Design, Synthesis and Biological evaluation of Reactive Selenium Species (RSeS)

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Von

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Dedicated to my

Lovely Family

Table of Contents

Table of Contents

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Abbreviations

Abbreviation	Definition
A. baumannii	*Acinetobacter baumannii*
BDE	Bond dissociation energy
B. oryziterrae	*Bacillus oryziterrae*
C. albicans	*Candida albicans*
Cys	Cysteine
E. coli	*Escherichia coli*
EGT	Ergothioneine
GPx	Glutathione peroxidase
K. pneumoniae	*Klebsiella pneumoniae*
L. brevis	*Lactobacillus brevis*
MDR	Multidrug resistance
Met	Methionine
MIC	Minimum inhibitory concentration
MSR	Methionine sulfoxide reductase
OCTN1	Organic cation / carnitine transporter 1
OS	Oxidative Stress
Prdx	Peroxiredoxin
ROS	Reactive Oxygen Species
RSeS	Reactive Selenium Species
RSS	Reactive Sulfur Species
s	seconds
S. aureus	*Staphylococcus aureus*

Abbreviations

S. carnosus	*Staphylococcus carnosus*
S. cerevisiae	*Saccharomyces cerevisiae*
SECIS	Selenocysteine insertion sequence
SeCys	Selenocysteine
SeMet	Selenomethionine
SeNPs	Selenium nanoparticles
tRNA	Transfer ribonucleic acid
TR	Thioredoxin reductase
Trx	Thioredoxin
VISA	Vancomycin-Intermediate *Staphylococcus aureus*

Summary

Naturally occurring RSeS form an interesting alcove of redox biology in living cells. These species provide interesting information for the design and synthesis of RSeS which modulate the redox state of cells and further grant important insights on lead structures in the hunt for active agents against infectious diseases associated with Oxidative Stress (OS).

In the present study, several synthetic RSeS were prepared and, thereafter, evaluated biologically against a plethora of targets. The synthetic compounds belong to different classes of organo-selenium compounds ranging from simple aromatic selenocyanates to ebselen-like selenazolinium salts and even rather complicated multi-component hybrid redox catalysts. These organic RSeS and some inorganic salts of selenium and tellurium were evaluated against a broad spectrum of microorganisms, including Gram-positive and Gram-negative bacteria of the notorious ESKAPE family, yeasts and multicellular nematodes. Some of the compounds, such as aryl methyl selenocyanates, were also investigated for cytotoxic activity against normal and cancer cell lines.

Generally, all the synthetic RSeS exhibited excellent activity against the selected targets. The preliminary mechanistic studies revealed that such compounds interact with the cellular thiolstat of the target organism. The interaction of selenium-based agents with intracellular thiolstat sets forth novel possibilities to tailor potent, efficient and target-oriented multifunctional RSeS.

Zusammenfassung

Natürlich vorkommende RSeS bilden eine interessante Nische der Redox-Biologie in lebenden Zellen. Diese Spezies liefern interessante Informationen für das Design und die Synthese von RSeS, die den Redox-Zustand der Zellen modulieren und wichtige Erkenntnisse über Leitstrukturen bei der Suche nach Wirkstoffen gegen Infektionskrankheiten im Zusammenhang mit Oxidativem Stress liefern.

In der vorliegenden Arbeit wurden mehrere synthetische RSeS vorbereitet und anschließend gegen eine Vielzahl von biologischen Targets bewertet. Die synthetischen Verbindungen gehören zu verschiedenen Klassen von Organoselenverbindungen, die von einfachen aromatischen Selenocyanaten über ebselenartige Selenazolinium Salze bis hin zu komplexeren mehrkomponentige Hybrid-Redoxkatalysatoren reichen. Diese Verbindungen wurden anhand eines breiten Spektrums an Mikroorganismen bewertet, darunter Gram-positiv und Gram-negativ Bakterien der berüchtigten ESKAPE-Familie, Hefen und Nematoden. Einige der Verbindungen, wie beispielsweise Arylmethylselenocyanate, wurden auch auf zytotoxische Aktivität gegen normale und Krebs-Zell linien untersucht.

Im Allgemeinen zeigten alle synthetischen RSeS eine ausgezeichnete Aktivität gegenüber den ausgewählten Zielen. Die mechanistischen Vorstudien zeigten, dass solche Verbindungen mit dem zellulären Thiolstat des Zielorganismus interagieren. Diese Interaktionen eröffnen neue Möglichkeiten, potente, effiziente und zielgerichtete multifunktionale RSeS zu gestalten.

Publications Included in this thesis

The following publications have been selected for this cumulative thesis out of a total 28 manuscripts published during the last four and half years (see “List of Publications”)

1. Pronounced activity of aromatic selenocyanates against multidrug resistant ESKAPE bacteria.

   Muhammad Jawad Nasim, Karolina Witek, Annamária Kincses, Ahmad Yaman Abdin, Ewa Żesławska, Małgorzat Anna Marć, Márió Gajdács, Gabriella Spengler, Wojciech Nitek, Gniewomir Latacz, Elżbieta Karczewska, Katarzyna Kieć-Kononowicz, Jadwiga Handzlik and Claus Jacob

   New Journal of Chemistry, 2019, Volume 43 (15), 6021-6031
2. Selenazolinium Salts as "Small Molecule Catalysts" with High Potency against ESKAPE Bacterial Pathogens.

   Karolina Witek, **Muhammad Jawad Nasim**, Markus Bischoff, Rosmarie Gaupp, Pavel Arsenyan, Jelena Vasiljeva, Małgorzata Anna Marć, Agnieszka Olejarz, Gniewomir Latacz, Katarzyna Kieć-Kononowicz, Jadwiga Handzlik, Claus Jacob

   Molecules, 2017, Volume 22(12), 2174, 1-16
3. Aspects of a Distinct Cytotoxicity of Selenium Salts and Organic Selenides in Living Cells with Possible Implications for Drug Design.

   Ethiene Castellucci Estevam, Karolina Witek, Lisa Faulstich, **Muhammad Jawad Nasim**, Gniewomir Latacz , Enrique Domínguez-Álvarez, Katarzyna Kieć-Kononowicz, Marilene Demasi, Jadwiga Handzlik and Claus Jacob.

   Molecules, 2015, Volume 20(8), 13894-13912

1. Introduction

1.1. The rise of the Greek moon

Selenium was discovered in 1817 by Jöns Jacob Berzelius (1779-1848), a Swedish chemist. The history of the discovery of this unique element is interesting and dates back to the beginning of the 18th century with the establishment of a chemical factory in the vicinity of the Castle of Gripsholm in Mariefred, Södermanland, Sweden, for the production of white lead paint. The production of paint involved the utilisation of acetic acid and sulfuric acid. The preparation of sulfuric acid involved the reaction of sulfur dioxide (by burning pyrite) with nitrogen dioxide in the presence of water in lead-lined chambers. The factory was soon liquidated due to several unfavourable conditions and was subsequently acquired by a team of entrepreneurs and chemists, namely Johan Gottlieb Gahn (1745-1818), Hans Peter Eggertz (1781-1867), and Jöns Jacob Berzelius [1,2]. The former proprietor of the chemical factory had observed the formation of reddish sludge in the lead chamber whenever the pyrite from Falun (Sweden) was utilized as a source of sulfur for the preparation of sulfuric acid. The reddish sludge was assumed to be an arsenic compound which was considered toxic and dangerous, so the utilization of pyrite from Falun was discontinued. The new owners Gahn and Eggertz insisted on utilizing the pyrite from Falun for the production of sulfuric acid. The reddish sludge was subsequently investigated by Gahn and Berzelius. The initial chemical analysis revealed the possibility of presence of tellurium also. Berzelius pursued his surprising investigations and reached a conclusion that the sludge must also contain a novel element. He named this element as “Selenium” (Greek: selḗnē, moon) which was chosen due to its similarity with tellurium (Latin: tellus, earth) which had been discovered a couple of decades earlier, in 1783, by the Austrian chemist Franz-Joseph Müller von Reichenstein (1740-1826) [3,4]. It is curious to note that Jöns Jacob Berzelius referred to the Greek, and not Latin name,

despite the fact that “tellus” was in essence a “latino”, otherwise he may have named the element as “lunatium” and this thesis would then address quite a few lunatic issues concerning “Reactive Lunatic Species”. The name was mentioned as:

“*skola beskrifvas vara en egen förut okänd brännbar mineralkropp, hvilken jag har kallat Selenium af ∑ελην η, måna, för at dermed utmerka dess nära slägtskap med Tellurium*“ [2].

1.2. Selenium: a “sensitive” element

Since its discovery, selenium has attracted the attention of numerous scientists who, later on, employed it in various areas of Science. Willoughby Smith (1828-1891) was the first one who discovered the photo-sensitive nature of elemental selenium in 1873 [5]. The famous German inventor Werner von Siemens (1816-1892) designed the first semiconductor based on selenium in construction in 1875 which was subsequently employed by Alexander Graham Bell (1847-1922) in the invention of the famous photo phone in 1880 which used to employ light for the transmission of sound [6,7]. The sequence of discoveries continued with the development of selenium cells by Charles Fritts (1850-1903) in 1884 which served as exposure meter for photographic instruments for several years [8,9].The photosensitive nature of selenium was employed to design solar panels and first solar arrays was installed by Charles Fritts on a New York city rooftop in 1884 [8,10]. Another interesting application of elemental selenium was established in the field of photography. The sensitive nature of selenium continued attracting scientists from various areas of Science and Industry. In fact, the element was discovered by a chemist, and explored by various physicists who channelled the sensitivity in the development of diverse fields of science and revolutionised the world with innovative inventions ranging from electrical semiconductors or solar cells all the way towards xerography in photography and graphic reproduction [11].

1.3. From photo sensitivity to redox sensitivity

The "sensitive" nature of selenium continued to lay the foundation for various other areas of Science. The redox-sensitive nature of selenium has recently contributed enormously to the field of organic synthetic chemistry. Various redox-active and also redox-modulating molecules based on selenium have been designed and synthesized for treating diseases associated with Oxidative Stress (OS). OS has been defined as "an imbalance between oxidants and antioxidants in the favour of oxidants, potentially leading to damage" by Helmut Sies in 1985 [12-14]. Organo-selenium compounds can selectively target oxidatively stressed cells as these cells have elevated levels of Reactive Oxygen Species (ROS) as compared to normal cells. The redox sensitive nature of selenium has led to the design of various classes of organo-selenium compounds and several lead structures have been synthesized.

1.4. From Toxin to Therapy

Selenium had always been considered dangerous and toxic for human consumption. The history of toxicity of selenium dates back to 13^{th} century when Marco Polo (1254-1324) observed that the hoofs of animals dropped off by the consumption of selenoferous plants in the mountainous region of western China although no one was aware of presence of selenium at that time, of course [15]. Maurice I. Smith and his colleagues reported the toxic effects of selenium on the health of people living on selenoferous soils in 1936 [16]. Its role as an essential nutrient was later recognised in animals in the 1950s and in human beings in 1973, in fact, with the discovery of selenium as an integral component of redox enzyme glutathione peroxidase [17,18]. Selenium forms an integral part of various proteins (selenoproteins) which play various important roles not only in growth and development. They also serve as essential components of antioxidant defence systems [19]. Many selenoproteins and selenoenzymes

contain selenocysteines (SeCys) and are mostly involved in oxidoreduction reactions which are extremely important for the intracellular redox homeostasis [20]. Glutathione peroxidase 1 (GPx 1), for instance, being the first selenoprotein discovered in 1957, is a crucial antioxidant enzyme which eliminates ROS and protects the cell against oxidative damages [21]. The redox biology of this unique protein has been studied extensively in the context of oxidative stress-related diseases *i.e.* prevention and therapy for cancer, cardio and neuro protection [22]. Other selenoproteins contain the amino acid selenomethionine (SeMet), the prime nutritional representative form of selenium. Since the tRNA responsible for the incorporation does not discriminate between Met and SeMet, SeMet assimilates into proteins as effectively as methionine (Met) [23,24]. Proteins containing SeMet, in contrast to other selenoproteins, do not participate in cell growth and are directly metabolized by the organisms to other forms of selenium *i.e.* SeCys, dimethyl selenide and trimethyl selenonium salts [25,26]. The unique role of selenium as a redox modulator in these selenoproteins and enzymes has inspired scientists to design and synthesise selenium-based GPx mimics. These endeavours have led to the development of multifunctional drugs, such as ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one), which have been studied extensively in the context of antioxidant activity and cancer prevention. Ebselen is currently undergoing clinical trials in the treatment of mania and hypomania [27,28].

1.5. Reactive Selenium Species (RSeS): an emerging concept

The concept of “Reactive Sulfur Species” (RSS) was postulated by Claus Jacob, Gregory I. Giles and Karen M. Tasker in 2001. It elaborates the roles of various sulfur containing metabolites in the context of OS [29]. Today, the analogy between sulfur and selenium provides grounds for postulation of the presence of similar Reactive Selenium Species (RSeS). Both sulfur and selenium are able to undergo redox modulation and interfere with the

redox-sensitive signalling proteins, thereby, interfering with the cellular thiolstat. RSS are more abundant still less reactive whilst RSeS are more reactive and less common. A brief comparison for different characteristics between sulfur and selenium atoms as well as RSS and RSeS is provided in Table 1.

Table 1. Comparison of the different physical, chemical and biochemical properties of elemental sulfur and selenium as well as RSS and RSeS.

Physicochemical Properties	Sulfur /RSS	Selenium/RSeS
Weight	light	heavy
Size	100 pm	115 pm
Polarizability	low	higher
Bond strengths	strong	weak
Bond breaking reactions	slower	faster
Energy level of sigma orbital	higher	lower
Stability at higher oxidation state	higher	lower
Acidity	less acidic	more acidic
Nucleophilicity	lower	higher
To act as leaving group	lower	higher
Hypervalency	less stable (*e.g.* sulfuranes, R_4S decompose at -67 °C)	more stable (*e.g.* selenuranes, R_4Se decompose at 0 °C)
Electrophilicity	lower	higher
π-Bonding	stronger	weaker
Chemical reactivity	slower	faster
Bonding with oxygen	strong	weak
Dipolar character of chalcogen-oxygen bond	weak	strong
Feasibility to form chalcogen oxides	highly favoured	less favoured
Preference for lower oxidation state	lower	higher
Nature of chalcogen dioxide	SO_2 is a mild reducing agent	SeO_2 is a mild oxidizing

		agent
Oxidation of chalcogen oxides	easy	difficult
Nucleophilicity of lone pair	high	Low
Redox potential	thiols have higher redox potentials	selenols have lower redox potentials
Rate of formation of di-chalcogenides	low	high
Bond dissociation energy (BDE) of O-H and chalcogen-H bond in sulfenic and selenenic acids	BDE of S-H bond (366.52 kJ mol^{-1}) is higher than O-H bond (287.02 kJ mol^{-1}) in sulfenic acid	BDE of Se-H bond (330.12 kJ mol^{-1}) is lower than O-H bond (339.74 kJ mol^{-1}) in selenenic acid
Nature of sulfinic and seleninic acids	sulfinic acids are mild reducing agents	seleninic acids are mild oxidizing agents
Interaction of sulfinic and seleninic acids with thiols	rate of reaction of thiols with sulfinic acids is 10^6 times slower as compared to seleninic acids acids	rate of reaction of thiols with seleninic acids is 10^6 times faster as compared to sulfinic acids
Reduction of sulfinic/seleninic acids to the oxidation states of 0 and -2	slow	fast
One electron redox reactions	faster	slower
Catalysis for enzyme	less. Cysteine is less catalytic than selenocysteine	more. Selenocysteine is more catalytic than cysteine

The term RSeS has been chosen to highlight the redox modulating, mostly oxidizing nature of certain organo-selenium compounds [30]. In comparison to the concept of RSS, the concept of RSeS is rather new and evolving. RSeS primarily include simple naturally occurring selenium-containing molecules, such as selenium nanoparticles, hydrogen selenide, selenocysteine, selenomethioneine and selenoneine. Nonetheless the term RSeS is quite broad and could also include several synthetic organo-selenium compounds, such as ebselen, selenocyanates, selenazolinium salts and selenium-based redox catalysts. The biochemical analogy comes from the fact that RSeS interfere with the cysteine (Cys) residues of the cellular thiolstat and trigger cellular signalling pathways which may either lead towards the

activation of antioxidant response or apoptosis just as often observed in the case of RSS. The presence of selenium donates more oxidizing or even catalytic characteristics to RSeS and therefore, these species, are often biologically active and, for instance, destroy certain target cancer cells effectively and selectively. The concepts of RSS and RSeS also provide a strong basis for the design and development of target based organo-selenium compounds (Figure 1).

