Synthesis of Novel Selenocyanates and Evaluation of Their Effect in Cultured Mouse Neurons Submitted to Oxidative Stress

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Oxidative Medicine and Cellular Longevity

Synthesis of novel selenocyanates and evaluation of their effect in cultured mouse neurons submitted to oxidative stress

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Abstract

Herein we report the synthesis of novel selenocyanates and assessment of their effect on the oxidative challenge elicited by hydrogen peroxide (H₂O₂) in cultured mouse neurons. First, α -methylene- β -hydroxy esters were prepared as precursors of allylic bromides. A reaction involving the generated bromides and sodium selenocyanate was conducted to produce the desired selenocyanates (**3a-f**). We next prepared cultures of neurons from 7-day-old-mice ($n = 36$). H₂O₂ (10⁻⁵ M) was added into the culture flasks as an oxidative stress inducer, alone or combined with one of each designed compounds. PhSe)₂ was used as positive control. It was carried out assessment of lipid (thiobarbituric acid reactive species, 4-hydroxy-2'-nonenal, 8-isoprostane), DNA (8-hydroxy-2'-deoxyguanosine) and protein (carbonyl) modification parameters. Finally, catalase and superoxide dismutase activities were also evaluated. Among the compounds, **3b**, **3d** and **3f** exhibited the most pronounced pattern of antioxidant activity, similar to (PhSe)₂. These novel aromatic selenocyanates could be promising to be tried in most sophisticated *in vitro* studies or even at preclinical level.

Introduction

Selenium (Se) is a trace element regarded essential for human and other mammals [1]. Main biological role of this metalloid is related to its incorporation into selenoproteins, most of which participates in redox homeostasis (particularly glutathione peroxidase [GPx, EC 1.11.1.9] and thioredoxin reductase [EC 1.8.1.9]), metabolism of thyroid hormones, biosynthesis of other Se-containing proteins [2] and in the spermatogenesis [3]. In addition, adequate supplementation with Se contributes to the detoxification of heavy metals, including mercury and related compounds [4]. At least in part, this effect is ascribable to the marked antioxidant property of Se and its organic analogues [5].

In this regard, a number of papers have pointed out the synthetic versatility of compounds termed organochalcogens [6–13]. Selenium-containing organochalcogenides seems to be of great therapeutic relevance, mostly due to the ability of these compounds to mimic natural substances with antioxidant, antitumor, antimicrobial and antiviral activities [14–20]. One of most known drugs of such class is called Ebselen (**Figure 1**), which presents GPx-like activity and have been searched for therapy of several human disorders [21]. In relation with the current pandemic of corona virus (COVID-19), a very interesting study just appeared in the literature where organoselenium compound, Ebselen presented the strongest antiviral effect at a concentration of 10 μ M treatment in COVID-19 virus infected Vero cells [22]. Diphenyl diselenide ((PhSe)₂) is other organoselenium compound with GPx-like activity (**Figure 1**) [23].

Figure 1: Structure of Ebselen and diphenyl diselenide

By considering the biological roles of these compounds, development of new and efficient routes for the synthesis of organoselenides is a tempting research area [12, 24, 25]. Therefore, as an additional step of the ongoing efforts aiming the design of novel organoselenides and medicinal chemistry [26–28], present study reports the synthesis of novel aromatic selenocyanates, comprising a second generation of chalcogenide esters. Furthermore, assessment of the effect of these compounds on the oxidative challenge elicited by hydrogen peroxide (H₂O₂) in mixed cultures of mouse neurons was undertaken, in order to provide insightful cues on their biological activity.

Materials and Methods

General procedure for the synthesis of allylic bromide 2

5.0 mmol of LiBr was added to a stirred solution of 2.5 mmol of α -methylene- β -hydroxy esters (Morita-Baylis-Hillman adduct) **1** in 10.0 mL of acetonitrile at 0–5 °C, followed by the addition of 6.3 mmol of 96% H₂SO₄. Subsequently, the reaction mixture was allowed to attend the room temperature and the stirring was continued until the complete consumption of **1** (monitored by TLC). The reaction mixture was diluted by CH₂Cl₂ (20 mL) and the organic phase was successively extracted with H₂O, saturated NaHCO₃, brined and dried over MgSO₄. The organic phase was concentrated under reduced pressure and the resulting residue was purified

by column chromatography (hexane/ethyl acetate 9:1) using flash silica gel, resulting the corresponding allylic bromides **2a-f**. (Figure S13-S18, FTIR for compound **2a-f** in supplementary materials, pg 11-13)

General procedure for the synthesis of allylic selenocyanates **3**

To a stirred solution of allylic bromide **2a-f** (1.0 mmol) in 4.0 mL of acetone/H₂O (4:1 v/v) at 25 °C was added 1.25 mmol of KSeCN. After stirring for 10 h, the final mixture was diluted with CH₂Cl₂ and washed with H₂O and brine. The organic extract was dried over Na₂SO₄, filtered and concentrated under reduced pressure. The resulting residue was purified by chromatography (hexane/ethyl acetate 9:1) to give the corresponding compounds **3a-f**.

