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Synthesis of a New Seleninic Acid Anhydride and Mechanistic Studies into Its Glutathione Peroxidase Activity

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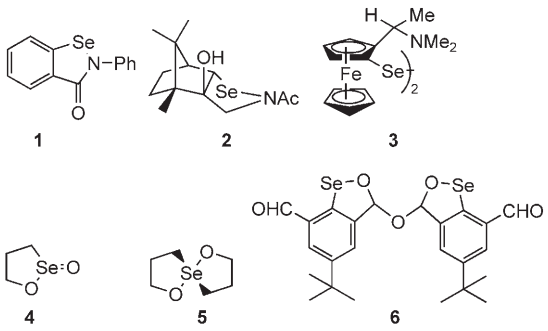
Abstract: Starting from low toxic salicyloylglycine, a new seleninic acid anhydride **7** that lacks Se···N or Se···O non-bonded interactions was synthesized. This compound exhibits a fourfold higher glutathione peroxidase-like (GPx-like) activity than ebselen and inhibits plant and mammalian 12/15-lipoxygenases at lower micromolar concentrations. Because of these pharmacological properties, **7** may constitute a new lead compound for the development of anti-inflammatory low-molecular-weight seleno-organic compounds. Analyzing the redox products of **7** with glutathione (GSH) and *t*BuOOH, we identified three potential catalytic cycles (**A**, **B**, **C**) of GPx-like activity that are interconnected by key metabolites. To study the relative contribution of these cycles to the catalytic activity, we prepared selected reaction intermediates and found that the activity of seleninic acid anhydride **7** and of the corresponding diselenide **11** and selenol **14** compounds were in the same range. In contrast, the GPx-like activity of monoselenide **9** was more than one order of magnitude lower. These data suggested that cycles **A** and **B** may constitute the major routes of GPx-like activity of **7**, whereas cycle **C** may not significantly contribute to catalysis.

Keywords: enzymes · inflammation · reaction mechanism · redox reactions · selenium

Introduction

Selenium is an essential trace element that plays an important role in the metabolism of pro- and eukaryotic cells.[1,2] It constitutes a functional element of selenium-containing enzymes, such as glutathione peroxidase (GPx), iodothyronine deiodinase (ID) and thioredoxin reductase (TrxR), which have been implicated in antioxidative defense, iodine homeostasis, and regulation of gene expression.[1,2] Selenium-containing GPx-isoforms are members of the antioxidative network in mammalian cells capable of detoxifying peroxides at the expense of reduced glutathione, but they also exhibit more specific functions.[2,3] Because of the beneficial activities of GPx-isoforms, nonenzymatic GPx mimics have been developed as potential drugs and the seleno-organic compound ebselen (**1**) has been well characterized with respect to its pharmacological profile.[4] In addition, there were numerous attempts to develop other seleno GPx mimics, such as ebselen analogues,[5,6] camphor-derived selenamides (**2**),[7] dicyclo-dextrinyl diselenides,[8] diferrocenyl diselenides (**3**),[9] cyclic seleninate esters (**4**),[10] spirodioxaselenanonane (**5**),[11] and cyclic selenenate ester (**6**).[12] Seleno-organic compounds with dendrimer cores[13] exhibit a high GPx-like activity, but owing to their low water solubility biological applications may be limited.



For the catalytic activity of most of the seleno GPx mimics, Se···N or Se···O non-bonded interactions are re-

