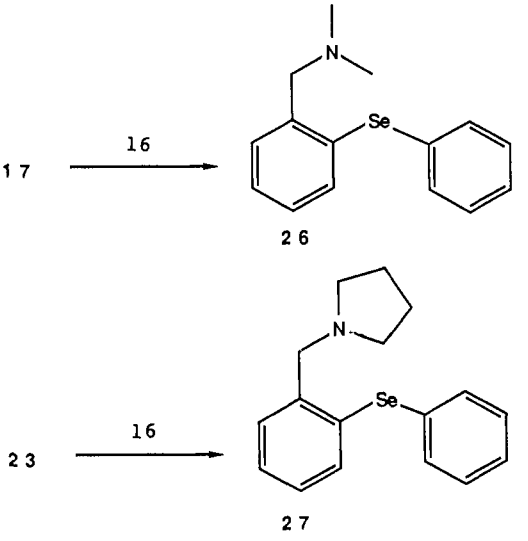
Scheme II



to handle than the corresponding diselenides and could thus be purified by chromatography.



The synthesis of the diaryl selenide **27** was achieved by the reaction of aryllithium **23** with diphenyl diselenide.



Results and Discussion

Glutathione peroxidase activity of the compounds was determined with a modification of the method of Wendel[9] using hydrogen peroxide as the substrate in the presence of GSH. Glutathione reductase was used to reduce the oxidized GSH with

(9) Wendel, A. *Methods Enzymol.* **1981**, *77*, 325–333.

Scheme III

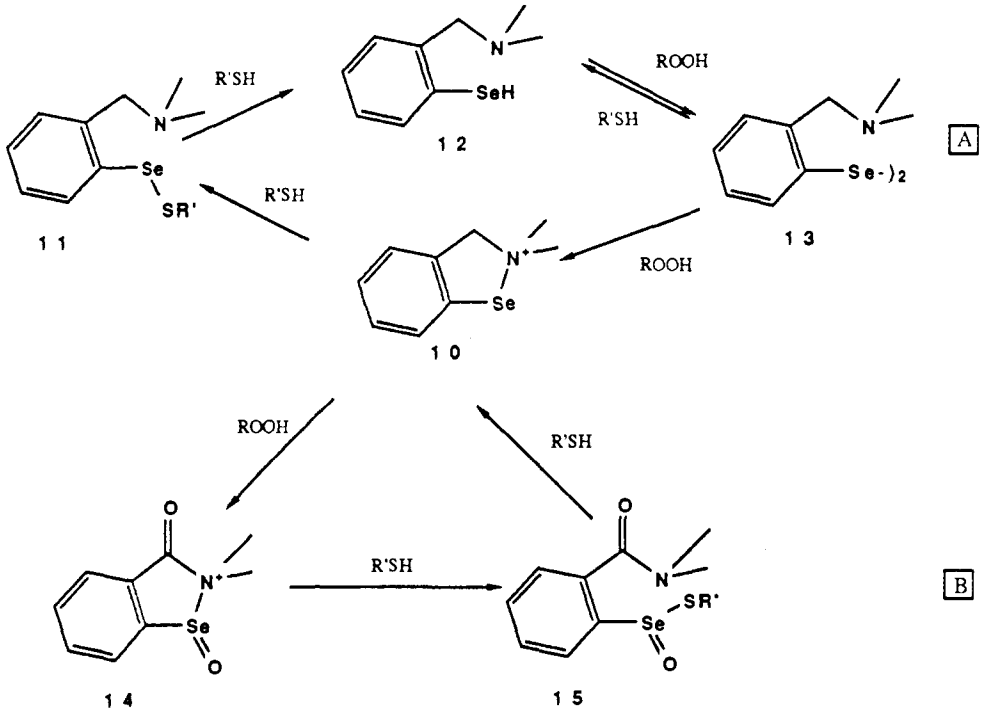


Table I

compd	act.[a]	rel GSH peroxidase act.[b]
2	0.034	0.03
3	0.99	0.88
16	1.95	1.7
26	0.050	0.043
27	0.046	0.04
19	10.5	9.1
25	11.1	9.7

[a] Micromoles of NADPH utilized per minute per micromole. [b] Based upon GSH peroxidase activity equal to 10000.

