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Selenium-containing polyphenols: antioxidant properties

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Selenium-containing polyphenols: antioxidant properties.

Resumo

Apesar de não ser recente, o interesse em compostos orgânicos contendo selénio na sua composição tem vindo a ser cada vez maior, em grande parte devido ao seu potencial antioxidante e anticarcinogénico. Nesse sentido, a introdução de um átomo de selénio em moléculas onde este não existe inicialmente é vista como uma técnica promissora na busca de antioxidantes mais potentes e versáteis para possíveis fins biomédicos.

Este trabalho teve como objectivo a introdução de um átomo de selénio em diferentes estruturas moleculares, de forma a obter um painel de compostos que pudessem ser testados pela sua potencial actividade antioxidante. Para tal, foi inicialmente testado um método de síntese de purinas selenadas com recurso a microondas. No entanto, as condições reaccionais não permitiram obter produtos passíveis de análise, pelo que a estratégia de síntese foi alterada, tendo sido escolhido um método sintético baseado na selenação directa de estilbenos com recurso a pó de selénio, que originou vários benzo-selenofenos, em rendimentos baixos na maioria dos casos. Estes foram totalmente caracterizados estruturalmente, nomeadamente por Ressonância Magnética Nuclear e Espectrometria de Massa.

De modo a comprovar o pretendido aumento da potencial actividade antioxidante relativamente aos estilbenos de partida, os benzo-selenofenos sintetizados foram testados pelas suas actividades de armadilha de radicais livres e actividade mimética da glutationa peroxidase. Relativamente ao primeiro ensaio, dos três compostos sintetizados, tanto o **31a** como o **36** apresentam melhorias no que diz respeito à capacidade de transferência de um átomo de hidrogénio, quando comparados com o respectivo material de partida. A energia de dissociação da ligação O-H foi calculada computacionalmente. Os resultados mostram que após a selenação, tanto os compostos **35** como **36** apresentam uma diminuição na energia de ligação O-H para os grupos presentes nas respectivas estruturas. Relativamente ao composto **31a**, verifica-se a diminuição da energia de ligação O-H na posição R_1, estruturalmente mais perto do átomo de selénio, e um aumento dessa mesma energia para as ligações O-H nas posições R_3 e $R_{4'}$. Em relação ao segundo teste, todos os quatro benzo-selenofenos sintetizados apresentam um aumento significativo na capacidade de redução de peróxido de hidrogénio, por comparação com o estilbeno de partida.

Os resultados apresentados mostram-se promissores, e indicam que a selenação de compostos com potencial antioxidante pode promover um aumento dessa mesma actividade, sendo esta uma técnica a ter em conta quanto à formulação de novas aplicações com fins terapêuticos.

Palavras-Chave: selénio, organoselenados, selenação, antioxidante.

Abstract

Interest in organic compounds containing selenium in its composition has grown, mainly due to its antioxidant and anti-carcinogenic potential. For that reason, introducing a selenium atom in molecules where it is not present initially is viewed as a promising technique in order to attain more powerful and versatile antioxidants with possible biomedical purposes.

The present work was aimed at introducing a selenium atom in different molecular structures, in order to obtain several compounds able to be tested for their potential antioxidant activity. As such, a methodology based on microwave synthesis was initially considered for the preparation of selenated purines. However, reaction conditions did not allowed to obtain products able to be analysed. A new synthetic methodology was then selected, based on the direct selenation of stilbenes using selenium powder, which afforded the desired benzoselenohene compounds in low yields, for most cases. These were structurally completely characterized, namely by the Nuclear Magnetic Resonance and Mass Spectrometry techniques.

In order to confirm the desired increase of antioxidant activity, synthesized compounds were tested for their free radical scavenging and GPx-like activities. For the first test, of the three tested compounds, **31a** and **36** presented improvements regarding the H-atom transfer activity, when compared to their respective starting materials. O-H bond dissociation energy (BDE) was computationally calculated. Results show that selenation for both compounds **35** and **36** leads to a decrease in O-H bond energy for all the OH groups present in those compounds. Compound **31a** presents a reduction of the O-H bond energy for the OH group at position R_1, structurally closer to the new selenium atom, and an O-H bond energy increase for the OH groups at positions R_3 and $R_{4'}$. For the second test, all four synthesized compounds showed a significant increase in the reduction of hydrogen peroxide, when compared with the parent stilbene.

Results presented in this work are promising, and indicate that the antioxidant activity of stilbenes is boosted upon selenation, making this a technique to consider when developing new drugs for therapeutic purposes.

Keywords: selenium, organoselenium, selenation, antioxidant.

Acknowledgments

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Glossary

[Aox]i - initial antioxidant concentration
Abs. - visible absorbance at time *t*
Abs.initial - initial absorbance
Abs.final - final visible absorbance
BDE - bond dissociation enthalpy
^{13}C NMR - Carbon 13 Nuclear Magnetic Resonance
COSY - correlation spectroscopy
d - duplet
DMAP - 4-dimethylaminopyridine
DMF - dimethylformamide
DMSO - deuterated dimethylsulfoxide
DNA - deoxyribonucleic acid
DPPH - 2,2-diphenyl-1-picrylhydrazyl
DTT_{red} - reduced dithiotreitol
EDTA - Ethylenediamine tetraacetic acid
ESI - electrospray ionization
GSH - glutathione
GSSG - glutathione dissulfide
GPx - Glutathione Peroxidase
^{1}H NMR - proton Nuclear Magnetic Resonance
HMBC - heteronuclear multiple bond correlation
HSQC - heteronuclear single quantum coherence
IC_{50} - half maximal inhibitory concentration
IMPDH - inosine 5'-monophosphate dehydrogenase
m/z - mass-to-charge ration
met-tRNA - methionine transfer ribonucleic acid
min. - minutes
ppm - parts per million
MS - Mass spectrometry
NADPH - reduced Nicotinamide adenine dinucleotide phosphate
n_{tot} - total stoichiometry
$^{\bullet}OH$ - hydroxyl radical
$(O_2)^{-}$ - superoxide anion radical
ρ - para
prep. T. L. C. - preparative thin layer chromatography
ROS - Reactive oxygen species
^{77}Se NMR - Selenium 77 Nuclear Magnetic Resonance
$t_{1/2}$ - half-life
t - triplet
THF - tetrahydrofuran
T. L. C. - thin layer chromatography
TrxR1 - thioredoxin reductase 1
Trx - thioredoxin reductase
v_0 - initial rate
W.R. - Woolins' reagent
δ - chemical shift

I. Introduction

I.1. Selenium

Despite having a crucial role in human metabolism, selenium (Se) has a low natural occurrence[1,2] and therefore is classified as a trace element. During the past 40 years it went from being known as a toxin that caused serious health problems, to an essential micronutrient, with benefits for both human and animal wellbeing, when used in proper concentration[3]. The recent realization of its role in human metabolism and the effects it may have as an antioxidant and anticancer element, led to an increase in its scientific interest and subsequent research.

Selenium was discovered in 1817 by the swedish scientist Jöns Jakob Berzelius. He was investigating an illness affecting workers in a chemical factory at Gripsholm, Sweden, where acetic, nitric, and sulfuric acids were produced. Berzelius guess was that this illness could be related to arsenic contamination from the sulfur ore, and further analysis led him to discover an unknown element that had properties similar to tellurium. For that it was named after the greek goddess of the moon, *Selene*, since tellurium was a reference to the latin word for "earth", *tellus*.

Selenium is a chemical element of the group 16 of the periodic table and along with oxygen, sulfur, tellurium, and polonium, all are referred to as chalcogens or the oxygen family. Se atomic number is 34, giving it an [Ar] $3d^{10}$ $4s^2$ $4p^4$ electronic distribution, and common oxidation states of +6, +4, -2 [4]. There are six naturally occurring stable Se isotopes (Table I.1)[5].

Table I.1 – Selenium isotopes with a known natural abundance. Adapted from [5].

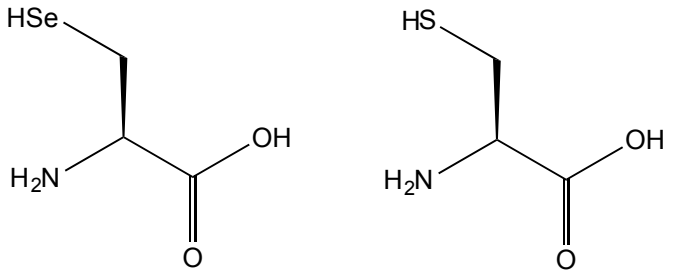
Atomic Mass Number	Natural Abundance (%)	Half-life
74	0.89	Stable
76	9.37	Stable
77	7.63	Stable
78	23.77	Stable
80	49.61	Stable
82	8.73	Stable

I.1.1. Selenium and its biological role

Selenium has had a controversial part regarding its biological role since it was discovered. The toxicological role of selenium was initially identified in the 1930s, as cause of livestock death in some parts of the U.S.A. due to high concentrations in cereal grains[6]. Later, in 1957, it was recognized as essential to human metabolism[7] and, in 1973, its key role against oxidative stress was revealed.

Selenium is acquired mainly through the diet, being found in different food sources such as meat, cereals, grains, milk, fruits and vegetables[8]. The average recommended daily intake is 60 µg (for men) and 53 µg (for women), with dosages above 400 µg considered to be toxic. Excess selenium consumption can lead to a condition named selenosis, with main signs consisting of hair and nail loss. Other, more serious symptoms include skin and nervous system lesions, nausea, diaharrea, skin rashes, mottled teeth, fatigue, irritability, and nervous system abnormalities[9].

Se is an important element to the human metabolism, and that is verified by its presence in most mammalians in the form of seleno-containing proteins. At least 25 distinct mammalian selenoproteins [8] are currently known and are characterized by possessing in its structure the amino acid residue selenocysteine (Fig. I.1, **1**), structurally very similar to cysteine (Fig. I.1, **2**), where the side chain sulfur atom is replaced for a selenium atom.

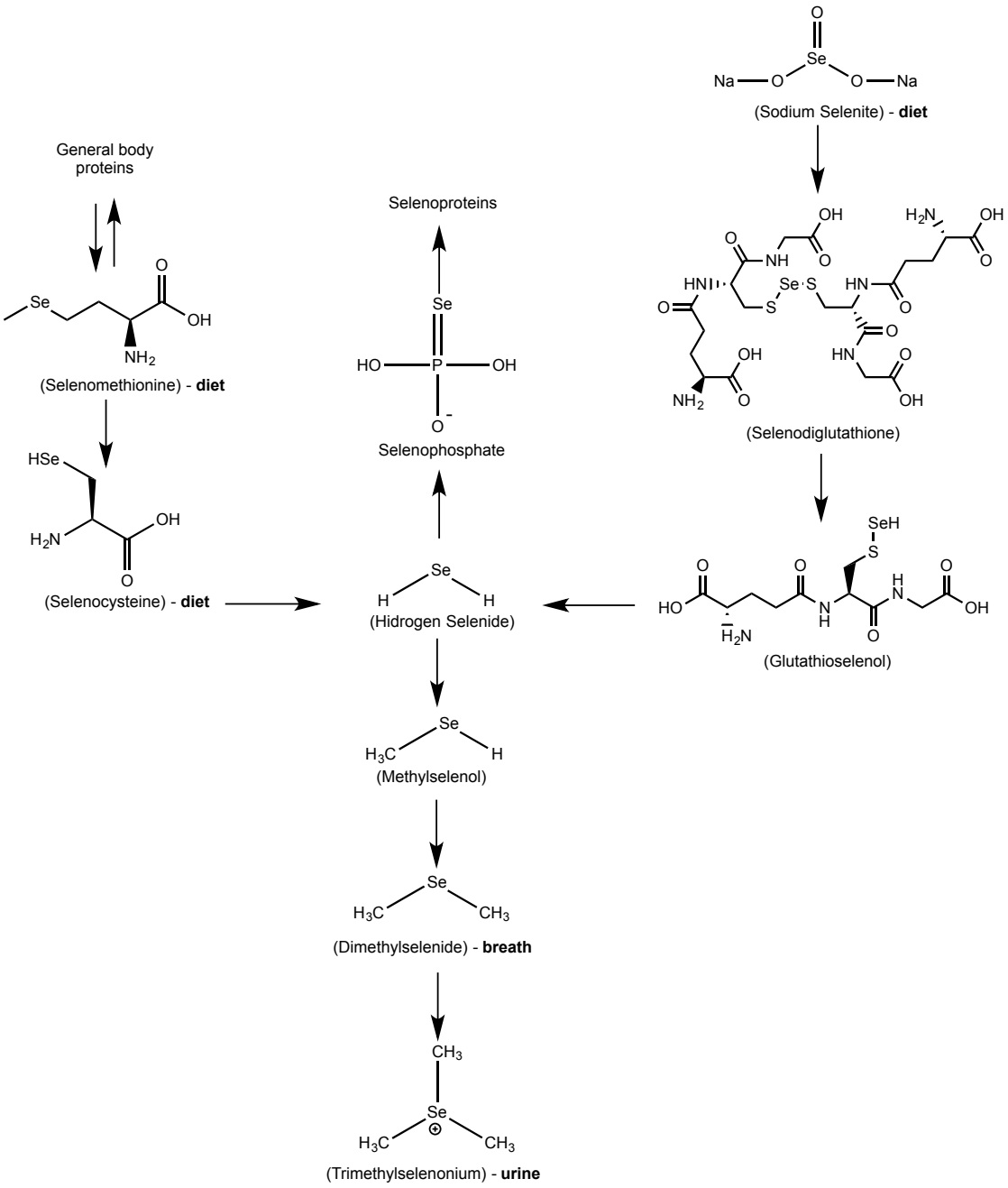


1 **2**

Figure I.1 - Selenocysteine (1) and cysteine (2) structures.

Selenium can be incorporated into human metabolism through more than one way. As shown [10,11](Scheme I.1), selenomethionine can be incorporated into general body proteins instead of methionine since it readily acylates met-tRNA. Alternatively, it can be converted through the trans-sulfuration mechanism to selenocysteine, which is degraded to hydrogen selenide (H_2Se). Additionally, selenite is metabolized to hydrogen selenide via selenodiglutathione and glutathione selenopersulfide. Hydrogen selenide is considered to be

the precursor for supplying Se in an active form for the synthesis of selenoproteins. It can then be excreted via exhalation in the form of dimethylselenide ($(CH_3)_2Se$) or via urine as trimethylselenonium ($(CH_3)_3Se^+$).



Scheme I.1 - Selenium metabolic pathway. Adapted from [10,11].

The majority of Se-containing proteins are synthesized in the liver, making it a key organ in Se metabolism [12]. There are only few selenoproteins that still do not have a known function in the human metabolism, but most of them are well studied and have extremely important enzymatic functions. A few examples are presented (Table I.2).

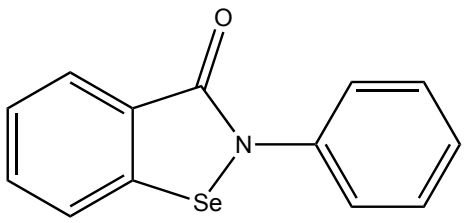
Table I.2 - Some selenoproteins and their function. Adapted from [8,12].

Selenoprotein	**Function**
Iodothyronine deiodinase	Regulation of thyroid hormone activity by reductive deodination.
Glutathione peroxidase (GPx)	Glutathione-dependent detoxification of hydrogen peroxide.
Thioredoxine reductase (TRx)	Reduction of the oxidized form of cytosolic thioredoxin.