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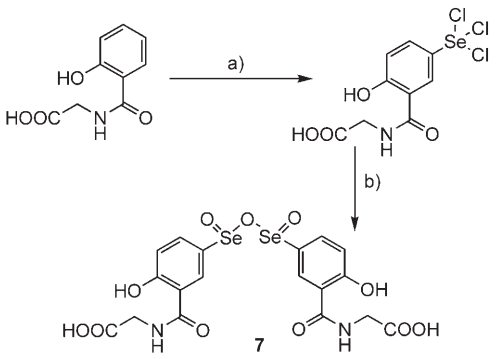
quired and these interactions may be established by reductive cleavage of Se–N or Se–O bonds.[14] Seleninic acid anhydrides form a well-characterized family of stable selenium-containing compounds. They contain covalent Se–O bonds, which cannot be converted to Se···O non-bonded interactions when reacting with glutathione (GSH). Thus, direct GPx-like activity is rather unlikely. On the other hand, seleninic acid anhydrides can be reduced to the corresponding diselenides,[15] selenenic acids, or selenol derivatives, which have been reported before to function as GPx mimics. Thus, seleninic acid anhydrides were expected to exhibit GPx-like activity, but the reaction mechanisms might differ from those of other seleno-organic compounds.

Results and Discussion

We have synthesized the novel seleno-organic compound salicyloylglycine seleninic acid anhydride (**7**) and explored the complex mechanism of its GPx-like activity. The salicyloylglycine moiety was selected as the substituent for two reasons: 1) It exhibits a low in vivo toxicity and 2) the anti-inflammatory properties of ebselene analogues were improved when amino acid derived functional groups were introduced.[16]

Salicyloylglycine seleninic acid anhydride (**7**) was synthesized (Scheme 1) by reacting salicyloylglycine with selenium tetrachloride in THF at room temperature for 1 h. The



Scheme 1. a) $SeCl_4$, THF, RT, 1 h; b) H_2O, RT, 18 h.

major reaction product was precipitated by addition of an excess of water and the precipitate was backwashed with THF. The final reaction product was purified by silica-gel column chromatography and the analytical data (elementary analysis, selenium content, ^1H-, ^{13}C-, and ^{77}Se NMR spectroscopy, HRMS, UV/Vis spectroscopy, and RP-HPLC) indicated its chemical structure.

To compare the GPx-like activity of **7** with that of the standard seleno-organic compound ebselen, the catalytic activities of the two substances were assayed spectrophotometrically.[17] Under our experimental conditions, **7** exhibited a more than fourfold higher catalytic activity than ebselen ((9.98 ± 0.50) for **7** versus (2.40 ± 0.30) μM min^{-1} for ebselen). When normalized to the selenium content, a twofold higher activity was determined. Moreover, we found that **7** inhibited mammalian ($IC_{50} = 8.6$ for **7** versus 0.56 μM for ebselen) and plant 15-lipoxygenases ($IC_{50} = 9.3$ for **7** versus 7.3 μM for ebselen), which suggests potential anti-inflammatory activities (Figure 1).

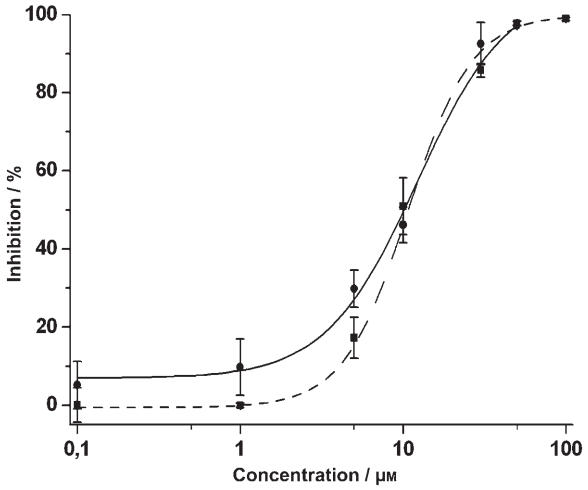


Figure 1. Inhibition of the soybean LOX I by **7** (-----) and ebselen (——). The extent of enzyme inhibition (%) calculated for the different inhibitor concentrations is plotted against the logarithm of the inhibitor concentration