Methyl (Z)-3-phenyl-2-(selenocyanatomethyl)acrylate (**3a**)

Yield 96%; m.p 72-73°C. IR (KBr): $\nu_{\text{max}}/\text{cm}^{-1}$ 305, 3003, 2956, 2935, 2849, 2141, 1693, 1619, 1435, 1346, 1255, 1077, 754, 482. ¹H NMR (200 MHz, CDCl₃): δ 7.88 (s, 1H), 7.59 – 7.36 (m, 5H), 4.17 (s, 2H), 3.86 (s, 3H). ¹³C NMR (50 MHz, CDCl₃): δ 166.8, 143.0, 133.7, 129.5, 129.1, 128.8, 126.9, 102.4, 52.5, 25.5. Anal. Calcd for C₁₂H₁₁NO₂Se: C, 51.44; H, 3.96; Br, 22.25; N, 5.00; O, 11.42; Se, 28.18. Found: C, 50.53; H, 3.70. (Figure S1, ¹H and ¹³C NMR Spectra and Figure S7, FTIR for compound **3a** in supplementary materials, pg 2 and 8, respectively.)

Methyl (Z)-3-(4-bromophenyl)-2-(selenocyanatomethyl)acrylate (**3b**)

Yield 86%; m.p 72-74°C: $\nu_{\text{max}}/\text{cm}^{-1}$ 3062, 2952, 2840, 2156, 1710, 1625, 1487, 1435, 1273, 1198, 1154, 1068, 1008, 808, 503. ¹H NMR (400 MHz, CDCl₃): δ 7.82 (s, 1H), 7.61 (d, *J* = 8.4 Hz, 2H), 7.31 (d, *J* = 8.0 Hz, 2H), 4.14 (s, 2H), 3.89 (s, 3H). ¹³C NMR (101 MHz, CDCl₃): δ 166.8, 141.9, 132.7, 132.4, 130.8, 127.8, 124.2, 102.4, 52.9, 25.4. Anal. Calcd for C₁₂H₁₀BrNO₂Se: C, 40.14; H, 2.81; Br, 22.25; N, 3.90; O, 8.91; Se, 21.99. Found: C, 40.13; H, 2.80. (Figure S2, ¹H and ¹³C NMR Spectra and Figure S8, FTIR for compound **3b** in supplementary materials, pg 3 and 8, respectively.)

Methyl (Z)-3-(2-bromophenyl)-2-(selenocyanatomethyl)acrylate (**3c**)

Yield 98%; white solid, mp 71.5-73.0 °C. IR (KBr): $\nu_{\text{max}}/\text{cm}^{-1}$ 3032, 2953, 2926, 2850, 2150, 1678, 1438, 1363, 1295, 1090, 758, 586. ¹H NMR (400 MHz, CDCl₃): δ 7.88 (s, 1H), 7.66 (dd, *J* = 8.1, 1.3 Hz, 1H), 7.48 – 7.39 (m, 2H), 7.31 – 7.26 (m, 1H), 4.00 (s, 2H), 3.91 (s, 3H). ¹³C NMR (101 MHz, CDCl₃): δ 166.7, 142.1, 134.5, 133.2, 130.9, 130.0, 129.1, 127.8, 123.9, 102.7, 52.9, 25.5. Anal. Calcd for C₁₂H₁₀BrNO₂Se: C, 40.14; H, 2.81; Br, 22.25; N, 3.90; O, 8.91; Se, 21.99. Found: C, 40.12; H, 2.78. (Figure S3, ¹H and ¹³C NMR Spectra and Figure S9, FTIR for compound **3c** in supplementary materials, pg 4 and 9, respectively.)

Methyl (Z)-3-(4-chlorophenyl)-2-(selenocyanatomethyl)acrylate (**3d**)

Yield 94%; white solid, mp 73-75 °C. IR (KBr): $\nu_{\text{max}}/\text{cm}^{-1}$ 3088, 2952, 2854, 2149, 1716, 1583, 1453, 1287, 1197, 1082, 793. ¹H NMR (400 MHz, CDCl₃): δ 7.84 (s, 1H), 7.45 (d, *J* = 8.7 Hz, 2H), 7.37 (d, *J* = 8.2 Hz, 2H), 4.15 (s, 2H), 3.88 (s, 3H). ¹³C NMR (101 MHz, CDCl₃): δ 166.8, 141.8, 135.8, 132.2, 130.6, 129.3, 127.7, 102.4, 52.8, 25.4. Anal. Calcd for C₁₂H₁₀ClNO₂Se: C, 45.81; H, 3.20; Cl, 11.27; N, 4.45; O, 10.17; Se, 25.10. Found: C, 45.80; H, 3.18. (Figure S4, ¹H and ¹³C NMR Spectra and Figure S10, FTIR for compound **3d** in supplementary materials, pg 5 and 9, respectively.)

Methyl (Z)-3-(2,4-dichlorophenyl)-2-(selenocyanatomethyl)acrylate (**3e**)

Yield 89%; white solid, mp 75.0-78.0 °C. IR (KBr): $\nu_{\text{max}}/\text{cm}^{-1}$ 3088, 2952, 2854, 2149, 1716, 1583, 1435, 1287, 1167, 1082, 763. ¹H NMR (400 MHz, CDCl₃): δ 7.86 (s, 1H), 7.50 (d, J = 0.9 Hz, 1H), 7.41 – 7.37 (m, 2H), 3.98 (s, 2H), 3.91 (s, 3H). ¹³C NMR (101 MHz, CDCl₃): δ 166.5, 138.8, 136.3, 135.0, 131.1, 130.8, 130.1, 130.0, 127.7, 102.6, 53.3, 25.4. Anal. Calcd for C₁₂H₉Cl₂NO₂Se: C, 41.29; H, 2.60; Cl, 20.31; N, 4.01; O, 9.17; Se, 22.62. Found: C, 41.31; H, 2.58. (Figure S5, ¹H and ¹³C NMR Spectra and Figure S11, FTIR for compound **3e** in supplementary materials, pg 6 and 10, respectively.)

