Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 4623–4630

BIOORGANIC & MEDICINAL CHEMISTRY

Design, Synthesis and Glutathione Peroxidase-Like Properties of Ovothiol-Derived Diselenides

Fabrice Bailly,[a,*] Nathalie Azaroual[b] and Jean-Luc Bernier[a]

[a]*Laboratoire de Chimie Organique Macromoléculaire, CNRS UMR 8009, USTL, Bâtiment C3, UST Lille I, 59655 Villeneuve d'Ascq, France*

[b]*Laboratoire de RMN, Faculté de Pharmacie, CNRS UMR 8009, BP83, 59006 Lille Cedex, France*

Received 7 February 2003; accepted 25 July 2003

Abstract—Eleven imidazole diselenides derived from the naturally occurring family of antioxidants, the ovothiols, were synthetised by cyclisation of selenoamides with trimethylsilyltrifluoromethanesulfonate or refluxing of cyanoamines in a selenium/sodium borohydride mixture. These compounds were assayed for their glutathione peroxidase-like (GSH Px-like) activity and their capacity to be reduced by glutathione. The most active molecules of the series were 4 times more potent in the GSH Px-like test than the widely known reference compound, ebselen. This catalytic activity was mediated by the formation of the antioxidant selenol intermediate upon partial but significant exchange reaction with glutathione.

Introduction

For several years, there has been an intensive search for the use of organoselenium compounds in enzymology and bioorganic chemistry. The development of these novel compounds was based on the knowledge of the various biological functions of selenium,[1,2] on the synthetic effort leading to the discovery of new useful reactions and on the pharmacological evaluation of the synthetised compounds.[3–5] The principal role of selenium in mammals was established as its implication in the catalytic site of the antioxidant enzyme glutathione peroxidase (GPx). The enzyme catalytic site includes a selenocysteine residue in which the selenium undergoes a redox cycle involving the selenolate anion as the active form which reduces hydroperoxides.[6,7] This led to the development of organoselenium compounds designed to mimic this enzymatic activity in vitro. They can be used for the treatment of a certain number of pathologies in which an over-production of cytotoxic hydroperoxides contributes to the functional impairments of cells or tissues and more particularly for the treatment of atherosclerosis, inflammatory and/or ischaemic cardiovascular, cerebrovascular, digestive, respiratory and ophtalmic pathologies.[8–10]

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) was the first compound suggested for hydroperoxide-inactivating therapy in the presence of glutathione.[11,12] Then several attempts have been made to prepare simple synthetic organoselenium compounds with glutathione peroxidase-like activity like ebselen, which include ebselen analogues,[13] benzoselenazolinones,[14] selenamides,[15] diaryldiselenides[14,16–21,43] and the semi-synthetic selenosubtilisin.[22] The activities of various selenides like diarylselenides,[23] α-(phenylselenyl) ketones[24] and more recently 3-hydroxypropylselenide[25] were also investigated. Two excellent reviews described the chemical and pharmacological aspects of these organoselenium compounds.[26,27] All these researches led to the obtention of compounds with enzymatic activities much higher than that of the original ebselen.

Recently, we reported the synthesis[28] and antioxidant properties of 4-mercaptoimidazoles derived from the naturally occurring ovothiols (Fig. 1).[28,29] These compounds were shown to be powerful scavengers of hydrogen peroxide, hydroxyl radicals and hypochlorous acid and to inhibit copper-induced LDL peroxidation.[29] Thiyl radicals were characterised upon radical scavenging.[30] Protection against peroxynitrite-induced damage and glutathione peroxidase-like activity were also evidenced.[31] According to the in vitro experiments, these aromatic heterothiols were shown to be largely better antioxidants than the aliphatic thiol, glutathione.

*Corresponding author. Tel.: +33-3-2033-7231; fax: +33-3-2033-6309; e-mail: fabrice.bailly@univ-lille1.fr

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doi:10.1016/S0968-0896(03)00504-2

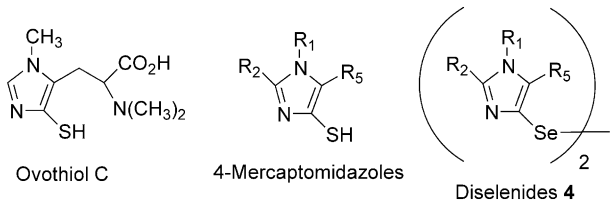


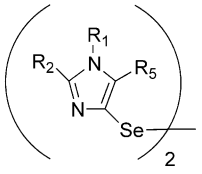
Figure 1. Structures of ovothiol C, 4-mercaptoimidazoles and imidazole diselenides.

In continuation of this work, we present in this paper the synthesis of the organoselenium analogues obtained as the diselenides **4** (Fig. 1) and their ability to catalyse decomposition of hydrogen peroxide in the presence of glutathione. The glutathione peroxidase-like activity was assessed in vitro by means of the NADPH–glutathione reductase system. Exchange reactions with glutathione of these new derivatives were also investigated by means of two tests: measurement of glutathione disulphide (GSSG) formed via the NADPH–glutathione reductase system and measurement of selenol formed via scavenging by the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH).

Results

Chemistry

The synthesis of aromatic diselenides **4a–k** (Fig. 2) was accomplished by a method similar to that developed by Spaltenstein et al. for the synthesis of 4-mercaptoimidazoles[28,32] as shown in Scheme 1. Strecker condensations gave the aminonitriles **1**. Transformation of amines in amides and conversion of nitriles to selenoamides gave the precursors **3**. For the third step, two reactions were performed: to produce hydrogen selenide: hydrolysis of aluminium selenide[33,34] and reaction of selenium with sodium borohydride (molar ratio 3:2) in ethanol.[35] Owing to the well-known instability of primary alkyl selenoamides when compared to the aromatic ones,[36,37] the precursors **3** could not be conveniently isolated and purified. Attempts to isolate the selenoamides **3** by extractive workup and silica gel chromatography led to a major recovery of the nitrile forms **2** by hydrogen selenide evolving as judged by thin-layer chromatography (TLC). The selenoamides **3** were identified on TLC plates as strongly absorbing spots under UV light and as orange spots after iodine revelation.