I.2. Organoselenium compounds

Organoselenium chemistry has become a matter of great interest in recent past, mostly due to its wide utility and biological beneficial effects. In particular, a considerable number of organoselenium compounds have been reported as promising candidates for cancer therapy and prevention due to their ability to modulate multiple physiological functions implicated in cancer development and progression, by presenting antioxidant [13], chemopreventive [14–16] , or apoptotic [17] activities. Regarding antioxidant activity, the protective effects of selenium-containing compounds are related to their ability of scavenging one electron (radical) and two-electron oxidants; rapid and ready repair of oxidized selenium species; binding to metal ions; and their role as catalytically active residues in multiple protective enzymes [13], being these the characteristics of organoselenium compounds responsible for their anticarcinogenic activity.

One of the most successful examples of organoselenium compounds is 2-phenyl-1,2-benzisoselenazole-3 (2H)-one, commonly referred to as ebselen (Fig. I.2, **3**).



3

Figure I.2 – Ebselen (3) structure.

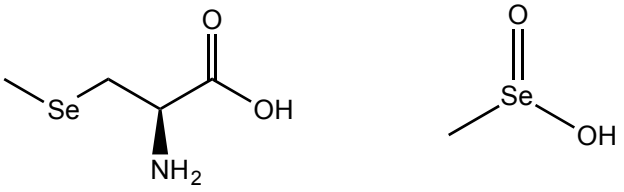
It is a compound that has been intensively studied for its glutathione peroxidase-like (GPx-like) and radical scavenging activities, among other characteristics that make it a very interesting potential drug.

I.2.1. Organoselenium compounds as anticarcinogenic and chemopreventive agents

Se status of a possible anti-carcinogenic is relatively recent. In fact, during the 1940's different Se forms were reported as carcinogens in rat livers [18], and in 1969 elemental Se was associated with the risk of cancer [19]. The hypothesis that Se could reduce experimental carcinogenesis lead to an intensive study [20–24] that resulted in solid evidence of an inverse relationship between Se intake and risk of cancer in humans.

Based on the effects that selenoenzymes have on antioxidant defences, several mechanisms have been proposed for the anti-carcinogenic activity of Se, such as GPx-like and radical scavenging activities, programmed cell death, DNA repair, carcinogen detoxification, regulation of cell proliferation, and tumour cell invasion [25,26]. It is also most likely that Se does not reduce carcinogenesis by a single mechanism, but instead by combined use of the ones mentioned above.

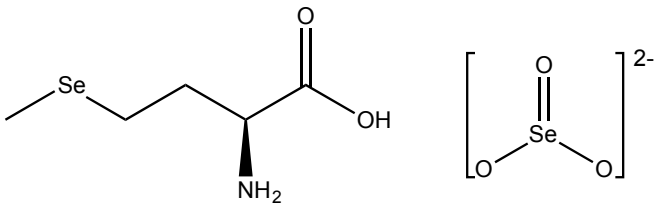
One remarkable characteristic about selenium-containing molecules is that they are not organ specific, since tumour inhibition has been reported in mammary gland, liver, skin, pancreas, oesophagus, colon, and a few other sites. Instead, there is a dose-dependent response regarding the site [11]. Some examples of molecules that have been tested for their anti-carcinogenic properties are methylselenocysteine (Fig. I.3, **4**) and methyl-seleninic acid (Fig. I.3, **5**).



4 **5**

Figure I.3 - Methylselenocysteine (4) and methyl-selenenic acid (5) structures.

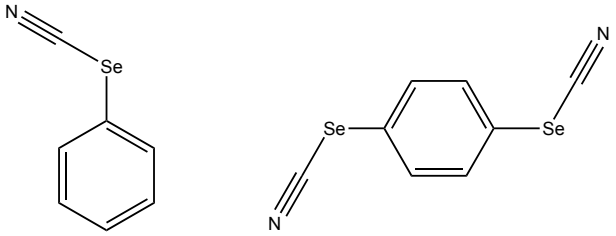
Methylselenocysteine (**4**) is reported to be more effective then selenomethionine (Fig. I.4, **6**), a natural occurring amino acid, in supressing the development of paramalignant lesions and the formation of adenocarcinomas in the mammary gland of carcinogen-treated rats [27], and is also reported to be a better chemopreventive agent than both selenomethionine (**6**) or selenite (**7**) [28]. Additionally, methyl-seleninic acid (**5**) has been reported to significantly suppress the growth of human premalignant breast cancer at levels of 5-10 µmol/L [29], while a concentration of 200 µmol/L of methylselenocysteine (**4**) was required to produce the same decrease in cell number.



6 **7**

Figure I.4 – Selenomethionine (6) and selenite (7) structures.

Examples of synthetic selenium-containing molecules with possible chemopreventive activity are benzylselenocyanate (Fig. I.5, **8**) and *ρ*-phenylenebis(methylene)selenocyanate (Fig. I.5, **9**).

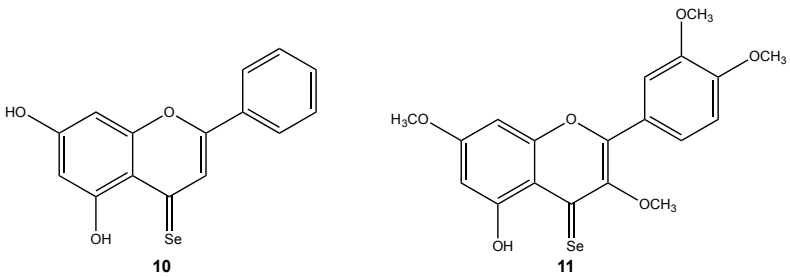


8 **9**

Figure I.5 – Benzylselenocyanate (8) and ρ-phenylenebis(methylene)selenocyanate (9) structures.

Benzylselenocyanate has proven to be a versatile chemopreventive agent in different model systems [30,31] and has been reported to inhibit the development of colon [32] and mammary [33] tumors in rats. Whereas ρ-phenylenebis(methylene)selenocyanate was developed [34] and proven [35] to be less toxic than benzylselenocyanate. Its efficiency for carcinogenesis in colon [36], lung [37], and tongue [38] cancer among others, such as its chemopreventive effects [15], have been reported.

Other examples of synthetic selenium-containing molecules with potential cancer chemotherapy applications are selenium-containing chrysin (SeChry) (Fig. I.6, **10**) and 3,7,3',4'-tetramethylquercetin (SePQue) (Fig. I.6, **11**).



10 **11**

Figure I.6 - Selenium-containing chrysin (10) and 3,7,3',4'-tetramethylquercetin (11) structures.

These compounds have been evaluated in nine human cancer cell lines of multiple origins, namely melanoma cells, colorectal adenocarcinoma cells, pancreatic

adenocarcinoma cells, breast adenocarcinoma cells, multidrug resistant breast adenocarcinoma cells, cervical adenocarcinoma cells, cisplatin-resistant cervical adenocarcinoma cells, and ovarian adenocarcinoma cells. Results show that replacing the oxygen atom of the carbonyl group by selenium presented a marked increase of cytotoxicity towards malignant cells, both in **10** and in **11**, when compared to their non-selenated analogues and also cysplatin, one of the most widely used anticarcinogenic drugs. In fact, **11** presented to be about 9-fold more cytotoxic towards malignant cells than its unprotected non-selenated derivative, and 3-fold more than cysplatin, one of the most widely used anticarcinogenic drugs.

As referred before, the anticancer and chemopreventive properties of organoselenium compounds are considered to be, at least in part, due to their antioxidant activity, which can be mediated by their ability to scavenge free radicals and/or by their capacity of affecting or mimicking the function of key redox enzymes [39].

I.3. Antioxidant activity of organoselenium compounds

Oxidative stress is a harmful condition characterized by a shift towards the pro-oxidant in the pro-oxidant/antioxidant balance in living cells, and this is an implied condition for a number of diseases where Alzheimer, Parkinson, and myocardial infarction are some examples [40].

Reactive oxygen species (ROS) are produced in human metabolism due to more than one cause. They can be generated by cellular energy production and also through environmental agents such as ultraviolet radiation, thermal stress, and tobacco smoke, among others. The cellular energy process production undergoes a sequence of reactions having oxygen as the final electron acceptor, originating ROS such as the superoxide anion radical ($(O_2)^{\cdot-}$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) among others [41]. Amongst ROS, superoxide anion radical and hydroxyl radical are free radicals due to having one unpaired electron and a short half-life, quickly reacting with multiple other cellular targets.

Oxidative reactions occur naturally in the human organism, and are balanced through antioxidants, that can be endogenous or attained by diet. Oxidative stress results in damage to cells [42], and exposure of a healthy cell to free radicals is known to damage structures and consequently to interfere with functions of enzymes and critical macromolecules. A free radical produced within a cell will react with surrounding molecules also within the cell to become paired and stable, resulting in oxidation of nucleic acids, lipids, carbohydrates, and proteins [43].

The human body has mechanisms in order to keep the equilibrium between pro- and antioxidants. Some mechanisms involve substances attained through human diet (carotenoids, alpha-tocopherol, ascorbic acid, and phenolic compounds are good examples)

that are able of inactivating free radicals. Other defence mechanisms involve endogenous enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (GPx).

I.3.1. Scavenging activity of organoselenium compounds

One mechanism through which the human body counteracts reactive oxygen species is by scavenging and neutralizing radicals, preventing them to cause any damage to healthy cells.

ROS can be generated through different processes. Superoxide anion radical is highly reactive [44] (Fig. I.7, (1)) and occurs upon reduction of O_2. It causes the inactivation of GPx, among others enzymes, and oxidation of intracellular components such as glutathione, due to its long half-life (0.05 s in the absence of scavengers) [45]. Hydrogen peroxide is not a radical species and is relatively stable [46], but its importance comes from the ability to generate ROS, particularly hydroxyl radical. Hydrogen peroxide is generated by the disproportionation of superoxide anion [47] (Fig. I.7, (2)). Hydroxyl radical is generated by the reduction of hydrogen peroxide through redox-active metal ions (Fig. I.7, (3) and (4)), and it is considered to be the most reactive and harmful ROS [46]. Its lifetime is limited, meaning it will react with molecules immediately after formation and subsequent release. It is reported to cause oxidation of lipids, proteins, nucleic acids, and also DNA modifications [48].

(1) $O_2 + e^- \rightarrow O_2^{-\bullet}$

(2) $2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$

(3) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$

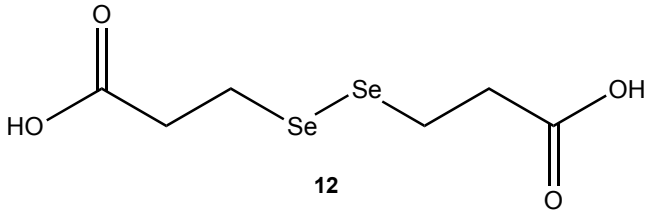
(4) $Cu^+ + H_2O_2 \rightarrow Cu^{2+} + {}^{\bullet}OH + OH^-$

Figure I.7 – ROS generation and decomposition reactions.

A balance between the formation of free radicals and protection against cellular damage induced by these species is essential for normal cellular function. When this balance is disrupted as a result of excessive generation of damaging species or low levels of antioxidants, a cell will enter a state of oxidative stress. Following this, the cell either repairs the damage or dies. However, if the damage persists, the cell will enter a state of genetic instability that can lead to chronic diseases or carcinogenesis [49].

Selenium compounds are well known for their ability to scavenge ROS. Indeed, ebselen (Fig. I.2, **3**), mentioned before, has been reported to enhance the antioxidant activity of the human enzyme thioredoxin reductase (Trx) system by acting as a substrate for the selenium-containing enzyme [50]. Additionally, among others, 3,3-diselenobispropionic acid

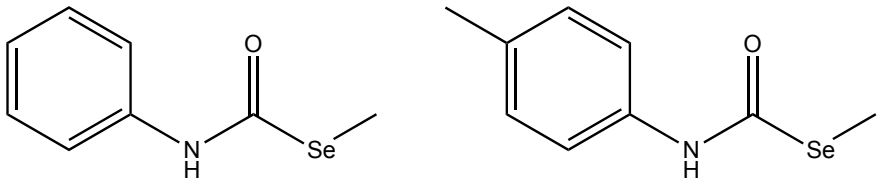
(Fig. I.8, **12**) has been reported [51] to scavenge peroxyl radical (CCl_3O_2), causing its antioxidant activity to be attributed to radical scavenging.



12

Figure I.8 – 3,3-diselenobispropionic acid (12) structure.

Selenocarbamates (Fig. I.9) analogues have also been reported [52] to present high radical scavenge activity towards superoxide radical, giving them importance as possible treatments for pathologies associated with superoxide radical and oxidative stress.

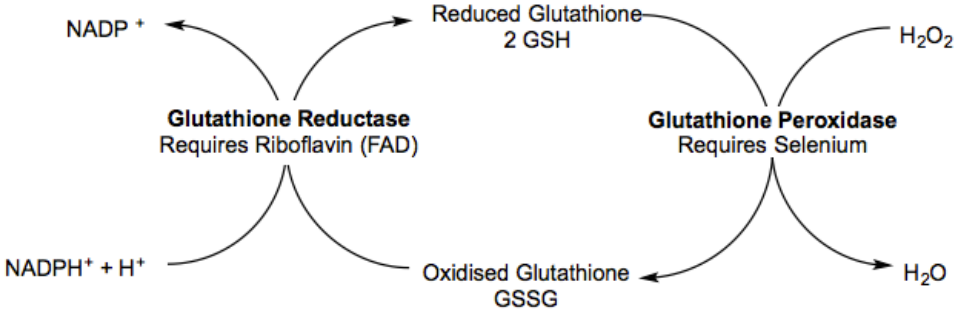


13 **14**

Figure I.9 – Selenocarbamate analogues. Methyl-*N*-phenylselenocarbamate (13) and methyl-*N*-(4-methylphenyl)selenocarbamate (14) structures.

I.3.2. GPx-like activity of organoselenium compounds

Another defence mechanism regarding oxidative stress involves glutathione peroxidase (GPx) (Scheme I.2). GPx is a selenoprotein with the ability to reduce free hydrogen peroxides to water, using glutathione as a co-factor, and can also contribute to cellular defence system against oxidative stress [53] .



Scheme I.2 - Glutathione reduction/oxidation mechanism.

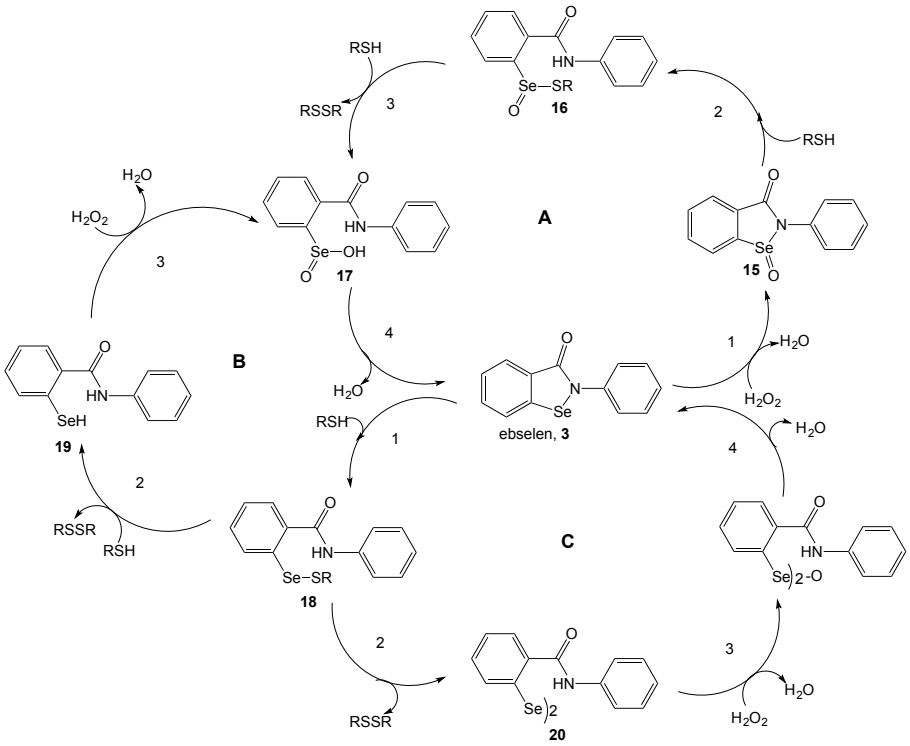
This is an important intracellular mechanism, allowing maintenance of the oxidation balance attained by GPx though oxidation of glutathione (GSH), in a process that reduces

hydrogen peroxide and generates glutathione dissulfide (GSSG) (Scheme I.2). Intracellular ratio between reduced GSH to GSSG is indicative of cellular oxidative stress.