Methyl (Z)-3-(4-nitrophenyl)-2-(selenocyanatomethyl)acrylate (3f)

Yield 95%; yellow solid, m.p. 83-84°C. IR (KBr): $\nu_{\text{max}}/\text{cm}^{-1}$ 3106, 2955, 2846, 2150, 1721, 1599, 1516, 1429, 1342, 1272, 1202, 1158, 854, 769. ¹H NMR (400 MHz, CDCl₃): δ 8.32 (d, J = 8.9 Hz, 2H), 7.93 (s, 1H), 7.61 (d, J = 8.8 Hz, 2H), 4.10 (s, 2H), 3.92 (s, 3H). ¹³C NMR (101 MHz, CDCl₃): δ 166.2, 147.9, 140.3, 140.1, 130.5, 130.0, 124.1, 102.1, 53.0, 24.8. Anal. Calcd for C₁₂H₁₀N₂O₄Se: C, 44.32; H, 3.10; N, 8.61; O, 19.68; Se, 24.28. Found: C, 44.29; H, 3.11. (Figure S6, ¹H and ¹³C NMR Spectra and Figure S12, FTIR for compound **3f** in supplementary materials, pg 7 and 10, respectively.)

Animals

Thirty-six, 7-day-old mice (*Mus musculus*; Balb/C strain) were obtained from the Central Animal House of the University of Southern Santa Catarina (UNESC). Animals received *ad libitum* water and chow and were kept in a colony room with 21 ± 1 °C temperature and a 12 hours light/dark cycle. Experimental groups were as follows: positive control (culture medium and sample); negative control or stress (culture medium, sample and H₂O₂); and a group for each tested **3a–f** compounds (culture medium, sample, H₂O₂ and the corresponding compound – seven groups); it was used four animals per group. Mice were killed by decapitation without anesthesia, the skull was opened and total brain was excised and cleaned. All experimental procedures were performed with approval by the UNESC's Ethical Committee (protocol # 012/2016-1).

Cell culture and hydrogen peroxide challenge

Immediately after euthanasia of the animals, the brain was placed in a chamber with constant air flux and ultraviolet illumination for incubation. Cells from both cerebral hemispheres were dissociated in phosphate-buffered saline (0.9%) and plated at a density of 10⁵ cells/cm² in 75 cm² culture flasks with Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 20% antibiotics. H₂O₂ (10⁻⁵ M) was added soon after as an oxidative stress inducer according to the reference [29]. Each **3a–f** compound was also added to the medium, aiming to reach a 10 μ M concentration, based on the study carried out by Posser and coworkers [23]. (PhSe)₂, which presents GPx-like activity, was used as positive control [23]. Plate was kept in a carbon dioxide (CO₂)-incubator during 24 hours. Thereafter, samples were stored at –80 °C for the subsequent analyses.

Assessment of lipid peroxidation

Quantification of thiobarbituric acid reactive species (TBARS) was carried out on the basis of malondialdehyde (MDA) content through the reference [30]. Briefly, samples (200 μ L aliquot) were mixed with 1 mL 10% trichloroacetic acid and 1 mL 0.67% thiobarbituric acid and heated in boiling water during 30 minutes. MDA equivalents absorbance was measured at λ = 532 nm,

160 using 1,1,3,3-tetramethoxypropane as standard. Data were expressed as MDA equivalents
161 (nmol/mg protein).

162 4-Hydroxy-2-nonenal (4-HNE) content was determined using the assay kit from Cell Biolabs
163 (Cell Biolabs, Inc., San Diego, California, USA). 8-Isoprostane (8-ISO) level was measured
164 using the ACE™ Competitive EIAs Kit (Cayman) with 8-isoprostane-acetylcholinesterase (EC
165 3.1.1.7) conjugate as a tracer and 8-isoprostane-specific rabbit anti-serum. Adducts of 4-HNE
166 with lysine, histidine or cysteine residues in proteins were quantified according to the
167 immunoassay described by Kimura and coworkers [31].

168 **DNA and protein modification parameters**

169 Nuclear DNA was isolated from the cells using the PureGenome™ On-Spot Tissue DNA Kit
170 (EMD Millipore, Burlington, Massachusetts, USA). DNA content in the extracts was measured
171 by using NanoDrop (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Levels of 8-
172 hydroxy-2'-deoxyguanosine (8-OHdG), a compound generated by oxidation of 2'-
173 deoxyguanosine residues, were determined using the OxiSelect™ Oxidative DNA Damage
174 ELISA Kit (Cell Biolabs, Inc., San Diego, USA).

175 Protein oxidative damage was estimated on the basis of carbonyl content determination
176 according to the method described by Levine and coworkers [32]. Protein precipitation was
177 conducted by addition of 20% trichloroacetic acid to the samples (400 µL aliquot), which were
178 then dissolved in a diphenylhydrazine (DNPH) solution. Data were expressed as nmol/mg
179 protein. Sample absorbance was read at $\lambda = 370$ nm.