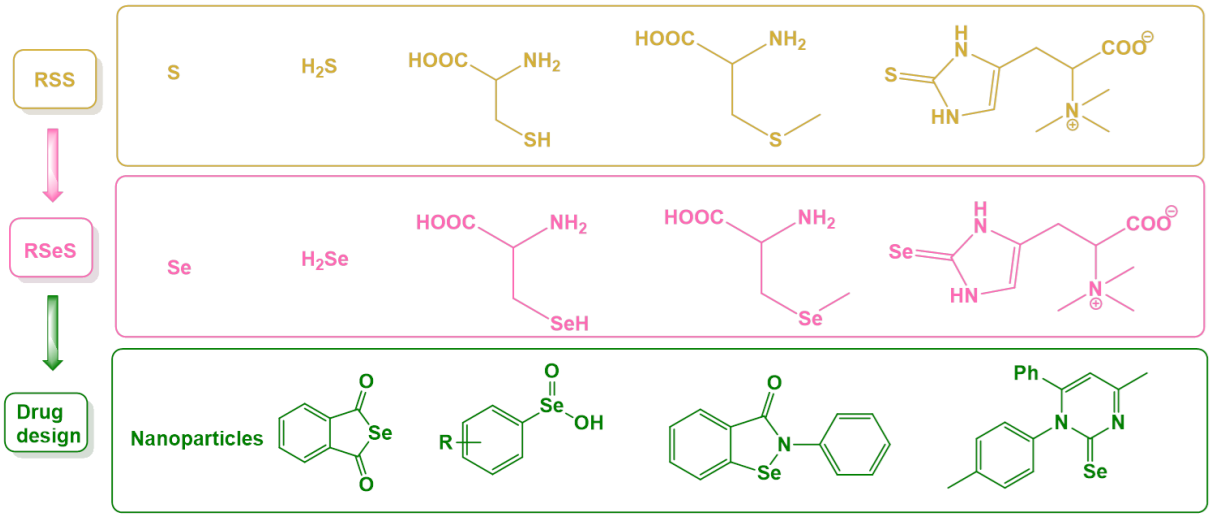

Figure 1. The figure provides an insight how the concepts of reactive sulfur and selenium species could be exploited in the development of innovative drug molecules. Some of these avenues are explored as part of this thesis.

The concept of RSeS is not only confined to naturally occurring organo-selenium agents *e.g.* elemental selenium (Se^0), hydrogen selenide (H_2Se), selenocysteine, selenomethionine and selenoneine, rather it also includes synthetic compounds inspired from nature *e.g.* selenocyanates and redox modulating hybrid-molecules *e.g.* seleno-quinones and seleno-lapachones.

1.5.1. Selenocysteine

Among the various RSeS, certain molecules are abundantly dispersed in nature and some of them are even considered as essential amino acids, such as selenocysteine (SeCys) which is

the selenium analogue of cysteine. SeCys is one of the most important RSeS which is incorporated into proteins following a certain specific mechanism which involves contributions from the DNA, a specific selenocystein insertion sequence (SECIS) and tRNA [31,32]. The redox chemistry of SeCys is associated with the selenenol/selenolate group. SeCys consumes thiols and converts peroxides (ROS) to alcohols and water catalytically, thereby protecting cells from oxidative damages [33]. The peroxidase cycles of SeCys and Cys involve the nucleophilic interaction of selenolate / thiolate with the oxidant (such as H_2O_2) to generate selenenic/ sulfenic acid which subsequently reacts with cellular thiols to produce mixed selenosulfide/disulfide. The interaction of these mixed selenosulfides or disulfides with further thiols regenerates the active enzyme (Figure 2). An interesting difference between the two peroxidases is the high reactivity of selenium compared to sulfur which enables the over-oxidized form of SeCys peroxidase (SeCys-Se(O)O^-) to oxidize exogenous thiols and return back to the catalytic cycle (Figure 2). In contrast, the over-oxidized form of cysteine peroxidase (Cys-S(O)O^-) is unable to oxidize the exogenous thiols and sulfinic acid formation (Cys-S(O)OH) therefore often leads to "self-inactivation". Peroxiredoxin (Prdx) is an exception as it can count on a special repair enzyme to reduce the over-oxidized form of Cys peroxidase and bring it back to the normal catalytic cycle [34]. This catalytic removal of peroxide leads to the antioxidant mechanism which is also employed by several other selenoenzymes, such as glutathione peroxidase (GPx) and even some organo-selenium compounds, such as ebselen, for instance, which in certain respects mimic the catalytic cycle of GPx.

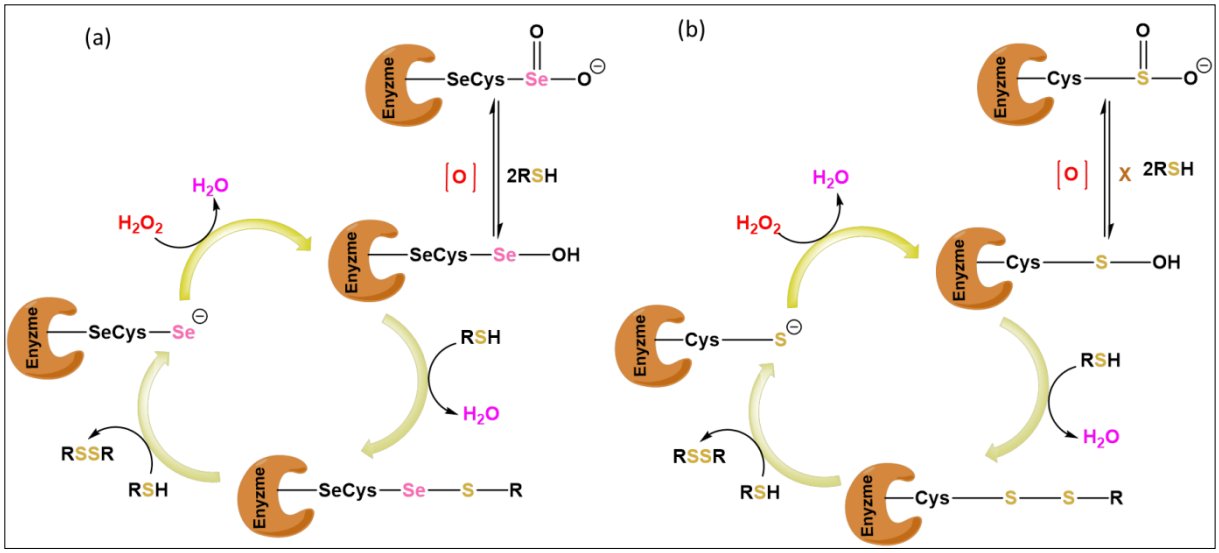

Figure 2. Comparison of the peroxidase cycles of (a) SeCys and (b) Cys commonly employed by enzymes such as GPx and Prdx, respectively. Isosteric replacement of sulfur for selenium plays a crucial role and enhances the peroxidase activity considerably.

1.5.2. Selenomethionine

Selenomethionine (SeMet) is also an interesting naturally occurring amino acid which can be integrated easily into cellular proteins and enzymes. It has been reported that SeMet constitute 20% and SeCys constitute 80% of the selenoproteins in human serum [35]. In contrast to animal kingdom, SeMet is the major form of selenium found in plants as it has been observed that tRNA responsible for the incorporation of methionine (Met) to the proteins does not discriminate between the sulfur and the selenium analogues. Although SeMet is not essential for the growth and development of plants, it is synthesized along with Met in quantities depending upon the amount of selenium available [36,37]. Intriguingly, a very high content of SeMet (ranging from 81-82%) was observed in some plants, such as seleniferous corn, wheat and soybeans [38]. Moreover, backer's yeast *Saccharomyces cerevisiae*, which is able to assimilate up to 3 mg/g of Se, incorporates more than 90 % of the total Se in the form of SeMet [39-41]. SeMet protects proteins and enzymes by providing an additional selenium-

based “antioxidant shield”. The redox chemistry of SeMet is rather interesting, as it combines the redox chemistry of selenides, selenoxides and selenones as depicted in Figure 3.

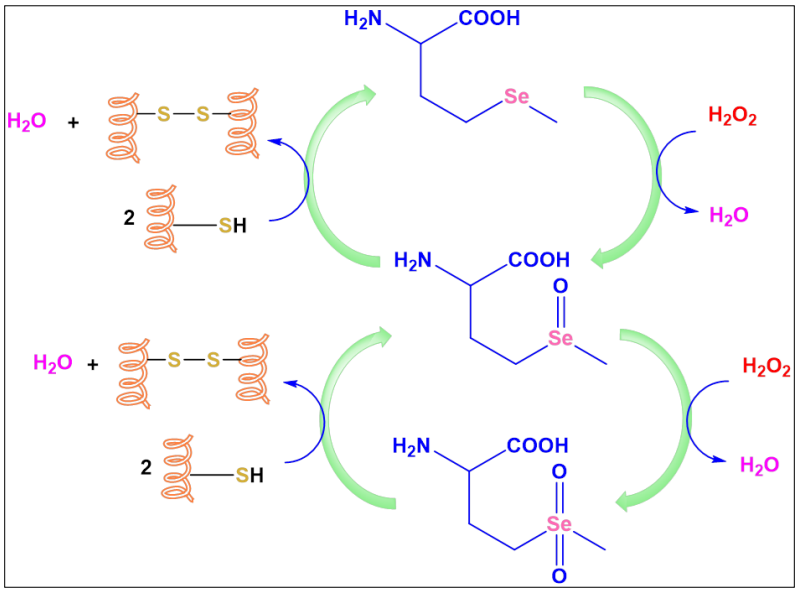

Figure 3. Selenium imparts strong antioxidant activity to SeMet. The oxidation of SeMet leads to the formation of selenoxide as a result of a catalytic and reversible cycle. The overoxidation of selenoxide to "selenone" does not occur under physiological conditions, which is different from its sulfur analogue.

The oxidation of SeMet leads to the formation of selenoxides and selenones which serve as oxidizing species and interact spontaneously with the proteins of cellular thiolstat, and may even enter a specific catalytic cycle. There are specific reductants such as methionine sulfoxide reductase (MSR) A and B which reduce methionine sulfoxide(s) to methionine(s) following a specific redox mechanism [42,43]. The redox pathway involves the reduction of oxidized form of MSR (A or B) which itself is mediated by simultaneous oxidation of reduced form of thioredoxin (Trx). The oxidised form of Trx is subsequently reduced by Thioredoxin Reductase (TR) which oxidises NADPH to $NADP^+$ (Figure 4) [42,43]. It is interesting to note that mammalian TR contains SeCys at the C-terminal extension [44].

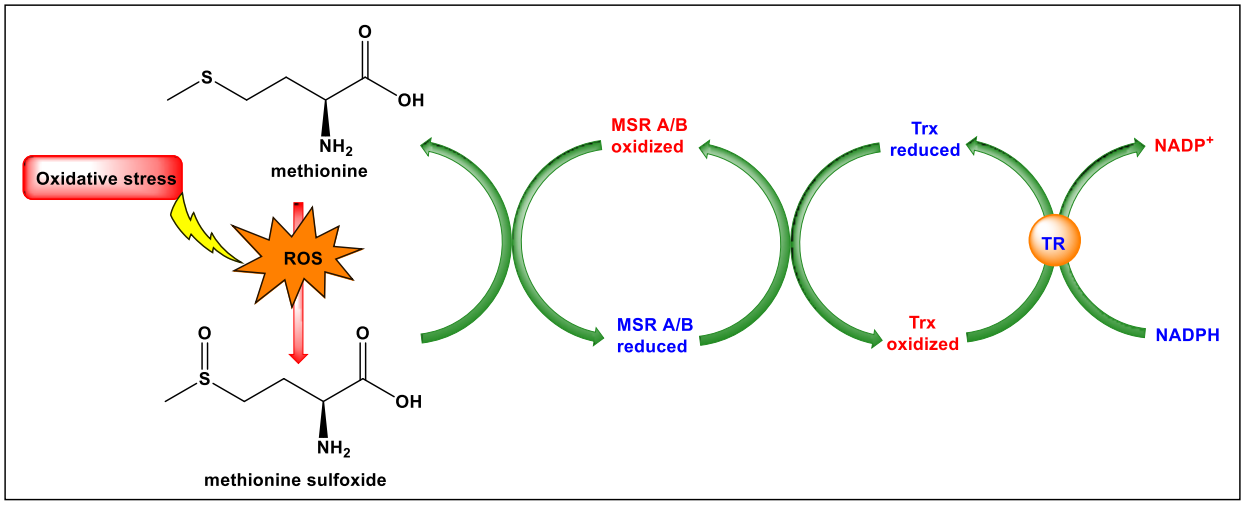

Figure 4. A Schematic overview of the redox cycle of oxidized and reduced forms of Met mediated by MSR, Trx and TR.

Selenoxides serve as an important participant for the selenium redox cycle in the fight against oxidizing species, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) [45]. Further oxidation of selenoxides leads to the formation of selenones $R_1Se(O_2)R_2$, the selenium analogue of sulfones, not to be confused with the other equivocal but chemically dramatically different selenone ($R_1C(=Se)R_2$) the selenium analogue of thione. In contrast to sulfones, selenones are rather unstable molecules and are, therefore, relatively rare in nature [30].

1.5.3. Selenoneine

Selenoneine is a naturally occurring selenium analogue of ergothioneine (EGT). EGT was first isolated by a French pharmacist and chemist Charles Tantet (1847-1917) about more than a century ago, in 1909, from the ergot of rye [46]. Several physiological functions of EGT have been reported in the literature including chelation of cations, regulation of the immune system and gene expression [47-50]. A very strong antioxidant and cytoprotective effect of EGT has been reported in the literature [51-60].

The selenium analogue of EGT, Selenoneine, has been discovered recently and isolated by Yamashita and his colleagues from the blood and tissues of tuna fish [61]. Chemically, selenoneine is a thoroughly unique naturally occurring molecule due to the presence of a selenourea motif. The molecule is highly sensitive towards oxidation due to the presence of the C=Se double bond or C-SeH in the tautomeric form respectively (see Figure 5). The presence of the lone pair of electrons on the adjacent nitrogen plays an important role in the chemistry of such selenoureas. Similar to the sulfur analogue EGT which exists in a thiol-thione equilibrium, selenoneine occurs in a specific selenol-selenone equilibrium (Figure 5) [62].

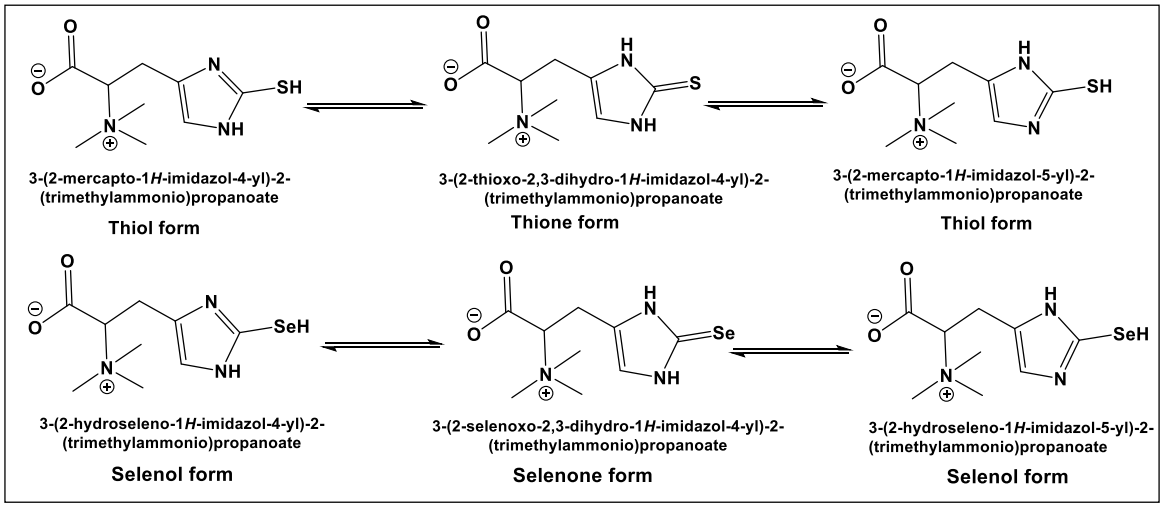

Figure 5. Ergothioneine and selenoneine exist in two tautomeric forms each, namely 3-(2-mercapto-1*H*-imidazol-4-yl)-2-(trimethylammonio)propanoate (thiol form), 3-(2-thioxo-2,3-dihydro-1*H*-imidazol-4-yl)-2-(trimethylammonio)propanoate (thione form), 3-(2-hydroseleno-1*H*-imidazol-4-yl)-2-(trimethylammonio)propanoate (selenol form) and 3-(2-selenoxo-2,3-dihydro-1*H*-imidazol-4-yl)-2-(trimethylammonio)propanoate (selenone form).

Selenoneine is apparently involved in the detoxification and metabolism of mercury compounds, such as methyl mercury (MeHg) which highlights the metal binding properties of this compound. The integration, incorporation and excretion of selenoneine is regulated by the

organic cation / carnitine transporter 1 (OCTN1) which is also an interesting transporter of EGT, the sulfur analogue of selenoneine [63]. Selenoneine also binds with other metals, including iron and has been found to attach to the haemoglobin (in blood) and myoglobin (in muscles) and protects them from auto-oxidation induced by iron ions in hypoxia. The antioxidant ability of cells and tissues is enhanced in the presence of GPx and several other selenoproteins [64]. The major antioxidant mechanism of selenoneine involves its interaction with ROS where it serves as an excellent radical scavenger and protects the cells and tissues against oxidative damages (Figure 6) [61].