NADPH as a cofactor (eq 2). The decrease in NADPH monitored spectrophotometrically at 366 nm is a measure of GSH peroxidase activity (see eq 2–4). The assay mixture (700 μL)

$$2GSH + H_2O_2 \xrightarrow[\text{peroxidase}]{GSH} GSSG + 2H_2O \quad (2)$$

$$GSSG + NADPH + H^+ \xrightarrow[\text{reductase}]{GSH} 2GSH + NADP^+ \quad (3)$$

$$H^+ + NADPH + H_2O_2 \rightarrow NADP^+ + 2H_2O \quad (4)$$

contains 50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM sodium azide, 1 mM GSH, 0.25 mM NADPH, 1 unit of GSSG reductase, and an appropriate amount of test compound (usually 2–20 μM final concentration). The absorbance at 366 nm was recorded for a few minutes to estimate the background and stability of the preparation. Reaction was initiated by the subsequent addition of hydrogen peroxide at concentrations up to 500 μM. Appropriate blanks were run in the absence of test compound and in some cases with test compound and H_2O_2 in the absence of NADPH. The results are shown in Table I. The relative activities of the compounds prepared in this study are compared to that of GSH peroxidase. Values for Ebselen (compound **3**) and compound **2** are included.

For most compounds, the initial rate of NADPH utilization was determined at six concentrations ranging over more than a 10-fold difference in concentration. The rates in all cases were found to be a linear function of concentration, suggesting that the compounds were saturated with substrate. Under such conditions, the initial rates can be assumed to be maximum rates and $V_{max} = kE_T$, where V = maximum velocity of the reaction and E_T represents the total concentration of the GSH peroxidase analogue. The k value represents the turnover number, i.e., the number of H_2O_2 molecules degraded per minute per molecule of analogue. These values are shown in Table I. It was found that the reaction rates remain linear for at least 5 min. Thus, for example, for compound **25**, 55.5 molecules of H_2O_2 are degraded per molecule of compound without any loss of catalytic activity. No indication of loss of catalytic activity was observed. While these turnover numbers are very low compared to that of GSH peroxidase, they are in the same range as have been reported for enzymes such as lysozyme (30 μmol μmol^{-1} min^{-1}).

Several conclusions can be drawn from these results. The observation that diphenyl diselenide (**16**) exhibits approximately 2 times the activity of Ebselen rules out the assumption that a selenium–nitrogen bond is necessary for glutathione peroxidase activity. This conclusion was tentatively suggested by the activity found in compound **2**. It is also consistent with the observations of Reich and Jasperse[6] since one could reasonably propose that the catalytic cycle A (Scheme II) completely bypasses the selenenamide **4** with nucleophilic attack of thiolate occurring on the diselenide **7**. Support of this view is found in the observation that oxidation of **6** led to the formation of **7** and not **4**.[6]

Also clear from the results in the table is the requirement that the disubstituted selenium atom have at least one selenium–heteroatom bond. The diaryl selenides **26** and **27** show small amounts of activity. This activity may be due to trace amounts of diphenyl diselenide present in the compounds as impurities remaining from their syntheses.

Compounds **19** and **25**, the tertiary amine analogues of **16**, are approximately 5.5-fold more active than **16**. The role of the tertiary amine in the catalysis of the reaction may be that which was suggested earlier. The amine may serve to deprotonate the thiol sulfhydryl group and thus provide a high local concentration of nucleophilic thiolate anion. Another possible mode of catalysis might be that the conjugate acid of the amine, the ammonium ion, obtained upon abstraction of a proton from a sulfhydryl group serves as a proton source, facilitating the reduction of hydrogen peroxide to water.

The most active compounds, **19** and **25**, are approximately 10 times more active than Ebelson (**3**) and indicate that it is possible to produce relatively simple compounds that have significant GSH peroxidase activity.

Experimental Section

All reactions were carried out under a nitrogen atmosphere. Melting points were determined in a Thomas capillary melting point apparatus

and are uncorrected. PMR spectra were recorded on a General Electric QE-300 Spectrometer using the specified solvent. GC–MS analyses were obtained on a Hewlett-Packard 5992B GC/MS system equipped with a capillary column. Exact masses were obtained from the NIH Rockefeller Mass Spectrometry Biotechnology Resource at Rockefeller University, New York, NY, by positive chemical ionization (CI). Gas chromatographic (GC) analyses were performed on a Varian Model 3700 gas chromatograph (flame ionization detector) using $^1/_8$-in. columns packed with 2% OV-101–0.2% Carbowax on Chromasorb G. The standard temperature program is 60 °C (1 min) up to 300 °C at 20 °C/min.