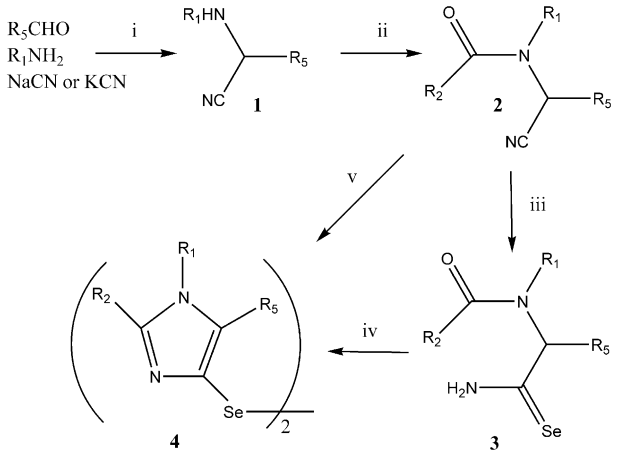
4a: R_1 = p-C_6H_4-OCH_3, R_2 = H, R_5 = H
4b: R_1 = m-C_6H_4-Cl, R_2 = H, R_5 = H
4c: R_1 = p-C_6H_4-Cl, R_2 = H, R_5 = H
4d: R_1 = m-C_6H_4-CF_3, R_2 = H, R_5 = H
4e: R_1 = p-C_6H_4-CF_3, R_2 = H, R_5 = H
4f: R_1 = CH_3, R_2 = H, R_5 = p-C_6H_4-OCH_3
4g: R_1 = CH_3, R_2 = H, R_5 = o-C_6H_4-Cl
4h: R_1 = CH_3, R_2 = H, R_5 = p-C_6H_4-Cl
4i: R_1 = CH_3, R_2 = H, R_5 = o-C_6H_4-CF_3
4j: R_1 = CH_3, R_2 = H, R_5 = p-C_6H_4-CF_3
4k: R_1 = CH_3, R_2 = m-C_6H_4-Cl, R_5 = H

Figure 2. Structures of the imidazole diselenides **4a–k**.

Thus, in the first method (method A), hydrogen selenide generated in situ by hydrolysis of aluminium selenide was bubbled into a solution of the precursors **2** and triethylamine in dichloromethane until there was not any significant evolution of the solution content as judged by TLC. Then the cyclisation step (step iv) was rapidly performed by treating at −10 °C the selenoamides **3** with 6 equiv of triethylamine (TEA) and 4 equiv of trimethylsilyltrifluoromethanesulfonate (Me_3SiOTf).

In the second method, the precursors **2** were treated at room temperature with a 2:3 molar ratio of sodium borohydride to selenium in ethanol. The crude product obtained after concentration in vacuo and filtration of the solid residues was used as well without further purification for the next cyclisation step.

A serendipituous observation led to an alternative method (step v). A by-product formed during the reaction of sodium borohydride with selenium in presence of precursors **2**, which was identified by NMR of the crude product as the cyclised diselenides **4**. It seemed that the initial heat of the reaction between selenium and sodium borohydride provoked the cyclisation of the selenoamides in the mixture. This was corroborated by the fact that the proportion of cyclised product was strongly diminished when the reaction was conducted at 0 °C. Thus, slight heating at reflux under the experimental conditions led to the progressive cyclisation of a major part of the selenoamide present in solution. This alternative method (method B) was greatly advantageous since the overall yields for conversion of nitriles **2** into diselenides **4** were similar to those obtained by combining step iii and step iv (method A). Yields ranged from 15 to 45%.



Scheme 1. Reagents and conditions: (i) H_2O–MeOH, HCl or AcOH; (ii) HCO_2H–Ac_2O or RCOCl, Et_3N; (iii) Al_2Se_3, H_2O, Et_3N; (iv) Me_3SiOTf, CH_2Cl_2, Et_3N, −10 °C; (v) Se, $NaBH_4$, EtOH, Et_3N, room temperature then slight reflux.

Antioxidant assays

Glutathione peroxidase-like activity evaluation. Figure 3 and Table 1 show the abilities of the diselenides **4** to catalyse the reduction of hydrogen peroxide in presence of glutathione. The most active compounds of the series were compounds **4f** with 2397% catalysis and **4h** with 2337% catalysis. They were about 4 times more potent than the reference compound, ebselen (613% catalysis). A large part of the remaining diselenides presented roughly the same catalytic activities (1800–1900%), except for compounds **4e**, **4i** and **4k**, which are only two times as effective as ebselen (1400% catalysis).

Exchange reactions with glutathione. As encountered for ovothiols and imidazole disulphides,[31,38] diselenides are readily reduced by glutathione (Table 1). In presence of a 2-fold glutathione excess, 70–80% of the initial diselenide concentration (compounds **4f**, **4h**) reacted with glutathione to yield glutathione disulphide. For the other test compounds, exchange rates ranged from 40 to 60% and here again, compounds **4e** and **4k** were the less reactive of the series. A thin precipitate appeared in the cuvette during experiments with **4e**, which could explain the particularly low exchange rate observed for this compound.