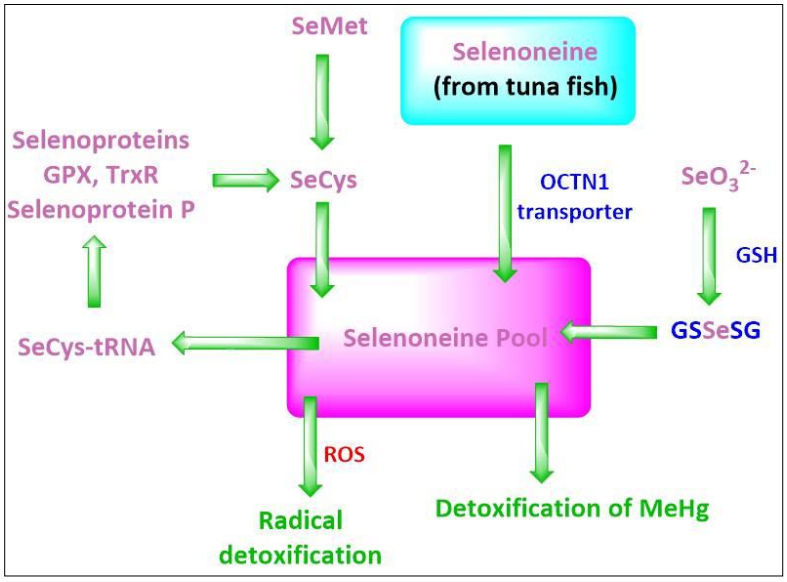

Figure 6. The naturally occurring small molecule selenium compound selenoneine serves as an excellent antioxidant and protects the cells by scavenging harmful radicals which belong to the family of ROS. As an excellent complexing agent / ligand system for many heavy metals, it also detoxifies methyl mercury in fish.

These natural RSeS have been explored extensively as redox modulators, antioxidants, chemopreventive and anti-proliferative or anticancer agents [30]. There are several other examples of RSeS, such as elemental selenium or hydrogen selenide (H_2Se), which have also been studied in the last couple of years in the context of OS and related diseases [65,66].

1.6. From RSeS to drug design

RSeS provide a solid base for designing selenium-based multifunctional redox modulators to achieve better selectivity and reactivity. As described earlier the term RSeS refers to the redox modulating and often oxidizing nature of selenium-based organic compounds. Elemental selenium and several classes of synthetic organo-selenium compounds could also be considered in this context. The application of elemental selenium in the fields of nutrition, medicine and agriculture has been investigated in detail [67-70]. Nanotechnology imparts several beneficial features to elemental selenium, such as an increased surface area, improved bioavailability and enhanced biological activity. Selenium Nanoparticles (SeNPs) can be generated mechanically by grinding utilizing a mill and homogenizer, chemically by the reduction of SeO_3^{2-} and also biologically by employing bacteria, such as *Staphylococcus carnosus* and *Bacillus oryziterrae* [70]. Among all the types of procedures employed to generate SeNPs, biologically produced SeNPs are of particular interest as these particles are (i) environmentally friendly, (ii) enhancing the bioavailability of elemental selenium, (iii) often covered by a protein coat which inhibits the agglomeration of such particles, and (iv) active against their targets, such as a wide spectrum of microbes [70].

The redox chemistry of SeCys is rather similar to the one of selenides due to presence of the selenol/ selenolate group, while the reodox chemistry of SeMet involves selenides, selenoxides and selenones. Chemically and also biologically, selenides and selenols are interrelated. It has been reported that the metabolic conversion of selenide to selenol results in enhanced nucleophilic and cytotoxic activity [71]. Selenoneine is rather exceptional as it is neither a classical selenol nor selenide. It contains an interesting selenourea motif in the ring which results in a selenone-selenol tautomerism. Unlike thiourea this selenone urea is not very stable but can be stabilized by altering the ring or employing certain functional groups to stabilize the structure. This information regarding the oxidation state, stability, nucleophlicity,

interaction with the cellular cysteine and proteins could be utilised to design synthetic RSeS. The synthetic organo-selenium compounds can range in complexity from simple selenocyanates to the products of complicated multicomponent reactions, such as the Click combinations or Biginelli reactions. The concept of RSeS could therefore be exploited to design synthetic molecules which may serve as promising lead structures from the perspective of drug design, as summarised in Figure 7.

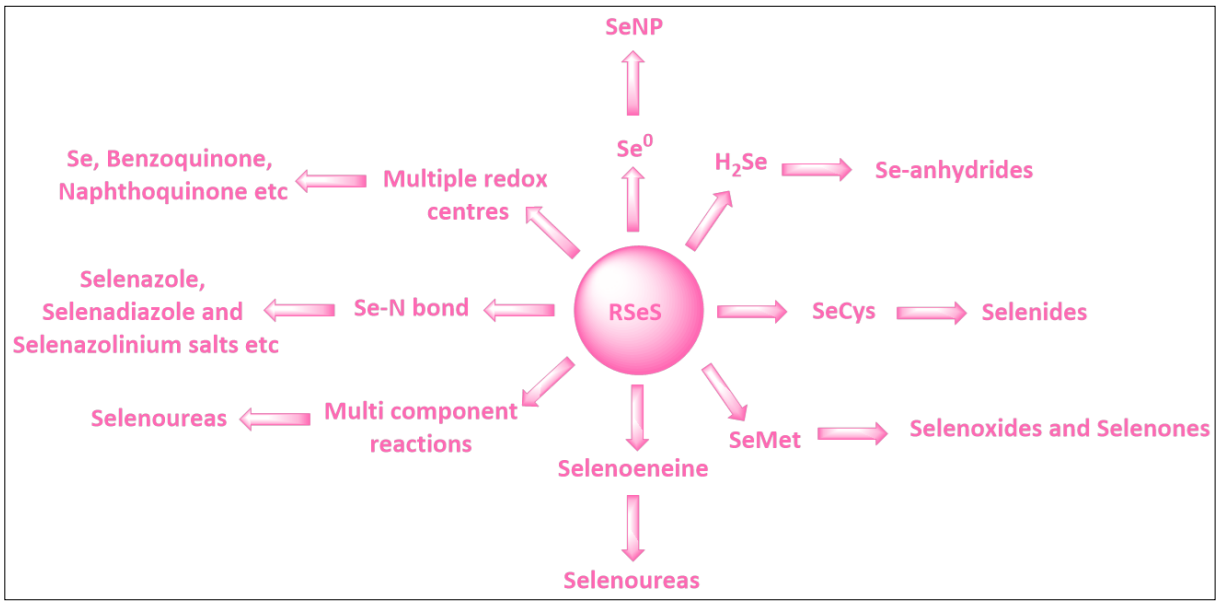

Figure 7. The concept of RSeS can be exploited from the perspective of drug design.

1.7. Seleno-nitrogen compounds

Seleno-nitrogen compounds represent an interesting class of organo-selenium compounds due to the presence of two hetero atoms in the aromatic ring. Ebselen is one of the most important members of this class as it is the only selenium containing organic molecule which so far has reached clinical trials [27]. Ebselen is a multifunctional agent which interacts with the cellular thiols of cysteine proteins, catalyses the reduction of ROS, similar to GPx and serves as an excellent oxidant of reduced thioredoxin [28,72]. Ebselen has been explored to serve as antioxidant, antimicrobial and cytoprotective agent [28,72]. Selenazolinium salts represent

another interesting class of compounds with Se-N bond which have been synthesized recently by Pavel Asrenyans and his colleagues [73]. These compounds resemble ebselen in structure, and possess an additional positive charge which considerably enhances the electrophilic affiliation of the compounds towards nucleophiles including thiols. These selenazolinium salts are therefore also more reactive biologically [73].

Besides ebselen and selenazolinium salts, there are several other classes of organoselenium compounds with different arrangements of selenium and nitrogen, including selenazoles, selenadiazoles, selenocyanates, isoselenocyanates and selenoureas.

1.7.1. The mystery of the selenamide/selenenamide bond (Se-N)

A cyclic selenamide, also referred to as selenenamide, has been proposed to be formed during the catalytic mechanism of GPx-1 as a result of complicated interactions of selenenic acid with a particular amide moiety of the protein backbone. This five membered selenamide ring serves as an excellent electrophile and subsequently interacts with glutathione to form mixed selenosulfides [33]. Based on these considerations several selenenamide-based mimics of GPx have been synthesized and their activities have been evaluated [74]. A recent study has demonstrated the mechanism of antioxidant activity of several selenenamide mimics based on their interactions with nucleophilic aromatic thiols [75].

Interestingly, the same selenenamide bond also exists in ebselen which belongs to the class of selenazolines. Ebselen (Figure 8) contains one nitrogen in the ring and, from a design perspective, addition of a second nitrogen atom may lead to the activation of these compounds for increased biological activities. Selenadiazoles are heterocyclic compounds which contain two nitrogen and one selenium in the heteroatom ring structure. The addition of a second nitrogen may decrease the electron density and subsequently enhance the electrophilic reactivity of the compounds which is usually desired for biological activity [30].

Selenadiazoles have been proposed to interact with the thiol residues of cellular cysteine proteins and thereby exert antimicrobial activity [76]. Several selenazoles have also been reported to induce apoptosis in cancer cells by increasing intracellular levels of ROS [77]. The generalised structures of selenazoles and selenadiazoles are shown in Figure 8

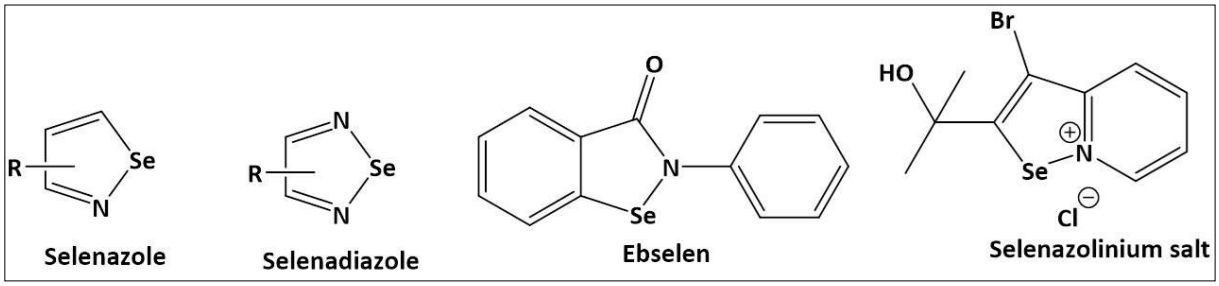

Figure 8. General representation of selenazoles, selenadiazoles, ebselen and structure of most active compounds from the family of selenazolinium salts.

1.7.2. Selenocyanates

Selenocyanates represent another attractive class of seleno-nitrogen compounds. Traditionally, cyanates have been an interesting class of compounds as they comprise of a unique blend of carbon, nitrogen and an element from the chalcogen family with the exception of polonium (Po). Inorganic cyanates, such as sodium and potassium cyanates, have been considered to be toxic due to several factors, including interactions with components of the central and the peripheral nervous system and irreversible binding with iron proteins, such as haemoglobin [78-81]. In contrast to inorganic cyanates, the organic cyanates or nitriles, have been found to be very useful for biological systems. Organic cyanates have been facilitated as an interesting pharmacophore for designing drug molecules. A huge number of medicines have been produced employing this interesting motif. The nitrile functional group is very strong and stays intact even after metabolism, as observed in several nitrile-containing drugs. Vildagliptin, Fadrozole monohydrochloride, Letrozole (with two nitrile groups),

Neratinib and Olprinone are a few rather prominent examples of nitrile containing drugs (Figure 9) [82,83] .

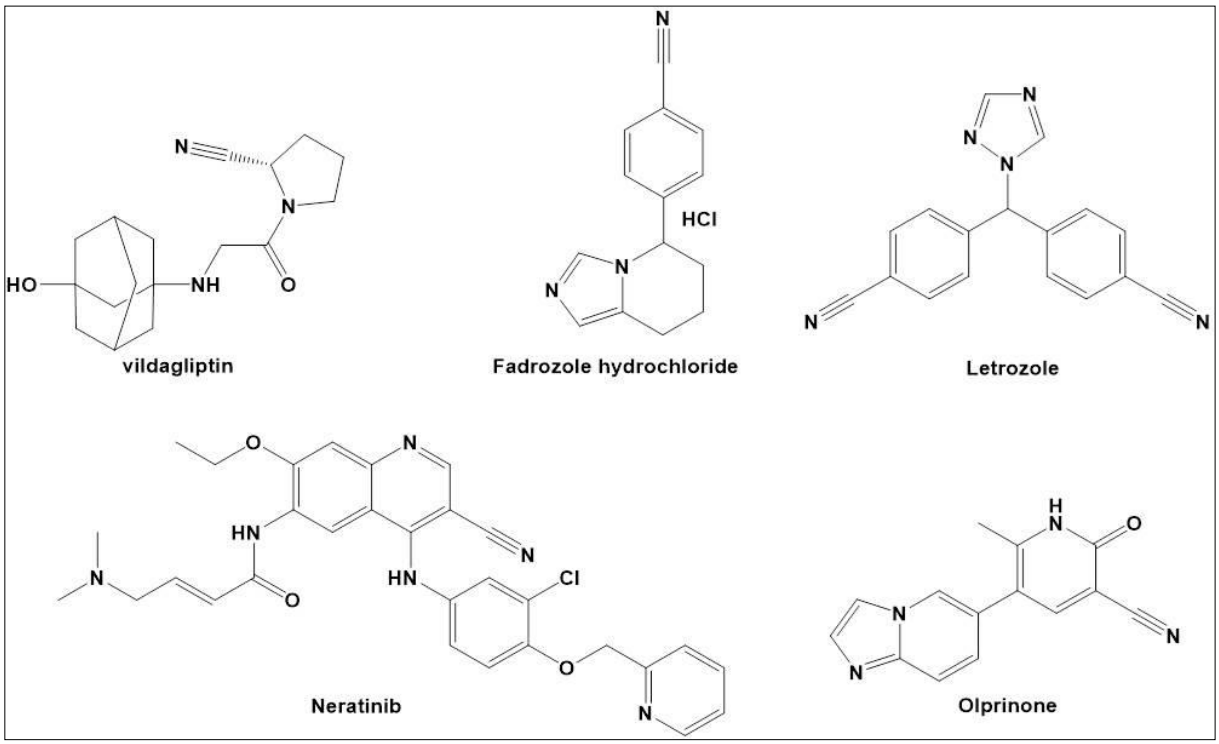

Figure 9. A few selected examples of nitrile containing drugs.

It has also been observed that addition of a chalcogen element confers some special characteristics to molecules. Thiocyanates, *i.e.* cyanates containing a sulfur atom, represent an interesting class of organic cyanates as they occur naturally in the extracellular fluids of mammals. Thiocyanates are either synthesized enzymatically by sulfur transferase, an enzyme which transfers sulfur to cyanides, or taken from the diet into the human system. These thiocyanates play an efficient role in the defence system of the host by inhibiting the metabolism of pathogenic organisms [84,85]. Moreover, they also serve as an integral part of the host antioxidant system [86]. Thiocyanates have the ability to interact with the cellular thiolstat and redox modulate transformations reversibly [87]. Furthermore, inorganic thiocyanates have also been employed in the shampoos for the regeneration of hair [88]. Organic thiocyanates, allyl (iso)thiocyanate, benzyl (iso)thiocyanate, phenethyl

(iso)thiocyanate and sulforafanes for instance, are widely dispersed in nature, especially in cruciferous plants, and are known for their excellent antimicrobial and anticancer activities [89-91]. Moreover, allyl isothiocyanate has been reported to serve as an efficient multifunctional agent for the treatment of neurodegenerative diseases, cardiovascular diseases, diabetes, chronic obstructive pulmonary disease, renal fibrosis and dermatitis (Figure 10) [92]. Interestingly, the thiocyanate motif has also been observed in marine natural products, such as fasiculin, psammaplin B and 9-thiocyanatopupukeanane (Figure 10) [93-95].

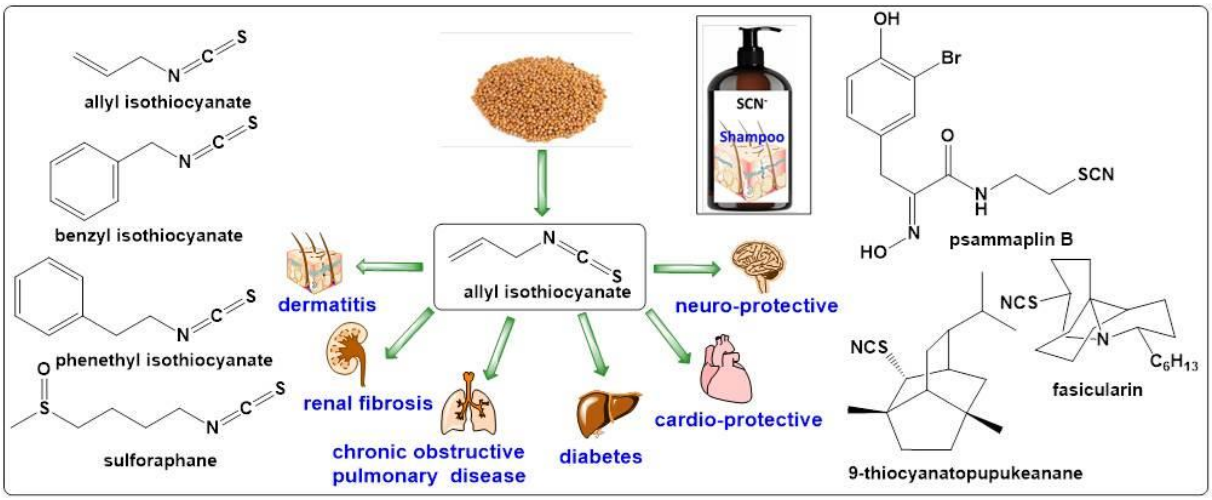

Figure 10. Nature itsef is an affluent source of (iso)thiocyanates *e.g.* allyl isothiocyanate, benzyl isothiocyanate, phenethyl isothiocyanate, sulforafane, fasiculin, psammaplin B and 9-thiocyanatopupukeanane, representing excellent agents able to treat various diseases. Inorganic salts of thiocyanate, such as potassium thiocyanate and sodium thiocyanate are commonly employed in shampoos for the regeneration of hair.