180 **Catalase activity**

181 Catalase (CAT; EC 1.11.1.6) activity was assessed on the basis of *in vitro* H₂O₂ decomposition
182 according to standardized method [33]. Brain tissue was sonicated in 50 mM phosphate buffer
183 (pH 7.0), and the resulting suspension was submitted to centrifugation (3,000 g for 10 minutes).
184 A 20 µL sample aliquot was added to 980 µL substrate mixture, which contained 0.3 mL H₂O₂
185 in 50 mL 0.05 M phosphate buffer (pH 7.0). Initial and final absorbance values were recorded
186 at $\lambda = 240$ nm after 1 and 6 minutes, respectively. A standard curve was established using
187 purified CAT (Sigma-Aldrich, St. Louis, Missouri, United States) at the same experimental
188 conditions of the samples.

189 **Superoxide dismutase activity**

190 Measurement of superoxide dismutase (SOD; EC 1.15.1.1) activity was performed based on
191 its ability to spontaneously inhibit oxidation of adrenaline to adrenochrome [34]. Sodium
192 carbonate buffer (2.78 mL; 0.05 mM; pH 10.2), 100 µL EDTA (1.0 mM) and 20 µL supernatant
193 or sucrose solution (blank) were incubated at 30 °C for 45 minutes. Thereafter, reaction was
194 initiated by adding 100 µL adrenaline solution (9.0 mM). Change in absorbance was recorded
195 at $\lambda = 480$ nm for 8 minutes. Temperature was maintained at 30 °C throughout the assay
196 procedure. One unit of SOD produced 50% of adrenaline auto-oxidation. Data were expressed
197 as units/mg protein.

Determination of protein content in the samples

Biochemical analyses were related to the protein content in the samples, for normalization. A 10 μ L aliquot of each sample was used in this procedure. Measurements were carried out according to the Lowry and coworkers' method [35]. Bovine serum albumin was used as standard.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation (S.D.). Differences between groups were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Software used for the comparisons was the Statistical Package for the Social Sciences (SPSS) 20 (IBM, Armonk, New York, USA). Differences were rated as statistically significant at $p < 0.05$.

Results

Synthesis of the selenocyanates (**3**)

α -Methylene- β -hydroxy esters for organic synthesis were readily prepared by the Morita–Baylis–Hillman reaction. Such compounds are building blocks for the synthesis of several substances, by converting their hydroxyl group into acetates, bromides and thiocyanates, acting like acceptors in many useful synthetic approaches. Thus, allylic bromides (**2**) were obtained according to the method described by Sá and coworkers [36]. In this procedure, lithium bromide (LiBr) and H₂SO₄ were added to a previously stirred solution of Baylis–Hillman adducts (i.e., α -Methylene- β -hydroxy esters, **1**) in acetonitrile at 0–5 °C (**Table 1**). The reaction mixture stirred until the consumption of the starting material. After the completing of the reaction, dichloromethane (CH₂Cl₂) was added and washed with water, saturated with sodium bicarbonate (NaHCO₃) and brine, dried over magnesium sulfate (MgSO₄), filtered and concentrated under reduced pressure. Resulting residue was purified by column chromatography to yield the corresponding 2-bromomethyl-2-alkenoates **2**.

In the next step, a reaction was conducted involving the generated **2** and potassium selenocyanate (KSeCN) to produce the desired allylic selenocyanates [37]. Briefly, process occurs by nucleophilic displacement of bromide with KSeCN in acetone, without the use of an external base. More specifically, aromatic-substituted allylic selenocyanates (**3**) could be obtained by mixing the corresponding bromides (**2**) with 1.25 molar equiv. of KSeCN in acetone at 25 °C in 10 min. The corresponding products (**3**) were then isolated in high yields after purification in a short plug of silica gel using acetone as eluent (**Table 1**). Assignment of the organoselenium structures (**3a–f**) was based on the characteristic signals for the selenocyanate (SeCN) functional group at infrared (IR; sharp band at 2140–2157 cm⁻¹) and carbon-13 nuclear magnetic resonance (¹³C NMR, 102 ppm) spectra of all purified products (supplementary materials).

Table 1: Synthesis of organoselenium compounds (**3a–f**) from allylic bromides (**2a–f**).

$ \begin{array}{c} \text{OH} \quad \text{O} \\ \quad \\ \text{R}-\text{C}-\text{C}-\text{OCH}_3 \\ \\ \text{H} \end{array} \xrightarrow[\text{H}_2\text{SO}_4]{\text{LiBr, CH}_3\text{CN}} \begin{array}{c} \text{O} \\ \\ \text{R}-\text{C}-\text{C}=\text{CH}-\text{Br} \\ \\ \text{H} \end{array} \xrightarrow[\text{Acetone : H}_2\text{O}]{\text{NaSeCN, 25}^\circ\text{C, 1h}} \begin{array}{c} \text{O} \\ \\ \text{R}-\text{C}-\text{C}=\text{CH}-\text{SeCN} \\ \\ \text{H} \end{array} $			
1a–f		2a–f	3a–f
Entry	R	Yield (%) ^[a] of 2	Yield (%) ^[a] of 3
1	C ₆ H ₅	2a , 85%	3a , 96%
2	4-Br-C ₆ H ₄	2b , 89%	3b , 86%
3	2-Br-C ₆ H ₄	2c , 76%	3c , 98%
4	4-Cl-C ₆ H ₄	2d , 80%	3d , 94%
5	2,4-(Cl) ₂ -C ₆ H ₃	2e , 88%	3e , 89%
6	4-NO ₂ -C ₆ H ₄	2f 87%	3f 95%

^[a] Isolated Yields.