In the second test, the same amounts of diselenides (50 μM) and glutathione (100 μM) as in the precedent study were incubated with DPPH (100 μM). If the exchange reaction is total, all the initial DPPH quantity should be reduced by the strongly reactive selenol form. Indeed, the characteristic DPPH absorbance at 517 nm was extinguished in some extent in presence of the selenol formed upon exchange reactions (Fig. 4; Table 1). Scavenging of DPPH by glutathione was extremely weak and did not interfere in the test. Here again, the most effective compounds were **4f** and **4h** (65% DPPH

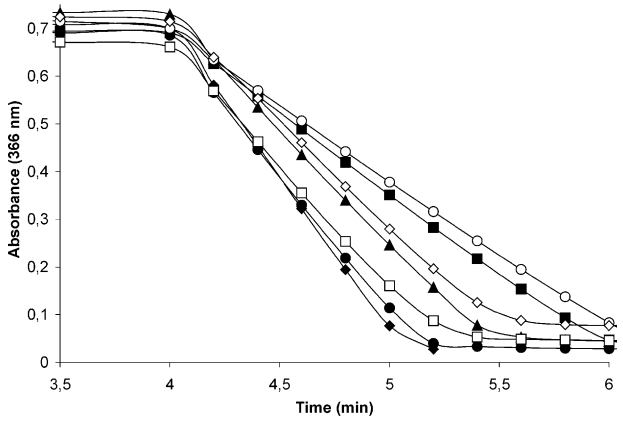


Figure 3. GSH Px-like activity of diselenides. Reactions were initiated by addition of 1.0 mM H_2O_2 to the assay mixture containing 1.0 mM GSH, 0.26 mM NADPH, 1U/mL GSSG reductase and 50 μM compound **4f** (◆), compound **4h** (●), compound **4j** (▲), compound **4i** (■), compound **4k** (○), compound **4b** (□) and compound **4e** (◇).

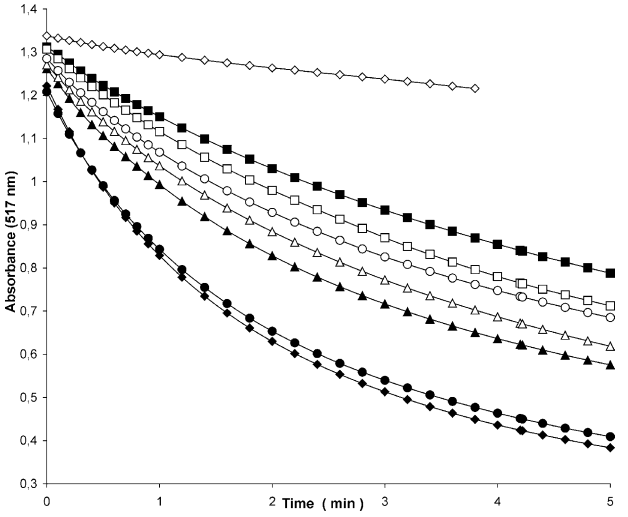


Figure 4. DPPH scavenging (initial concentration 100 μM) by an ethanolic solution of 100 μM glutathione and 50 μM compound **4f** (◆), compound **4h** (●), compound **4j** (▲), compound **4i** (■), compound **4a** (△), compound **4d** (□), compound **4k** (○) and diphenyldiselenide (◇).

Table 1. Antioxidant activities of diselenides **4a–k** and reference compounds

Compd	% Catalysis[a]	% Ebselen[b]	% Exchange[c]	DPPH scavenging	
				% 2 min[d]	% 4 min[e]
4a	1828	298	37.6	34.6	48.7
4b	1928	314	48.5	28.3	42.2
4c	1877	306	45.6	27.0	39.7
4d	1800	293	31.6	28.4	42.6
4e	1487	242	16.6	33.6	48.5
4f	2397	391	72.8	53.2	67.0
4g	1893	309	62.5	35.1	49.6
4h	2337	381	80.4	51.2	64.7
4i	1400	228	45.8	25.1	37.7
4j	1916	312	59.8	39.3	53.0
4k	1028	167	27.6	30.9	43.9
Ebselen	613	100	—	—	—
Diphenyl diselenide	1600	261	23.0	7.7	13.0

[a]The catalyst's percentage increase of the basal reaction rate between GSH and H_2O_2 was calculated as: (rate of NADPH consumption + catalyst)/(rate of NADPH consumption + vehicle) × 100.
[b]Calculated as: (rate of NADPH consumption + catalyst)/(rate of NADPH consumption + ebselen) × 100.
[c]Fraction of test compound (initial concentration 50 μM) reduced in presence of glutathione (100 μM) after 4-min incubation.
[d]Fraction of DPPH (initial concentration 100 μM) reduced in presence of test compound (50 μM) and glutathione (100 μM) after 2-min incubation.
[e]As for d, after 4-min incubation.

scavenging after 4-min incubation). DPPH scavenging rates were very close for all the other diselenides (40–50% DPPH scavenging after 4-min incubation). This must be explained by the rapid reaction between DPPH and the selenol forms, which displaced in some extent the exchange equilibriums to the right. All the imidazole diselenides were largely superior to diphenyl selenide (13% DPPH scavenging). For ebselen no selenol could be detected as previously reported.[39] The major products formed under the experimental conditions were the selenenylsulfide conjugate and the diselenide.