Since **7** constitutes a seleninic acid derivative, the catalytic mechanism was expected to be different from that of ebselen and other seleno-organic compounds, in which selenium is present as monoselenide, diselenide, or selenamide. To explore the sequence of reactions involved in the catalytic cycle(s), **7** was reacted at a concentration of 10 mM with different amounts of reductant. To mimic biological conditions, the reaction was carried out in 0.1 M phosphate buffer (pH 7.4) at physiological salt concentrations and reduced GSH was employed as a reducing agent. The reaction products were quantified by RP-HPLC (Table 1) and their chemical structures were confirmed by selenium analysis, MS, and ^{77}Se NMR spectroscopy after a 10 min reaction period. When **7** was incubated with GSH at an equivalent ratio of 1:0.5, most (96%) of the starting material **7** was recovered.

Table 1. Product composition [%][a] of the GSH-dependent reduction of **7**.

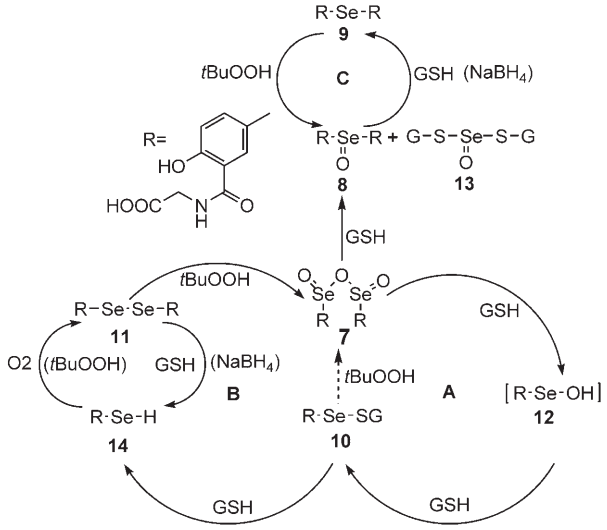
Ratio **7**/GSH	**7**	**8**	**9**	**10**	**11**	**13**
1:0.5	96	1	1	1	1	–
1:1	41	11	7	40	1	–
1:2	1	–	18	77	5	–
1:3	–	–	25	37	24	15
1:4	–	–	22	29	31	18
1:5	–	–	22	30	30	18
1:20	–	–	24	38	24	13

[a] Determined on the basis of areas of RP-HPLC peaks (absorbance at 240 nm) considering the different molar extinction coefficients.

Only small amounts of the corresponding monoselenoxide **8**, monoselenide **9**, selenenyl sulphide **10**, and diselenide **11** derivatives were identified by RP-HPLC. When we increased the GSH concentration to an equivalent ratio of 1:1, the product mixture still contained large amounts of **7** (41%) and the major reaction product was identified as the corresponding selenenyl sulphide derivative **10**. Formation of this product was confirmed by ^{77}Se NMR spectroscopy and the ratio of the signal intensities at $\delta = 1147.65$ (**7**) and 467.36 ppm (**10**) was 1.0:1.4. In addition, smaller amounts of the corresponding monoselenoxide **8** and monoselenide **9** derivatives were detected by RP-HPLC and MS (see the Supporting Information).