Glutathione peroxidase acts as an antioxidant enzyme in plasma, inactivating free radicals and decomposing hydrogen peroxides and lipid peroxides [43]. Its activity and concentration have been shown to increase according to selenium consumption, until the dose-response ratio reaches a plateau, on serum selenium level between 70 and 90 ng/ml [54].

GPx catalyzes the reduction of a considerable number of hydroperoxides, but has some restraints, such as instability, poor availability, and high molecular weight, features that result in a limited therapeutic application [40]. With that in mind, considerable efforts have been made to find or synthesize organoselenium compounds capable of mimicking the enzymatic properties of glutathione peroxidase and with the least possible drawbacks. Ebselen, mentioned before, is an interesting example of an organoselenium compound with GPx-like activity, among others presented later.

In fact, ebselen is known for being the first organoselenium compound reported as a GPx mimic, with a proposed [55] catalytic mechanism (Scheme I.3) kinetically identical to that of the GPx enzyme. Ebselen catalyzes the reduction of different hydroperoxides and assists cellular defence system against oxidative stress [53]. Another relevant characteristic of ebselen and other organoselenium compounds is the ability to utilize a variety of thiols in addition to glutathione as a substrate [56,57], unlike GPx which contains binding sites conferring substrate specificity.



Scheme I.3 – Mechanism of the catalytic reduction of hydroperoxides by ebselen. Adapted from [40,55].

As shown, ebselen (**3**) can be oxidized to the selenoxide derivative **15** by the reaction

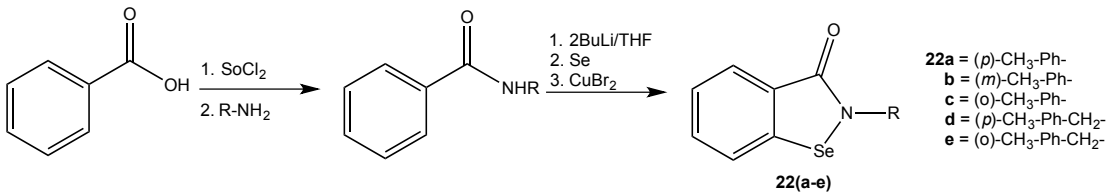
with hydrogen peroxide (cycle A, step 1). In the presence of excess thiol, the Se-N bond in the ebselen selenoxide is immediately cleaved by the thiol to produce the corresponding thiolseleninate **16** followed by the formation of selenenic acid **17** (cycle A, step 3). The nitrogen nucleophilic attack to the selenium atom regenerates ebselen (**5**) and water (cycle A, step 4). The thiol group can cleave ebselen to give selenenyl sulfide **18**, which reacts with the thiol group and gives the ebselen selenol **19** (cycle B, step 2). Ebselen selenol **19** can be oxidized to selenenic acid **17** (cycle B, step 3), participating in cycle A. The simultaneous reduction and oxidation of the selenenyl sulfide **18** produces the corresponding diselenide **20** (cycle C, step 2), that can regenerate ebselen through H_2O_2 with the formation of water.

Additionally, there is evidence showing that supplementation, *in vitro* and *in vivo,* with inorganic selenium or various forms of organoselenium compounds inhibited both chemically and physically induced oxidative damage [58–60], due to the fact that it is incorporated in GPx and other selenium enzymes [61]. However, the role of selenium-containing enzymes in preventing diseases is not yet clearly defined, and it has been reported that excessive intake of Se may result in oxidative damage leading to genomic instability [62].

I.4. Synthesis of biologically significant organoselenium compounds

Since ebselen presents such potential and remarkable characteristics regarding its use as an anticarcinogenic drug, the search and design for selenium-containing heterocyclic molecules has become extremely relevant, and several compounds have been reported and studied with the goal of duplicating or improving those same characteristics. Some of those compounds are here presented.

Ebselen (Fig. I.4, **5**), mentioned before, was first prepared in 1924, being reported as an anti-inflammatory with antioxidant and GPx-like activity. Preparation of this molecule has undergone several methods. In the earliest approach, 2,2'-diselenobis(benzoic acid) was converted to selenyl chloride benzoyl chloride, which was treated with aniline to produce ebselen. More recent advances involve ortholithiation of benzanilide, subsequent insertion of selenium into benzanilide-derived dianion and cyclization of selenium-containing dianion to ebselen (Scheme I.4).



Scheme I.4 – Synthetic route described [63] by Hsu for the preparation of ebselen derivatives.

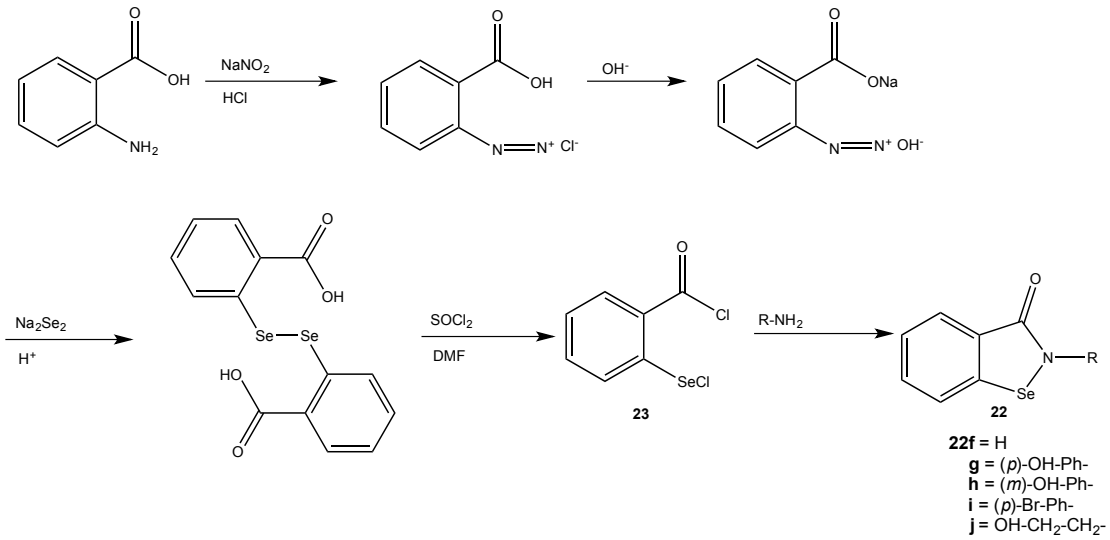
Since it presented several interesting and promising possibilities, ebselen derivatives have been developed and studied. In order to enhance its solubility and to increase its activity, Hsu and co-workers synthesized [63], using the latter method described, five ebselen derivatives (**22a-e**) that were tested for their GPx-like activity, and all displayed slightly higher activity than ebselen (Table I.3).

Table I.3 – GPx-like activity of ebselen and derivatives synthesized by Hsu.

Compound	GPx-like activity (relative to ebselen)
Ebselen	1.00
22a	1.36
22b	1.47
22c	1.17
22d	1.60
22e	1.60

* *Assay conditions*: The consumption of NADPH upon addition of H_2O_2 in the absence of the compounds tested was 0.8 μM/min and the consumption of NADPH for ebselen was 10.9 μM/min.

Several ebselen derivatives were synthesized by Bhabak and Mugesh, and evaluated for their antioxidant activity [64]. The selenenyl chloride (Scheme I.5, **23**), synthesized from anthranilic acid and disodium selenite (Na_2Se_2), was used as intermediate for the synthesis of most of the ebselen derivatives (Scheme I.5, **22f-j**) reported by these authors. Compounds **22g-j** were synthesized upon treatment of **23** with appropriate primary amines in dry acetonitrile.



Scheme I.5 – Synthetic route described by Bhabak and Mugesh [64] for the preparation of ebselen derivatives.

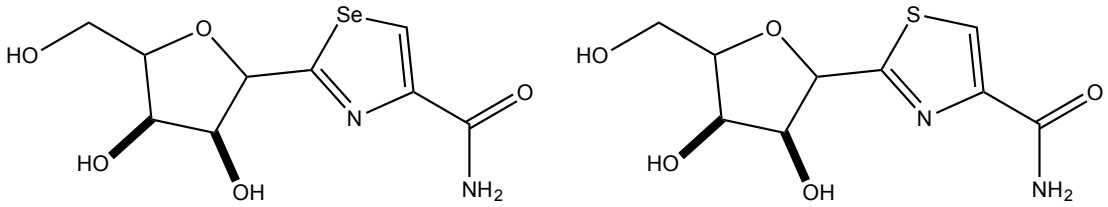
All exhibited excellent GPx-like activity in the presence of glutathione (Table I.4), and some even presented higher activity than ebselen. Lower activity for **22f** suggests that a substitution at the N atom is required for higher GPx activity.

Table I.4 – Initial rates (v_0) for the reduction of hydrogen peroxide by glutathione (2 mM) in the presence of ebselen derivatives (80 μM) at 23 ºC.

	Initial rates, v_0 [μM.min^{-1}]
Compound	**H_2O_2**
Ebselen	140.3±1.6
22f	103.3±0.5
22g	278.0±1.3
22h	257.7±0.3
22i	71.2±0.8
22j	179.1±1.7

* *Assay conditions:* phosphate buffer (100mM), glutathione reduced (2 mM), NADPH (0.4 mM), EDTA (1 mM), glutathione reductase (1 unit), peroxide (1.6 mM) and test compound (80 μM)

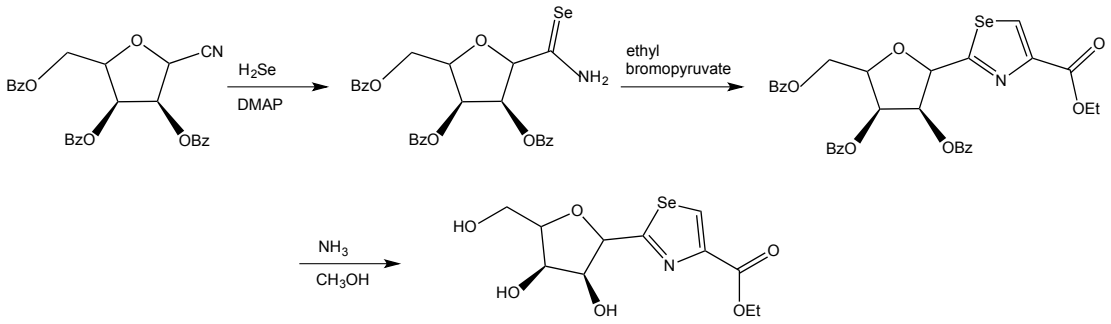
Selenazofurin (Fig. I.10, **24**), the selenium-derivative of tiazofurin (Fig. I.10, **25**), is reported [65] to have anti-tumor activity in animals and broad spectrum *in vitro* anti-viral activity, and has also been reported [65,66] to be 5-10 fold more potent than tiazofurin in different *in vitro* and *in vivo* anti-tumor screenings.



24 **25**

Figure I.10 – Selenazofurin (24) and tiazofurin (25) structures.

Its synthetic route (Scheme I.6) was developed similarly to the preparation method reported [66] by Srivastava and Robins for tiazofurin. It began by treating the precursor 2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl-1-carbonitrile with hydrogen selenide, and 4-dimethylaminopyridine (DMAP) as catalyst, resulting in 2,5-anhydro-3,4,6-tri-*O*-benzoyl-D-allonoselenoamide. It was then treated with ethyl bromopyruvate to produce ethyl 2-(2,3,5-tri-*O*-benzoyl-*D*-ribofuranosyl)selenazole-4-carboxylates as a mixture of α,β-anomers, which were readily separated by silica gel column chromatography. Selenazofurin (**24**) was obtained by the deprotection and amination reaction of the β-anomer with metallic ammonia.

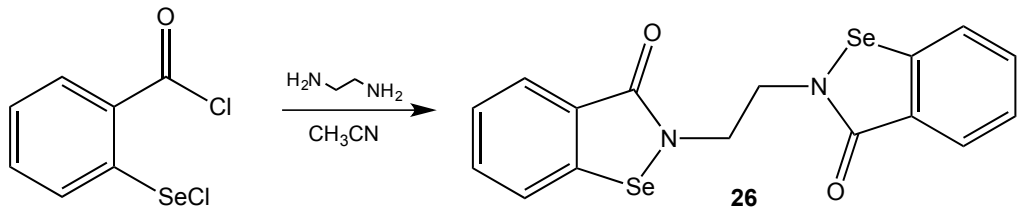


24

Scheme I.6 – Synthetic route described by Srivastava and Robins [66] for the preparation of selenazofurin (24).

Although selenazofurin, a synthetic nucleoside analogue, is a potent broad-spectrum anti-viral agent, not much is known about its metabolism and mechanism of action because of its development as an anti-tumor agent. Selenazofurin is thought to demonstrate anti-viral activities by inhibiting inosine 5'-monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme, which results in inhibition of tumor cells proliferation [67].

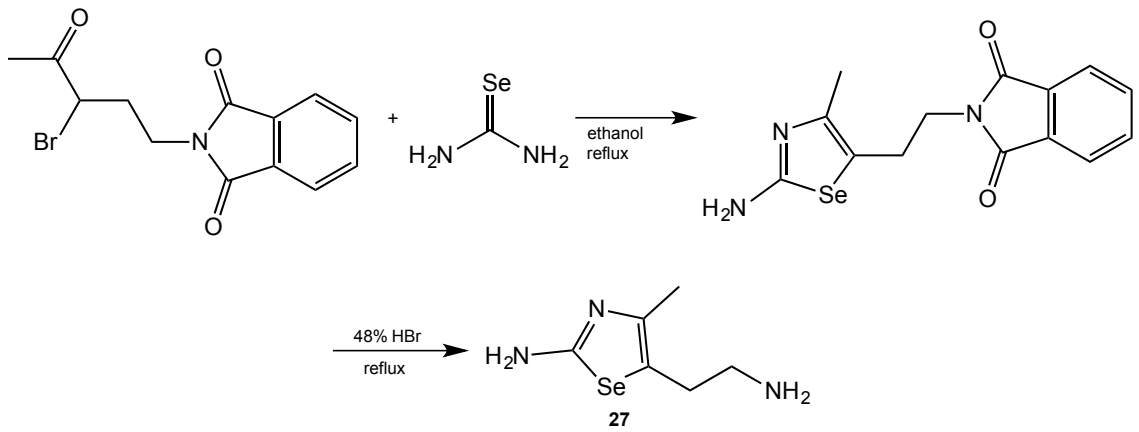
Ethaselen (1,2-bis[1,2-benzisoselenazolone-3(2*H*)-ketone)]ethane) (Scheme I.7, **26**) demonstrated [68] significant anti-tumor effects with slight toxicity and immune regulating characteristics in several tumor models.