Moving down the Periodic Table from sulfur to selenium, there is a remarkable change in the physical characteristics, chemical reactivity and biochemical and antimicrobial activity of the relevant compounds. This could be attributed to the more nucleophilic nature of selenium. Moreover, the valence shell electrons in selenium are loosely held as compared to sulfur.

Furthermore, selenium cannot form all the kinds of π bonds as sulfur and the bond strength is also not very strong as compared to sulfur [96]. Elemental selenium has been proven to be biologically more active in various systems compared to sulfur. In the context of cyanates, there is not much literature available for comparison, especially in biological systems. Among the few reports Krishnegowda *et al.* have demonstrated that selenocyanates exhibit a higher cytotoxic activity against MCF-7 breast cancer cell lines compared to thiocyanates [97].

Apart from these activities, selenocyanates represent an interesting class of organo-selenium compounds which serve as a precursor for various other types of organo-selenium compounds classes, such as seleninic acids and diselenides. They have been investigated extensively from chemical and biological perspectives but little has been reported about their anti-microbial activity. Moreover, the literature also reveals that selenocyanates have mostly been studied when attached to some "bioactive" scaffolds which may also influence and mask their very own biological activity [98].

1.8. Selenium based intelligent redox catalysts

The significance of redox catalysis could be estimated by considering the redox chemistry underlying the biological activities of seleninic acids. From the perspective of drug design, seleninic acids and their respective deprotonated forms and salts are generally easily soluble in many solvents, including aqueous media. Seleninic acids are easy to synthesize, acidic, rather reactive and tend to be highly specific for the thiols in general and thiols of the cellular thiolstat in particular [99,100]. Seleninic acids are relatively weaker acids than their corresponding sulfur analogues by around two p*K*a units *e.g.* the p*K*a values of benzeneseleninic acid ($PhSeO_2H$) and Benzenesulfinic acid ($PhSO_2H$) are 4.79239 and 2.76240, respectively. The relatively higher acidic nature of sulfinic acid is attributed to the intrinsic higher electronegativity and enhanced π- electrons accepting tendency of sulfur when

compared to selenium. Although, at the moment, the literature lacks information concerning the basicity of seleninic acids *i.e.* p*K*a of $PhSe(OH)^{2+}$, it can be envisaged that they would be significantly more basic in nature than sulfinic acids (as is the case with selenoxides and sulfoxides). Hence, acid catalysed substitution reactions of seleninic acid are favoured and therefore the reactions involving the reduction of such selenenic acids by thiols are rapid [101-103]. Studies performed by the colleagues of Hans J. Reich supported this notion by demonstrating that benzeneseleninic acid was rapidly reduced by thiols *i.e.* in less than 30 s at −90 °C, whilst its sulfur analogue did not react with the thiols for weeks even at room temperature [96,103]. Interestingly, the literature reveals that the reactions of alkaneseleninic acid with aromatic thiols are around 200-fold faster as compared to seleninic acids which are part of selenoenzyme, such as selenosubtilisin [102]. These rapid interactions also insinuate towards the brief lifespan of the seleninic acids *i.e.* seconds whilst thiols are found in the millimolar concentrations in the cells. The literature also reveals that the rate of reduction of seleninic acids by thiols is at least 10^6 fold faster as compared to their sulfur analogues [96]. Moreover, these compounds interact spontaneously with thiols to generate disulfide, selenylsulfide and selenol (in some cases only) in several steps (Figure 11). Seleninic acids have the potential to modify a total of four cysteine residues by oxidation, still usually their reactivity is discontinued with the formation of the selenylsulfide. It is interesting to note that at the same time, the final reduced form (selenol) and other intermediates could, theoretically, enter a catalytic cycle in the presence of an appropriate oxidizing agent such as hydrogen peroxide (H_2O_2) (Figure 11).

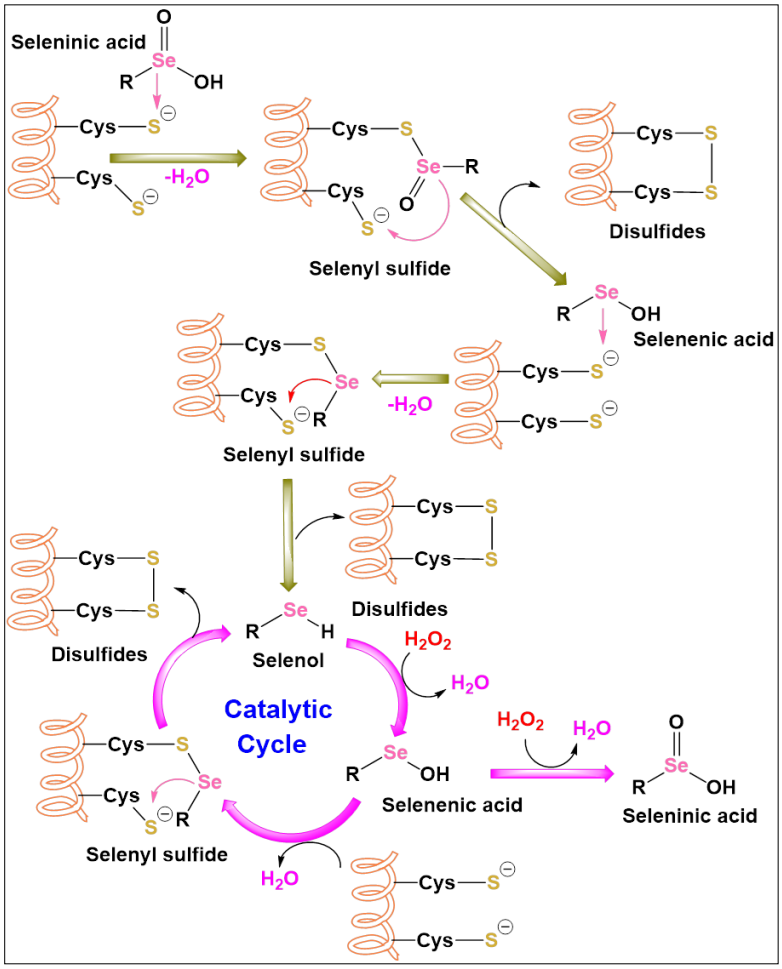

Figure 11. The catalytic cycle of seleninic acids represents the significance of selenium-based redox catalysts which may serve as intelligent multifunctional agents as they interfere specifically with the thiol residues of the proteins of cellular thiolstat.

The catalytic cycle can be divided into several steps:

- In the very first step, the nucleophilic selenium atom of seleninic acids interacts with the thiol residue of the cysteine protein which leads to the formation of mixed species, *i.e.* selenyl sulfides
- In the second step, the nucleophilic selenium of mixed selenyl sulfide oxidises another thiol of the cysteine protein leading to the formation of disulfides and the selenenic acid

- Selenenic acid interacts with another thiol of the cysteine protein to form selenyl sulfides
- The mixed selenyl sulfide oxidises one more thiol of the cysteine proteins with the formation of disulfides of cysteine proteins and the selenol
- The selenol may be oxidized by certain ROS, such as H_2O_2 to selenenic acid
- Selenenic acid interacts with another thiol of the cysteine protein to form selenyl sulfides
- The mixed selenyl sulfide oxidises one more thiol of the cysteine proteins with the formation of disulfides of the cysteine proteins and recycling of the selenol

This catalytic cycle occurs frequently in the selenium enzymes, such as GPx. Similar catalytic cycles have also been observed for some organo-selenium compounds, such as ebselen, and involve other selenium species with higher oxidation states [104]. Generally, the mechanism of catalysis involves the interaction of substrates with the specific catalyst to generate related products. In the context of biological applications at the cellular level, the nature, concentration and the impact of these substances *i.e.* substrate, catalyst and also products on the cellular process should be considered.

Such intracellular catalysis therefore combines several interesting aspects which are extremely important from the perspective of drug design. The most distinct features include selective targeting of cells under the condition of pre-existing OS, specific interactions with the proteins of the cellular thiolstat, absence of intrinsic toxicity and the efficiency even at low concentrations. Several organo-selenium catalysts have been reported to exert their cytotoxic activity at sub-micromolar concentrations due the presence of excessive amount of substrate, in this case ROS in the target cells *e.g.* cancer cells without damaging the normal cells due to the low concentrations of ROS present there [105,106]. Not surprisingly, several studies support the notion of employing specific catalysts as potential drugs which sense a

certain biochemical signature *i.e.* the substrate and reveal their cytotoxic or antioxidant potential in response to and by employing the presence of this substrate [106].

As a part of this thesis, several compounds from different classes of organo-selenium compounds which fall under the umbrella of the RSeS have been designed and synthesized. The biological activity of these compounds has been evaluated against a plethora of microorganisms and several other targets.

2. Aims of the thesis

The analogy between RSS and RSeS provides interesting insights from the perspective of target oriented drug design and synthesis. The first and foremost aim of the current study is to employ the concept of RSeS to produce a) selenium analogues of naturally occurring RSS, such as thiocyanates, and b) multifunctional redox catalysts, in this case, selenium-based hybrid molecules. The second aim of the current study is to determine the biological significance of these organo-selenium compounds together with some other RSeS, such as ebselen-like selenazolinium salts and inorganic salts of chalcogens. Initially the compounds were, therefore, evaluated against a wide spectrum of targets, such as Gram-positive and Gram-negative ESKAPE bacteria including the multidrug resistant (MDR) strains, pathogenic and non-pathogenic yeasts, multicellular nematodes and certain normal and cancer cell lines.

The third aim of this study intents to investigate the underlying mode and mechanisms of actions of selected compounds. The inorganic salts of chalcogens and selenazolinoium salts were subjected to detailed investigations to explore their interactions with cellular targets. The fourth aim of this study involves the investigation of drug-like properties and the safety profile of some highly effective and (re)active RSeS.

3. Results

3.1. Publication 1

Pronounced activity of aromatic selenocyanates against multidrug resistant ESKAPE bacteria.

Muhammad Jawad Nasim, Karolina Witek, Annamária Kincses, Ahmad Yaman Abdin, Ewa Żesławska, Małgorzat Anna Marć, Márió Gajdács, Gabriella Spengler, Wojciech Nitek, Gniewomir Latacz, Elżbieta Karczewska, Katarzyna Kieć-Kononowicz, Jadwiga Handzlik and Claus Jacob

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Pronounced activity of aromatic selenocyanates against multidrug resistant ESKAPE bacteria†

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Selenocyanates represent an interesting class of organic selenium compounds. Due to their similarity with better known natural (iso-)thiocyanates, they promise high biological activity and may also be metabolized to other Reactive Selenium Species (RSeS), such as selenols, diselenides and seleninic acids. Thirteen arylmethyl selenocyanates (**1–13**) have been synthesized and evaluated for potential antimicrobial, nematicidal and cytotoxic activity. The compounds exhibit pronounced antimicrobial activity against various strains of Gram-positive and Gram-negative bacteria and yeasts, including multidrug resistant strains. The results obtained so far demonstrate that these arylmethyl selenocyanates are also non-mutagenic and have limited cytotoxicity against human cells. Here, benzyl selenocyanate (**1**) represents the most active anti-ESKAPE agent, with potent activity against multidrug resistant MRSA strains (HEMSA 5) – with a competitive MIC value of just 0.76 μg mL^{-1} (3.88 μM), whereas it exhibits low(er) cytotoxicity (IC_{50} = 31 μM) and no mutagenicity against mammalian cells. Due to this selective antimicrobial activity, aromatic selenocyanates may provide an interesting lead in the development of antimicrobial agents, particularly in the context of drug resistance.

Introduction

Since the discovery of the first modern antibiotics almost one hundred years ago, substances such as penicillin have served as important and effective weapons in the prevention and treatment of a wide spectrum of infectious diseases. Nonetheless, over- and misuse of antibiotics, among various other reasons, have led to a surge of resistance in pathogenic bacteria, which has now become one of the biggest threats and challenges facing humanity.[1] The phenomenon of resistance to available antibiotics has attracted the attention of scientists for over two decades now, and has led to a considerable demand for the development of new types of antibiotics against such resistant strains of bacteria and fungi.

Fortunately, nature itself is an affluent source of antibiotics. Natural substances acquired from medicinal and culinary plants, such as garlic and mustard, have been employed extensively as antibiotics throughout history.[2–4] More recently, such secondary metabolites have become important leads in the development of new and effective medicines.[5–7] These phytochemicals include alkaloids, flavonoids, terpenes, polyphenols and, particularly, organosulfur compounds (OSCs).[8–11] OSCs are distributed widely in nature, for instance as allicin in garlic, ergothioneine in mushrooms, and thiocyanates and isothiocyanates in cruciferous vegetables, to name just a few (Fig. 1).[12–14] Isothiocyanates and thiocyanates are reactive, electrophilic substances belonging to the class of natural Reactive Sulfur Species (RSS) and are cherished for their ability to randomly modify cysteine proteins and enzymes of the cellular thiolstat.[15] Such a wider "oxidative onslaught" in microorganisms frequently results in pronounced toxicity, even in otherwise resistant organisms, and RSS are often seen as promising candidates in the battle against resistant bacteria and fungi.[14–18]

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† Electronic supplementary information (ESI) available. ESI and CCDC 1819893 and 1819894. For crystallographic data in CIF or other electronic format see DOI: 10.1039/c9nj00563c

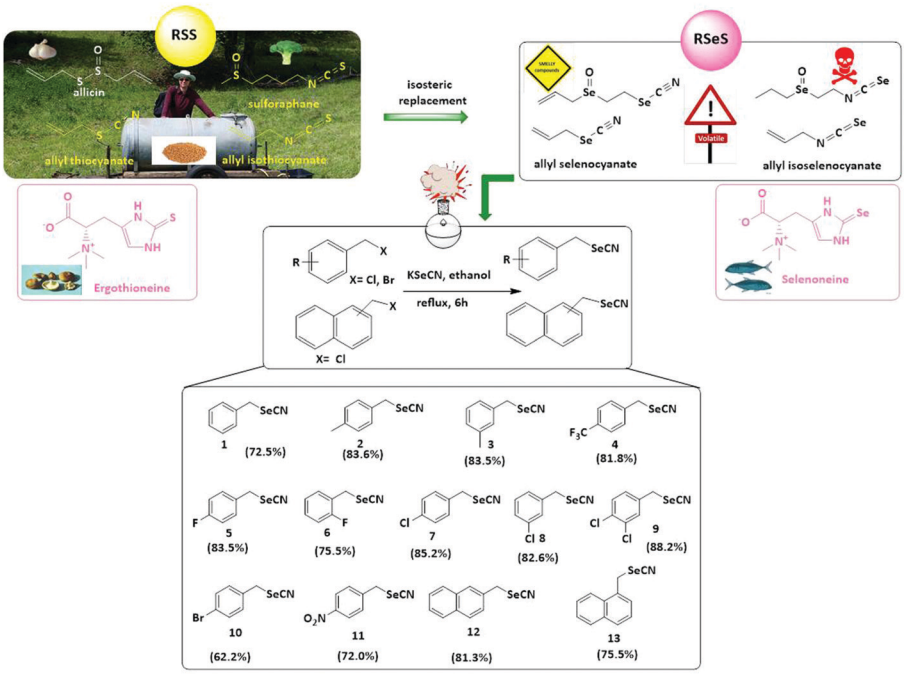

Fig. 1 An "isosteric replacement" of sulfur for selenium is found in nature where it leads from Reactive Sulfur Species (RSS), such as ergothioneine, to Reactive Selenium Species (RSeS), such as selenoneine. In pharmaceutical research, this strategy encompasses a range of (iso-)selenocyanates based on naturally occurring allyl(iso-)thiocyanates. Notably, the direct analogues of allyl(iso-)thiocyanates are rather unpleasant substances. The aromatic counterparts (**1–13**), shown here with their synthesis and respective percentage yields, are more promising candidates in the context of modern drug development.