When the GSH concentration was increased to an 1:2 equivalent ratio, we observed complete conversion of the starting material **7**. In this case, we identified selenenyl sulphide **10** as the major reaction product and these data were confirmed by the appearance of a highly abundant signal at $\delta = 466.84$ ppm in the ^{77}Se NMR spectrum. In addition, smaller amounts of monoselenide **9** and diselenide **11** derivatives (see the Supporting Information) were detected. In contrast, the corresponding monoselenoxide **8** was not detectable any more. Under these experimental conditions, we also detected small amounts of the selenenic acid derivative **12** by MS as indicated by the molecular ion at m/z: 289.5129. When **7** was incubated with three equivalents of GSH monoselenide **9**, selenenyl sulphide **10**, and diselenide **11** derivatives were identified as major reaction products by RP-HPLC (see the Supporting Information). Under these conditions, we also isolated an HPLC fraction containing diglutathione selenoxide **13** as indicated by the molecular ion at m/z: 704.6 in the mass spectrum. A previous report[15] on the reduction of phenyl seleninic acid with three equivalents of reduced thiols suggested the formation of 1 mol of the corresponding phenyl selenenyl sulphide, which was then slowly converted to the diphenyl diselenide derivative. Under our experimental conditions, the selenenyl sulphide derivative **10** was quickly formed even at a 1:1 ratio of the reactants (**7** and GSH) and its relative abundance was increased to a 1:2 ratio. The corresponding diselenide **11** was also present in the reaction mixtures (1:2 and 1:3) and we did not see any slow time-dependent increase in its relative abundance. When we further increased the concentration of the reductant in the reaction mixture (1:4 to 1:20), we did not observe major alterations in product composition (see the Supporting Information). In these samples, the selenenyl sulphide **10** and diselenide **11** derivatives were identified as major reaction products, whereas the corresponding monoselenide **9** and diglutathione selenoxide **13** were found in smaller amounts. Taken together, the results shown in Table 1 indicate that the salicyloylglycine derivatives of monoselenoxide **8** and of monoselenide **9** are preferentially formed at lower GSH concentrations. However, when the thiol content was increased, corresponding selenenyl sulphide **10**, diselenide **11**, and diglutathione selenoxide **13** derivatives became more abundant. Under these experimental conditions, we still observed significant amounts of the monoselenide **9**, whereas the corresponding monoselenoxide **8** was hardly detectable any more.

After we had identified the reduction products of **7** at various GSH concentrations, we attempted to close the catalytic cycle by oxidizing the major reaction products **9**, **10**, and **11** by addition of peroxide. For this purpose, we first added *t*BuOOH (final concentration 64 mM) to a 1:2 reaction mixture of **7** and GSH, in which monoselenide **9** and selenenyl sulphide **10** were the major reaction products. Analyzing the product mixture 1 h after addition of *t*BuOOH, we observed significant formation of salicyloylglycine seleninic acid anhydride (**7**) as indicated by RP-HPLC and ^{77}Se NMR spectroscopy (signal at $\delta = 1148.07$ ppm). The signal at $\delta = 466.12$ ppm in the spectrum was due to unconverted selenenylsulfide **10**, which also showed up in RP-HPLC (see the Supporting Information). In addition, significant amounts of the corresponding monoselenoxide **8** were found, whereas the monoselenide derivative **9** had disappeared. It should be stressed that neither **7** nor **8** were detectable in the reaction mixture before *t*BuOOH was added (Table 1). Oxidation of selenenyl sulfide **10** by *t*BuOOH under our experimental conditions appears to be a slow process since after 1 h only about 30% of **10** was converted to **7** (catalytic cycle **A** in Scheme 2). However, after 36 h we observed complete oxidation of **10**. In contrast, the monoselenide derivative **9** was quickly converted to monoselenoxide **8** (catalytic cycle **C** in Scheme 2) as indicated by the complete disappearance of **9** after a 1 h reaction period. Addition of *t*BuOOH to the mixture of reduction products of **7** closed the catalytic cycle of the GPx-like activity of **7**. To initiate the next cycle, we added an excess of reduced glutathione (final concentration 130 mM) to the mixture of oxidation products and observed the formation of **9**, **10**, and **11** (see the Supporting Information). Taken together, our data suggests the existence of three catalytic subcycles (**A**, **B**, and **C** in Scheme 2) for the



Scheme 2. Potential catalytic cycles of the GPx-like catalytic activity of **7**.

GPx-like activity of **7**, which are interconnected by the key metabolites **7**, **8**, and **10**.