26

Scheme I.7 – Synthetic route described by Młochowski and co-workers for the preparation of ethaselen (26).

Synthetic strategy (Scheme I.7) is similar to that of ebselen. The reaction of 2-chloroselenobenzoyl chloride with ethylenediamine was carried out under standard conditions to produce the corresponding ethaselen. It was reported that ethaselen induces tumor cells apoptosis by inhibition of wild-type mammalian thioredoxin reductase 1 (TrxR1), considered to be an important anticarcinogenic drug target, and involved in both carcinogenesis and cancer progression. Ethaselen specifically binds to the unique selenocysteine-cysteine redox pair in the C-terminal active site of mammalian Trx-R1. It has recently entered phase I clinical trials in China, and is considered to be a great candidate for development of anti-tumor and anticarcinogenic drug.



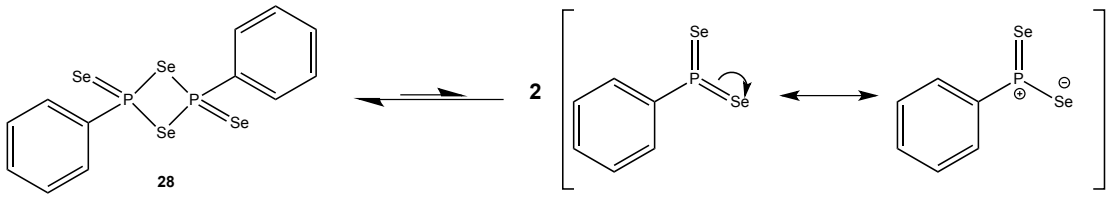
27

Amselamine (2-amino-5-(2-aminoethyl)-4-methyl-1,3-selenazole) (Scheme I.8, **27**) is a selenium analogue of amthamine, a very effective H_2-R-agonist.

Scheme I.8 – Synthetic route described for the preparation of amselamine (27).

Histamine is a biogenic amine that mediates its effects by four histamine receptor (HR) subtypes, being that the H_2-R subtype has a crucial physiological role in stimulating gastric acid secretion. Amthamine is a structure that can act as H_2-R-angonist with high efficiency rates. Studies show [69] that amselamine (**27**) is a more potent H_2-R-agonist than amthamine and histamine . Due to the selenazole ring of amselamine being more basic than the thiazole ring of amthamine, it is expected that amselamine has higher affinity for H_2-R than histamine.

Caffeine [1, 3, 7-trimethyl-1*H*-purine-2,6-(3*H*, 7*H*)-dione], a natural occurring methylxanthine commonly used as a component of tea, coffea, and soft drings, has been studied for its antioxidant and anticarcinogenic activity, and effects in cell cycle [70]. Since its basic scaffold is of unquestionable interest as lead compound for the development of new derivatives with enhanced activities and/or lower activities, the synthesis of a selenium derivative was reported [71] by replacing the oxygen of a carbonyl group by a selenium atom, using a microwave-based methodoly. Conversion of carbonyl groups to selenocarbonyl was achieved by using Woolins' reagent (Scheme I.9, **28**).

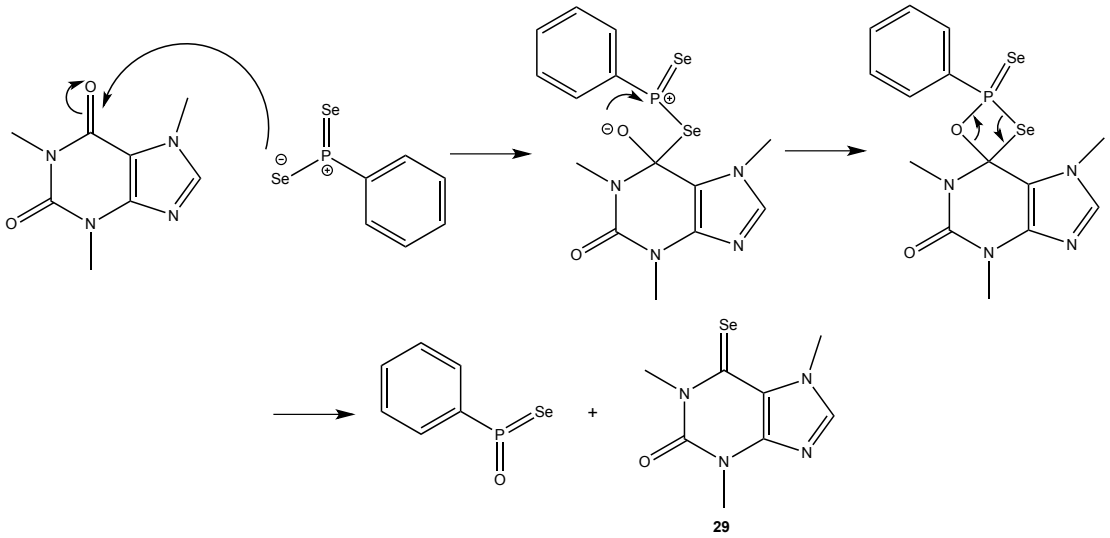


28

Scheme I.9 – Woolins' reagent (28) dissociation.

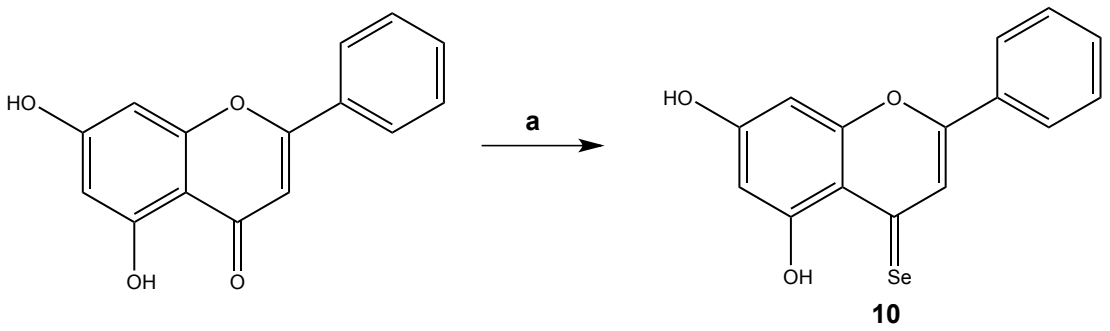
The proposed synthetic route presented (Scheme I.10) is consistent with structural characterization, and studies conducted showed that selenated-caffeine (Scheme I.10, **29**)

presented an improvement of the antioxidant effect when compared to caffeine, despite its scavenging capacity to be only moderate.



Scheme I.10 – Synthetic route proposed by Martins *et al* for the preparation of selenocaffeine (29) using Woolins' reagent.

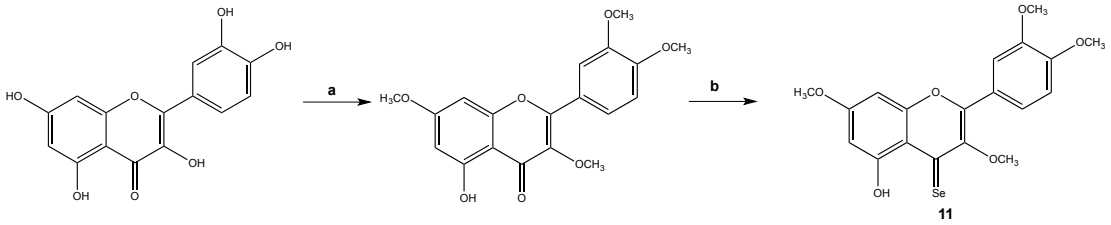
The same methodology was applied on different compounds [72] by Martins *et al*, namely chrysin (Scheme I.11) and quercetin (Scheme I.12). These are flavonoids, a common group of plant polyphenols, which have been extensively studied regarding their health promoting properties and potential pharmacological applications in cancer therapy and prevention.



Scheme I.11 – Synthetic route described by Martins *et al* for chrysin direct selenation.

Assay conditions: **a** = Woolins' reagent, acetonitrile, MW, 175 W, 150 ºC, 5 min.

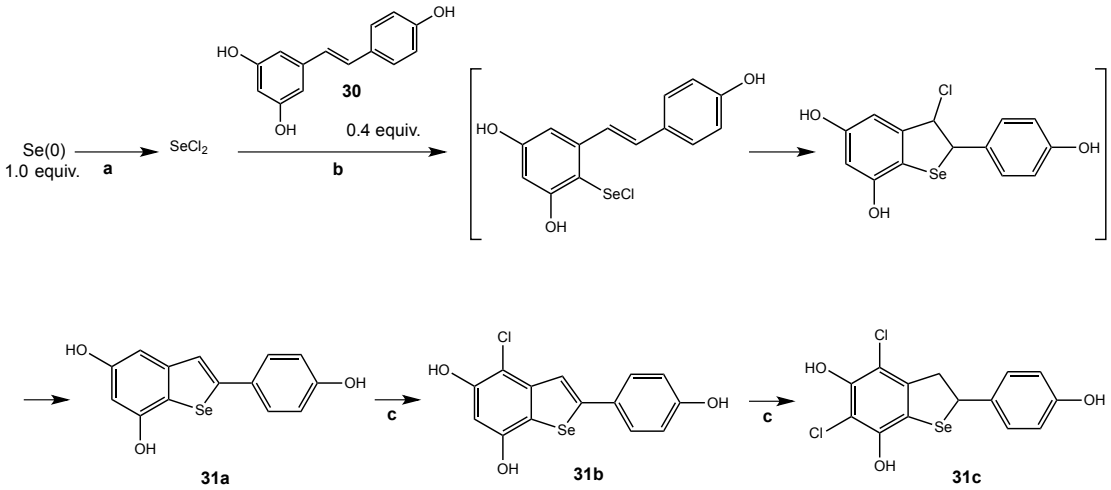
The preparation method for selenium-containing derivatives of chrysin was through direct selenation (Scheme I.11). For the quercetin compound, preparation involved initial protection of hydroxyl groups by methylation with dimethylsulfate (Scheme I.12, step a) followed by selenation of the carbonyl group (Scheme I.12).



Scheme I.12 – Synthetic route described by Martins *et al* for quercetin selenation. *Assay conditions*: **a** = $(CH_3)_2SO_4$, KH_2CO_3, acetone, Δ; **b** = Woolins' reagent, acetonitrile, MW, 175 W, 150 ºC, 5 min.

The preliminary evaluation of the free radical scavenging activity of seleno-derivatives crysin (Scheme I.11, **10**) and quercetin (Scheme I.12, **11**) was studied. It presented an improvement of the ability to scavenge the DPPH upon selenation of the carbonyl group, for 10, when compared to the non-selenated compound. The selenated-quercetin compound **11** exhibited a value of DPPH radical scavenging activity very similar to the non-selenated quercetin, a powerful radical scavenger. When tested for their catalytic properties as GPx mimics, results show that both tested compounds exhibit potential GPx-like activitiy.

In an effort to discover a new organoselenium compound with potential therapeutic use, selenation of the resveratrol (Scheme I.13, **30**) molecule was studied [73] . Resveratrol [(*E*)-5-(4-hydroxystyryl)benzene-1,3-diol] is a natural stilbene known for its antioxidant activity. Selenation as a way of boosting its antioxidant capacity presented three different selenated resveratrol derivatives (Scheme I.13, **31a-c**), attained due to the variable ratio between Se and SO_2Cl_2 [73].



Scheme I.13 – Synthetic route described by Tanini *et al* [73] for resveratrol selenation. *Assay conditions*: **a** = i) SO_2Cl_2 2.0 equiv, neat, 10 min, rt; ii) dry THF, 1h, rt; **b** = dry DMF, 24h, rt; **c** = *in situ* formed Cl_2.

The three selenium derivatives were tested for their antioxidant capacities using DPPH and ferric reducing/antioxidant power assay. DPPH assay results show that all the selenophene derivatives are more efficient than resveratrol when tested under the same conditions, and **31a** activity presented to be similar to that of the standard used. Results of the assay measuring reducing capacity of the selenophene derivatives show that **31a** presents an activity comparable to the standard used, while the chlorinated derivatives **31b** and **31c**, though more active than resveratrol, proved to be less efficient. With the aim of understanding the possible use of these derivatives as topical or systemic antioxidants, their cytotoxicity was evaluated in comparison with resveratrol by measuring the proliferation of human keratinocyte cells and human intestinal cells in a time-dependent assay. However, the results showed no significant decrease in cell survival after 24, 48, and 72 h of incubation following the addition of benzoselenophene derivatives at 5 μM final concentration, and no alteration in cell morphology was observed.

II. Results and Discussion

II.1. Microwave assisted seleno-purine synthesis

The initial approach towards synthesis of organoselenium compounds involved a microwave methodology, aiming to obtain selenated purine derivatives, with the procedure (**III.2.1.**) adapted from Martins *et al* [71] that consisted on converting a carbonyl into a selenocarbonyl using Woolins' reagent (W. R.) (Fig. II.1).

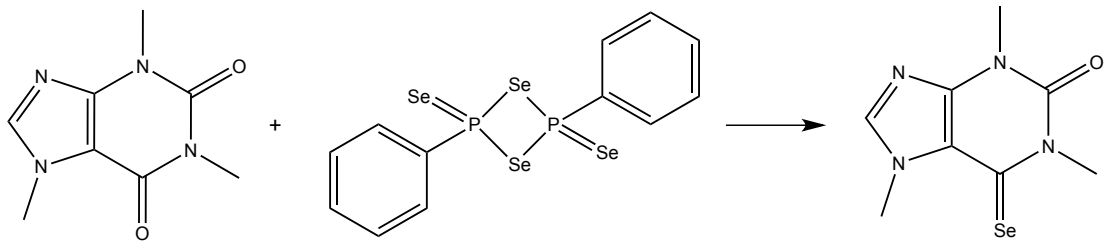


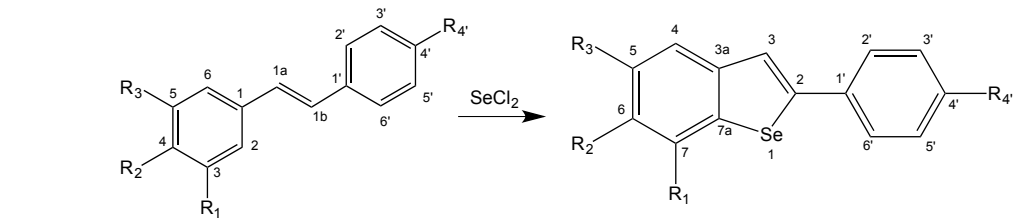
Figure II.1 – Generic scheme for the methodology reported by Martins et al [71].

However, despite the results presented by these authors, the experiments carried out presented some problems, with reagents insolubility to be the main issue. In an effort to overcome this, a number of modifications to the original protocol were tested, such as the use of different solvents and overall reaction time. Yet, all syntheses resulted in mixtures with dark aggregates and red powder residues with a strong odor, presumably arising from Woolins Reagent (W.R.), that were unable to be analyzed. For those reasons this procedure was disregarded, and a different synthesis procedure was considered.

II.2. Synthesis of benzoselenophene derivatives

The procedure followed for preparation of the benzoselenophene derivatives is based on a synthesis using resveratrol as the scaffold for selenium-containing resveratrol analogues reported by Tanini *et al* [73]. As shown before, introduction of a third-period or higher chalcogen atom is reported to effectively enhance the antioxidant capacity of phenolic compounds. With that in mind, selenium was used in this study as means of boosting the antioxidant activity of stilbenes *via* conversion to 2-phenylbenzoselenophene analogues, with potential antioxidant and GPx-like activity.