Conclusions

Our study provides evidence that diselenides derived from the naturally occurring ovothiols exhibit antioxidant properties expressed either by their glutathione peroxidase-like activity or their capacity to be reduced by glutathione. In this series no clear structure–activity relationship emerged from the antioxidant studies. The most effective compounds were the ones substituted on position 5 of the imidazole ring, close to the selenium atom. Thus compounds **4f** and **4h** were 4 times more potent than the reference compound, ebselen in the GSH Px-like test. The introduction of the selenium atom led to a major increase in the GSH Px-like activity since the ovothiol-derived 4-mercaptoimidazoles were approximately 8 times less effective at a 5-fold higher dose.[31]

The catalytic cycle of ebselen has been clearly detailed. It has been proved that ebselen reacts with one equivalent of glutathione to produce a selenenyl sulphide and then with a second equivalent of glutathione to produce a selenol. This predominant antioxidant species is responsible for the GSH Px-like activity of ebselen.[40–42] In presence of hydroperoxides it is oxidised to selenenic acid, which regenerates the selenenyl sulphide by reaction with glutathione. This catalytic cycle characterises glutathione peroxidase, certain diaryl diselenides,[16,19] diphenyldiselenide[19] and α-(phenylselenenyl)ketones.[24] Here we have also shown the intervention of the antioxidant selenol form as product from the reaction of imidazole diselenides with glutathione. The former catalytic cycle can only be hypothetically formulated for these imidazole diselenides. The catalytic intermediates remain to be further characterised. The main advantage of imidazole diselenides over diphenyldiselenide lies in their ability to be reduced by glutathione to a larger significant extent. The most potent candidates are 3–5 times more reactive towards glutathione than diphenyl diselenide, as evidenced by two complementary experiments. These selenol/diselenide exchange reactions of imidazole diselenides with glutathione may be relevant in vivo. Partial but significant reduction by GSH may be sufficient to maintain in living cells a concentration of the selenol form suitable for an effective antioxidant potency.

We are presently investigating in vitro the protective effects of these materials against the cytotoxic oxygen-derived species. Their lipophilicity will also lead us to evaluate their antioxidant potency in peroxidation systems.

Experimental

General chemical methods

Chemicals and reagents were of the highest quality available and were purchased from Sigma Aldrich Company. Dichloromethane and triethylamine were distilled from calcium hydride. Kieselgel 60 (70–230 mesh) and (40–63 mesh) of Merck were used for column chromatography and flash column chromatography, respectively. Analytical TLC's were performed on precoated Merck silica gel $60F_{254}$ plates and spots were detected under UV light and after iodine exposition. The proton and carbon nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded on a Bruker AM 300 WB (300 MHz) spectrometer. Chemical shifts are reported from tetramethylsilane as an internal reference and are given in δ/ppm units; coupling constants are given in Hz. Abbreviations used for signal patterns are: br s, broad singlet; s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet. The ^{77}Se-NMR spectra were recorded at 25 °C and at 52.22 MHz on a Bruker DPX 300 (300 MHz) spectrometer using DMSO-d_6 as NMR solvent and diphenyldiselenide as an external standard. Chemical shifts are reported relative to dimethylselenide (δ 0.0 ppm) by assuming that the resonance of the standard is at δ 461.0 ppm. UV measurements were made on a Uvikon 932 spectrophotometer. Mass spectral analyses were performed on an Applied Biosystems Voyager DE STR mass spectrometer (MALDI-TOF). High-resolution mass spectra were recorded on a Kratos MS80 instrument. Elemental analyses were determined by the CNRS microanalysis center. Compounds **1a**, **1f**–**1i**, **2a**, **2f**–**2i**, **2k** were prepared as we have described before.[28] Compounds **1b**–**1e** were synthesised according to a literature procedure.[44]

2-(3-Chlorophenylamino)acetonitrile (1b). Formaldehyde (37% solution; 2.70 mL, 36.2 mmol) was added dropwise to a mixture of 3-chloroaniline (3.0 g, 23.5 mmol) in acetic acid (15 mL) and potassium cyanide (1.94 g, 29.8 mmol) in water (3.5 mL) at 0 °C. The solution was stirred for 15 min at 0 °C and for 4 h at 30 °C. The solution was diluted with water (50 mL) and CH_2Cl_2 (150 mL). The organic layer was separated, washed with 1 M aq NaOH and water, dried over Na_2SO_4 and evaporated in vacuo. The residue was chromatographied on silica gel (solvent system: CH_2Cl_2) to give **1b** as a white solid in 75% yield; mp 64 °C; ^{1}H NMR ($CDCl_3$) δ 4.02 (d, J = 6.8 Hz, 2H), 4.30 (t, J = 6.8 Hz, 1H), 6.55 (d, J = 8.0 Hz, 1H), 6.67 (t, J = 2.2 Hz, 1H), 6.84 (d, J = 8.0 Hz, 1H), 7.16 (t, J = 8.0 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 32.2, 111.5, 113.3, 116.6, 119.7, 130.4, 135.1, 146.1.

2-(4-Chlorophenylamino)acetonitrile (1c). This compound was prepared in a similar manner to that reported for **1b**; white solid (70%); mp 66 °C; ^{1}H NMR ($CDCl_3$) δ 3.99 (d, J = 6.9 Hz, 2H), 4.15 (t, J = 6.9 Hz, 1H), 6.58 (d, J = 8.9 Hz, 1H), 7.19 (d, J = 8.9 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 32.7, 114.7, 116.5, 124.7, 129.3, 143.4.

2-(3-Trifluoromethylphenylamino)acetonitrile (1d). 18 h reaction at 30 °C. Purification by column chromatography was not necessary. Brown oil (89%); ^{1}H NMR

($CDCl_3$) δ 4.03 (d, $J=7.1$ Hz, 2H), 4.51 (t, $J=7.1$ Hz, 1H), 6.82 (d, $J=8.0$ Hz, 1H), 6.93 (s, 1H), 7.11 (d, $J=8.0$ Hz, 1H), 7.32 (t, $J=8.0$ Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 31.4, 109.2 (q, $^3J_{C-F}=4.0$ Hz), 115.4 (q, $^3J_{C-F}=4.0$ Hz), 115.9, 116.7, 123.8 (q, $^1J_{C-F}=272.5$ Hz), 129.6, 131.0 (q, $^2J_{C-F}=31.8$ Hz), 145.2.