238 Effect of different selenocyanates (**3**) towards lipid peroxidation elicited by H₂O₂

239 Initially, it was performed measurement of TBARS level in the samples (**Figure 2A**). As
 240 expected, TBARS content was significantly increased in the cell cultures only receiving H₂O₂
 241 (stress group), as compared to control group ($p < 0.05$). In addition, it was detected increased
 242 MDA levels in the cells subjected to the oxidative challenge and receiving Compound **3a** as
 243 well, in comparison to control group ($p = 0.000026$). In contrast, addition of Compound **3b**,
 244 Compound **3c**, Compound **3d**, Compound **3e**, Compound **3f** or (PhSe)₂ to the medium
 245 significantly reduced TBARS levels in cells exposed to H₂O₂, as compared to the cultures from
 246 stress group ($p < 0.05$).

Figure 2: Thiobarbituric acid reactive species (TBARS, **A**), 4-hydroxy-2'-nonenal (4-HNE, **B**) and 8-isoprostane (8-ISO, **C**) levels in cultures of neurons obtained from 7-day-old-mice exposed to hydrogen peroxide (H₂O₂) alone or in the presence of organoselenium compounds (diphenyl diselenide ((PhSe)₂) or one of each tested selenocyanates (**3a–f**)). The concentration of each organoselenium compound in the medium was 10 μM. Data were expressed as malondialdehyde equivalents nmol per milligram protein (MDA equivalents nmol/mg protein – TBARS), micrograms per milligram protein (μg/mg protein – 4-HNE) and picograms per milligram protein (pg/mg protein – 8-ISO). *n* = 4 animals per group. **p* < 0.05, as compared to control group; #*p* < 0.05, as compared to stress group. (Tukey's *post hoc* test).

The next step in the assessment of lipid peroxidation of the samples was determination of the 4-HNE content (**Figure 2B**). Significant increases in this parameter were detected in cell cultures exposed to H₂O₂ alone or in combination with Compound **3a** or Compound **3c**, as compared to control cells (H₂O₂: *p* = 0.01002; Compound **3a**: *p* = 0.0053; Compound **3c**: *p* = 0.00103). However, 4-HNE levels in cells exposed to H₂O₂ whose medium received Compound **3b**, Compound **3d**, Compound **3f** or (PhSe)₂ exhibited a trend to decrease in comparison to cells from stress group (Compound **3b**: *p* = 0.22; Compound **3d**: *p* = 0.74; Compound **3e**: *p* = 0.99; Compound **3f**: *p* = 0.16; (PhSe)₂: *p* = 0.61).

Finally, 8-ISO content was measured to provide further insight on the lipid peroxidation elicited by H₂O₂ (**Figure 2C**). Significantly increased levels of such marker were detected in cells exposed to H₂O₂ alone or combined with Compound **3a**, Compound **3c** or Compound **3e**, as compared to control cells (H₂O₂: *p* = 0.000004; Compound **3a**: *p* = 0.000008; Compound **3c**: *p* = 0.00019; Compound **3e**: *p* = 0.014). In contrast, addition of Compound **3b**, Compound **3d**, Compound **3f** or (PhSe)₂ into the medium of cells exposed to H₂O₂ produced decrease in the 8-ISO content, as compared to cells submitted to the oxidative challenge in a selenocyanate free medium (Compound **3b**: *p* = 0.000004; Compound **3d**: *p* = 0.000007; Compound **3f**: *p* = 0.00016; (PhSe)₂: *p* = 0.000051).

Effect of different selenocyanates on the H₂O₂-induced oxidative damage against DNA and proteins

In the present contribution, DNA damage was estimated on the basis of 2'-deoxyguanosine residue oxidation into 8-OHdG. There was increase in the levels of this by-product in cells receiving H₂O₂ alone or combined with Compound **3a** or Compound **3c**, in comparison to control cells (H₂O₂: *p* < 0.05; Compound **3a**: *p* < 0.05; Compound **3c**: *p* < 0.05). Nevertheless, reduced 8-OHdG content was detected in the cells submitted to the oxidative challenge but receiving one of each remaining selenocyanates in their medium, as compared to cell cultures from stress group (Compound **3b**: *p* < 0.05; Compound **3d**: *p* = 0.00044; Compound **3e**: *p* < 0.05; Compound **3f**: *p* = 0.000001; (PhSe)₂: *p* = 0.00025). **Figure 3A** depicts data obtained with determination of 8-OHdG levels in the cell cultures.

Figure 3: 8-Hydroxy-2'-deoxyguanosine (8-OHdG, **A**) and protein carbonyl (**B**) levels in cultures of neurons obtained from 7-day-old-mice exposed to hydrogen peroxide (H₂O₂) alone or in the presence of organoselenium compounds (diphenyl diselenide ((PhSe)₂) or one of each tested selenocyanates (**3a–f**)). The concentration of each organoselenium compound in the medium was 10 μ M. Data were expressed as nanograms per milligram protein (ng/mg protein) and nanomoles per milligram protein (nmol/mg protein). $n = 4$ animals per group. * $p < 0.05$, as compared to control group; # $p < 0.05$, as compared to stress group. (Tukey's *post hoc* test).