Compared to RSS, Reactive Selenium Species (RSeS) are considerably less common in biology. Despite the overarching importance of this trace element in humans, we actually find only a handful of selenium secondary metabolites in higher organisms, such as the ergothioneine–analogue selenoneine in blue tuna, whereas most selenium in higher organisms is incorporated in the amino acids selenocysteine and selenomethionine and forms part of a range of selenoproteins and selenoenzymes.[19–21]

The lack of a wider spectrum of selenium containing secondary metabolites is rather surprising and somewhat disappointing. A remarkable upsurge in activity and reactivity is often observed for the selenium analogues of organosulfur compounds, and "going for selenium" in the form of selenium analogues of naturally occurring RSS is quite attractive from the perspective of drug design, especially at a time when certain selenium-containing organic compounds are receiving renewed attention. Selenazole compounds, such as ebselen (2-phenyl-1,2-benzoselenazol-3-one), not only mimic the activity of the selenium enzyme glutathione peroxidase, they also have already entered clinical trials in the context of mania and hypomania.[22–25] Moreover, naturally occurring (iso-)thiocyanates, such as allyl isocyanate in mustard oil, and selenium analogues thereof, *e.g.* primarily (iso-)selenocyanates, represent a particularly promising class of compounds. Unfortunately, the most obvious selenium analogues of such natural antibiotics are either chemically unstable or extraordinarily difficult to synthesise and to handle because of intense odour and inherent toxicity.[26,27] This is particularly the case for the respective, chemically simple isoselenocyanates. Then again, replacement of sulfur with selenium in aromatic isothiocyanates has been reported to enhance the reactivity of compounds towards thiols in proteins.[28] Additionally, isoselenocyanates demonstrate a higher reactivity towards GSH, target several cellular proteins, possess a greater ability to modulate the redox cycle in cells and also induce elevated levels of apoptosis as compared to isothiocyanates.[28] Since isoselenocyanates have been explored already for biological applications, for instance against several kinds of cancers (*e.g.* lung, colon, liver, prostate and breast) and infective diseases (*e.g.* malaria, tuberculosis and leishmaniasis) we have shifted our focus to aromatic selenocyanates, which have received less attention and also promise greatly improved physico-chemical properties.[29–32] These compounds are intriguing as they are not only active on their own, being often metabolized to a range of other RSeS, such as selenols, diselenides and seleninic acids.[33–37] Once again, such organoselenium compounds are not "exotic" and – either directly or as metabolites – play significant roles in biology.[38] Although aromatic selenocyanates so far have mostly been studied when attached to some other "bioactive" scaffolds which may have influenced or even dominated their biological activity, they have been reported to exhibit leishmanicidal activity.[35,39,40] Moreover, the dietary intake of benzyl selenocyanate has been reported to inhibit the incidence of small intestinal and colon adenocarcinoma.[41] Since hardly any wider investigations of such – chemically quite stable – benzyl selenocyanates are found in the biological literature, we have turned our attention to this class of compounds first. Here we

report the synthesis of aromatic selenocyanate compounds, including four novel compounds (**2**, **8**, **9** and **13**), a pronounced and even somewhat selective antimicrobial activity against pathogenic and resistant microorganisms and a set of relevant pharmacological parameters which indicate a low(er) risk to human cells.

Results and discussion

Chemical synthesis

Thirteen arylmethyl selenocyanates (**1–13**, Fig. 1) were designed and synthesized based on basic pharmacokinetic and pharmacodynamic considerations.[42–44] The compounds were produced from appropriate commercially available arylmethyl halides and potassium selenocyanate (KSeCN) according to the general procedure described by Wheeler and Merriam, with minor modifications (Fig. 1).[45] All of the compounds – of which four are novel – were obtained in good yields (62–88%) and the structures of compounds **1–13** were confirmed by ^{1}H and ^{13}C-NMR spectroscopy. Molecular mass and purity were determined by LC-MS (see Experimental section and ESI†).

Crystallographic studies

In order to provide a deeper insight into the structural properties, two selected compounds (**1** and **12**) were studied by X-ray crystallographic analysis (Fig. 2 and Table S1 in ESI†). In both crystal structures, the unit cells consisted of four molecules. The values of bond lengths formed by the selenium atom for C(sp)–Se were 1.850 Å and 1.837 Å for compound **1** and **12**, respectively, whereas for C(sp^3)–Se the bond length was 1.991 Å for both structures. Similar values were observed in other crystal structures with selenium atoms. A search of the CSD demonstrated that the geometry of the methyleneselenocyanate moiety in **1** and **12** is not exceptional among structures containing this fragment. The crystal structure of **1** has been determined earlier lacking hydrogen atoms.[46]

Antimicrobial activity

Once available, compounds **1–13** were pre-screened for potential antimicrobial activity against selected Gram-positive and Gram-negative bacteria, fungi and multicellular nematodes. The antimicrobial activity was evaluated in terms of minimum inhibitory concentrations (MICs) and values were compared to standard reference antimicrobial agents (Table 1).[47–52]

Overall, a significant and on occasion astonishingly selective antimicrobial activity against highly pathogenic organisms has been observed, frequently overcoming the kind of multidrug resistance traditional antibiotics are faced with. The impact on cultured human cells was considerably more modest, pointing towards a possible selectivity against some – particularly nasty – pathogens. Indeed, most of the compounds displayed significant activities against both Gram-positive and Gram-negative members of a most obnoxious family of bacteria from the perspective of resistance, *i.e.* ESKAPE pathogens, which comprise of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species.[53]

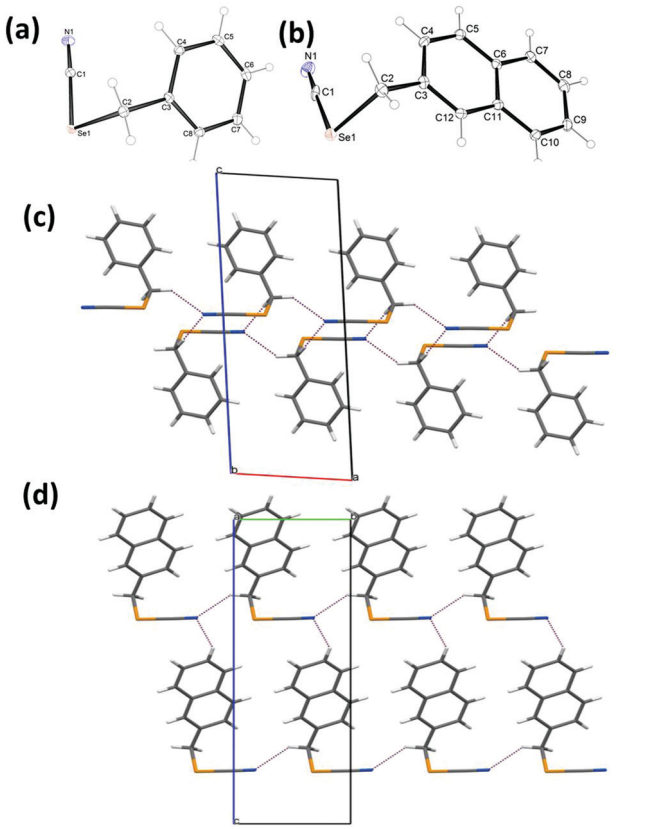

Fig. 2 The molecular structures of (a) **1** and (b) **12**, with the appropriate atomic numbering schemes. Displacement ellipsoids are drawn at the 30% probability level. Partial packing views, indicating the intermolecular interactions in a layer of (c) **1** and (d) **12**. The intermolecular interactions are depicted as dashed lines.

In the case of Gram-positive bacteria, the compounds were examined against the reference strain (ATCC 25923) and the multidrug resistant (MDR) clinical isolate of *Staphylococcus aureus* (*S. aureus*, HEMSA 5). It is noticeable that all compounds, except compound **11**, displayed MIC values against the MDR strain which were considerably lower than the ones for the reference antibiotic oxacillin.[47,48] In the case of the most active compound, *i.e.* benzyl selenocyanate (**1**) an excellent antimicrobial activity was observed against HEMSA 5. Amazingly, the potency of this compound against the resistant strain (MIC 0.76 μg mL^{-1} or 3.88 μM) was almost one hundred fold higher than the reference drug oxacillin (MIC 128 μg mL^{-1} or 318.86 μM) (Table 1).

Other compounds (**2**, **3**, **6**, **8**, **9**, **12** and **13**) also demonstrated anti-*Staphylococcal* activity with MIC values below 10 μg mL^{-1} (62.50 μM). Once more, these selenocyanates did not discriminate between reference and resistant MDR strains of *S. aureus*, displaying similar antibacterial potency against both, with an activity comparable to or even slightly higher against the MDR strain (**12**). Hence, selenocyanates appear to overcome bacterial MDR, most likely by circumventing the components responsible for resistance. Still, the exact biochemical causes for this

Table 1 Antimicrobial activity of arylmethyl selenocyanates against selected bacteria from non-ESKAPE and ESKAPE families of bacteria and yeasts (**1–13**)

	MICs (μg mL^{-1})						
Compound	*S. carnosus*	*S. aureus* ATCC25923	MRSA[a] HEMSA 5	*A. baumannii* 4184/2/5	*P. aeruginosa* 4600	*S. cerevisiae*	*C. albicans*
1	24.48	**≤0.76**	**0.76**	**1.53**	**6.12**	24.48	**12.24**
2	26.27	**6.57**	**6.57**	**6.57**–13.14	52.54	52.54	52.54
3	**13.19**	**6.59**	**6.59**	**13.19**	26.37	**6.59**	**13.19**
4	264.08	**16.51**	33.02	33.01	33.01	66.04	264.08
5	53.52	≥26.76	>26.76	26.76	26.76	53.52	**6.69**
6	**6.69**	**6.69**	**6.69**	**13.38**	107.05–210.10	**6.69**	**6.69**
7	230.4	**14.4**–28.8	28.8	14.4–28.8	28.8	460.8	**7.4**
8	115.28	**7.2**–14.4	**7.2**–14.4	**7.2**–14.4	115.28	28.82	28.82
9	33.13	**8.28**	**8.28**	**8.28**–16.56	66.25	66.25	33.13
10	275.04	34.38	>34.38	34.38	34.38	68.76	275.04
11	30.125	482	482	>482	>482	60.25	**7.53**
12	123.09	**15.44**	**7.72**	**15.44**	246.17	123.09	61.55
13	**15.39**	**7.69**	**7.69**	**15.39**	246.16	61.56	30.78
Ref.	0.03[b]	0.45[c]	128[c]	<4[c]	≤16[d]	0.03–2[f]	0.09–4[e]

[a] MRSA; methicillin-resistant *S. aureus*. [b] MIC values for reference antibiotics: ampicillin. [c] MIC values for reference antibiotics: oxacillin. [d] MIC values for reference antibiotics: piperacillin. [e] MIC values for reference antibiotics: fluconazole. [f] MIC values for reference antibiotics: itraconazole.[48–53] Bold denotes significant values.

"resistance busting activity" are unclear, and, as mentioned earlier, may also involve reactive metabolic products of selenocyanates, such as selenols, diselenides and seleninic acids, which together with the original selenocyanate may interfere with microbial targets to overcome resistance. Notably, most of these compounds were considerably less active against the harmless *Staphylococcus* strain *S. carnosus*, with almost twenty to thirty-fold selectivity for HEMSA 5 over *S. carnosus* observed for compounds **12** and **1**, respectively. Except for compounds **3**, **6** and **13** which inhibited the growth of *S. carnosus* at the concentrations of 13.19, 6.69 and 15.39 μg mL^{-1} (62.50 μM, 31.25 μM and 62.50 μM), respectively, no significant activity against this reference strain could be found.

The antibacterial activity of the selenocyanates was not limited to Gram-positive bacteria. Compound **1** also demonstrated excellent antimicrobial activity against pathogenic *Acinetobacter baumannii* (*A. baumannii*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). Once again, compound **1**, with a MIC of 1.53 μg mL^{-1} (7.80 μM) against *A. baumannii* and 6.12 μg mL^{-1} (31.25 μM) against *P. aeruginosa*, was more potent against these Gram-negative pathogens when compared to the respective reference drug, in this case piperacillin with a MIC of 16 μg mL^{-1} (30.92 μM) against *P. aeruginosa*.[49,50] Compounds **2**, **3**, **6**, **9**, **12** and **13** also demonstrated a significant, albeit slightly lower activity against *A. baumannii* (MIC values 6.57–15.44 μg mL^{-1}, *i.e.* 31.25–62.50 μM). Although the compounds exhibit selectivity for both Gram-positive and Gram-negative bacteria, the selectivity of the compounds against pathogenic strains of Gram-negative bacteria is particularly stimulating from a pharmaceutical perspective, as Gram-negative bacteria are generally more difficult to target, in part due to the specific structure and limited permeability of their cell wall.[54,55]

When evaluated against pathogenic yeast *Candida albicans* (*C. albicans*), compounds **1**, **3**, **5**, **7** and **11** revealed an encouraging growth inhibitory activity at concentrations below 20 μg mL^{-1} (62.50 μM). Compounds **5**, **7** and **11** prevailed with MIC values even below 10 μg mL^{-1} (31.25 μM). The fungistatic effect is lower compared to clinically relevant antifungal drugs, such as fluconazole and itraconazole (MIC values 0.09–4 μg mL^{-1} (0.29–13.06 μM) and 0.03–2 μg mL^{-1} (0.04–2.83 μM), respectively) (Table 1).[46,47] When compared to a non-pathogenic yeast, the fungicidal activity of compound **7** exhibited a high level of selectivity towards *C. albicans*, where it was more than 60-fold more active (MIC of 7.4 μg mL^{-1} (31.25 μM)) compared to baker's yeast *Saccharomyces cerevisiae* (*S. cerevisiae*)(MIC 460.8 μg mL^{-1} (2 mM)). Compounds **5** and **11** exhibited similar activities, although their selectivity declined noticeably when compared to compound **7**. Nonetheless, these two compounds still maintained remarkably low MIC values against pathogenic yeast (6.69 μg mL^{-1} (31.25 μM) and 7.53 μg mL^{-1} (31.25 μM), respectively) compared to non-pathogenic *S. cerevisiae* (MIC 53.52 and 60.25 μg mL^{-1} (31.25–250 μM), respectively). Compound **1**, which was extraordinarily active against bacteria, also exhibited some activity against unicellular fungi, with slightly higher fungicidal activity against pathogenic *C. albicans* (MIC 12.24 μg mL^{-1} (62.50 μM)) compared to *S. cerevisiae* (MIC 24.48 μg mL^{-1} (62.50–125 μM)). Such outcomes are promising in terms of utilizing these compounds as antifungal agents, for instance in the treatment of fungal infections, such as cutaneous, oropharyngeal and vulvovaginal candidiasis.[56–58]

Nematicidal activity

Although often ignored in developed countries, multicellular microorganisms, such as parasitic nematodes, still represent important targets in drug design which are particularly difficult to attack. In order to extend the scope of our preliminary studies, the series of aromatic selenocyanates (**1–13**) was evaluated for nematicidal activity against the agricultural nematode *S. feltiae*, which often serves as a simple and reliable representative model of a multicellular organism (Fig. 3).

After a pre-screen to determine the concentration range relevant for this organism, compounds were evaluated at four different concentrations, *i.e.* at 3.75, 7.5, 15 and 30 μM. A remarkable, concentration-dependent decrease in the viability of

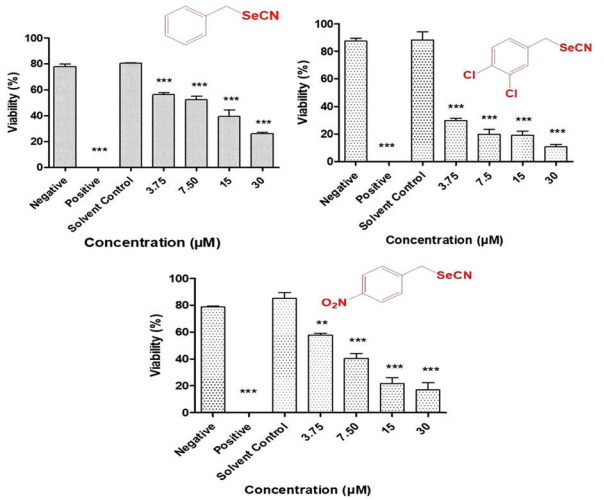

Fig. 3 Concentration-dependent activities of the most active selenocyanates (from left **1**, **9** and **11**) against *S. feltiae*. PBS buffer and ethanol (70% v/v) were employed as negative and positive controls, respectively. Values represent mean ± S.D. $***p < 0.001$ and $**p < 0.01$.

the nematodes was observed for all compounds, which was pronounced even at the lowest concentrations employed. Compounds **9** and **11** exhibited the most significant nematicidal activity and decreased the viability of nematodes to less than 40% at a concentration of 3.75 and 15 μM, respectively. These compounds exhibited LD_{50} values of 0.28 μM and 4.90 μM against *S. feltiae*, respectively. Compound **1**, which has shown considerable antibacterial and antifungal activity (see above), also exhibited significant nematicidal activity (LD_{50} = 6.85 μM) and decreased the viability of nematodes to less than 40% at the concentration of 15 μM. Other selenocyanates were less active.

Although practical applications are more speculative at this time, these results indicate that the aromatic selenocyanates may also serve as excellent nematicidal agents, possibly against pathogenic nematodes affecting plants, animals and humans – clearly a matter for further investigation (Fig. 4).