Selenol derivatives of seleno-organic compounds have previously been implicated in GPx-like reactions,[18,19] but we did not obtain major evidence for the formation of salicyloylglycine selenol **14** when the GPx-like reaction was carried out in the presence of oxygen (Table 1). However, when **7** was reduced by a molar excess of $NaBH_4$ and the reaction mixture was kept under an argon atmosphere, we observed dominant formation of selenol **14** (Figure 2). This

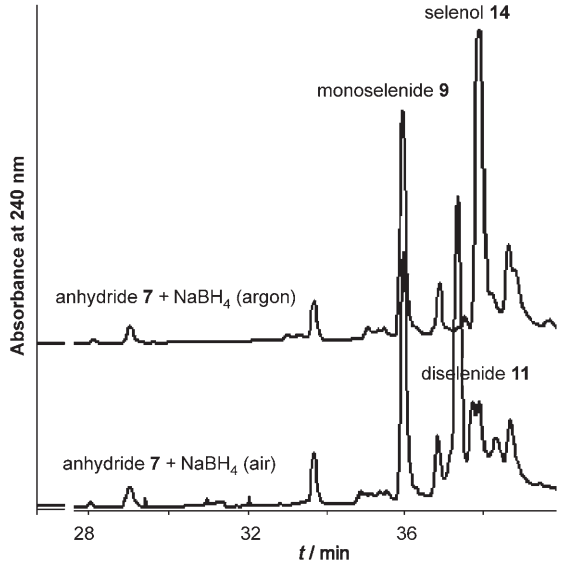


Figure 2. Partial RP-HPLC chromatograms of reduction products formed from **7** after addition of an excess of $NaBH_4$. After reduction, the sample was kept under argon (prevention of re-oxygenation of the sample) for about 16 h and thereafter under a normal atmosphere for 7 h.

result was confirmed by ^{77}Se NMR spectroscopic measurements, in which we detected strong signals at $\delta = 12.71$ and 14.17 ppm. When the reaction mixture was kept under argon for up to 16 h (Figure 2), only small amounts of the corresponding diselenide **11** were found. In contrast, after re-equilibration with air or after addition of *t*BuOOH, the selenol **14** was completely oxidized to the corresponding diselenide **11**. This oxidation was confirmed by the appearance of a signal at $\delta = 521.08$ ppm in the ^{77}Se NMR spectrum.

Seleninic acid anhydride **7** is partly converted at low GSH concentrations to the corresponding monoselenoxide **8** as indicated in Table 1. We hypothesized that this compound may be further reduced to the monoselenide **9**, which can subsequently be oxidized by *t*BuOOH to yield **8**. This reversible redox reaction (cycle **C**) between salicyloylglycine monoselenoxide **8** and the corresponding monoselenide **9** has not been implicated before in GPx-like activity of seleno-organic compounds. To obtain direct experimental evidence for the existence of that redox cycle, we followed the interconversion of chemically synthesized **8** and **9** by RP-HPLC. We found that the pure monoselenoxide derivative **8** was rapidly reduced to the corresponding monoselenide **9** when reacted with $NaBH_4$ or GSH. Inversely, the monoselenide derivative **9** is oxidized to the corresponding monoselenoxide **8** when reacted with an excess *t*BuOOH (see the Supporting Information). Thus, in principle, cycle **C** may be involved in the GPx-like activity of **7**.

After having identified the major reaction products formed during the interaction of salicyloylglycine seleninic acid anhydride **7** with GSH and *t*BuOOH, we aimed at investigating, which of these chemical entities might be involved in GPx-like activity. To do so, we first prepared selected key metabolites (monoselenide **9**, diselenide **11**, selenol **14**) and compared their GPx-like activities with that of the anhydride **7**. For this purpose, **7** was reduced with a 10-mollar excess of $NaBH_4$ under aerobic or anaerobic conditions and the intermediates were isolated by RP-HPLC. To quantify the different compounds, we determined the selenium content of the HPLC fractions by atomic absorption spectrometry and we found that the GPx-like activities of **7**, **11**, and **14** were in the same range (Table 2), whereas that of

Table 2. GPx-like activities of reaction intermediates.[a]

Catalyst	v [μM min^{-1}]
7	9.98 ± 0.50
9	0.24 ± 0.05
11	5.46 ± 0.63
14	9.69 ± 0.90

[a] Each measurement has been carried out in triplicate; a catalyst concentration of 5 μM was adjusted.

the monoselenide **9** was much lower. Unfortunately, we did not manage to isolate pure selenyl sulphide **10** since it partially decomposes to the corresponding diselenide **11** during the preparation procedure. Taken together, these data suggest that the catalytic activity of **7** involves cycles **A** and **B**, whereas cycle **C** may not play a major role.