[2-[(*N*,*N*-Dimethylamino)methyl]phenyl]lithium (17). *n*-Butyllithium (0.2 mol, 80 mL of a 2.5 M solution in hexanes) was placed in a round-bottomed flask under nitrogen. *N*,*N*-Dimethylbenzylamine (13.5 g, 0.1 mol) and 215 mL of dry ether were added, and the resulting solution was allowed to stir at room temperature for 20 h. This was used as the stock solution of **17** and was approximately 0.34 M in **17**.

2,2′-Diselenobis[[(*N*,*N*-dimethylamino)methyl]benzene] Bis(hydrochloride salt) (19). Under a nitrogen atmosphere, 2.0 g (25.3 mmol) of metallic selenium was added to 40 mL (13.6 mmol) of the stock solution of **17** and the mixture stirred at room temperature for 1 h. The resulting mixture (slightly green) was quenched by the addition of water and ether followed by bubbling air through it for 1 h. Layers were separated, and the aqueous phase was extracted with several portions of ether. The combined ethereal phases were washed with water and extracted with a 6 M HCl, solution. The combined extracts were concentrated in vacuo to give an oily residue, **18**. This oil solidified upon trituration with ethanol–benzene to give bis(hydrochloride salt) **19** as an orange powder. Precipitation from ethanol–benzene provided 621 mg (21.4%) of yellow powder (darkening at 219 °C, mp 224–225 °C dec): GC retention time (free base) = 8.56 min; TLC (free base, silica gel, 20% ethyl acetate–ligroin) R_f = 0.35; ^{1}H NMR (300 MHz, D_2O) δ 7.73 (d, 1 H, 4.2 Hz), 7.45 (m, 2 H), 7.36 (m, 1 H), 3.98 (s, 2 H), 2.67 (s, 6 H); HRMS (from free base/Cl) $(C_{18}H_{25}N_2Se_2)H^+$ 429.0222 [calculated for $(C_{18}H_{25}N_2Se_2)H^+$ 429.0348].

1-Bromo-2-(pyrrolidin-1-ylmethyl)benzene (22). 2-Bromobenzyl bromide (**21**) (3.0 g, 12 mmol) was dissolved in 5 mL of methylene chloride, and 2 equiv (22.4 mmol, 2.2 mL) of pyrrolidine (**20**) was added dropwise. The initial reaction was exothermic. After the addition was complete, the mixture was allowed to stir at room temperature overnight; it was then poured into water and ether, the layers were separated, and the ether layer was washed with water, dried (KOH), and concentrated to a yellow oil. Distillation provided 2.346 g (81%) of the amine **22** boiling at 104–107 °C (20 mmHg): GC retention time = 3.92 min; TLC (silica gel, 20% ethyl acetate–ligroin) R_f = 0.39; IR (neat) 2950, 2780, 1600, 1450, 1430, 1025, 750; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.54 (m, 2 H), 7.28 (m, 1 H), 7.09 (m, 1 H), 3.74 (s, 2 H), 2.60 (m, 4 H), 1.81 (m, 4 H); GC–MS (% abundance) 241 (70), 240 (100), 239 (71), 238 (93), 171 (43), 169 (45), 84 (57), 70 (20), 42 (43).