2-(4-Trifluoromethylphenylamino)acetonitrile (1e). 18 h reaction at 30 °C. Column chromatography on silica gel (solvent system: CH_2Cl_2–MeOH, 95:5). White solid (89%); mp 106 °C; ^{1}H NMR ($CDCl_3$) δ 4.14 (d, $J=6.8$ Hz, 2H), 4.21 (t, $J=6.8$ Hz, 1H), 6.73 (d, $J=8.6$ Hz, 2H), 7.50 (d, $J=8.6$ Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 32.0, 112.8, 116.3, 121.7 (q, $^2J_{C-F}=32.7$ Hz), 124.5 (q, $^1J_{C-F}=270.6$ Hz), 126.9 (q, $^3J_{C-F}=3.7$ Hz), 147.6.

2-(Methylamino)-2-(4-trifluoromethylphenyl)acetonitrile (1j). A solution of 4-(trifluoromethyl)benzaldehyde (3.00 g, 17.2 mmol) in methanol (5 mL) was added dropwise to a solution of potassium cyanide (1.35 g, 20.7 mmol) and methylamine hydrochloride (1.40 g, 20.7 mmol) in water (5 mL) at 0 °C. The solution was stirred for 1 h at 0 °C and for 24 h at 30 °C. After extraction with CH_2Cl_2, drying (Na_2SO_4) and concentration in vacuo of the extract, the residue was chromatographied on silica gel (solvent system: CH_2Cl_2) to give compound **1j** as a yellow oil (77%). ^{1}H NMR ($CDCl_3$) δ 1.75 (br s, 1H), 2.50 (s, 3H), 5.20 (s, 1H), 7.43 (d, $J=8.2$ Hz, 2H), 7.57 (d, $J=8.2$ Hz, 2H); ^{13}C NMR ($CDCl_3$) 27.0, 48.3, 114.1, 122.4 (q, $^1J_{C-F}=272.2$ Hz), 125.0 (q, $^3J_{C-F}=3.9$ Hz), 126.8, 130.6 (q, $^2J_{C-F}=32.7$ Hz), 134.7.

***N*-(Cyanomethyl)-*N*-(3-chlorophenyl)formamide (2b).** Acetic anhydride (36.1 mL, 383 mmol) was added dropwise to a 96% formic acid solution (14.4 mL, 383 mmol) at 0 °C. The mixture was stirred for 15 min at 60 °C. The mixed anhydride was then treated dropwise with the cyano amine **1b** (2.90 g, 17.4 mmol) in CH_2Cl_2 (15 mL) at 0 °C. Stirring was continued for 1 h at 0 °C and 2 h at room temperature. After several washings with 1 M aq NaOH and water, drying of the organic layer (Na_2SO_4) and removal of the volatiles in vacuo, the residue was chromatographied on silica gel (solvent system: CH_2Cl_2–MeOH, 98:2) to give compound **2b** as a colourless oil (74% yield). ^{1}H NMR ($CDCl_3$) δ 4.58 (s, 2H), 7.11 (d, $J=8.0$ Hz, 1H), 7.20 (t, $J=2.2$ Hz, 1H), 7.24 (d, $J=8.0$ Hz, 1H), 7.32 (t, $J=8.0$ Hz, 1H), 8.30 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 32.3, 114.6, 121.1, 123.0, 127.3, 130.6, 134.6, 139.6, 161.0.

***N*-(Cyanomethyl)-*N*-(4-chlorophenyl)formamide (2c).** Same method as for the preparation of **2b**. 15 h reaction at room temperature. Beige solid (80%); mp 53 °C; ^{1}H NMR ($CDCl_3$) δ 4.60 (s, 2H), 7.19 (d, $J=8.9$ Hz, 2H), 7.40 (d, $J=8.9$ Hz, 2H), 8.31 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 32.4, 114.5, 124.6, 129.5, 132.9, 136.9, 160.9.

***N*-(Cyanomethyl)-*N*-(3-trifluoromethylphenyl)formamide (2d).** Yellow oil (76%); ^{1}H NMR ($CDCl_3$) δ 4.67 (s, 2H), 7.44–7.54 (m, 2H), 7.58–7.60 (m, 2H) 8.39 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 32.7, 114.9, 120.2 (q, $^3J_{C-F}=3.90$ Hz), 123.2 (q, $^1J_{C-F}=272.50$ Hz), 124.2 (q, $^3J_{C-F}=3.90$ Hz), 126.8, 130.7, 131.8 (q, $^2J_{C-F}=32.70$ Hz), 139.5, 161.5.

***N*-(Cyanomethyl)-*N*-(4-trifluoromethylphenyl)formamide (2e).** Column chromatography on silica gel (solvent system: petroleum ether–ethyl acetate, 70:30). Beige solid (72%); mp 48 °C; ^{1}H NMR ($CDCl_3$) δ 4.65 (s, 2H), 7.32 (d, $J=8.3$ Hz, 2H), 7.62 (d, $J=8.3$ Hz, 2H), 8.43 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 32.3, 114.8, 122.5, 123.3 (q, $^1J_{C-F}=272.1$ Hz), 126.7 (q, $^3J_{C-F}=3.90$ Hz), 127.0 (q, $^3J_{C-F}=3.90$ Hz), 128.7 (q, $^2J_{C-F}=33.0$ Hz), 141.9, 161.2.