Tanini *et al* successfully reported the increase of resveratrol derivatives antioxidant activity [73] upon selenation, and a similar strategy (Scheme II.1) was chosen for synthetizing different benzoselenophene derivatives.

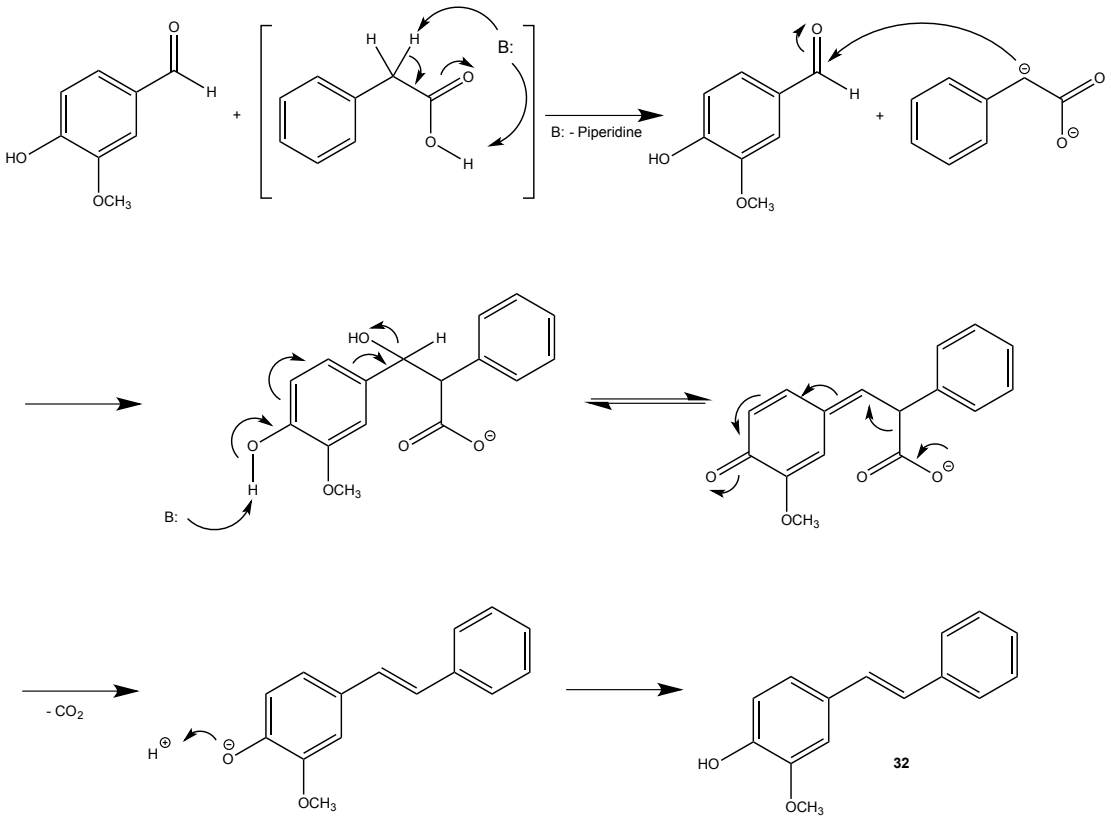


30: R_1 = OH R_2 = H R_3 = OH $R_{4'}$ = OH
32: R_1 = OCH_3 R_2 = OH R_3 = H $R_{4'}$ = H
33: R_1 = OH R_2 = H R_3 = OH $R_{4'}$ = H
34: R_1 = OCH_3 R_2 = H R_3 = OCH_3 $R_{4'}$ = H

31a: R_1 = OH R_2 = H R_3 = OH $R_{4'}$ = OH
35: R_1 = OCH_3 R_2 = OH R_3 = H $R_{4'}$ = H
36: R_1 = OH R_2 = H R_3 = OH $R_{4'}$ = H
37: R_1 = OCH_3 R_2 = H R_3 = OCH_3 $R_{4'}$ = H

Scheme II.1 – Generic scheme for the methodology reported by Tanini *et al* [73], and respective substituents.

All the stilbenes used as reagents were available in the laboratory and were previously synthesized by Dr. João Paulo Telo [74], except **32** which was unavailable in the laboratory, and was then synthesized using the same methodology refered (Scheme II.2., **32**).



Scheme II.2 – Reaction mechanism for the formation of (*E*)-2-methoxy-4-styrylphenol (32). Adapted from Sinha *et al* [75].

After column chromatography and prep. T. L. C., two fractions were separated and evaluated using T. L. C., with one revealing to be a somewhat pure fraction with the desired compound, and the other a mixture of compounds. Aiming to purify that same mixture, prep. T.L.C (dichloromethane) was used, resulting in a total of 1.156 g (17 % yield) of pure compound, (*E*)-2-methoxy-4-styrilphenol.

There are some reasons that may justify the low yield presented. One of them may be a not so effective purification. Indeed, when evaluated by T. L. C., the spots appeared to have a good separation when using dichloromethane as eluent, which led to its choice as the eluent for the prep. T. L. C. However, upon separation of the different observed fractions, they were too close to each other, even after a second elution. That may indicate some overlap between the fractions, resulting in losses during the process of extraction of the presumable pure compound

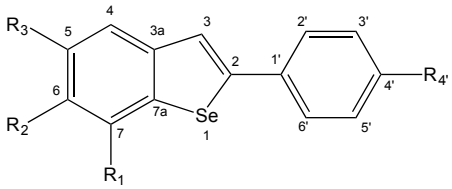
Structure characterization was attained by ^{1}H and ^{13}C NMR (Nuclear Magnetic Resonance) spectra (**III.2.2.1**) and is similar to the one reported upon synthesis of the same product by Sinha *et al* [75] .

Regarding benzoselenophene derivatives, the procedure followed is based on the efficient selenation of the reagents, involving *in situ* generation of $SeCl_2$, a convenient source of electrophilic Se^{2+} [76], through the reaction of Se(0) with SO_2Cl_2 (**Equation II.1**), followed by the reaction with the respective substituted stilbene in dry DMF.

$$Se + SO_2Cl_2 \longrightarrow SeCl_2 + SO_2$$

Equation II.1 – $SeCl_2$ formation reaction.

Similarly to the procedure described (**Scheme I.13**), the goal of this experiment was to attain different derivatives without any chlorine atom in the molecule, which lead to the use of a [Se : SO_2Cl_2 : substituted stilbene] ratio of [1 : 0.8 : 0.4]. This allowed isolation of all the desired benzoselenophene compounds (Fig. II.2) using silica gel chromatography or prep. T. L. C., confirmed after structural characterization, discussed later in this section.



31a: R_1 = OH R_2 = H R_3 = OH $R_{4'}$ = OH
35: R_1 = OCH_3 R_2 = OH R_3 = H $R_{4'}$ = H
36: R_1 = OH R_2 = H R_3 = OH $R_{4'}$ = H
37: R_1 = OCH_3 R_2 = H R_3 = OCH_3 $R_{4'}$ = H

Figure II.2 – Benzoselenophene structure and respective substituent groups.

Table II.1 – Starting materials and respective products, with corresponding reaction yield.

Starting Material	Product	Yield (%)
30	31a	59
32	35	1
33	36	13
34	37	8

This reaction is advantageous in terms of costs and expedient operational conditions, without the need for phenol group protection, sometimes required due to the structure of the starting material; therefore, this is a facile and scalable procedure. Solvents were used as described by the followed protocol. THF was used in the first step of the reaction since it significantly reduces disproportionation of monochalcogen dihalides into oligo-chalcogen dihalides [76,77]. For the second step of the reaction, protic solvents such as water or methanol were discarded due to their reactivity with SO_2Cl_2 and the formation of electrophilic seleno-species. All stilbenes presented good solubility in DMF, being a solvent compatible with the reaction conditions.

Yields obtained for benzoselenophenes were considerably low, as shown (**Table II.1**). In fact, despite the relatively higher yield for compound **31a**, all the other derivatives where isolated in low yields, and a reason that can explain this result is that the methodology used was developed specifically for resveratrol compounds, which was the reagent used in that specific reaction.

The fact that the reaction leading to compound **36** was the next best efficient reaction may be supportive of the specificity of the overall procedure, since compounds **31a** and **36** only differ in one OH group at the substituent position $R_{4'}$.

Introduction of the selenium atom involves an electrophilic aromatic substitution reaction, favoured by the presence of electron-donating groups, such as hydroxyl (–OH) and methoxy ($–OCH_3$) groups, at the *orto/para* position regarding the entering group, and even considering that $SeCl_2$ is a weak electrophilic species. Analysis of the data obtained seems to indicate that the presence of an –OH group at the *orto* position promotes a higher yield to the selenation reaction when compared to the presence of an $–OCH_3$ group, which is consistent with the fact that the –OH group is a better electron donor than $–OCH_3$.

As stated before, the proposed mechanism for the formation of benzoselenophenes derivatives (**Scheme I.13**) also predicts the formation of chlorinated products, when using a different ratio between Se and SO_2Cl_2. In fact, the reaction leading to **35** allowed the isolation of a product that would probably have one or more chlorine atoms in its structure. However, since that was not the intent of this work, and ^1H and ^{13}C NMR along with mass spectrometry (MS) analysis of that chlorinated product was not conclusive to the effective structure, its structural elucidation was set aside.

II.3. Structural characterization of benzoselenophene derivatives

Analysis of the isolated compounds revealed that the syntheses were successful, regarding the desired reaction products, despite low yields. The entire initial proposed structures were isolated and structural characterization was achieved using unidimensional ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra, as well as heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), and

correlation spectroscopy (COSY) experiments, along with mass spectrometry (MS). Regarding compound **31a**, NMR spectra acquired show that the chemical shifts presented here are similar and consisted to the ones already reported [73]. Assignment of the structure for compound **36**, for instance, was based on a ^{1}H, ^{13}C heteronuclear multiple bond correlation (HMBC) spectrum (Fig. II.3). Namely, proton H4 at 6.87 ppm presented 3-bond correlations with C7a quaternary carbon at 120 ppm and C3 carbon at 123 ppm; also highlighted is the the signal of H6 hydrogen proton at 6.32 ppm and C7a carbon at 120 ppm. Structural characterization of the remaining compounds was attained using the same methodology (Table II.2 and Table II.3).

Chemical shifts for ^{77}Se NMR spectra confirm the success of the selenation reaction, since analysis of three of the four tested compounds (Table II.4) present chemical shifts similar to the values reported by Tanini *et al* [73]. Regarding **35**, the amount of benzoselenophene available was not enough for analysis.

Additionally, all compounds were analysed by MS using electrospray ionization (ESI) (**III.2.3**). Spectra for compound **36** (Fig. II.4), for instance, present six of selenium's most abundant natural isotopes corresponding to the deprotonated molecule $[M-H]^-$, leaving no doubts as to the selenation efficacy. The same isotopic distribution is present for the remaining compounds.

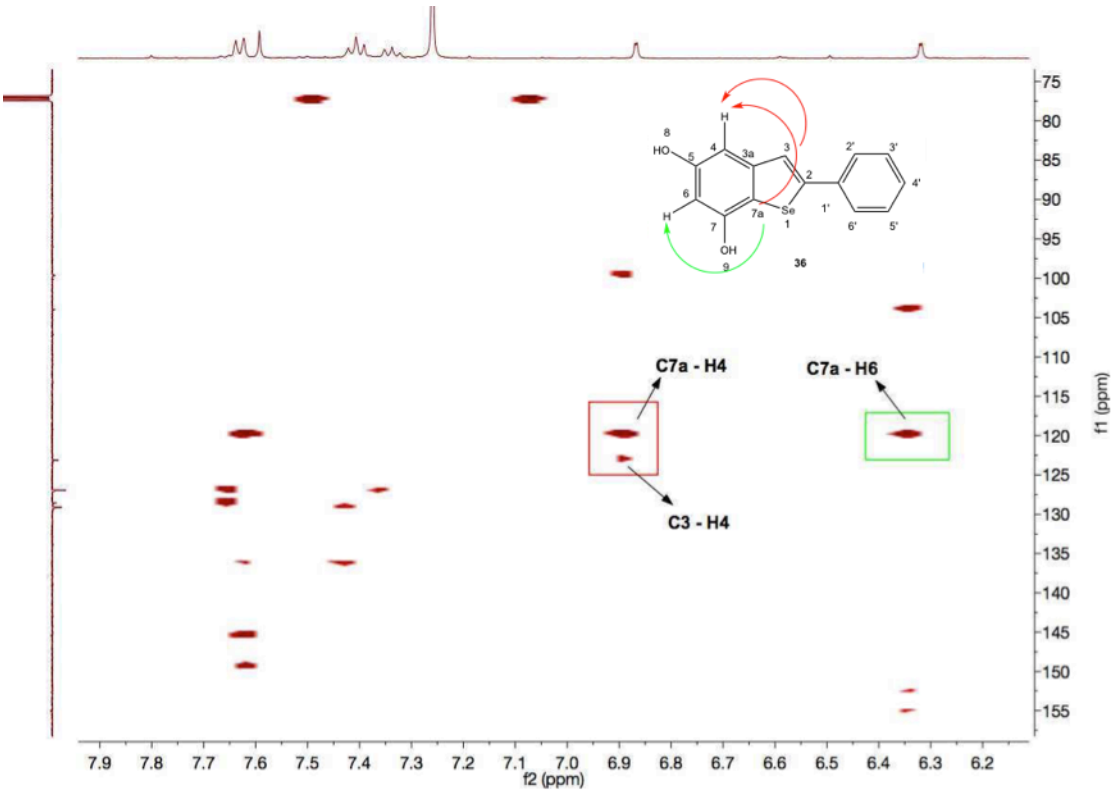


Figure II.2 – Three bond correlations on a HMBC spectrum for compound 36 ($CDCl_3$, 400 MHz).

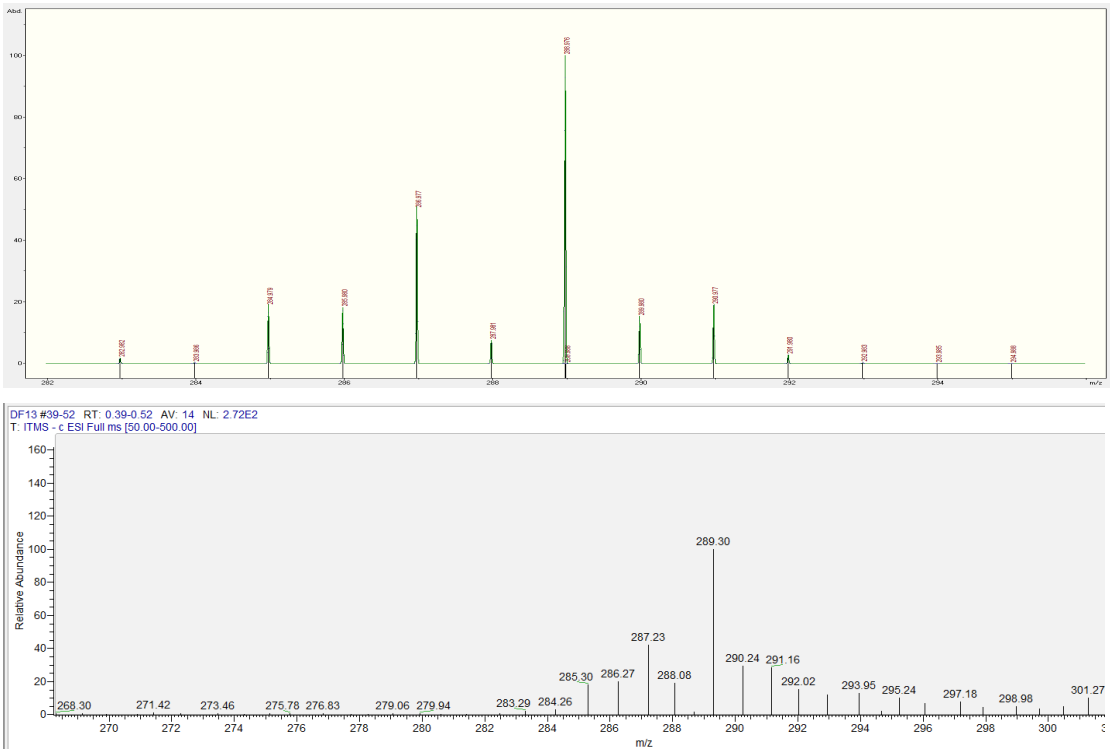


Figure II.3 – *Above*: calculated isotopic distribution for the deprotonated molecule of compound 36. *Below*: Full scan mass spectrum obtained by electrospray ionization in negative mode for the deprotonated molecule of compound 36 at m/z 289.