Despite our quantitative data on the relative catalytic activities of major reaction intermediates, it was not possible to reliably quantify the relative contribution of the different catalytic subcycles **A**, **B**, and **C** to overall GPx-like activity of **7**. When we quantified by HPLC the metabolite pattern during the time course of the GPx-like reaction (data not shown) we mainly detected metabolites **10** and **11**. In addition, small amounts of **8** were found suggesting minor importance of subcycle **C**. These data are consistent with the low catalytic efficiency of **9** (Table 2). On the other hand, our observation that oxidation of **10** by *t*BuOOH yielding **7** was rather slow may point towards a concerted action of cycles **A** and **B** and thus, catalysis may involve selenenic acid **12**, selenyl sulfide **10**, and selenol **14**, which have previously been implicated in the catalytic cascade of seleno-organic compounds. It should, however, be stressed that steady-state metabolite concentrations and catalytic efficiencies per se do not mirror flux rates along the different catalytic subcycles. To answer these questions, detailed kinetic experimentation and mathematic modeling must be performed.

Conclusion

Salicyloylglycine seleninic acid anhydride **7** is a novel seleno-organic compound that exhibits GPx-like activity in the absence of Se···N or Se···O non-bonded interactions. Its catalytic activity is fourfold higher than that of ebselen and it inhibits lipoxygenases at lower micromolar concentrations. The catalytic mechanism is more complex than that of previously investigated seleno-organic compounds and three potential catalytic subcycles have been identified. Unfortunately, in vivo activities of seleninic acid anhydrides have never been studied in detail and little is known about their pharmacokinetics and systemic toxicology. However, the simple synthetic procedure and the biochemical properties of **7** are promising.

Experimental Section

General information: Commercial reagents salicyloylglycine (o-hydroxy hippuric acid) (Merck) and selenium tetrachloride (Sigma-Aldrich) were used as received. All solvents and reagents used were of extra pure grade and purchased from Merck, Aldrich, or Roth (Germany). IR spectra were recorded on a Nicolet Magma IR 750. Kinetic measurements were performed on a Shimadzu UV-2102 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Brucker Biospin AV 400 (^{1}H: 400 MHz; ^{13}C: 100.5 MHz) by using [D$_6$]DMSO as a solvent. Chemical shift values (δ) are reported in ppm downfield from TMS ($\delta = 0.0$ ppm) as internal standard. Data are reported as follows: chemical shift (δ/ppm), multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), integration, coupling constant (Hz). ^{77}Se NMR spectra were recorded by using a JEOL JNMLA 400 instrument (76.20 MHz) in [D$_6$]DMSO-dimethylselenide as external standard. HPLC analysis was carried out on a Shimadzu LC-10Avp liquid chromatograph connected to SPD-10Advp UV detector. RP-HPLC analysis was performed on a Nucleosil C18-column; 250/4 mm, 5 μm particle size (Machery-Nagel, Düren, Germany) by using a linear gradient of MeOH in a water/MeOH solvent system (from 5 to 100 %) with a flow rate of 1 mL min^{-1}. HRMS was carried out on a Finnigan LTQST NSI.