***N*-[1-Cyano-1-(4-trifluoromethylphenyl)methyl]-*N*-methylformamide (2j).** Column chromatography on silica gel (solvent system: petroleum ether–ethyl acetate, 70:30). Yellow oil (83%); ^{1}H NMR ($CDCl_3$) δ 2.59, 2.74 (2s, ratio 1:4, 3H), 6.09, 6.74 (2s, ratio 1:4, 1H), 7.43 (d, $J=8.3$ Hz, 2H), 7.54 (d, $J=8.3$ Hz, 2H), 8.05, 8.37 (2s, ratio 4:1, 1H); ^{13}C NMR ($CDCl_3$) δ 26.0, 30.0, 45.7, 52.9, 114.5, 114.8, 123.2 (q, $^1J_{C-F}=272.2$ Hz), 125.5 (q, $^3J_{C-F}=3.90$ Hz), 125.9 (q, $^3J_{C-F}=3.90$ Hz), 127.1, 127.3, 130.9 (q, $^2J_{C-F}=32.7$ Hz), 131.2 (q, $^2J_{C-F}=32.7$ Hz), 134.7, 134.9, 156.1, 156.3.

Bis-4-[1-[(4′-methoxy)phenyl]-4-seleno-imidazole] (4a). Method A. The all-glass reaction apparatus consisted of a 100 mL two-neck reaction flask (A) in which hydrogen selenide was generated by the action of H_2O upon Al_2Se_3. This was connected to a 200 mL three-neck reaction flask (B) by means of a glass tube. A granular anhydrous calcium chloride cartridge was placed between the two flasks, which served to dry hydrogen selenide prior to its contact with the cyano amide compound **2a** in flask B. Flask A was charged with aluminium selenide (0.78 g, 2.7 mmol) and fitted with a dropping funnel containing 3 mL of water. A solution of compound **2a** (0.36 g, 1.9 mmol) and triethylamine (0.79 mL, 5.7 mmol) in CH_2Cl_2 (50 mL) was prepared in flask B. The apparatus was then evacuated and flushed with dry argon.

Water was added dropwise to the aluminium selenide so that the rate of evolution of H_2Se gas was constant. The mixture was stirred until there was not any evolution of the content of flask B (TLC analysis) and cooled at −10 °C. Then trimethylsilyltrifluoromethanesulfonate (0.69 mL, 3.8 mmol) was added dropwise and advancement of the reaction was followed by TLC. If the reactivity of the silane was too low after 1 h stirring, 3 equiv (0.79 mL) of TEA and 2 equiv (0.69 mL) of Me_3SiOTf could be further added and reaction was run for 15 h at 0 °C. The mixture was washed with water. The organic layer was dried (Na_2SO_4) and evaporated in vacuo. The residue was chromatographied on silica gel (solvent system: CH_2Cl_2–MeOH, 98:2) to give **4a**, which crystallised as an orange solid in ethyl acetate in 30% yield.

Method B. To a solution of compound **2a** (0.60 g, 3.1 mmol) and triethylamine (0.48 mL, 3.4 mmol) in dry ethanol (25 mL) were added finely ground gray selenium powder (1.5 g, 19.0 mmol) and sodium borohydride (0.48 g, 12.7 mmol). The resulting mixture was stirred at

room temperature until formation of selenoamide **3a** was maximal and was then slightly refluxed overnight. Ethanol was removed in vacuo and CH_2Cl_2 (100 mL) was added. The solid residues were filtered. The organic phase was extracted with 0.2 M aq HCl and water, dried over Na_2SO_4 and the solvent was removed in vacuo. Column chromatography of the residue on silica gel using 98/2 CH_2Cl_2/MeOH afforded the title compound **4a**, which crystallised in ethyl acetate in 36% yield. Orange powder; mp 127 °C; ^{1}H NMR ($CDCl_3$) δ 3.84 (s, 3H), 6.96 (d, J = 9.0 Hz, 2H), 7.30 (d, J = 9.0 Hz, 2H), 7.46 (s, 1H), 7.77 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 55.4, 114.8, 122.9, 124.2, 128.5, 129.8, 136.6, 159.0; ^{77}Se NMR (DMSO-d_6) δ 422.5; m/z (MALDI-TOF) 507.0 (MH^+, 100); HRMS calcd (M^+) 505.9760; Found: 505.9767. Anal. calcd for $C_{20}H_{18}N_4O_2Se_2$: C, 47.63; H, 3.60; N, 11.11; Found: C, 47.71; H, 3.58; N, 11.16.

Bis - 4 - [1 - (3′ - chlorophenyl) - 4 - selenoimidazole] (4b). Method A: 27% yield; Method B: 23% yield. Orange powder; mp 208 °C; ^{1}H NMR (DMSO-d_6) δ 7.43 (d, J = 8.0 Hz, 1H), 7.51 (d, J = 8.0 Hz, 1H), 7.65 (d, J = 8.0 Hz, 1H), 7.85 (t, J = 2.0 Hz, 1H), 8.05 (s, 1H), 8.44 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 112.4, 113.8, 117.3, 120.7, 121.2, 125.0, 127.8, 130.8, 130.9; ^{77}Se NMR (DMSO-d_6) δ 420.7; m/z (MALDI-TOF) 514.9 (MH^+, 100); HRMS calcd (M^+) 513.8769; Found: 513.8761. Anal. calcd for $C_{18}H_{12}Cl_2N_4Se_2$: C, 42.13; H, 2.36; N, 10.92; Found: C, 42.07; H, 2.30; N, 11.02.