Table II.2 – ^{1}H NMR chemical shift (δ, ppm) for the benzoselenophene derivatives.

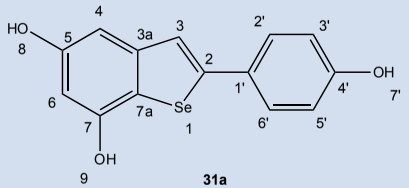
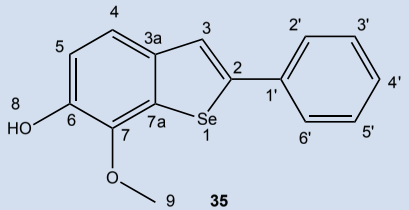
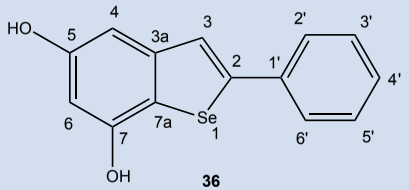
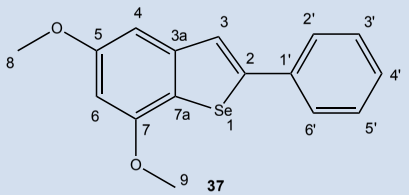
Structure / δ (ppm)	$H^{2'}$ + $H^{6'}$	$H^{3'}$ + $H^{5'}$	$H^{4'}$	H^{3}	H^{4}	H^{5}	H^{6}	H^{8}	H^{9}
 **31a**	7.48 (2H, d, *J* = 8.2 Hz)	6.81 (2H, d, *J* = 8.5 Hz)	-	7.58 (1H, s)	6.66 (1H, s)	-	6.26 (1H, s)	-	-
 **35**	7.78 (2H, d, *J* = 7.6 Hz)	7.50 (2H, t, *J* = 7.3 Hz)	7.59 (1H, t, *J* = 7.5 Hz)	7.54 (1H, s)	7.37 (1H, d, *J* = 8.4 Hz)	6.97 (1H, d, *J* = 8.4 Hz)	-	3.99 (3H, s)	-
 **36**	7.64 (2H, d, *J* = 6.6 Hz)	7.41 (2H, t, *J* = 7.5 Hz)	7.34 (1H, t, *J* = 7.5 Hz)	7.59 (1H, s)	6.87 (1H, d, *J* = 1.9 Hz)	-	6.32 (1H, d, *J* = 1.9 Hz)	-	-
 **37**	7.66 (2H, d, *J* = 7.2 Hz)	7.43 (2H, t, *J* = 7.4 Hz)	7.32 (1H, t, *J* = 7.3 Hz)	7.67 (1H, s)	6.92 (d, *J* = 2.0 Hz)	-	6.42 (d, *J* = 2.0 Hz)	3.98 (3H, s)	3.90 (3H, s)

Table II.4 – ^{13}C NMR chemical shift (δ, ppm) for the benzoselenophene derivatives.

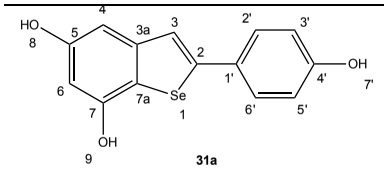
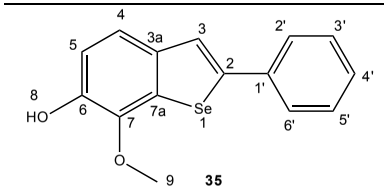
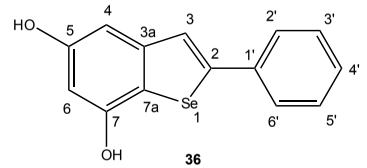
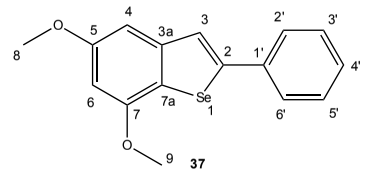
Structure / δ (ppm)	$C^{1'}$	$C^{2'} + C^{6'}$	$C^{3'} + C^{5'}$	$C^{4'}$	C^{2}	C^{3}	C^{3a}	C^{4}	C^{5}	C^{6}	C^{7}	C^{7a}	C^{8}	C^{9}
 **31a**	127.3	128.0	116.2	158.2	147.5	121.9	145.4	102.5	157.2 or 154.3	99.4	157.2 or 154.3	121.9	-	-
 **35**	138.3	129.7	128.2	126.3	146.6	111.8	129.0	131.9	123.5	150.1	146.6	Not assigned	-	56.1
 **36**	136.1	126.8	129.0	128.4	149.4	123.0	145.3	103.8	154.9 or 152.6	99.5	154.9 or 152.6	120.0	-	-
 **37**	136.4	126.7	128.9	128.2	148.8	123.3	144.2	99.9	159.6	95.8	156.4	121.5	55.79	55.70

Table II.3 – ^{77}Se NMR chemical shift (δ, ppm) for the benzoselenophene derivatives.

Structure	^{77}Se (δ, ppm)
31a	464.99
35	- [a]
36	469.20
37	479.91

[a] Not found due to the small amount of product obtained

II.4. DPPH Scavenging activity

In order to evaluate the antioxidant capacity of the synthesized compounds, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay in methanol was used. This assay is based on the ability of a given compound to transfer one or more hydrogen atoms to DPPH (Fig. II.5), a stable free radical.

As stated before, the chemopreventive properties of organoselenium compounds are considered to be related to their antioxidant activity, which can be mediated by their ability to scavenge free radicals. As means to understand whether selenation of stilbene derivatives enhances these compounds chemical properties with potential biological significance to cancer chemoprotection and therapy, a preliminary evaluation of the free radical scavenging activity was performed, allowing a comparison between the starting materials and their selenium derivatives. Compounds **34** and **37** were tested but presented no reaction. This may be related to the fact that there is no hydroxyl group in their structure, making them unable of donating any hydrogen atom, and therefore were not presented.

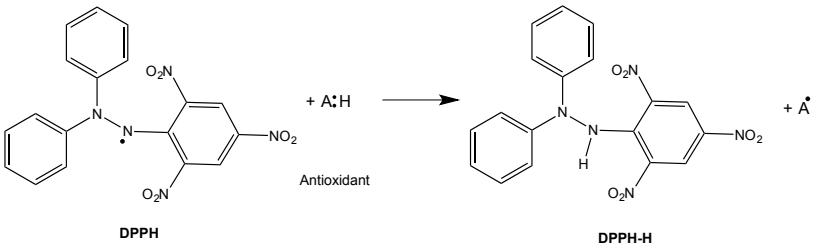


Figure II.5 – DPPH assay generic reaction.

Both starting materials (**30, 32, 33**) and benzoselenophenes (**31a, 35, 36**) were tested for a gradient of concentration (10 μM, 25 μM, 50 μM, 75 μM).

The time course of DPPH reaction for compounds **30** and **31a** (Chart II.1), **32** and **35** (Chart II.2), and **33** and **36** (Chart II.3) is presented, along with the same reaction for all compounds at concentration 75 μM (Chart II.4).

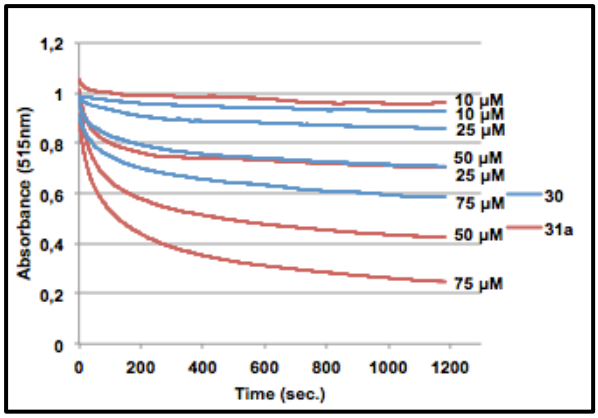


Chart II.1 – Decrease in the absorbance at 515 nm of 200 μM DPPH in the presence of different concentrations of the starting material (30) and benzoselenophene compound (31a), in methanol.

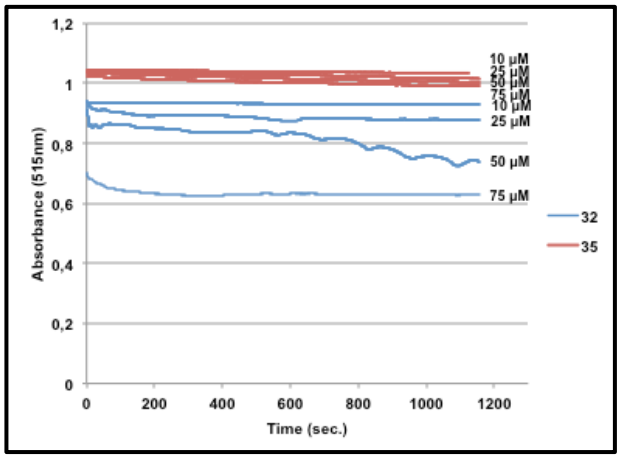


Chart II.2 - Decrease in the absorbance at 515 nm of 200 µM DPPH in the presence of different concentrations of the starting material (32) and benzoselenophene compound (35), in methanol.

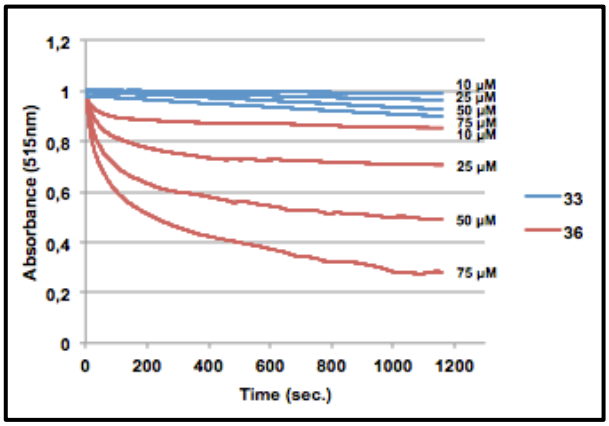


Chart II.3 - Decrease in the absorbance at 515 nm of 200 µM DPPH in the presence of different concentrations of the starting material (33) and benzoselenophene compound (36), in methanol.

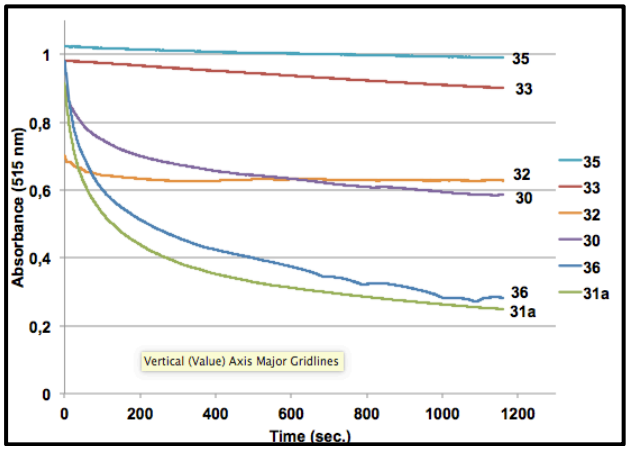


Chart II.4 - Decrease in the absorbance at 515 nm of 200 µM DPPH in the presence of all starting materials and all benzoselenophene compounds at 75 µM, in methanol.

Table II.5 – Percentage of DPPH reduction, IC_{50} values obtained for the free DPPH radical scavenging activity of the starting materials (30, 32, 33) and benzoselenophene compounds (31a, 35, 36) with DPPH (200 μM), rate constant, and number of H-atoms transferred at 20 min. of reaction.

Compound	[Aox.] (μM)	DPPH reduced (%) [a]	IC_{50} (μM)	k_{obs}	k_1 (M/s) [b]	n_{tot} [c]
30	10	6	108.54	0.0002	50.1	1.05
	25	11		0.0007		0.78
	50	24		0.0032		0.82
	75	35		0.0031		0.76
31a	10	8	49.80	0.0017	136.0	1.49
	25	28		0.0076		1.98
	50	55		0.0105		1.84
	75	74		0.0112		1.64
32	10	0	349.07	2.58E-5	29.4	0.05
	25	5		7.05E-4		0.30
	50	14		0.0037		0.45
	75	8		0.0013		0.13
35	10	1	1655.04	3.66E-5	0.3	0.21
	25	2		5.02E-5		0.18
	50	3		5.67E-5		0.11
	75	3		5.4E-5		0.08
33	10	1	476.82	2.41E-5	0.3	0.13
	25	3		9.62E-5		0.25
	50	6		6.89E-5		0.22
	75	8		6.26E-5		0.18
36	10	13	56.32	0.0025	121.4	2.25
	25	30		0.005		2.19
	50	51		0.008		1.84
	75	72		0.0105		1.67

[a] calculated after 20 min of reaction; [b] rate constant for the fast step, $k_{obs} = k_{1'}$ vs [Aox]; [c] number of H-atoms transferred after 20 min.

Structurally, these are relatively similar compounds (Fig. II.1). **31a** has three hydroxyl groups at carbons C5, C7, and C4' (Fig. II.1), all of them able to donate one H-atom. As expected, its starting material **30** revealed a considerable % of DPPH radical scavenging activity (Table II.5), while compound **31a** presented a much higher % of scavenging activity, when comparing the same concentration range, indicating that selenation has a significant boost on antioxidant activity. That boosting effect is also shown by the increase of the rate constant for the H-atom transfer in the fast step (k_1 value, Table II.5). Stoichiometry values higher than 1 (n_{tot}, Table II.5) for the benzoselenophene derivative indicate that it is still capable of reacting with DPPH and donate another one of the H atom present, under the reaction conditions. Also, there is a decrease in IC_{50} value, of almost 3-times, when compared to **30**, known for its antioxidant activity. IC_{50} values and n_{tot} are related and indicate whether the reaction is complete after 20 minutes. If so, $IC_{50} = [DPPH]/(2 \times n_{tot})$, meaning IC_{50} values around 100 μM for n_{tot}=1 or 50 μM for n_{tot}=2.

The same analysis can be made for **37**. In fact, it's structure is very similar to **31a**, except that **36** has only two –OH groups (Fig. II.1), at positions C5 and C7. In this case, selenation of **33** produces a remarkable increase in % of DPPH radical scavenging activity

(Table II.5) along with the IC_{50} value, with a decrease of about 9-times when compared to its starting stilbene. Also, there is a high increase for the k_1 value, such as for the n_{tot} indicating that there is still the possibility of donating the other H atom.