Synthesis of salicyloylglycine seleninic acid anhydride (7): Selenium tetrachloride (1.107 g, 5 mmol) was added to a solution of salicyloylglycine (0.975 g, 5 mmol) in THF (10 mL) and the mixture was stirred for 1 h at room temperature. After this time, water (100 mL) was added to the reaction mixture and the sample was stirred at room temperature for an additional 18 h. The white precipitate was filtered, back-washed with THF (3 × 30 mL), and air-dried at 30 °C to give salicyloylglycine seleninic acid anhydride (**7**). Yield: 0.939 g (1.58 mmol); m.p. 175–178 °C (decomp.); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 3.99$ (d, $J = 5.8$ Hz, 4H; CH$_2$), 7.11 (d, $J = 8.0$ Hz, 2H; 3-H), 7.81 (dd, $J = 10.0$ Hz, 2H; 4-H), 8.32 (d, $J = 2.0$ Hz, 2H; 6-H), 9.21 (t, $J = 11.0$ Hz, 2H; NH), 12.63 ppm (s, 2H; COOH); ^{13}C NMR (100.5 MHz [D$_6$]DMSO): $\delta = 041.31$ (CH$_2$), 116.41 (1-C), 117.96 (3-C), 127.31 (6-C), 131.17 (4-C), 139.01 (5-C), 161.54 (2-C), 167.22 (C=O), 170.95 ppm (COOH); ^{77}Se NMR (76.20 MHz): $\delta = 1182.08$ ([D$_6$]DMSO), 1150.08 ppm (NaOH); IR (KBr): 3303, 3071, 2925, 2706, 2577, 1715, 1599, 1551, 1426, 1300, 1249, 1067, 831, 671, 545 cm^{-1}; UV/Vis (0.1 N NaOH): $\lambda_{max} = 227.20$, 263.80 nm; NSI-MS: m/z: calcd for $C_{18}H_{15}N_2O_{11}{}^{80}Se_2$: 594.9012 [$M^+ - H$]; found: 594.9009; elemental analysis calcd (%) for $C_{18}H_{15}N_2O_{11}Se_2$: C 36.38, H 2.71, N 4.71; found: C 36.51, H 2.34, N 4.45.

Preparation of monoselenide 9 and diselenide 11: An excess of $NaBH_4$ (0.1 mmol) was added to a stirred solution of **7** (5.90 mg, 0.01 mmol) in 0.1 M phosphate buffer (pH 7.4). The mixture was vigorously mixed for 36 h under air, acidified with 0.1 mL AcOH (5 M) to pH 4.0, and purified by RP-HPLC to yield **11** and **9**.

*Data for **9***: Yield: 1.42 mg (31 %); ^{77}Se NMR (76.20 MHz, [D$_6$]DMSO): $\delta = 466.84.08$ ppm; HPLC: $t_R = 35.8$ min; NSI-MS: m/z: calcd for $C_{18}H_{16}N_2O_8{}^{80}Se$: 467.2882 [$M^+ - H$]; found: 467.0012.

*Data for **11***: Yield: 2.05 mg (38 %); ^{77}Se NMR (76.20 MHz, [D$_6$]DMSO): $\delta = 521.08$ ppm; HPLC: $t_R = 37.3$ min.

Preparation of selenol 14: An excess of $NaBH_4$ (0.1 mmol) was added to a solution of **7** (5.9 mg, 0.01 mmol) in 0.1 M phosphate buffer (pH 7.4). The mixture was kept under argon for 1 h and then **14** was purified by RP-HPLC. The HPLC solvent was evaporated in vacuum and the solid residue was reconstituted in DMSO under an argon atmosphere. White solid; yield: 2.13 mg (78 %); HPLC: $t_R = 37.8$ min; ^{77}Se NMR (76.20 MHz, [D$_6$]DMSO): $\delta = 12.71$, 14.17 ppm.