Cytotoxicity of arylmethyl selenocyanates against mammalian cells

In medicine, selenocyanates have been associated frequently with outright toxicity. In order to rule out any major cytotoxicity against mammalian cells and to investigate whether there may be any selectivity against microorganisms, compounds **1–12** were investigated for their activity towards two cancer cell lines of mouse T-lymphoma, *i.e.*, the sensitive (PAR) and the multidrug resistant cell line (MDR) transfected with the human MDR1 (ABCB1) gene which codes for the ABC transporter, and a normal NIH/3T3 mouse embryonic fibroblast cell line as a control.

Compounds **1–12** exhibited some cytotoxicity against the non-cancerous NIH/3T3 mouse fibroblast cell line at concentrations ranging from 24 μM to above 100 μM, which was around two to five-fold lower when compared to the anticancer reference drug, doxorubicin, and also significantly lower when pitched against the activity of these selenocyanates affecting various microorganisms (Table 2). Compounds **4**, **5**, **7** and **10** were the selenocyanates with the lowest cytotoxicity against both T-lymphoma cell lines, compared to doxorubicin. Intriguingly, compounds **1**, **2**, **3**, **6**, **8**, **9**, **11** and **12** were more cytotoxic – and also selective. They displayed significant cytotoxic activities against the parental and multidrug-resistant sublines of mouse T-lymphoma cells and were less cytotoxic against the non-cancerous NIH/3T3 cell line, pointing towards a slight, three- to ten-fold selectivity against these cancer cells.

It is also notable, that the concentrations required for cytotoxicity in non-cancerous NIH/3T3 cells are generally two to three-fold lower compared to the concentrations required for antimicrobial activity (Fig. 4, see below).

The substantial activity of compounds **1**, **3** and **12** against MDR cells is worth noticing, despite the lack of any selectivity for particular cell lines. Aromatic selenocyanates **1–3**, **6**, **8**, **9**, **11** and **12** may not only be of interest in the context of antimicrobial action – as anticipated initially – they may also represent a starting point in the search for anticancer agents with MDR-reversing properties. Such anti-cancer activity is a very complex issue and requires further investigations.

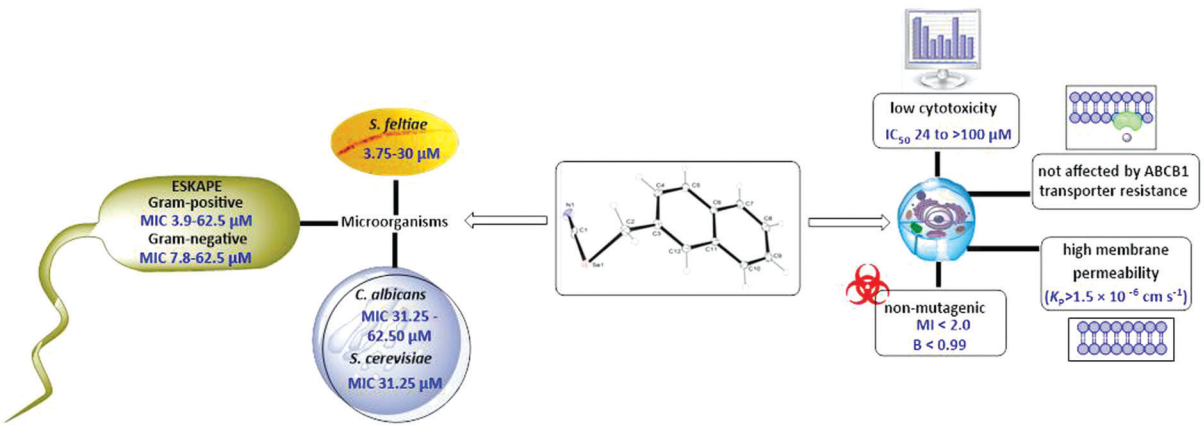

Fig. 4 Different targets affected by a typical aromatic selenocyanate and relevant concentrations required to affect these targets. One may note the distinct differences in concentrations required for resistant and non-resistant pathogens, and normal and cancer cell lines (see text for details).

Table 2 Cytotoxicity of arylmethyl selenocyanates against non-cancerous and cancer cells

Cytotoxicity against mammalian cell lines							
	Mouse T-lymphoma cells						
	PAR		MDR			Non-cancerous NIH/3T3	
Cpd	IC_{50} (μM)	SD±	IC_{50} (μM)	SD±	SI	IC_{50} (μM)	SD±
1	5.84	0.45	7.69	0.66	0.76	31.00	3.24
2	5.33	0.41	9.72	1.1	0.55	55.12	2.81
3	9.03	0	5.95	0.18	1.52	29.58	0.95
4	89.18	2.33	>100	—	≤0.89	>100	—
5	>100	—	>100	—	n.d.	>100	—
6	7.93	0.36	8.08	0.75	0.98	59.94	0.35
7	>100	—	>100	—	n.d.	>100	—
8	8.58	0.18	9.64	0.88	0.89	44.73	1.79
9	8.53	0.39	10.32	1.13	0.83	42.17	3.08
10	>100	—	>100	—	n.d.	>100	—
11	7.26	0.74	14.09	1.58	0.52	70.08	1.55
12	8.47	0.1	7.05	0.15	1.20	24.18	1.97
DOX	0.42	0.17	2.64	0.09	0.16	13.38	0.98
DMSO	>2% v/v	—	>2% v/v	—	n.d.	>2% v/v	—

PAR: parental T-lymphoma cells; MDR: multidrug resistant T-lymphoma cells overproducing efflux pump ABCB1; NIH/3T3: non-cancerous mouse embryonic fibroblast cells; DOX: doxorubicin; DMSO: dimethyl sulfoxide; SD: standard deviation; SI: selectivity index; n.d.: not determined.

Pharmacological parameters associated with activity

Since the series of selenocyanates under investigation demonstrated pronounced antimicrobial and nematicidal activities, it was considered important to investigate their ''drug-likeness'' profile. Four compounds (**1**, **2**, **4** and **13**) were selected for further investigations employing *in vitro* assays indicative of safety and absorption properties. The mutagenic potential and membrane permeability of the compounds were evaluated employing a modified Ames fluctuation assay and in parallel artificial membrane permeability assay (PAMPA) (see ESI† for details).[53,59–62]

Ames fluctuation assay

Since inorganic sodium selenite (Na_2SeO_3) is reputed for its toxic and mutagenic actions, the selenocyanates were evaluated for potential mutagenic potential. The Ames fluctuation assay was employed to calculate the mutagenic index (MI) and binomial *B*-values according to the method described in the literature.[53] Compounds are generally considered mutagenic if the MI is above 2.0 and *B* is equal to or above 0.99.[53,59,60] Neither the selenocyanates (**1**, **2**, **4** and **13**), nor the reference selenium compound ebselen displayed any mutagenic potential at concentrations of 1 μM and 10 μM (details in ESI†), therefore ruling out any major mutagenic potential. Compound **1** exhibited an MI value of 1.15 (B = 0.74) and 1.20 (B = 0.81) at 1 μM and 10 μM concentrations, respectively.

In vitro PAMPA permeability

The PAMPA permeability screening test imitates the structural and biological conditions of the cell membrane and allows for a rapid and simple determination of the passive transport of a compound through biological membranes, characterized by a permeability coefficient (K_p). Since some of the aromatic selenocyanates seem to enter cells readily and also appear to circumvent resistance based on efflux transporters, their transport properties were investigated employing a pre-coated PAMPA Plate System Gentest™ (Corning, Tewksbury, MA, USA), which provides good predictability and correlation of data for absorption in the human Caco-2 cell line. The concentrations of the compounds tested in the donor and acceptor compartments were estimated by capillary electrophoresis (CE) as described previously.[60–62] The data on permeability obtained was compared to that for selected reference drugs, *i.e.*, highly permeable caffeine and less permeable norfloxacin (see ESI† for details).

Notably, all compounds investigated, *i.e.* **1**, **2**, **4** and **13**, exhibited good permeability with K_p values above the threshold for highly permeable compounds (*i.e.* $>1.5 \times 10^{-6}$ cm s^{-1}).[62] In this context, the highest permeability was observed for compound **2** ($K_p = 3.17 \times 10^{-6}$ cm s^{-1}) and compound **1** ($K_p = 2.69 \times 10^{-6}$ cm s^{-1}) which are comparable in permeability to the reference drug caffeine ($K_p = 3.61 \times 10^{-6}$ cm s^{-1}).

Whilst permeability may explain the ability of the selenocyanates investigated to enter – and to remain inside – target cells, the mode(s) of action underlying the biological activities associated with these compounds are still elusive. Here, one may speculate that selenocyanates may act as strong electrophiles, hence widely modifying cysteine thiols and possibly also amine groups in proteins and enzymes (Fig. 5).[63] Such redox modulating interactions with proteins and enzymes of the cellular thiolstat may also explain, in part, some of the selectivity observed for certain bacteria and cell lines. Other activities, possibly exerted by various metabolic and breakdown products of the selenocyanates, may also be possible and clearly require further investigation.

Experimental

Chemical synthesis

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury-VX 300 MHz PFG system in DMSO-d_6 at ambient temperature employing the solvent signal as an internal standard. The values of the chemical shifts are expressed in δ values and the coupling constants (J) in Hz. Mass spectra were recorded on a UPLC-MS/MS system consisting of a Waters ACQUITY® UPLC® (Waters Corporation, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). The purity of the final products was confirmed by UPLC/MS to be higher than 95%. Retention times (t_R) are provided in min. Thin-layer chromatography (TLC) was performed on pre-coated Merck silica gel 60 F_{254} aluminium sheets.

General procedure for the synthesis of selenocyanates

Selenocyanates were synthesized following the general protocol described by Wheeler and Merriam with some modifications.[45] Alkyl halides (10–20 mmol) were treated with KSeCN (12–25 mmol) in ethanol (10–20 mL). The reaction mixture was refluxed for 6 h and progress of the reaction was monitored periodically by TLC.

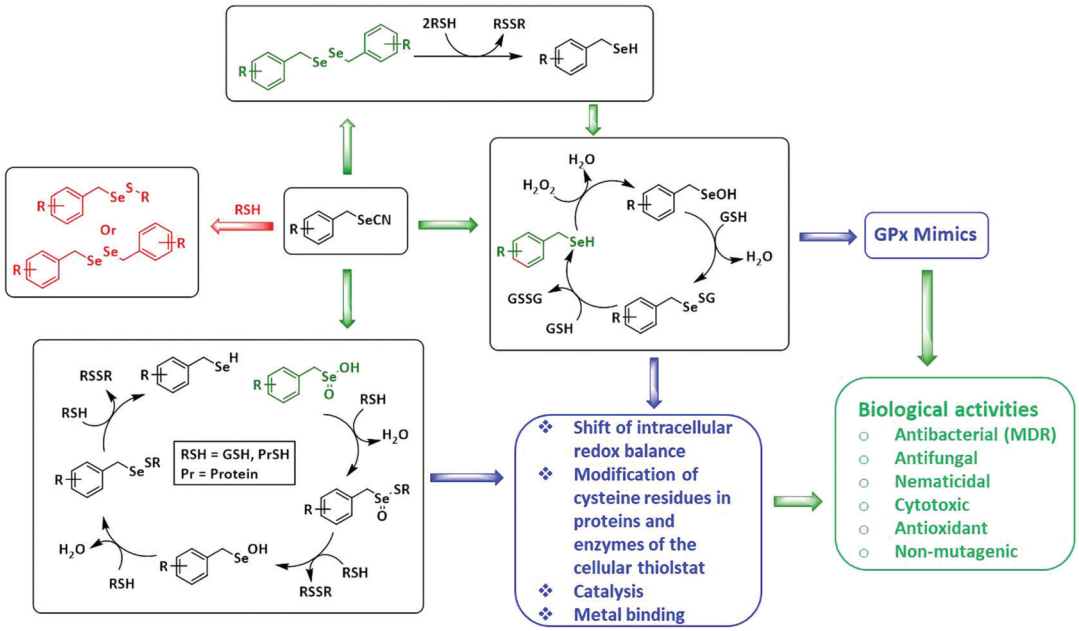

Fig. 5 Selenocyanates and their metabolites exhibit a wide spectrum of redox activity, which includes electrophilic attacks, oxidative modifications of thiol and selenol functions in proteins and enzymes, and catalytic metal binding. The considerable impact on the cellular redox homeostasis in general, and on the cellular thiolstat, in particular, may explain the pronounced biological activities observed as part of this study.

After completion, the inorganic salt was separated by filtration and the filtrate was purified with charcoal, condensed and crystallized with ethanol, to yield crystals of arylmethyl selenocyanates (**1–13**).

Compounds **1**, **3–7** and **10–12** have been described in the literature before and analytical data for these compounds is in agreement with the values reported (see ESI†).[40,64,65]

4-Methylbenzyl selenocyanate (2)

4-Methylbenzyl chloride (1.4 g, 10 mmol), KSeCN (1.73 g, 12 mmol) and ethanol (10 mL) were employed. Compound **2** was obtained as light crystals. Yield 83.6% (1.76 g, 8.36 mmol); m.p. = 43–45 °C, TLC R_f (DCM, 100%): 0.51. ^{1}H NMR (DMSO-d_6, ppm): δ 7.23 (d, J = 7.62 Hz, 2H, 2C–H), 7.17 (d, J = 8.21 Hz, 2H, 2C–H), 4.27 (s, 2H, CH_2), 2.28 (t, J = 9.10 Hz, 3H, CH_3). ^{13}C NMR (DMSO-d_6, ppm): δ 137.62, 135.65, 129.61, 129.22, 105.39 (Se–CN), 33.08, 21.22. LC-MS: purity 100%, t_R = 6.25, (ESI) m/z: calculated for C_9H_9NSe $[M + H]^+$: 105.07, found: 105.02.

3-Chlorobenzyl selenocyanate (8)

3-Chlorobenzyl chloride (3.22 g, 20 mmol), KSeCN (3.60 g. 25 mmol) and ethanol (20 mL) were employed. Compound **8** was obtained as light crystals. Yield 82.55% (3.81 g, 16.51 mmol); m.p. = 40–42 °C, TLC R_f (DCM, 100%): 0.75. ^{1}H NMR (DMSO-d_6, ppm): δ 7.42 (m, 1H, CH), 7.37 (m, 2H, CH), 7.33 (m, 1H, CH), 4.28 (t, J = 9.15 Hz, 2H, CH_2). ^{13}C NMR (DMSO-d_6, ppm): δ 141.45, 133.38, 130.95, 129.04, 128.17, 128.00, 105.23 (Se–CN), 31.96. LC-MS: purity 98.95%, t_R = 6.24, (ESI) m/z: calculated for C_8H_6ClNSe $[M + H]^+$: 125.02, found: 125.02.

3,4-Dichlorobenzyl selenocyanate (9)

3,4-Dichlorobenzyl chloride (3.91 g, 20 mmol), KSeCN (3.6 g. 25 mmol) and ethanol (20 mL) were employed. Compound **9** was obtained as yellow crystals. Yield 88.2% (4.673 g, 17.64 mmol); m.p. = 76–79 °C, TLC R_f (DCM, 100%): 0.60. ^{1}H NMR (DMSO-d_6, ppm): δ 7.62 (m, 2H, CH), 7.37 (dd, J_1 = 2.12 Hz, J_2 = 2.09 Hz, 1H, CH), 4.28 (t, J = 9.15 Hz, 2H, CH_2). ^{13}C NMR (DMSO-d_6, ppm): δ 140.24, 131.29 (4C), 129.67, 105.19 (Se–CN), 31.23. LC-MS: purity 99.49%, t_R = 6.86, (ESI) m/z: calculated for $C_8H_5Cl_2NSe$ $[M + H]^+$: 158.98, found: 158.97.

1-(Selenocyanatomethyl)naphthalene (13)

1-Chloromethyl naphthalene (3.533 g, 20 mmol), KSeCN (3.6 g. 25 mmol) and ethanol (20 mL) were employed. Compound **13** was obtained as yellow crystals. Yield 75.5% (3.71 g, 15.1 mmol); m.p. = 92–94 °C, TLC R_f (DCM, 100%): 0.64. ^{1}H NMR (DMSO-d_6, ppm): δ 8.27 (d, J = 8.21 Hz, 1H, CH), 7.96 (dd, J_1 = 7.03 Hz, J_2 = 7.03 Hz, 2H, CH), 7.49 (m, 4H, CH), 4.78 (t, J = 9.38 Hz, 2H, CH_2). ^{13}C NMR (DMSO-d_6, ppm): δ 134.02, 130.87, 129.21, 128.61, 126.77, 125.73, 124.54, 105.33 (Se–CN), 31.09. LC-MS: purity 94.69%, t_R = 6.59, (ESI) m/z: calculated for $C_{12}H_9NSe$ $[M + H]^+$: 141.07, found: 141.03.