Regarding compound **35** and its starting material **32**, results present the opposite tendency when compared to the other studied compounds. The structure of **35** differs from those presented before in the substituent groups, having an –OH group and an $–OCH_3$ group at positions C6 and C7. Like the other tested compounds, **35** also has spin delocalization along its aromatic ring similarly as **36**, although in this case there is no contribution from the $-OCH_3$ group. Results show a low radical scavenging activity of the stilbene **32**. However, contrary to expected, the presence of one selenium atom in the molecule's structure leads to a decrease in % of DPPH radical scavenging activity of **35**, along with the decrease of k_1 value and also n_{tot} (Table II.5).

Data presented indicates that when comparing compounds **31a** and **36** with their respective starting materials **30** and **33**, higher concentrations result in a higher % of DPPH radical scavenging activity (Chart II.1 and Chart II.3). When comparing the concentration range of 75 μM (Chart II.4), benzoselenophene **36** DPPH radical scavenging activity is similar to the one presented by compound **31a**, known for its antioxidant actitivy. Benzoselenophene **35** has however a different behaviour, since it's starting stilbene **32** presents low DPPH radical scavenging activity, and upon selenation **37** has near to zero activity. This seems to indicate that upon selenation, the presence of a –OH group at an *orto* or *para* position regarding the new selenium atom favours DPPH radical scavenging activity, while the presence of an $–OCH_3$ at the *orto* position does not.

In fact, calculated bond dissociation enthalpy (BDE) (Table II.6) seems to support that. It clearly shows that selenation has a considerable impact on O-H bond energy for the hydroxyl groups present.

Table II.6 – Bond Dissociation Enthalpy (BDE) for each O-H bond in kcal.mol^{-1}, computed with (B3LYP/6-31+G(d), Opt/Freq).

Compound	O-H R_1	O-H R_2	O-H R_3	O-H $R_{4'}$
30	86.89	-	86.89	82.22
31a	83.69	-	90.99	84.15
32	-	92.54	-	-
35	-	94.33	-	-
33	88.49	-	88.84	-
36	82.09	-	83.84	-

***** *Assay conditions*: **[a]** BDE O-H bond dissociation enthalpy, computed as BDE (ArOH) = BDE_{exp}(PhOH) + [E_{calc}(ArO•) - E_{calc}(ArOH)] - [E_{calc}(PhO•) - E_{calc}(PhOH)] [78], with BDE_{exp} (PhOH) = 87.6 kcal/mol [79].

Whereas the H atom most prone to abstraction in **30** was the one at position $R_{4'}$, as shown by its lower BDE, introduction of the selenium atom bonding to carbons C7a and C2 in compound **31a** leads to a decrease in the BDE of the O-H bond at position R_1 and an increase for the BDE of the other two O-H bonds. The low BDE for the H atom at the position

$R_{4'}$ of **30** is explained by spin delocalization throughout the entire molecule due to the network of conjugated double bonds (Fig. II.6), which favours radical stabilization.

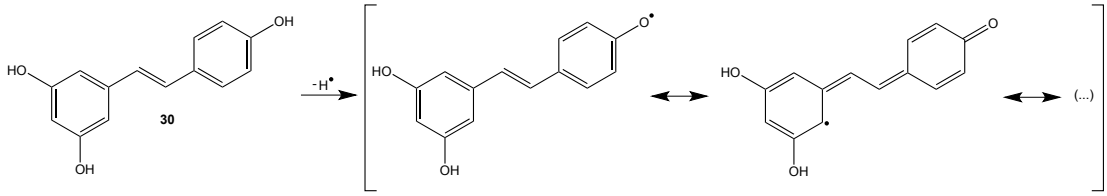


Figure II.6 – Canonical resonance for the radical delocalization possible on 30.

Again, the new selenium atom present at the molecule has a decreasing effect on the BDE for the O-H bond at the *orto* position next to it, and the same is verified for benzoselenophene **36**.

Stilbene **33**, much like **30**, is able to present spin delocalization althought not as extensive (Fig. II.7).

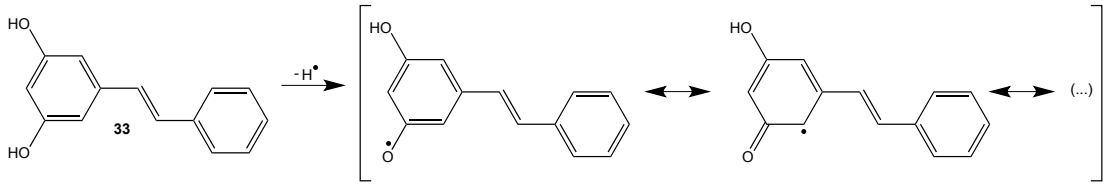


Figure II.7 – Canonical resonance for the radical delocalization possible on 33.

Data calculated shows that for **33** both OH groups have similar BDE values, which is not the case for the benzoselenophene **36**, with selenation leading to a lower BDE for the OH group at position R_1, *orto* relative to the selenium atom, clearly showing which one is the H atom most prone to abstraction.

The same effect is visible for the stilbene **32** and its benzoselenophene **35**. Despite only having one OH group in its molecule, there is a decrease in BDE value for **35** when comparing with **32**.

Results attained through the DPPH assay along with the calculated BDE values allow some insight as to which are the compounds with better antioxidant activity. High *k1* value and $n_{tot} > 1$ are indicative of a good antioxidant [80], and taking that into account, it is clear that upon selenation, compounds **31a** and **36** show a considerable improvement regarding those values, along with interesting results conferring % of DPPH reduced and IC_{50} values. Noteworthy, the fact that **36** presented a lower IC_{50} value than **30**, which is an already known antioxidant.

Still, compound **35** did not present the same results as the other tested compounds. In fact, low n_{tot} value is indicative of an incomplete reaction, even considering that the assay was conducted using increasing concentration values. Also, the starting material **32** presented better *k1* and n_{tot} values before selenation, which seems to indicate that the existence of an

OH group at the *orto* or *para* positions regarding the selenium atom favours the molecule regarding radical scavenging properties.

Regarding BDE values, it is clear that selenation promotes a decrease in the H atom most prone to abstraction, which seems to be the one at the *orto* position regarding the selenium atom, from analysis of the values attained for compounds **31a** and **36**. Despite **32** only having one hydroxyl group on its structure, there is also a decrease of the BDE value following selenation.

II.5. GPx-like assay

Nowadays there is a considerable number of organoselenium compounds known to have antioxidant properties mediated by the ability of mimicking the catalytic activity of the selenoenzyme GPx. Thus, GPx-like activity, characterized by the capacity of catalyzing the reduction of harmful hydroperoxides using thiols as cofactors, has attracted significant interest for this type of compounds as potential therapeutic agents. Aiming at a preliminary evaluation of the potential catalytic properties of **31a**, **35**, **36**, and **37** as GPx mimics, a ^{1}H-NMR-based methodology reported by Iwaoka *et al* [81]. This methodology consists on monitoring the oxidation of reduced dithiotreitol (DTT_{red}) into oxidized dithiotreitol (DTT_{ox}) mediated by H_2O_2 in the presence of a catalytic amount (10%) of the tested compound.

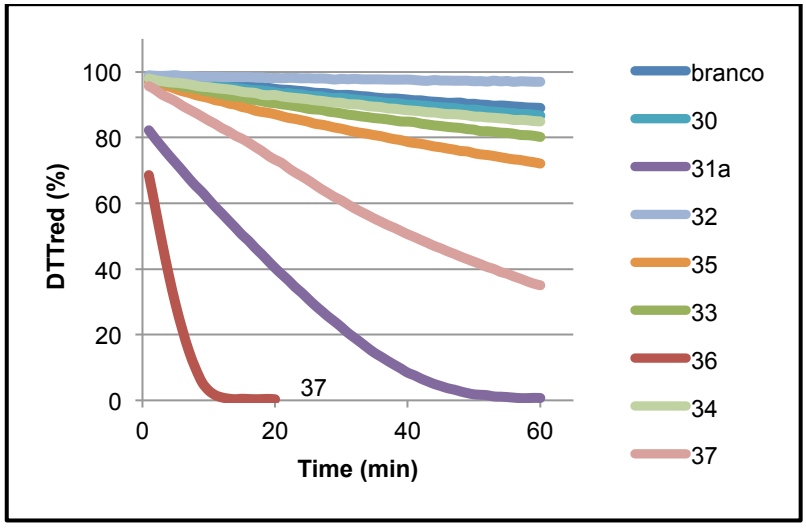


Chart II.4 – Glutathione peroxidase-like activity of the tested compounds: plot of percentage of DTT_{red} vs time. Oxidation of DTT_{red} mediated by H_2O_2 in the presence of a catalytic amount (10%) of the tested compounds was monitored by ^{1}H NMR.

Results obtained (Chart II.5) show that all the selenium derivatives have increased potential GPx-like activity, when compared with their respective starting materials, supporting the fact that selenation induces a modulation of this activity. Nonetheless, **31a** and **36** are the ones that completely reduced DTT within the time frame considered. In order to evaluate the relative efficiency of these compounds, the time required to oxidize 50% of the DTT_{red} ($t_{1/2}$) was estimated by linear regression. This shows thatd **36** is a more efficient reducing species ($t_{1/2}$ = 2.48 min), when compared to **31a** ($t_{1/2}$ = 15.79 min). This test also suggests that the presence of a hydroxyl group at the *para* position of the aromatic ring at position 2 of

benzoselenophenes results in a decrease of GPx-like activity. Moreover, taking into consideration that product **37** also exhibits some activity, suggests that the protection (as a methyl ether) of the hydroxyl group at position 7 and 5 of benzoselenophene results in a decrease of GPx-like active, albeit this activity is not completely hampered by this protection. In contrast, the absence of a hydroxyl group (or hydroxymethyl) at position 5 of benzoselenophene is determinant for the GPx-like activity. In fact, from the tested compounds, **35** is the only product where this group is absent and is the benzoselenophene presenting lower GPx-like activity.

II.6. Conclusions

The results presented in this work are, for most part, considered to be promising, regarding selenation as means of increasing potential antioxidant activity of the tested compounds.

Although the initial methodology, aiming at converting a carbonyl into a selenocarbonyl of purine derivatives did not achieve the success in producing selenated compounds, the second procedure proved to be a right choice. The reaction of direct selenation of stilbenes leading to benzoselenophene derivatives proved to be successful, producing compounds with one selenium atom in their structure, as was proved by structural characterization attained by Nuclear Magnetic Resonance and Mass Spectrometry.

As means to evaluate the antioxidant potential of the selenated derivatives, two assays were applied. DPPH scavenging activity showed a very interesting increase of the free radical activity for two of the three tested compounds, along with a decrease in BDE values for the H-atom most prone to abstraction. GPx-like activity was also tested, for all of the synthetized compounds, and results showed that upon selenation there is a drastic increase in this activity.

Overall conclusions lead to the belief that introducing one selenium atom on molecules with a structure already prone to have antioxidant activity will benefit from the presence of this new element, and gain a boost in its antioxidant activity.

III. Experimental Section

III.1. Materials and Methods

Chemicals

All reagents and solvents, commercially available, were acquired from Sigma-Aldrich CHEMIE Gmbh, BDH Chemicals, and May & Baker, and were used without any purification or previous treatment. Whenever necessary, solvents were purified and dried accordingly to standard methods [82].

Woolins' Reagent (W.R.) was either purchased from Sigma-Aldrich or synthesized according to the method described in literature [83]. (*E*)-5-styrylbenzene-1,3-diol (Fig. III.1, **33**) and (*E*)-1,3-dimethoxy-5-styrylbenzene (Fig. III.1, **34**) were synthesized by research group members and used in the subsequent experiments. (*E*)-2-methoxy-4-styrylphenol was synthesized according to the method described in literature [74]. The synthesized products were fully characterized using the techniques described below.

Nuclear Magnetic Resonance Spectroscopy (NMR)

Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectra were acquired in Bruker Avance III 300, Avance III 400, and Avance III^{+} 500 (Bruker), operating at 300 MHz, 400 MHz, and 500 MHz, respectively. Carbon Nuclear Magnetic Resonance (^{13}C-NMR) spectra were acquired in the same spectrometers operating at 100.6 MHz and 120.8 MHz, respectively. Selenium-77 Nuclear Magnetic Resonance (^{77}Se-NMR) spectra were acquired on a Bruker Avance III 500 at a resonance frequency of 95.4 MHz.

Chemical shifts are expressed in parts per million (ppm) down from tetramethytlsilane (TMS) for ^{1}H and ^{13}C NMR experiments, and H-H coupling constants (*J*) in Hertz (Hz). Dimethylselane (Me_2Se) was used as external reference for ^{77}Se-NMR spectra.

Structural elucidation of all synthesized molecules was based on results obtained by bidimensional experiments (heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC) and homonuclear correlation spectroscopy (COSY)) acquired with standard pulse programs. Data acquisition and processing was performed with Bruker Topsin v3.5 pl 5 software.

Mass Spectrometry (MS)

Mass spectra were obtained with by direct infusion in a ion trap LCQ Fleet Mass Spectrometer (Thermo Scientific) with the electrospray ionization source (ESI) in the negative mode optimized with the following settings: sheath gas flow rate, 40 (arbitrary units); auxiliary gas flow rate, 10 (arbitrary units); spray voltage, 4.5 kV; capillary voltage, -18/16 V; tube lens voltage, -125/63 V; skimmer voltage, 28 V; vaporizer temperature: 185 °C; capillary

temperature, 300 °C. Data were acquired using Xcalibur software (ThermoFischer Scientific). Internal calibration was performed for sodium formate/acetate clusters.

Thin layer chromatography

Thin layer chromatography (T.L.C.) and preparative thin layer chromatography (prep T.L.C.) were performed respectively on 0.2 mm and 0.5 mm thick plates, with Merck GF_{254} silica gel. Whenever necessary, the eluent is mentioned, with the indication of the component's proportion when using a mixture.

Flash column chromatography

Flash column chromatography was performed using Sigma-Aldrich silica gel (pore size 60 Å, 200-400 mesh particle size). Whenever necessary, the eluent is mentioned, with the indication of the component's proportion when using a mixture.

Microwave Synthesis

All reactions were conducted using a Anton Paar Monowave 300 Microwave Synthesis Reactor.

UV Spectroscopy

UV measurements were conducted in a Shimadzu UV-3101PC UV-VIS-NIR Scanning Spectrophotometer using UVPC software.

III.2. Synthesis

III.2.1. Microwave assisted seleno-purine synthesis

The following procedure was adapted from the one described in [71] as follows:

Woolins' Reagent (W.R.) (83 mg, 0.156 mmol) was added to a solution of purine (0.257 mmol) (1,3,7-trimethyl-3,7-dihydro-1*H*-purine-2,6-dione; 3,5-dihydro-4*H*-imidazo[4,5-*d*][1,2,3] triazin-4-one) in 3 ml of different solvents (dry *p*-xylene, N-methyl-2-pyrrolidone, dry sulfolane, 1,1,1-trichloroethane). The microwave vial was sealed, and the resulting solution was irradiated at 300 W reaching 170 ºC during 180 minutes. Following cooling to room temperature, solvent was evaporated under reduced pressure. The resulting residue was

dissolved in dichloromethane, and the mixture was purified by prep. T.L.C. (9:1 dichloromethane/methanol).