Additionally, increased carbonyl content – an important parameter correlated with protein damage, was found in cells receiving H₂O₂ alone or in combination with Compound **3a** or Compound **3c**, in comparison to control cells (H₂O₂: $p = 0.000022$; Compound **3a**: $p = 0.0018$; Compound **3c**: $p = 0.00012$). In contrast, addition of Compound **3b**, Compound **3d**, Compound **3e**, Compound **3f** or (PhSe)₂ to the cell medium exposed to H₂O₂ significantly mitigated increased carbonyl levels, as compared to the cell culture submitted to oxidative challenge with no addition of selenocyanate (Compound **3b**: $p = 0.000004$; Compound **3d**: $p = 0.0021$; Compound **3e**: $p = 0.00027$; Compound **3f**: $p = 0.00028$; (PhSe)₂: $p = 0.00047$). **Figure 3B** shows the findings obtained with determination of carbonyl content in the cultures.

Effect of the synthesized compounds on activity of antioxidant enzymes

Figure 4 depicts effect of selenocyanates (**3**) on the CAT activity. Oxidative challenge elicited by H₂O₂ produced significant increase in this parameter ($p < 0.05$, **Figure 4A**). In comparison to neurons only receiving H₂O₂, decreased CAT activity was detected in all cultures in which there was addition of any organoselenium compound in particular (**Figure 4A**; $p < 0.05$).

In contrast, no significant differences in SOD activity were found between control cultures and neurons receiving H₂O₂ (**Figure 4B**; $p = 1.00$). Interestingly, addition of Compound **3a**, Compound **3b**, Compound **3c**, Compound **3d** or (PhSe)₂ elicited upregulation of SOD activity, as compared to the control or H₂O₂ cultures (**Figure 4B**; $p < 0.05$). A similar effect was not observed when Compound **3e** or Compound **3f** was added into the medium (**Figure 4B**; $p > 0.05$).

Figure 4: Catalase (A) and superoxide dismutase (B) activities in cultures of neurons obtained from 7-day-old-mice exposed to hydrogen peroxide (H₂O₂) alone or in the presence of organoselenium compounds (diphenyl diselenide ((PhSe)₂) or one of each tested selenocyanates (3a–f)). The concentration of each organoselenium compound in the medium was 10 μM. Data were expressed as enzyme units per milligram protein (U/mg protein). *n* = 4 animals per group. **p* < 0.05, as compared to control group; #*p* < 0.05, as compared to stress group. (Tukey's *post hoc* test).

Discussion

Administration of compounds bearing the selenocyanate functional group in their structure was significantly associated to inhibition of lipid peroxidation and enhancement of antioxidant enzyme defences in the liver of mice receiving cadmium [38]. In addition, antioxidant activity was implicated in at least part of therapeutic properties of organoselenium compounds [39-40]. This effect was further showed in studies performed with cell culture [41-43] and animal species such as rats [44-45], fishes [46], *Drosophila melanogaster* [47], and *Caenorhabditis elegans* [48].

Present study reported that cells submitted to oxidative challenge with H₂O₂ in culture medium containing Compound 3b, Compound 3c, Compound 3d, Compound 3e, Compound 3f or the control compound (PhSe)₂ exhibited lower TBARS levels than cells in similar conditions but without addition of selenocyanates; Compound 3a was the only that failed to prevent this oxidative modification elicited by H₂O₂. Furthermore, all compounds failed to prevent increase in the 4-HNE levels elicited by H₂O₂. However, a trend to decrease in such parameter was observed in cells subjected to oxidative challenge in the presence of Compound 3b, Compound 3d, Compound 3f or (PhSe)₂. With respect to 8-ISO content, Compound 3a, Compound 3c or Compound 3e was not effective in normalization of the aberrant marker levels detected in cells into a pro-oxidant culture environment. Presence of Compound 3b, Compound 3d, Compound 3f or (PhSe)₂ in the medium collaborated to lowering 8-ISO levels in cells at oxidative challenge. In addition, increased 8-OHdG levels were detected in cells exposed to H₂O₂ alone or combined with Compound 3a or Compound 3c, whereas decreased content of this marker was found in cells receiving Compound 3b, Compound 3d, Compound 3e, Compound 3f or (PhSe)₂. Similarly, it was showed higher carbonyl content in cells receiving H₂O₂ alone or combined with Compound 3a or Compound 3c; in contrast, presence of Compound 3b, Compound 3d, Compound 3e, Compound 3f or (PhSe)₂ in the medium triggered significant decrease in this parameter. All the synthesized compounds were implicated in significant attenuation of CAT activity, whereas four compounds (Compounds 3a-d) and (PhSe)₂ produced up regulation of SOD activity when added to the cultured neurons exposed to H₂O₂.

Thus, Compound **3b**, Compound **3d** and Compound **3f** exhibited the most favorable antioxidant profile, potentially with higher scavenging activity towards reactive oxygen species. Since (PhSe)₂ presents GPx-like antioxidant activity and so was used in present study as reference [23], the three aforementioned compounds could in part act mimicking this enzyme activity. On the other hand, Compound **3a** and Compound **3c** were the substances with the worst performance. Compound **3e**, in turn, showed moderate antioxidant profile, due to fact of this compound did not counteract alterations in the levels of 4-HNE and 8-ISO. Regarding R groups attached to the aromatic ring bound to a five-carbon branch bearing a selenocyanate group, nitro or bromine functional group at *para* orientation and chlorine group at *orto* orientation could be pivotal to the antioxidant profile of the molecules designed with such R groups. Accordingly, compounds with bromine group at *orto* orientation or chlorine group at *para* orientation showed weaker antioxidant activity, which substantiates the importance of R group position on the activity of each selenocyanate. Indeed, minimal structural alterations in selenocyanates may strictly influence their antioxidant activity, as showed in the paper performed by Ibrahim and coworkers [40]. The precise mechanisms underpinning marked differences in the antioxidant profile of compounds with so similar structure are unknown, but they may be partly driven by the experimental observation indicating that bromine is a weaker functional group to withdraw electrons, as compared to chlorine or nitro groups, keeping a higher electron density at the selenocyanate group [49]. Although (PhSe)₂ is not a selenocyanate, its antioxidant activity may be related to the phenylselenenyl group, which was recently described by Vogt and colleagues [50] as pivotal for the antioxidant roles of 7-chloro-4-phenylselenenyl-quinoline.