Measurements of glutathione peroxidase (PPx-like) acitivity: The glutathione peroxidase activity (GPx-like activity) of the test compounds (ebselen, **7**, **9**, **11**, **14**) was assayed spectrophotometrically by measuring the decrease in absorbance at 340 nm (coupled optical test). The catalytic reaction was run at 37 °C in a 1 mL reaction mixture consisting of 100 mM Tris-HCl buffer (pH 7.4) containing 5 mM ethylenediaminetetraacetic acid, 0.1 % Triton X-100, 3 mM GSH, 0.2 mM NADPH (nicotinamide adenine dinucleotide phosphate), 1 U of glutathione reductase, and 5 μM of the test compound. The assay sample was equilibrated for 10 min in the absence of peroxide substrate and the GPx-like reaction was initiated by addition of 0.5 mM *t*BuOOH. The time-dependent decrease in absorbance at 340 nm (10 to 70 s after the addition of *t*BuOOH) was recorded by using a Shimazu U-2102 PC UV/VIS spectrophotometer. During the GPx-like reaction reduced GSH is oxidized and the resulting disulfide (GSSG) is back-reduced by the glutathione reductase reaction consuming stoichiometric amounts NADPH. Since NADPH exhibits a local absorbance maximum at 340 nm, but its oxidized counterpart (NADP) does not, NADPH oxidation can be quantified by measuring the decrease in absorbance at 340 nm. A blank assay (solvent control) was run in the absence of catalysts and this control rate was subtracted. The rate of NADPH oxidation was calculated by using a molar absorbance coefficient for NADPH of 6.22×10^3 M^{-1} cm^{-1}. Measurements were carried out in triplicate and ebselen was used as a reference compound. The test compounds were added as DMSO solutions at a final concentration of 5 μM.

Catalytic mechanism of the GPx-like activity of salicyloylglycine seleninic acid anhydride (7): To investigate the catalytic mechanism of the GPx-like activity of salicyloylglycine seleninic acid anhydride (**7**), we first analyzed the composition of the reduction products of **7** formed during the reaction with different amounts of reduced GSH. Next, selected key metabolites were prepared and re-oxidized with *t*BuOOH. Reaction products were analyzed by RP-HPLC on a Shimadzu LC10 HPLC system that was connected to a SPD-M19AVP diode array detector. Analytes were resolved on a CC 250/4 Nucleosil 120–5 C18 column (Macherey and Nagel, Düren, Germany) with a linear water/methanol gradient containing 0.1 % acetic acid. For analysis, the column was equilibrated for 15 min with a water/methanol mixture (95:5, by volume) containing 0.1 % acetic acid. Aliquots (20–50 μL) of the reaction mixtures were injected and the reaction products were separated by the following analytical profile: 5 min isocratic elution at a water/methanol ratio of 95:5 (0.1 % acetic acid). This pre-elution phase was followed by a linear gradient elution of increasing methanol concentration starting at 5 % and reaching 100 % after 30 min (0.1 % acetic acid). This gradient elution phase was followed by a 10 min isocratic elution at 100 % methanol (0.1 % acetic acid). To start the next analysis, the column was re-equilibrated at starting conditions (5 % methanol in water containing 0.1 % acetic acid). Major eluting compounds were identified by retention times in RP-HPLC, by their UV-spectral properties (diode array detector) and NSI-MS.

Inhibition of 15-lipoxygenase isoforms: The pure rabbit reticulocyte 12/15-lipoxygenase (2 μL of a 1.7 mg mL^{-1} enzyme solution) or soybean LOX-1 (Merck, 10 μM, 20 μL) were preincubated with the inhibitors (final concentration ranging between 0.05–100 μM) either at 22 °C in 0.1 M phosphate buffer (pH 7.4) or at 37 °C in 0.1 M borate buffer (pH 9.0) for 5 min. The reaction was started by the addition of linoleic acid as the substrate (20 μL of a methanolic stock solution, 100 μM final concentra-

tion, assay volume 1 mL). The inhibitors were dissolved in DMSO. The catalytic activity of the enzyme was assayed spectrophotometrically following the increase in absorbance at 235 nm over a time interval of 30 s.

Acknowledgements

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