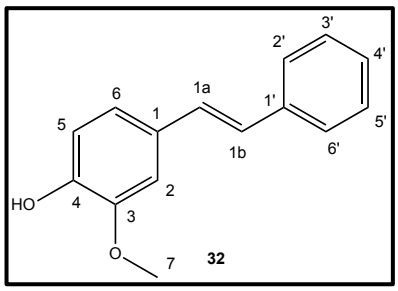
III.2.2. Synthesis of substituted stilbenes

III.2.2.1. (*E*)-2-methoxy-4-styrilphenol (32)

The following procedure was adapted from the one described in [74] as follows:

Phenylacetic acid (4.0 g, 0.029 mol) and 4-hydroxy-3-methoxybenzaldehyde (vanillin) (4.6 g, 0.03 mol) were dissolved in 0.5 ml of piperidine, and heated to 120 ºC for 18 hours. The mixture was treated with 5% aqueous NaOH and neutralized with aqueous hydrochloric acid (HCl), and extracted with ethyl acetate. The organic layer was dried over magnesium sulfate and concentrated in vaccum, affording the crude product. Application of the crude product to chromatography on silica gel (petroleum ether/toluene 1:1) resulted in two fractions that were evaluated using T. L. C., with one revealing to be a pure fraction of (*E*)-2-methoxy-4-styrilphenol (**32**) after confirmation with ^{1}H and ^{13}C NMR, and the other a mixture of compounds, which was then separated using prep. T. L. C.

III.2.2.1. (*E*)-2-methoxy-4-styrilphenol (32) – white crystalline solid, (1.156g, 17% yield). NMR: δ_H (500 MHz, $CDCl_3$, Me_4Si): 7.52 (2H, d, *J* = 7.6 Hz, ArC$^{2'}$H and ArC$^{6'}$H), 7.38 (2H, t, *J* = 7.6 Hz, ArC$^{3'}$H and ArC$^{5'}$H), 7.27 (1H, t, *J* = 7.1 Hz ArC$^{4'}$H), 7.09 (1H, d, *J* = 8.95 Hz, ArC^{6}H), 7.07 (1H, d, *J* = 7.3 Hz, ArC^{5}H), 7.00 (1H, s, C^{2}H), 6.96 (1H, d, *J* = 8.2 Hz, C^{1a}H), 6.94 (1H, d, *J* = 8.05 Hz, C^{1b}H), 3.98 (3H, s, C^{7}H). δ_C ($CDCl_3$): 146.8 (C^{3}), 145.6 (C^{4}), 137.6 (C$^{1'}$), 129.9 (C^{1}), 128.9 (C$^{3'}$ and C$^{5'}$) 128.6 (C$^{2'}$ and C$^{6'}$), 127.9 (C$^{4'}$), 126.5 (C^{6}), 126.3 (C^{1a} and C^{1b}), 120.5 (C^{5}), 114.6 (C^{2}), 55.8 (C^{7}).



III.2.3. Synthesis of benzoselenophene derivatives

The following procedure was adapted from the one described in [73] as follows:

All the reactions were carried out using oven-dried glassware under an inert atmosphere (N_2). Freshly distilled SO_2Cl_2 (0.2 ml, 0,8 mol) was added dropwise to selenium powder (237 mg, 3 mmol) and stirred at room temperature for 10 min, followed by the addition of 7.5 ml of dry THF. After 1 hour, a solution of each substituted stilbene (Fig. III.1) (1.2 mmol) in 2.4 ml of dry DMF was added and the resulting mixture was stirred for 24 hours at room temperature. The reaction mixture was diluted with THF and passed through a short column packed with Celite. The filtrate was then extracted with ethyl acetate and the organic phase was sequentially washed with water and brine, dried over anhydrous magnesium sulfate and

concentrated in vacuum, affording the crude product. Flash column chromatography over silica gel or preparative T.L.C. were used to further purify the desired products.

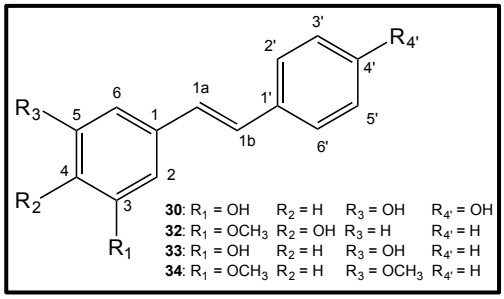
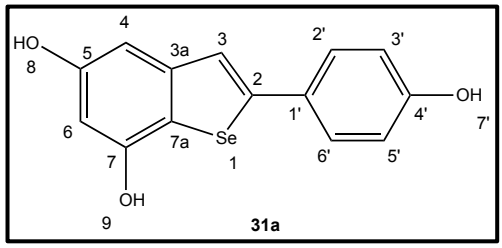
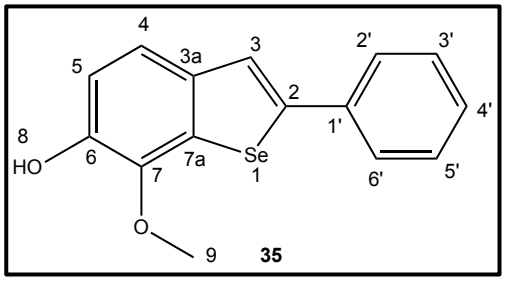


Figure III.1 – Generic structure and respective substituent groups used as reagents for benzoselenophene derivatives syntehesis.

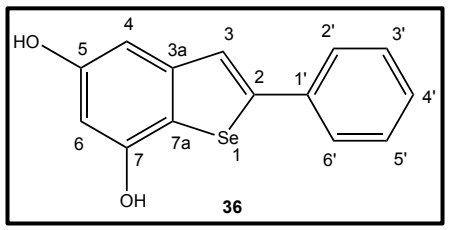
III.2.3.1. 2-(4'-hydroxyphenyl)benzo[*b*]selenophen-5,7-diol (31a) – Dark brown solid (105 mg, 59 % yield). NMR: δ_H (400 MHz, DMSO, Me_4Si): 7.58 (1H, s, $C^3\underline{H}$), 7.48 (2H, d, *J* = 8.2 Hz, $ArC^{2'}\underline{H}$ and $ArC^{6'}\underline{H}$), 6.81 (2H, d, *J* = 8.5 Hz, $ArC^{3'}\underline{H}$ and $ArC^{5'}\underline{H}$), 6.66 (1H, s, $C^4\underline{H}$), 6.26 (1H, s, $C^6\underline{H}$). δ_C (DMSO): 158.2 ($C^{4'}$), 157.2 (C^5 or C^7), 154.3 (C^5 or C^7), 147.5 (C^2), 145.4 (C^{3a}), 128.0 ($C^{2'}$ and $C^{6'}$), 127.3 ($C^{1'}$), 121.9 (C^3 or C^{7a}), 116.2 ($C^{3'}$ and $C^{5'}$), 102.5 (C^4), 99.4 (C^6). δ_{Se} (DMSO): 464.99. MS-ESI (-): *m/z* rel. int. (%) 308 [M(^{82}Se)-H]$^-$ (20.5), 306 [M(^{80}Se)-H]$^-$ (100), 304 [M(^{78}Se)-H]$^-$ (49.6), 303 [M(^{77}Se)-H]$^-$ (17.9), 302 [M(^{76}Se)-H]$^-$ (18), 300 [M(^{74}Se)-H]$^-$ (1.8).



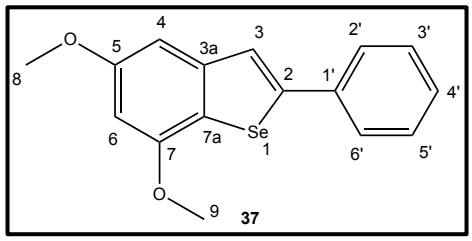
III.2.3.2. 7-methoxy-2-phenylbenzo[*b*]selenophen-6-ol (35) – Dark orange solid (5 mg, 1% yield). NMR: δ_H (500 MHz, $CDCl_3$, Me_4Si): 7.78 (2H, d, *J* = 7.6 Hz, $ArC^{2'}\underline{H}$ and $ArC^{6'}\underline{H}$), 7.59 (1H, t, *J* = 7.5 Hz, $ArC^{4'}\underline{H}$), 7.54 (1H, s, $C^3\underline{H}$), 7.52 (2H, t, *J* = 7.3 Hz, $ArC^{3'}\underline{H}$ and $ArC^{5'}\underline{H}$), 7.37 (1H, d, *J* = 8.4 Hz, $ArC^4\underline{H}$), 6.97 (1H, d, *J* = 8.4 Hz, $ArC^5\underline{H}$), 3.99 (3H, s, $C^8\underline{H}$). δ_C ($CDCl_3$): 150.1 (C^6), 146.6 (C^7 and C^2), 138.3 ($C^{1'}$), 131.9 (C^4), 129.9 (C^2), 129.7 ($C^{2'}$ and $C^{6'}$), 129.0 (C^{3a}), 128.2 ($C^{3'}$ and $C^{5'}$), 126.3 ($C^{4'}$), 123.5 (C^5), 111.8 (C^3), 56.1 (C^9). MS-ESI (-): *m/z* rel. int. (%) 306 [M(^{82}Se)-H]$^-$ (20.4), 304 [M(^{80}Se)-H]$^-$ (100), 302 [M(^{78}Se)-H]$^-$ (49.8), 301 [M(^{77}Se)-H]$^-$ (18.1), 300 [M(^{76}Se)-H]$^-$ (18), 298 [M(^{74}Se)-H]$^-$ (1.8).



III.2.3.3. 2-phenylbenzo[b]selenophene-5,7-diol (36) – Yellowish solid (45 mg, 13 % yield). NMR: δ_H (500 MHz, $CDCl_3$, Me_4Si): 7.64 (2H, d, *J* = 6.6 Hz, ArC$^{2'}$H and ArC$^{6'}$H), 7.59 (1H, s, C^{3}H), 7.41 (2H, t, *J* = 15.1 Hz, ArC$^{3'}$H/ArC$^{5'}$H), 7.34 (1H, t, *J* = 7.5 Hz, ArC$^{4'}$H), 6.87 (1H, d, *J* = 1.9 Hz, C^{4}H), 6.32 (1H, d, *J* = 1.9 Hz, C^{6}H). δ_C ($CDCl_3$): 154.9 (C^5 or C^7), 152.6 (C^5 or C^7), 149.4 (C^2), 145.3 (C^{3a}), 136.1 (C$^{1'}$), 129 (C$^{3'}$ and C$^{5'}$), 128.4 (C$^{4'}$), 126.8 (C$^{2'}$ and C$^{6'}$), 123.0 (C^3), 120.0 (C^{7a}), 103.8 (C^4), 99.5 (C^6). δ_{Se} ($CDCl_3$): 469.20. MS-ESI (-): *m/z* rel. int. (%) 292 [M(^{82}Se)-H]$^-$ (20.3), 290 [M(^{80}Se)-H]$^-$ (100), 288 [M(^{78}Se)-H]$^-$ (49.6), 287 [M(^{77}Se)-H]$^-$ (17.9), 286 [M(^{76}Se)-H]$^-$ (18), 284 [M(^{74}Se)-H]$^-$ (1.8).



III.2.3.4. 5,7-dimethoxy-2-phenylbenzo[b]selenophene (37) – Orange solid (29 mg, 8% yield). NMR: δ_H (500 MHz, $CDCl_3$, Me_4Si): 7.67 (1H, s, ArC^{3}H), 7.66 (2H, d, *J* = 7.2 Hz, ArC$^{2'}$H and ArC$^{6'}$H), 7.43 (2H, t, *J* = 7.4 Hz, ArC$^{3'}$H and ArC$^{5'}$H), 7.32 (1H, t, *J* = 7.3 Hz, ArC$^{4'}$H), 6.92 (1H, d, *J* = 2.0 Hz, ArC^{4}H), 6.42 (1H, d, *J* = 2.0 Hz, ArC^{6}H), 3.98 (3H, s, C^{8}H), 3.90 (3H, s, C^{9}H). δ_C ($CDCl_3$): 159.6 (C^5), 156.4 (C^7), 148.8 (C^2), 144.2 (C^{3a}), 136.4 (C$^{1'}$), 128.9 (C$^{3'}$ and C$^{5'}$), 128.2 (C$^{4'}$), 126.7 (C$^{2'}$and C$^{6'}$), 123.3 (C^3), 121.5 (C^{7a}), 99.9 (C^4), 95.8 (C^6), 55.79 (C^8), 55.70 (C^9). δ_{Se} ($CDCl_3$): 479.91. MS-ESI (-): *m/z* rel. int. (%) 320 [M(^{82}Se)-H]$^-$ (20.6), 318 [M(^{80}Se)-H]$^-$ (100), 316 [M(^{78}Se)-H]$^-$ (49.9), 315 [M(^{77}Se)-H]$^-$ (18.3), 314 [M(^{76}Se)-H]$^-$ (18), 412 [M(^{74}Se)-H]$^-$ (1.8).



III.3. Computational Studies

All calculations were performed using the Gaussian 09 Revision A.01 [84] suite of Quantum Chemical programs at the B3LYP/6-31+G(d) level (using the UB3LYP approach for radicals) [85–87]. The geometry of each species was fully optimized on the gas phase, using a quadratically convergent SCF procedure [88] followed by a single-point frequency and energy calculation; no imaginary frequencies were observed.

III.4. DPPH Scavenging Activity

The procedure for DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity was adapted from [80] . Briefly:

In a typical procedure, 4, 10, 20, or 30 µL of a freshly prepared 1 mM solution of the test compound in methanol, resulting in final concentrations of 10 µM, 25 µM, 50 µM, and 75 µM, were added to a 0.5 cm quartz cell containing 300 µl of freshly prepared DPPH solution (200 µM) in the same solvent. Ultraviolet spectra were recorded at 515 nm every 0.7 s over 1 – 2 min for the determination of rate constants and stoichiometries, and over 20 min for the determination of total stoichiometries. The blank was performed with 300 µL of DPPH in methanol without adding the antioxidant solution. Inhibition of free radical DPPH in percent was calculated using the following equation:

$$\text{DPPH radical scavenging activity } (\%) = \frac{\text{Abs.}\,initial - \text{Abs.}\,final}{Abs.\,initial} \text{ x } 100$$

Equation III.1 – DPPH radical scavenging activity.

Total stoichiometry was calculated using **Equation III.2** (*Abs.initial*, initial absorbance; *Abs.final*, final visible absorbance; *[Aox]i*, initial antioxidant concentration):

$$n_{tot} = \frac{\text{Abs.}\,initial - \text{Abs.}\,final}{\varepsilon\ x\ [Aox]i}$$

Equation III.2 – n_{tot} value calculation.

k_1 value for the fast step (~60 seconds) was calculated by plotting **Equation III.3** as a function of time, using a straight line with zero intercept. The slope of the line returns k_1 value (*Abs.*, visible absorbance at time *t*):

$$ln\ \frac{1 - \frac{Abs.\,final}{Abs.}}{1 - \frac{Abs.\,final}{Abs.\,initial}} = -\ \frac{k_1\ x\ [Aox]}{\frac{Abs.}{Abs.\,final} - 1}$$

Equation III.3 – k_1 value calculation.

III.5. GPX-like Assay

The procedure for GPx-like assay was adapted from [81]. Briefly:

In a typical procedure, 41 µM of $DTT_{red.}$ and a catalytic amount of the tested compound (0.41 µM) were dissolved in CD_3OD (300 µL), and the reaction was initiated by the addition of H_2O_2 (41 µM). ^1H NMR spectra were recorded at distinct time points at 25 ºC, using 3 mm tubes. A similar reaction was performed in the absence of the catalyst. Relative concentration of $DTT_{red.}$ and $DTT_{ox.}$ were determined by the area of the corresponding ^1H NMR signals.

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