Bis(2-hydroxyphenyl) diselenide and other diphenyl diselenides possess remarkable radical trapping activity, preventing increase in the contents of protein carbonyls and lipid hydroperoxides elicited by oxidative imbalances [51]. The reference compound, (PhSe)₂, has been tested to manage several pathological conditions characterized by oxidative disturbances. To illustrate, the antiviral property of the compound was associated to its antioxidant action in mice infected with type 2, *Herpes simplex* virus in the paper performed by Sartori and coworkers [52]; this antioxidant activity was characterized by decrease in MDA content and alleviation of CAT and SOD inhibition – parameters assessed in the present study. Furthermore, (PhSe)₂ was showed to counteract reductions in the content of total thiol and activity of antioxidant enzymes as well the release of reactive oxygen species and TBARS elicited by acute intoxication with manganese in *Drosophila melanogaster* [47]. The compound was also effective to mitigate protein and lipid oxidative modifications and collaborated to normalize CAT and SOD activities in colon of rats submitted to dextran sulfate-induced colitis [53]. Moreover, (PhSe)₂ markedly decreased levels of reactive oxygen species and alleviated inflammation in the spleen of rodents chronically infected with *Toxoplasma gondii* [47]. Therefore, our findings on the effect of (PhSe)₂ corroborate its previously reported antioxidant activity.

Since marked antioxidant effect was assigned to Compound **3b**, Compound **3d** and Compound **3f** and the fact of antioxidant activity was showed to contribute to the therapeutic properties of organoselenium compounds [40], it is expected that these novel compounds could be promising in experimental studies performed in pathological scenarios in which oxidative stress is strongly implicated. We cannot ascertain the precise mechanisms involved in this antioxidant effect. Possible ways may include the direct trapping (and eventual decomposition) of the reactive oxygen species in the cell medium, mimicking the activity of antioxidant enzymes, as described for (PhSe)₂ and Ebselen. On the other hand, compounds can act indirectly, triggering signaling cascades aiming to abrogate oxidative stress. Indeed, several antioxidants strongly

activate a signaling molecule involved in response to oxidative stress and cell survival termed nuclear factor erythroid 2-related factor 2 (Nrf2) [55]. For example, supplementation with the antioxidant *p,p'*-methoxyl-diphenyl diselenide was implicated in Nrf2 activation in frontal cortex of rats submitted to experimental pain-depression dyad [44]. 3-Selena-1-dethiacephem and (PhSe)₂ were also involved in Nrf2 pathway activation, contributing for the antioxidant roles of these organoselenium compounds [56,57]. Thus, Nrf2 activation by the novel selenocyanates cannot be ruled out in the present study, potentially substantiating the reported antioxidant properties.

It is noteworthy that selenocyanates have been tested for therapy of oxidative stress-driven diseases, including cancer [58]. In this regard, supplementation with 1,4-phenylenebis(methylene)selenocyanate (*p*-XSC) was showed to abrogate the tumorigenesis process triggered by nitrosamine and enhance antioxidant defenses in mouse lung [59]. In addition, the 8-OHdG levels, a parameter measured in the present contribution, provide an estimation of cancer risk. Recently, Wu and colleagues [60] detected significant increase in the 8-OHdG content in leucocytes from cancer patients, as compared to healthy subjects. Evidence concerning a potential role of selenocyanates in the reduction of DNA oxidative modification, estimated as 8-OHdG levels, is scarce yet. Nevertheless, *p*-XSC was also showed decrease 8-OHdG levels in rat mammary glands, 6 hours after intragastric administration of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine – a well known carcinogenic agent [61,62]. Other organoselenium compound able to decrease 8-OHdG content in animals is (PhSe)₂ (5 µmol/kg body weight), as described in the brain of mice orally receiving methylmercury, which was accompanied by decreased brain-derived neurotrophic factor level, oxidative disturbances and histological modifications in the cerebral cortex [63]. Selenocyanates can also reduce genotoxicity induced by certain compounds *in vivo*. To illustrate, oral administration of diphenylmethyl selenocyanate produced significant decrease in the DNA damage in hepatocytes of mice acutely receiving carbon tetrachloride [64]. Thereby, Compound **3b**, Compound **3d**, Compound **3e** and Compound **3f** have potential antigenotoxic activity by mitigate the increase in 8-OHdG content induced by H₂O₂ in the cell cultures.