X-ray crystallography

Single crystals suitable for X-ray analysis were obtained in ethanol for **1** and in butan-2-ol for **12** by slow evaporation of the solvent at room temperature. Intensity data of **1** was collected on a Bruker-Nonius Kappa CCD four circle diffractometer, whereas data for **12** was collected on an Oxford diffraction SuperNova diffractometer equipped with a Mo (0.71069 Å) Kα radiation source. The positions of non-hydrogen atoms were

determined by the direct method employing the SIR-2014 programme.[66] Hydrogen atoms bonded to carbons atoms were included at idealized positions and were refined utilising a riding model. The aryl hydrogen atoms were constrained with C–H 0.93 Å, the methylene groups with C–H 0.97 Å and $U_{iso}(H) = 1.2U_{eq}$. The final refinements were performed employing the SHELXL programme. ORTEP and MERCURY programmes were employed for molecular graphics.[67–69]

Compound 1. C_8H_7NSe, M_r = 196.11, crystal size 0.08 × 0.16 × 0.46 mm^3, monoclinic, space group $P2_1/c$, a = 5.9880(1) Å, b = 7.4440(2) Å, c = 17.4880(5) Å, β = 96.277(2)°, V = 774.85 Å^3, Z = 4, T = 100(2) K, 6844 reflections collected, 1786 unique reflections [R_{int} = 0.0326], R_1 = 0.0226, wR_2 = 0.0521 [$I > 2\sigma(I)$], R_1 = 0.0226, wR_2 = 0.0536 [all data].

Compound 12. $C_{12}H_9NSe$, M_r = 246.16, crystal size 0.25 × 0.48 × 0.60 mm^3, monoclinic, space group Ia, a = 8.2486(2) Å, b = 5.9838(1) Å, c = 20.4158(7) Å, β = 93.097(3)°, V = 1006.23 Å^3, Z = 4, T = 130(2) K, 4415 reflections collected, 2048 unique reflections [R_{int} = 0.0300], R_1 = 0.0368, wR_2 = 0.0921 [$I > 2\sigma(I)$], R_1 = 0.0397, wR_2 = 0.0947 [all data].

CCDC 1819893 and 1819894.†

Antimicrobial activity

The minimal inhibitory concentrations (MICs) were determined by the standard microdilution method in cation-adjusted Mueller-Hinton II Broth (MHB, Becton-Dickinson, Germany) according to the recommendations of the Clinical and Laboratory Standard Institute (CLSI).[70] The compounds (**1–13**) were evaluated for their antimicrobial activity against a broad spectrum of microorganisms, including Gram-positive bacteria (*S. carnosus* and *S. aureus*), Gram-negative bacteria (*A. baumannii* and *P. aeruginosa*) and yeasts *(C. albicans* and *S. cerevisiae*). The values of MIC were recorded after 20 h and 24 h of incubation with the compounds for bacteria and yeasts, respectively. Experiments were performed in triplicate and on three different occasions (*i.e.*, a total of nine repeats for each individual measurement).

Nematicidal activity

S. feltiae was obtained from Sautter and Stepper GmbH (Ammerbuch, Germany). The assay was performed according to an established literature protocol.[71,72] Results are provided as means ± SD. GraphPad Prism 5 was employed to perform the statistical analysis. Statistical significances were calculated by employing one-way ANOVA, with $p < 0.05$ considered to be statistically significant.

Cytotoxicity assays

L5178 mouse T-cell lymphoma cells (PAR) (ECACC Cat. No. 87111908, obtained from FDA, Silver Spring, MD, USA) were transfected with pHa MDR1/A retrovirus, as described previously by Cornwell *et al.*[73] The NIH/3T3 mouse embryonic fibroblast cell line (ATCC CRL-1658) was purchased from LGC Promochem, Teddington, UK. The cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, containing 4.5 g L^{-1} glucose) supplemented with 10% heat-inactivated fetal bovine serum. The cell line was incubated at 37 °C in a 5% CO_2, 95% air atmosphere. Cytotoxicity assays were performed following the procedures described in the literature.[74,75]

Mutagenicity assay

The *Salmonella typhimurium* TA100 strain with base pair substitution (hisG46 mutation, whose target is GGG) was purchased from Xenometrix, Allschwil, Switzerland, and employed in the Ames 384-well microtiter assay.[76] Prior to the experiment, the *Salmonella typhimurium* TA100 strain was cultivated overnight (NB-2 liquid medium in the presence of 25 μg mL^{-1} ampicillin). Then, all of the compounds were assayed according to the microtitre liquid Ames fluctuation protocol described in the literature.[76] NQNO was utilized as a positive control in the mutagenicity assays. This reagent causes point mutations in the genome as it induces G:C → A:T transitions in the *Salmonella typhimurium* TA-100 strain.[76]

In vitro PAMPA permeability assay

Compounds **1**, **2**, **4** and **13,** and reference substances were dissolved in PBS buffer (pH = 7.4) from 10 mM DMSO stocks, according to a protocol described previously.[60] The concentrations of compounds and references drugs – in this case caffeine, and norfloxacin – were estimated in the donor and acceptor compartments employing capillary electrophoresis (CE), and calibration curves were determined accordingly. Finally, the permeability coefficients (K_p, (cm s^{-1})) of the compounds tested were calculated employing the formula provided by the PAMPA plate system manufacturer.[60,62]

Conclusions

The comprehensive studies presented in the previous sections have provided new insights into the chemistry and biological activity of small aromatic selenocyanates, which may be valuable in the search for new antimicrobial agents. It is particularly noteworthy that several of the compounds investigated, in particular benzyl selenocyanate (**1**), exhibit considerable activity against Gram-positive and Gram-negative bacteria at concentrations below 1 μg mL^{-1}, *i.e.* at concentrations comparable to or even below the ones of traditional antibiotics, such as ampicillin, oxacillin and piperacillin. In the case of the most aggressive drug-resistant strains of *S. aureus*, the activity of some of these selenocyanates even seems to supersede that of oxacillin, which is not active against these dangerous pathogens. Similarly, an excellent activity has been noted against Gram-negative organisms, such as *A. baumannii* and *P. aeruginosa*, and against the pathogenic yeast, *C. albicans*. In the case of yeasts, a surprisingly high activity against infectious *C. albicans* and a surprisingly low activity against baker's yeast have been observed for several compounds, which besides an astonishing antibacterial action also promises some interesting selectivity within the fungal kingdom.

Additional studies are now required to elucidate the underlying mode(s) of action and to enhance the activity and

selectivity of these agents. Here, the notion of a random attack of such selenocyanates – and their respective metabolites – against a range of redox-sensitive cysteine proteins and enzymes composing the cellular thiolstat of the target organisms may serve as a first hypothesis (Fig. 5). Such a random attack is not uncommon within the realm of chalcogen redox chemistry and may also explain the ability of such electrophiles to overcome the traditional, more focussed mechanisms of drug resistance.

Here, it should be emphasized that such seemingly indiscriminate modifications of certain proteins and enzymes are not necessarily preventing selectivity, as the defence mechanisms against such oxidative onslaughts tend to differ dramatically between different target organisms, and also between targets and healthy human cells. Notably, our initial studies *de facto* hint at a low(er) (cyto-)toxicity against human cells, and relevant toxicity studies in higher organisms are clearly warranted now. As for other redox modulating drugs, such a low(er) activity may be due to the presence of a pronounced antioxidant defence in human cells, which is often lacking in smaller organisms. Still, this is speculative at this time and provides space for further studies, also addressing questions concerning any indiscriminatory activity and toxicity.

Furthermore, sulfur and selenium compounds are notorious for their unpleasant odour and one may indeed ''sniff a rat'' here when considering the smell of several naturally occurring compounds, such as polysulfanes from garlic and allyl isothiocyanate in mustard oil.[26,77,78] Since such a smell is due to high volatility of compounds, and aromatic selenocyanates are fairly stable solids, odour, and any apparent toxicity which may traditionally be associated with it, are not of any major concern. In fact, this aspect has been considered carefully as part of the selection of suitable compounds, as shown in Fig. 1.

In any case, selenocyanates and their closely related isoselenocyanates represent an interesting addition to the menu of selenium agents able to break through the resistance mechanisms of dangerous pathogens.[47] Once developed and studied in more detail, they may spice up the search for the next generation of effective antibiotics with a specific culinary note of matured selenium enriched broccoli-mustard.

Author contributions

M. J. N., K. W., G. S., J. H. and C. J. conceived and designed the experiments; M. J. N. synthesized the compounds; E. Ż. and W. N. performed crystallographic studies; M. J. N. and K. W. performed the experiments with microbes; A. K., M. G. and G. S. performed the experiments with mammalian cells; M. A. M. and M. J. N. performed ADMET studies *in vitro*; G. L. and K. K.-K. supervised ADMET studies *in vitro*; E. K. supervised microbiological studies; and M. J. N., K. W., A. Y. A, C. J. and J. H. wrote the paper.

Conflicts of interest

There are no conflicts to declare.

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3.2. Publication 2

Selenazolinium Salts as "Small Molecule Catalysts" with High Potency against ESKAPE Bacterial Pathogens.

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Selenazolinium Salts as "Small Molecule Catalysts" with High Potency against ESKAPE Bacterial Pathogens

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Abstract: In view of the pressing need to identify new antibacterial agents able to combat multidrug-resistant bacteria, we investigated a series of fused selenazolinium derivatives (**1**–**8**) regarding their in vitro antimicrobial activities against 25 ESKAPE-pathogen strains. Ebselen was used as reference compound. Most of the selenocompounds demonstrated an excellent in vitro activity against all *S. aureus* strains, with activities comparable to or even exceeding the one of ebselen. In contrast to ebselen, some selenazolinium derivatives (**1**, **3**, and **7**) even displayed significant actions against all Gram-negative pathogens tested. The 3-bromo-2-(1-hydroxy-1-methylethyl)[1,2]selenazolo[2,3-*a*]pyridinium chloride (**1**) was particularly active (minimum inhibitory concentrations, MICs: 0.31–1.24 μg/mL for MRSA, and 0.31–2.48 μg/mL for Gram-negative bacteria) and devoid of any significant mutagenicity in the Ames assay. Our preliminary mechanistic studies in cell culture indicated that their mode of action is likely to be associated with an alteration of intracellular levels of glutathione and cysteine thiols of different proteins in the bacterial cells, hence supporting the idea that such compounds interact with the intracellular thiolstat. This alteration of pivotal cysteine residues is most likely the result of a direct or catalytic oxidative modification of such residues by the highly reactive selenium species (RSeS) employed.

Keywords: selenazolinium salts; ebselen; RSeS; multidrug resistance; MRSA; ESKAPE pathogens; antibacterial agents

1. Introduction

The emergence and spread of antimicrobial resistance among pathogenic bacteria represents a major global healthcare problem in the 21st century [1]. A number of common pathogens are reported to develop resistances against virtually all types or classes of antibiotics [2]. The most troublesome bacteria

that pose a growing challenge for healthcare practitioners due to their antimicrobial resistance are referred as ESKAPE pathogens and include vancomycin-resistant *enterococci* (VRE), methicillin resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and extended-spectrum β-lactamase (ESBL)-producing or carbapenem-resistant species of the family *Enterobacteriaceae* (CRE). The acronym ESKAPE was first proposed by Rice et al. in 2008 to emphasize the great capacity of these bacteria to "escape" from common antibacterial treatment through rapid acquisition or development of resistance determinants allowing them to tolerate the antimicrobial substance(s) [3–6]. Each member of the ESKAPE species is a major source of severe and frequently lethal diseases in hospitalized patients, and some of them have also successfully spread to community settings, affecting otherwise healthy individuals [3,7]. Among ESKAPE pathogens, MRSA strains are the most prevalent Gram-positive bacteria, causing nosocomial infections throughout the world [8,9]. Because of their considerable ability to acquire resistance mechanisms against any antibiotics introduced into clinical use, the appearance of MRSA strains in hospitals has been associated with substantial morbidity and mortality rates [9–11]. For multidrug-resistant (MDR) Gram-negative bacteria—namely *A. baumannii*, *P. aeruginosa*, *K. pneumoniae*, and *E. coli*, the situation is even more complex and worrisome as "they represent the problem of multidrug resistance to the maximum" [11]. The therapeutic options for infections caused by these MDR pathogens are often so extremely limited that, in many cases, clinicians are left with practically no rational choice of antibiotic treatment [12]. The present situation is even more threatening when considering the stagnation in the development and approval of novel antimicrobial agents to treat these pathogens [13]. Therefore, an immediate and continual search for new antimicrobial agents effective against drug-resistant bacteria, preferentially with a lower risk of resistance formation, is urgently needed.

In the last few years, selenium-based compounds have received significant attention due to their unique biological properties which could have multiple prospective applications in clinical practice [14]. The organoselenium compound ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one, Figure 1), in particular, is a promising agent for the therapy of various health disorders [15,16] and is presently undergoing Phase III clinical trials in patients suffering from acute ischemic stroke and cortical infarct due to its pharmacological efficacy and favourable safety profile [17,18]. Ebselen, like many other organic selenium compounds, is redox-active and able to modify cysteine residues in proteins and enzymes effectively and selectively. In the presence of elevated levels of reactive oxygen species (ROS), it can also "turn catalytic", and its pronounced glutathione peroxidase (GPx)-like activity promotes widespread interactions with cysteine residues of proteins belonging to the cellular thiolstat [19], hence resulting in significant decreases of intracellular protein thiols and activation of various redox-controlled cellular pathways [20]. In addition to various pro- and antioxidant actions associated traditionally with ebselen, recent studies have also discovered an excellent bactericidal action against the Gram-positive ESKAPE species *E. faecalis/E. faecium* and *S. aureus*. Curiously, ebselen lacks any major activity against Gram-negative ESKAPE pathogens [21]. This may not be extraordinarily surprising, as ebselen is not the most reactive amongst the selenium compounds frequently discussed today.

The overarching rationale of this study has therefore been the evaluation of ebselen-like selenium compounds with similar structural features and specificity towards nucleophilic attack by thiols, yet with improved reactivity. Within this context, one particular strategy to "improve" the reactivity of the Se–N bond towards cysteine thiols is the introduction of a positive charge which increases the electrophilic behaviour of the bond.

As part of this strategy, and during the search for new ebselen analogues containing fused rings with an endocyclic Se–N bond, a series of selenazolinium salts were obtained [22]. Eight of these compounds (**1**–**8**, Figure 1), have been investigated here for their antibacterial properties against a variety of *S. aureus* clinical isolates, as well as representative strains of clinically relevant Gram-negative ESKAPE bacteria. In addition, initial pharmaceutical safety studies have been carried out, and the most active compounds have been investigated regarding their potential mechanisms of action.

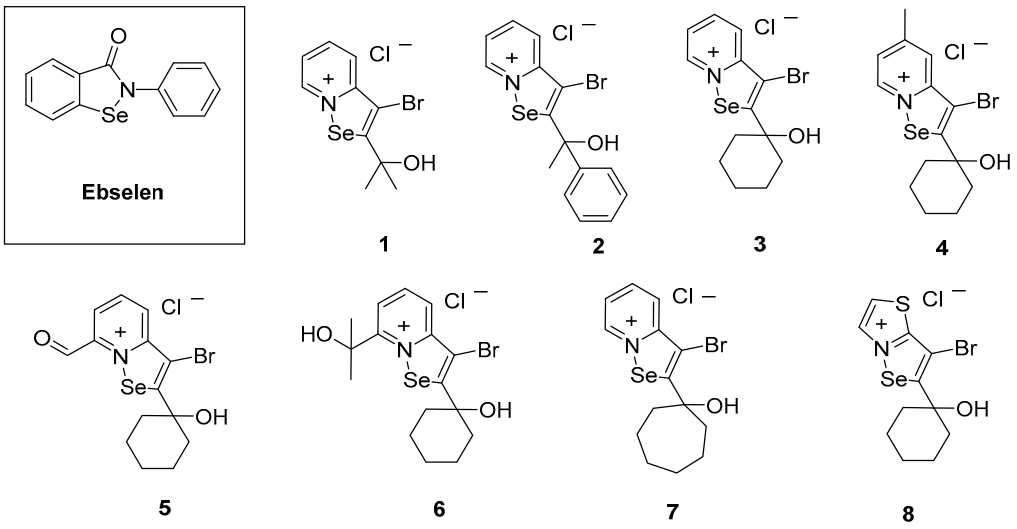

Figure 1. Chemical structures of ebselen (in the insert) and the selenazolinium salts **1**–**8** investigated.

2. Results and Discussion

2.1. Chemical Synthesis

Synthesis of 3-bromo-selenazolopyridinium chlorides **1**–**7** and 3-bromo-2-(1-hydroxycyclohexyl)[1,2]selenazolo[2,3-*b*]thiazolinium chloride **8** (Figure 1) was described elsewhere [22].

2.2. In Vitro Antibacterial Activity

The in vitro antibacterial activities of the selenazolinium compounds (**1**–**8**) were evaluated for 25 strains of ESKAPE bacteria, including 11 Gram-positive strains (Table 1) and 14 Gram-negative microbes (Table 2) with a variety of clinical characteristics.

Table 1. Characteristics of the ESKAPE Gram-positive strains used in this study.