2,2′-Diselenobis[(pyrrolidin-1-ylmethyl)benzene] Bis(hydrochloride salt) (25). The bromide **22** (480 mg, 2 mmol) was dissolved in 4 mL of dry THF and the resulting solution cooled to −78 °C. *n*-Butyllithium (2.0 mL of a 2 M solution in hexanes, 2 equiv) was added dropwise via syringe. After the addition was complete, the mixture was stirred at −78 °C for 1 h. Selenium (2 equiv, 4 mmol, 316 mg) was added followed by anhydrous $MgBr_2$ (4 mmol). This mixture was allowed to stir overnight. Selenium (2 portions of 35 mg each) was added, and the mixture turned dark red. The mixture was again stirred overnight. The mixture was then poured into water and ether, the ether layer was separated, and the aqueous phase was extracted with ether. The combined ethereal extracts were dried (Na_2SO_4) and concentrated to give an oily residue. The residue was taken up in 6 M HCl solution and the solution washed with methylene chloride several times. The aqueous solution was then concentrated in vacuo to give a resinous residue (432 mg). This material was dissolved in the minimum amount of ethanol, benzene was added, and the compound was allowed to precipitate. This procedure provided 250 mg (45%) of **25** as a yellow powder (darkening at 216 °C, mp 229–230 °C dec): GC retention time (free base) = 10.12 min; TLC (free base, silica gel, 20% ethyl acetate–ligroin) R_f = 0.21; ^{1}H NMR (300 MHz, D_2O) δ 7.73 (d, 1 H, 4.2 Hz), 7.45 (m, 2 H), 7.36 (m, 1 H), 4.09 (s, 2 H), 3.30 (m, 2 H), 2.95 (m, 2 H), 1.99 (m, 2 H), 1.83 (m, 2 H); HRMS (from free base/Cl) $(C_{22}H_{28}N_2Se_2)H^+$ 481.0661 [calculated for $(C_{22}H_{28}N_2Se_2)H^+$ 481.0654].

[[2-[(*N*,*N*-Dimethylamino)methyl]phenyl]seleno]benzene (26). The aryllithium reagent **17** (20 mL 6.6 mmol) solution was placed in a flask under nitrogen. Diphenyl diselenide (2.375 g, 7.6 mmol) in 20 mL of dry ether was added dropwise over the course of 5 min. The resulting solution was allowed to stir at room temperature for 1 h. The solution was diluted with ether, washed well with water, dried (Na_2SO_4), and concentrated. The yellow residual oil was distilled (Kugelrohr, 0.5 Torr) at 145–155 °C (yield 1.302 g, 68%). Material obtained in this way contained a small amount of diphenyl diselenide. This impurity could be removed by purification by preparative TLC on silica gel eluting with 20% ethyl acetate–ligroin containing 1% triethylamine (R_f = 0.38). In this way 145 mg of distilled material gave 101 mg of purified **26** as a tan, mobile oil. Upon standing at room temperature, the oil solidified to a waxy, tan solid (mp 48 °C): GC retention time = 5.88 min; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.56–7.0 (m, 9 H), 3.52 (s, 2 H), 2.24 (s, 6 H); GC–MS (% abundance) 291 (82), 276 (30), 165 (52), 132 (59), 121 (100), 91 (32), 58 (57).

[[2-(Pyrrolidin-1-ylmethyl)phenyl]seleno]benzene (27). The bromide **22** (240 mg, 1 mmol) was dissolved in 3 mL of dry THF and the resulting solution cooled to −78 °C under nitrogen. *n*-Butyllithium (2 equiv, 2 mmol, 1 mL of a 2.0 M solution in hexanes) was added dropwise via syringe and the mixture stirred for an additional hour at −78 °C after the addition was complete. Diphenyl diselenide (1 equiv, 1 mmol, 212 mg) In 5 mL of THF was added dropwise and the mixture stirred overnight. A few drops of saturated ammonium chloride solution were added, salts were filtered, and the solution was concentrated to give the crude **27**. Purification by preparative TLC on silica gel, eluting with 20% ethyl acetate–ligroin containing 1% triethylamine (R_f = 0.42), gave pure **27** (104 mg, 33%): GC retention time = 7.12 min; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.56–7.0 (m, 9 H), 3.75 (s, 2 H), 2.52 (m, 4 H), 1.77 (m, 4 H).

Acknowledgment. We acknowledge the National Institutes of Health for supporting this work (NEI EY00423, A.S.; GM-29259, S.R.W.). P.A.Z. thanks Alcon Laboratories, Inc., and the NYU University/Industry fund for fellowship support.

Registry No. 2, 119747-35-2; **3**, 60940-34-3; **16**, 1666-13-3; **17**, 27171-81-9; **18**, 119747-36-3; **19**, 119747-37-4; **20**, 123-75-1; **21**, 3433-80-5; **22**, 91130-46-0; **24**, 119747-38-5; **25**, 119747-39-6; **26**, 119747-40-9; **27**, 119747-41-0; glutathione peroxidase, 9013-66-5; *N*,*N*-dimethylbenzylamine, 103-83-3; selenium, 7782-49-2.