Bis - 4 - [1 - (4′ - chlorophenyl) - 4 - selenoimidazole] (4c). Method A: 38% yield; Method B: 29% yield. Orange brown powder; mp 215 °C; ^{1}H NMR ($CDCl_3$) δ 7.29 (d, J = 8.7 Hz, 2H), 7.38 (s, 1H), 7.44 (d, J = 8.7 Hz, 2H), 7.79 (s, 1H); ^{13}C NMR (CDCl3) δ 115.9, 117.6, 121.4, 123.5, 125.3, 128.8, 130.9; ^{77}Se NMR (DMSO-d_6) δ 421.2; m/z (MALDI-TOF) 514.9 (MH^+, 100); HRMS calcd (M^+) 513.8769; Found: 513.8766. Anal. calcd for $C_{18}H_{12}Cl_2N_4Se_2$: C, 42.13; H, 2.36; N, 10.92; Found: C, 42.16; H, 2.44; N, 10.97.

Bis-4-[1-[(3′-trifluoromethyl)phenyl]-4-selenoimidazole] (4d). Method A: 37% yield; Method B: 26% yield. Lemon powder; mp 181 °C; ^{1}H NMR ($CDCl_3$) δ 7.60–7.64 (m, 5H), 7.90 (s, 1H); ^{13}C NMR (CDCl3) δ 118.3 (q, $^3J_{C-F}$ = 3.9 Hz), 122.1, 123.2 (q, $^1J_{C-F}$ = 272.0 Hz), 123.6, 124.6 (q, $^3J_{C-F}$ = 3.9 Hz), 129.9, 130.8, 132.6 (q, $^2J_{C-F}$ = 33.0 Hz), 136.4, 137.1; ^{77}Se NMR (DMSO-d_6) δ 420.2; m/z (MALDI-TOF) 582.9 (MH^+, 100); HRMS calcd (M^+) 581.9296; Found: 581.9288. Anal. calcd for $C_{20}H_{12}F_6N_4Se_2$: C, 41.40; H, 2.08; N, 9.66; Found: C, 41.50; H, 1.99; N, 9.63.

Bis-4-[1-[(4′-trifluoromethyl)phenyl]-4-selenoimidazole] (4e). Method A: 32% yield; Method B: 29% yield. Lemon powder; mp 189 °C; ^{1}H NMR (DMSO-d_6) δ 7.82 (d, J = 8.9 Hz, 2H), 7.87 (d, J = 8.9 Hz, 2H), 8.07 (s, 1H), 8.51 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 120.9, 124.0 (q, $^1J_{C-F}$ = 272.2 Hz), 124.1, 127.2 (q, $^3J_{C-F}$ = 3.9 Hz), 127.6 (q, $^2J_{C-F}$ = 32.3 Hz), 128.1, 137.5, 139.3; ^{77}Se NMR (DMSO-d_6) δ 420.2; m/z (MALDI-TOF) 582.9 (MH^+, 100); HRMS calcd (M^+) 581.9296; Found: 581.9301. Anal. calcd for $C_{20}H_{12}F_6N_4Se_2$: C, 41.40; H, 2.08; N, 9.66; Found: C, 41.34; H, 2.07; N, 9.71.

Bis-4-[5-[(4′-methoxy)phenyl]-4-seleno-1-methylimidazole] (4f). Method A: 41% yield; Method B: 53% yield. Orange powder; mp 160 °C; ^{1}H NMR ($CDCl_3$) δ 3.47 (s, 3H), 3.55 (s, 3H), 6.84 (d, J = 8.8 Hz, 2H), 7.10 (d, J = 8.8 Hz, 2H), 7.46 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 32.8, 55.3, 113.6, 121.3, 125.7, 131.7, 137.5, 138.4, 159.6; ^{77}Se NMR (DMSO-d_6) δ 430.2; m/z (MALDI-TOF) 534.9 (MH^+, 100); HRMS calcd (M^+) 534.0073; Found: 534.0067. Anal. calcd for $C_{22}H_{22}N_4O_2Se_2$: C, 49.64; H, 4.17; N, 10.52; Found: C, 49.73; H, 4.21; N, 10.57.

Bis-4-[5-[(2′-chloro)phenyl]-4-seleno-1-methylimidazole] (4g). Method A: 43% yield; Method B: 37% yield. Orange powder; mp 210 °C; ^{1}H NMR ($CDCl_3$) δ 3.46 (s, 3H), 7.14–7.48 (m, 4H), 7.51 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 32.7, 125.9, 126.6, 126.8, 128.1, 129.6, 130.8, 133.8, 135.3, 139.0; ^{77}Se NMR (DMSO-d_6) δ 420.3; m/z (MALDI-TOF) 542.9 (MH^+, 100); HRMS calcd (M^+) 541.9082; Found: 541.9078. Anal. calcd for $C_{20}H_{16}Cl_2N_4Se_2$: C, 44.39; H, 2.98; N, 10.35; Found: C, 44.48; H, 2.93; N, 10.34.

Bis-4-[5-[(4′-chloro)phenyl]-4-seleno-1-methyl-imidazole] (4h). Method A: 31% yield; Method B: 42% yield. Orange powder; mp 238 °C; ^{1}H NMR ($CDCl_3$) δ 3.52 (s, 3H), 7.15 (d, J = 8.4 Hz, 2H), 7.32 (d, J 8.4 Hz, 2H), 7.62 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 33.0, 125.9, 127.3, 128.5, 131.7, 134.6, 136.3, 138.9; ^{77}Se NMR (DMSO-d_6) δ 425.9; m/z (MALDI-TOF) 542.9 (MH^+, 100); HRMS calcd (M^+) 541.9082; Found: 541.9089. Anal. calcd for $C_{20}H_{16}Cl_2N_4Se_2$: C, 44.39; H, 2.98; N, 10.35; Found: C, 44.36; H, 3.02; N, 10.32.