Conclusions

In conclusion, we have described the synthesis and preclinical antioxidant assessment of novel selenocyanates (**3a–f**) using standardized and widely available methodologies. All selenocyanates were evaluated for their antioxidant ability *in vitro*. Compound **3b**, Compound **3d** and Compound **3f** showed significant activity when tested in cultured mouse neurons exposed to H₂O₂, with a pattern resembling to (PhSe)₂. Measurement of CAT and SOD activities provided further evidence that most of the synthesized compounds are endowed of marked antioxidant activity. We did not use various concentrations of each compound in culture aiming determine the best value with minimal potential adverse events (or a dose-response curve), which is one limitation of the present work. We argue that further *in vitro* studies using these promising selenocyanates will pave the way for their use in most sophisticated pathological experimental scenarios and potentially as a novel platform of antioxidant drugs with therapeutic properties.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Authors' Contributions

Jamal Rafique, Tiago E. A. Frizon, José H. Cararo, Sumbal Saba, Felipe Dal-Pizzol, and Samira S. Valvassori conceived the project and wrote the manuscript. Tiago E. A. Frizon, Sumbal Saba, and Jamal Rafique performed the synthesis and characterization analysis (NMR, FTIR, HRMS). Tairine Pimentel, and Hugo de C. Braga performed the purification of **3a-f**. Gustavo C. Dal-Pont, Monique Michels, Felipe Dal-Pizzol, and Samira S. Valvassori performed the biological evaluation.

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Supplementary Materials

Figure S1, ¹H and ¹³C NMR Spectra for compound **3a** in supplementary materials, pg 2.
Figure S2, ¹H and ¹³C NMR Spectra for compound **3b** in supplementary materials, pg 3.
Figure S3, ¹H and ¹³C NMR Spectra for compound **3c** in supplementary materials, pg 4.
Figure S4, ¹H and ¹³C NMR Spectra for compound **3d** in supplementary materials, pg 5.
Figure S6, ¹H and ¹³C NMR Spectra for compound **3e** in supplementary materials, pg 6.
Figure S6, ¹H and ¹³C NMR Spectra for compound **3f** in supplementary materials, pg 7.
Figure S7, FTIR Spectra for compound **3a** in supplementary materials, pg 8.
Figure S8, FTIR Spectra for compound **3b** in supplementary materials, pg 8.
Figure S9, FTIR Spectra for compound **3c** in supplementary materials, pg 9.
Figure S10, FTIR Spectra for compound **3d** in supplementary materials, pg 9.

478 Figure S11, FTIR Spectra for compound **3e** in supplementary materials, pg 10.
 479 Figure S12, FTIR Spectra for compound **3f** in supplementary materials, pg 10.
 480 Figure S13, FTIR Spectra for compound **2a** in supplementary materials, pg 11.
 481 Figure S14, FTIR Spectra for compound **2b** in supplementary materials, pg 11.
 482 Figure S15, FTIR Spectra for compound **2c** in supplementary materials, pg 12.
 483 Figure S16, FTIR Spectra for compound **2d** in supplementary materials, pg 12.
 484 Figure S17, FTIR Spectra for compound **2e** in supplementary materials, pg 13.
 485 Figure S18, FTIR Spectra for compound **2f** in supplementary materials, pg 13.

486 Keywords

487 antioxidants • drug design • oxidative stress • selenium • selenocyanates.

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The synthesis and preclinical antioxidant assessment of novel aromatic selenocyanates using standardized and widely available methodologies. Furthermore, assessment of the effect of these compounds on the oxidative challenge elicited by hydrogen peroxide (H₂O₂) in mixed cultures of mouse neurons was undertaken, in order to provide insightful cues on their biological activity.

SUPPORTING INFORMATION

Synthesis of novel selenocyanates and evaluation of their effect in cultured mouse neurons submitted to oxidative stress

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(I) NMR spectra of the products 3a-f:

Figure S1- Nuclear Magnetic Resonance Spectra: **(a)** ^1H NMR (200 MHz) in CDCl_3 and **(b)** ^{13}C NMR (50 MHz) in CDCl_3 for compound **3a**.

Figure S2- Nuclear Magnetic Resonance Spectra: **(a)** ^1H NMR (300 MHz) in CDCl_3 and **(b)** ^{13}C NMR (101 MHz) in CDCl_3 for compound **3b**.

Figure S3- Nuclear Magnetic Resonance Spectra: **(a)** ^1H NMR (400 MHz) in CDCl_3 and **(b)** ^{13}C NMR (101 MHz) in CDCl_3 for compound **3c**.

Figure S4- Nuclear Magnetic Resonance Spectra: **(a)** ^1H NMR (300 MHz) in CDCl_3 and **(b)** ^{13}C NMR (100 MHz) in CDCl_3 for compound **3d**.

Figure S5- Nuclear Magnetic Resonance Spectra: **(a)** ^1H NMR (400 MHz) in CDCl_3 and **(b)** ^{13}C NMR (101 MHz) in CDCl_3 for compound **3e**.

Figure S6- Nuclear Magnetic Resonance Spectra: **(a)** ^1H NMR (400 MHz) in CDCl_3 and **(b)** ^{13}C NMR (101 MHz) in CDCl_3 for compound **3f**.

(II) FTIR spectra of the products 3a-f:

Figure S7- Infrared spectra of compound 3a.

Figure S8- Infrared spectra of compound 3b.

Figure S9- Infrared spectra of compound **3c**.

Figure S10- Infrared spectra of compound **3d**.

Figure S11- Infrared spectra of compound **3e**.

Figure S12- Infrared spectra of compound **3f**.

(III) FTIR spectra of the starting materials 2a-f:

Figure S13- Infrared spectra of starting material 2a.

Figure S14- Infrared spectra of starting material 2b.

Figure S15- Infrared spectra of starting material **2c**.

Figure S16- Infrared spectra of starting material **2d**.

Figure S17- Infrared spectra of starting material **2e**.

Figure S18- Infrared spectra of starting material **2f**.

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