Bis-4-[5-[(2′-trifluoromethyl)phenyl]-4-seleno-1-methyl imidazole] (4i). Method A: 30% yield; Method B: 26% yield. Lemon powder; mp 224 °C; ^{1}H NMR ($CDCl_3$) δ 3.29 (s, 3H), 6.87 (s, 1H), 7.25–7.71 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 32.0, 123.4 (q, $^1J_{C-F}$ = 274.0 Hz), 126.2 (q, $^3J_{C-F}$ = 5.0 Hz), 127.7, 129.7, 130.8 (q, $^2J_{C-F}$ = 33.0 Hz), 131.2, 131.7, 134.0 (q, $^3J_{C-F}$ = 5.0 Hz), 138.2, 138.5;77SeNMR (DMSO-d_6) δ 419.4; m/z (MALDI-TOF) 611.0 (MH^+, 100); HRMS calcd (M^+) 609.9609; Found: 609.9615. Anal. calcd for $C_{22}H_{16}F_6N_4Se_2$: C, 43.44; H, 2.65; N, 9.21; Found: C, 43.52; H, 2.60; N, 9.27.

Bis-4-[5-[(4′-trifluoromethyl)phenyl]-4-seleno-1-methyl imidazole] (4j). Method A: 28% yield; Method B: 33% yield. Lemon powder; mp 180 °C; ^{1}H NMR ($CDCl_3$) δ 3.53 (s, 3H), 7.35 (d, J = 8.5 Hz, 2H), 7.49 (s, 1H), 7.62 (d, J = 8.5 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 33.0, 123.9 (q, $^1J_{C-F}$ = 272.2 Hz), 125.1 (q, $^3J_{C-F}$ = 3.9 Hz), 126.7, 130.3 (q, $^2J_{C-F}$ = 33.0 Hz), 130.7, 132.7, 136.0, 139.2; ^{77}Se NMR (DMSO-d_6) δ 423.7; m/z (MALDI-TOF) 611.0 (MH^+, 100); HRMS calcd (M^+) 609.9609; Found: 609.9605. Anal. calcd for $C_{22}H_{16}F_6N_4Se_2$: C, 43.44; H, 2.65; N, 9.21; Found: C, 43.37; H, 2.72; N, 9.15.

Bis-4-[2-[(3′-chloro)phenyl]-4-seleno-1-methyl-imidazole] (4k). Method A: 23% yield; Method B: 30% yield. Lemon powder; mp 154 °C; ^{1}H NMR ($CDCl_3$) δ 3.75 (s, 3H), 7.32 (s, 1H), 7.36–7.51 (m, 3H), 7.67 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 34.7, 126.6, 127.6, 128.8, 128.9, 129.0, 129.7, 131.5, 134.5, 147.4; ^{77}Se NMR (DMSO-d_6) δ 420.3; *m*/*z* (MALDI-TOF) 542.9 (MH^+, 100); HRMS calcd (M^+) 541.9082; Found: 541.9089. Anal. calcd for $C_{20}H_{16}Cl_2N_4Se_2$: C, 44.39; H, 2.98; N, 10.35; Found: C, 44.36; H, 3.05; N, 10.43.

Antioxidant assays

Drug preparation. Compounds were solubilised in *N,N*-dimethylformamide (DMF) to yield 10 or 5 mM stock solutions. Ebselen and diphenyldiselenide were used as references.

Measurement of the glutathione peroxidase-like (GSH Px-like) activity. GSH Px-like activity was determined by the reduction of GSSG formed via the NADPH–glutathione reductase system as a continuous indicator system.[11] Experiments were conducted at room temperature (23 °C). The decrease in NADPH monitored spectrophotometrically at 366 nm (ε = 3300/M/cm) is a measure of GSH Px activity. The assay mixture (1.0 mL) contained 50 mM phosphate buffer (prepared with Milli-Q water, conductivity $<10^{-18}/\Omega$/cm), pH 7.6, 0.1 mM EDTA and 1.0 mM NaN_3. Test compound (50 μM final concentration), glutathione (1.0 mM), NADPH (280 μM) and GSSG reductase (1 unit) were added and the absorbance was recorded for 4 min to estimate the background and stability of the preparation. Reaction was initiated by the subsequent addition of hydrogen peroxide at concentration 1.0 mM. Appropriate blanks were also run in the absence of test compound and in the presence of H_2O_2. For compounds **4b–e** and diphenyldiselenide, DMF (200 μL) was included in the incubations to improve the solubility of test compounds in buffer. Catalyst activity (% catalysis) was determined as the increase of the basal reaction between GSH and H_2O_2 in presence of test compound (as compared with the uncatalysed process in presence of vehicle).

Exchange reaction with glutathione. Two methods were used to investigate the exchange reaction of diselenides with glutathione. The first one used the NADPH-glutathione reductase system, which reduced GSSG formed upon exchange reaction. For this purpose, diselenide (50 μM) and glutathione (100 μM) were incubated for 4 min at room temperature. NADPH (100 μM) and GSSG reductase (1 U/mL) were subsequently added. The absorbance decrease at 366 nm was measured and compared to that obtained in presence of glutathione disulphide alone (50 μM). The ratio of both NADPH losses gave the amount of glutathione/diselenide exchange. The second method used the stable radical DPPH. Diselenide (50 μM) and glutathione (100 μM) were added to 1.0 mL of a solution of DPPH (100 μM) in ethanol. The absorbance decrease at 517 nm was measured for 10 min. Control was also run in absence of test compound.

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