Bacterial Strain	Relevant Phenotype *
Staphylococcus aureus	
ATCC 25923	Reference strain, CC5, MSSA
MM-O058	Clinical isolate, CC121, MSSA, MDR
MM-N072	Clinical isolate, CC152, MSSA, MDR
HEMSA 5	Clinical isolate, MRSA, XDR
LG-N017	Clinical isolate, CC5, MRSA, MDR
MM-O021	Clinical isolate, CC8, MRSA, MDR
R45-CC45	Clinical isolate, CC45, MRSA
R46-CC22	Clinical isolate, CC22, MRSA
USA300 LAC	Clinical isolate, CC8, CA-MRSA, MDR
5328	Clinical isolate, CC398, LA-MRSA
Mu50	Clinical isolate, CC5, MRSA, VISA

* CC, clonal complex; MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; CA-MRSA, community-acquired MRSA; LA-MRSA, livestock-associated MRSA; VISA, vancomycin-intermediate *S. aureus;* MDR, multidrug-resistant; XDR, extensively drug-resistant [23].

Table 2. Characteristics of the ESKAPE Gram-negative strains used in this study.

Bacterial Strain	Relevant Phenotype
Klebsiella pneumoniae	
NRZ-00103	Reference strain, KPC-2, MDR, 4 MRGN
KP 21513017	Clinical isolate
KP 1963584	Clinical isolate, OXA-2, MDR, 4 MRGN
Acinetobacter spp.	
AC 2151300	Clinical isolate, *A. ursingii*
AC 1995594	Clinical isolate, *A. baumannii complex*
AB 4184/2/5	Clinical isolate, *A. baumannii*
Pseudomonas aeruginosa	
ATCC 27853	Reference strain
PA T18	Clinical isolate, MDR, 3 MRGN
PA 54	Clinical isolate, MDR
PA 58	Clinical isolate, MDR
Escherichia coli	
NCTC 13351	Reference strain, TEM-3
EC 2151612	Clinical isolate, MDR, 3 MRGN
EC 1995591	Clinical isolate, MDR, 3 MRGN
EC 1227107	Clinical isolate, MDR, 3 MRGN

3/4 MRGN bacteria, multidrug-resistant Gram-negative bacteria. The description 3 or 4 MRGN refers to the classification of multidrug-resistant Gram-negative bacteria created by the Commission for Hospital Hygiene and Infectious Disease Prevention (KRINKO) of the Robert Koch-Institute (RKI) in order to outline the resistance pattern of these pathogens. Bacteria identified as 3 or 4 MRGN are resistant to three or four out of four classes of antibiotic groups used in the treatment of infections that they cause, respectively [24]; KPC-2, carbapenemase KPC-2; OXA-2, β-lactamase OXA-2; TEM-3, extended-spectrum β-lactamase TEM-3.

2.2.1. Antibacterial Activity against *S. aureus* Strains

Antimicrobial activities of compounds **1**–**8** were investigated against 11 strains of *S. aureus* (Gram-positive bacteria) in comparison to ebselen and oxacillin. (Table 3). Results demonstrate that all selenocompounds (**1**–**8**) exhibited excellent antibacterial activities against all *S. aureus* strains employed in this study, with MIC values (0.31–3.44 μg/mL) in a similar range as that of ebselen (0.28–2.24 μg/mL). The same antibacterial effect of compounds **1**–**8** was observed against both the methicillin-susceptible *S. aureus* isolates ATCC 25923, MM-O058, and MM-N072 (MICs = 0.35–3.44 μg/mL) as well as the methicillin-resistant *S. aureus* isolates USA300 LAC (CA-MRSA), 5328 (LA-MRSA), LG-N017, MM-O021, R45 CC22, R45 CC45, HEMSA 5 (HA-MRSA), and Mu50 (VISA) (MIC = 0.31–3.44 μg/mL). Most of the compounds, except the aldehyde analogue (5), were more active against all MDR and XDR strains as compared to the reference strain (ATCC 25923, Table 3). This selectivity for the MDR and XDR (extensively drug-resistant) strains—paired with high activity at low concentrations—turns these compounds into a promising tool in the fight against drug-resistant bacteria [23]. Moreover, in all cases, compounds **1**–**8** exhibited significantly higher antimicrobial activity against MRSA clinical isolates when compared to the standard drug oxacillin. Among selenazolinium salts, compounds **1** and **6** represented the most effective antimicrobials with MIC values below 1 μg/mL against all but one MRSA strain, namely HEMSA 5.

Table 3. Antibacterial activities (MICs) of compounds **1–8** against different strains of *S. aureus*.

***S. aureus* Strain**	**MIC (μg/mL)**									
	OXA	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Ebselen**
ATCC 25923	0.25	1.24	1.72–3.44	1.4	1.45	0.72–1.44	0.36–0.72	1.44–2.88	2.88	0.56
MM-O058 *	0.25	0.62	0.86	0.35–0.7	0.36–0.73	0.72	0.36–0.72	0.36–0.72	0.36–0.72	0.56–1.12
MM-N072 *	0.25	0.62–1.24	3.44	0.7–1.4	0.73–1.46	0.72	0.36–0.72	0.72	1.44–2.88	0.56–1.12
USA300 LAC *	12	0.62	3.44	0.7	0.73	0.72–1.44	0.72–1.44	0.36–0.72	0.72–1.44	0.56–1.12
5328	4	0.62–1.24	3.44	1.4	1.45	1.44–2.88	0.72–1.44	0.72–1.44	1.44–2.88	1.12–2.24
LG-N017 *	12	0.62–1.24	0.86–1.72	0.35–0.7	0.73–1.45	0.72–1.44	0.36–0.72	0.36–1.44	0.72–1.44	1.12–2.24
MM-O021 *	64	0.31–0.62	1.72–3.44	0.35–0.7	0.36–0.73	0.36–0.72	0.36–0.72	0.36–0.72	0.72–1.44	0.56–1.12
R45 CC22	64	0.31	0.86–1.72	0.35	0.36–0.73	0.36–0.72	0.36	0.36–0.72	0.36–0.72	1.12
R45 CC45	4	0.31–0.62	1.72–3.44	0.7–1.4	0.36–0.73	0.72	0.36–0.72	0.72	1.44	0.56
HEMSA 5 **	128	1.24	1.72	1.4	1.45	1.44	0.72	0.72–1.44	2.88	2.8
Mu50 ***	256	0.31–0.62	0.43–0.86	0.7–1.4	0.36–0.73	0.36–0.72	0.36	0.36–0.72	0.72–1.44	0.28

* MDR, multidrug-resistant isolates; ** XDR, extensively drug-resistant isolate; *** VISA strain. Oxacillin (OXA) was used as reference β-lactam antibiotic.

2.2.2. Antibacterial Activity against Gram-Negative Bacteria

Subsequently, selenocompounds **1**–**8** were evaluated for their possible inhibitory effects against four particularly problematic Gram-negative bacterial species belonging to ESKAPE pathogens (Table 2), and compared to ebselen (Table 4). At least three isolates of *K. pneumoniae*, *Acinetobacter* spp., *P. aeruginosa*, and *E. coli*, including 3 or 4 MRGN strains, were employed in the MIC experiments. Bacteria identified as 3 or 4 MRGN are resistant to three or four out of four classes of antibiotic groups used in the treatment of infections that they cause, respectively [24].

Compounds with MICs < 5 μg/mL were considered as highly active ones. In stark contrast to ebselen, all selenoazolinium salts (**1**–**8**) exhibited significant antibacterial activities against at least two Gram-negative ESKAPE strains. The highest sensibility for compounds **1**–**8** was observed for *Acinetobacter* spp., whereas the reference strain of *P. aeruginosa* (ATCC 27853) was sensitive only to compound **1**. Interestingly, compounds **1**–**8** were significantly more active in case of MDR *P. aeruginosa* strains when compared to the reference strain. Apart from the ATCC 27853 strain, all selenoazolinium compounds (**1**–**8**) exhibited MICs significantly lower than those of ebselen. Among the selenazolinium salts, compound **1** demonstrated excellent activities against all ESKAPE strains tested (MICs = 0.31–2.48 μg/mL). A high antibacterial potency was also observed for compounds **3**, **4**, and **7**, as these selenium compounds were active against 3 MRGN strains of *E. coli* and the 4 MRGN *K. pneumoniae* isolate (Table 4).

2.3. Results of Pharmaceutical Safety

In order to assess some central drug-safety properties for the selenium agents (**1**–**8** and ebselen), and bearing in mind that certain inorganic-selenium compounds such as selenite (SeO_3^{2-}) interact unfavourably with DNA, all relevant compounds were investigated for their possible mutagenic properties in vitro employing the microtiter Ames test [25–29].

Each experiment was performed in triplicate, and results were given in terms of mutagenic index (MI), which is the quotient of the number of revertant colonies induced in a test sample and the number of revertants in a negative control (media with 1% DMSO). A compound is considered mutagenic if MI is above 2.0 [30,31]. According to the results obtained (Figure 2), neither selenazolinium salts (**1**–**8**) nor ebselen appeared to cause any significant mutagenic changes at the concentrations used (1 μM and 10 μM), resulting in MI below 2.0, whereas the MI value for reference NQNO (0.5 μM) was 7.24 (Table 5). When comparing the MI values obtained between the two concentrations tested (1 μM and 10 μM), however, an evident decrease of MI values for the higher concentration of compounds **4**–**8** can be noted (Table 5). This decrease is connected with the cytotoxic activity of **4**–**8** against the *Salmonella typhimurium* TA100 strain, confirmed at 10 μM by Binominal B-values calculated according the manufacturer protocol, where $B \leq 0.01$ indicates the occurrence of cytotoxic events (Figure 2, Table 5).

Table 4. MIC values of the compounds **1–8** and ebselen against Gram-negative ESKAPE strains.

	Bacterial Strain	MIC (μg/mL)								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Ebselen**
K. pneumoniae	1	1.24 *	14	1.4–2.8	2.88–5.76	46	46	2.88	5.76	≥143
	2	0.62–1.24	6.88–14	1.4	2.88	35	17	2.88	5.76	108
	3	0.62	6.88	1.4	2.88	17	17	1.44–2.88	2.88	72
Acinetobacter spp.	4	0.31–0.62	0.86	0.35–0.7	0.36–0.72	2.88	1.44	0.36–0.72	1.44	18
	5	0.62	1.72–3.44	1.4	0.72	2.88–5.76	2.88	0.72	1.44–2.88	27
	6	0.31	0.86	0.35	0.36	2.88	0.72–1.44	0.36	1.44	18
P. aeruginosa	7	2.48	110	5.60–11	5.76–12	69	138	17	23	72
	8	0.31	1.72	0.7	1.44	12	12	0.72	1.44–2.88	18
	9	0.62–1.24	21	1.4–2.8	5.76	12	69	5.76	5.76–12	108
	10	0.62	6.88–14	1.4	2.88	17	23	2.88	5.76	27
E. coli	11	1.24–2.48	6.88–14	1.4	2.88	17	12	2.88	5.76	108
	12	1.24–2.48	6.88	1.4	2.88	17	12	1.44–2.88	5.76	72
	13	2.48	6.88–14	1.44–2.8	2.88	23	12	2.88	5.76	72
	14	1.24–2.48	14	2.8	5.76	23	17	2.88	5.76–12	54

* Particularly potent antibacterial activities (MIC < 5 μg/mL) are underlined. In case of MIC ≥ 10 μg/mL, the MIC values are expressed rounded to integers (see Supplementary for details). Bacterial strains—*K. pneumoniae*: (1) NRZ-00103, (2) KP 21513017, (3) KP 1963584; *Acinetobacter* spp.: (4) AC 2151300, (5) AB 1995594, (6) AB 4184/2/5; *P. aeruginosa*: (7) ATCC 27853, (8) PA T18, (9) PA54, (10) PA58; *E. coli*: (11) NCTC 13351, (12) EC 2151612, (13) EC 1995591, (14) EC 1227107.

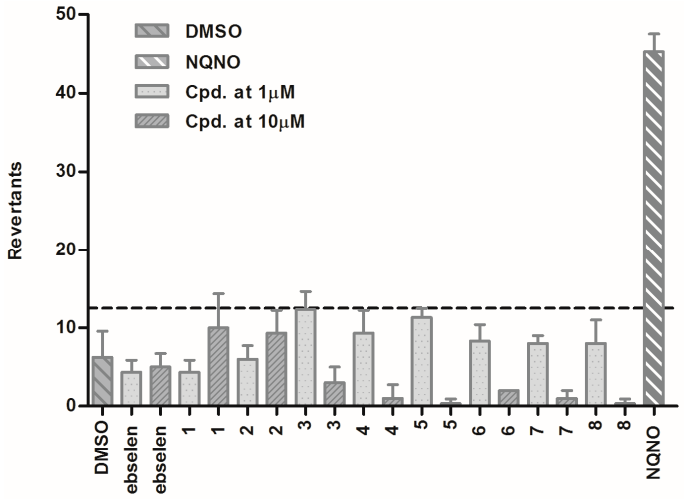

Figure 2. Results of the Ames liquid microtiter test and determination of the mutagenic potential; DMSO (1%)—negative control; ebselen-reference compound; NQNO (4-nitroquinoline-*N*-oxide)—mutagenic agent at concentration 0.5 μM; **1**–**8**—selenocompounds at concentrations 1 μM and 10 μM, respectively; —baseline defining the mutagenicity threshold (over the line).

Table 5. Mutagenic index (MI) values for ebselen and tested compounds (**1**–**8**).

Cpd.	MI (1 μM)	B	MI (10 μM)	B
Ebselen	0.69	0.26	0.80	0.46
1	0.69	0.35	1.60	1.00
2	0.96	0.83	1.49	1.00
3	1.97	0.99	0.48	0.06
4	1.49	0.71	0.16	0.00
5	1.81	0.96	0.05	0.00
6	1.33	0.97	0.32	0.00
7	1.28	0.94	0.16	0.00
8	1.28	0.98	0.05	0.00

MI—mutagenic index values for ebselen and selenocompounds **1**–**8**, B—Binomial B-value.

2.4. Studies on the Possible Mode of Antimicrobial Action

Whilst the results obtained so far point towards a considerable and widespread antibacterial activity associated with virtually all of the selenazolinium compounds under investigation, they do not reveal any information regarding the possible underlying mode(s) of action. Based on the chemical structures of these compounds, and their particular reactivity as thiol-selective electrophiles, one may expect a certain "redox link" that has been investigated in more detail. Such a link may constitute, for instance, in the production of ROS, a loss of thiols or a—possibly catalytic—oxidation of specific thiol groups in particularly redox-sensitive proteins and enzymes of the cellular thiolstat [32–34].

2.4.1. Evaluation of ROS Formation

In order to analyse the effect of the selenazolinium salts (**1**–**8**) on intracellular oxidative stress production in *S. aureus*, the 2′,7′-dichlorofluorescein diacetate assay (DCFH-DA assay) was performed. This assay is used routinely in the context of human cell culture and can also be applied to bacteria. For this purpose, the impact of the most active compounds identified in the MICs assays (**1** and **6**) and ebselen on intracellular ROS concentrations was determined with the reference *S. aureus* strain ATCC 25923 and the clinical XDR-MRSA isolate HEMSA 5 (Figures S1–S3, Supplementary section).

Rather unexpectedly, we observed that neither compound **1** nor compound **6** were able to increase ROS levels in the *S. aureus* isolates tested.

2.4.2. Reactivity of the Compounds with Thiol Groups

Recent studies have shown that many chalcogen compounds do not generate ROS per se but instead interfere with their removal by consuming thiol groups [35–37]. Indeed, selenium-based molecules have been reported to spontaneously react with various biological thiols, including GSH and cysteine-containing proteins. We therefore performed a standard Ellman's Reagent DTNB assay in order to explore the consumption of thiols as possible mode of antibacterial activity of the compounds tested (Figure 3).

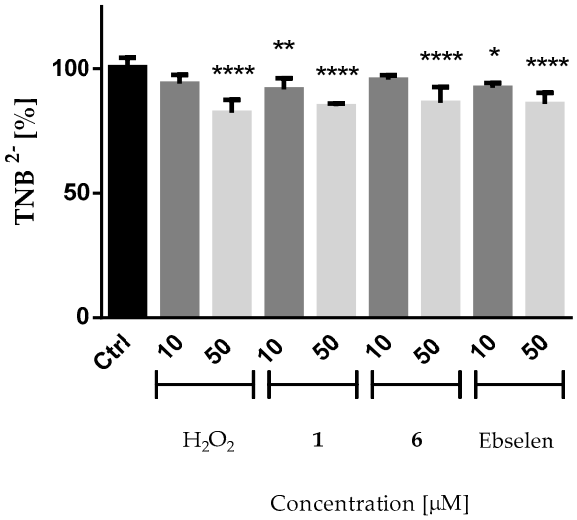

Figure 3. Estimation of levels of thiol residues of 100 μM *N*-acetyl-L-cysteine in the presence of different concentrations of compounds **1**, **6**, or ebselen by the DTNB assay. Hydrogen peroxide served as positive control in the study. The decrease in 2-nitro-5-thiobenzoate (TNB) indicates the ability of the compounds/H_2O_2 to modify free sulfhydryl groups of various amino acids of the proteins. It should be noted that compounds have been added in substoichiometric amounts compared to *N*-acetyl-L-cysteine to account for possible thiol oxidation cascades. Statistical significance was assessed by one-way ANOVA (mean ± SD, $n = 5$ replicates) followed by Dunnett's multiple comparisons test. * $p \leq 0.05$, ** $p \leq 0.01$ and **** $p \leq 0.0001$ compared with the control.

The ability of the selenium-based compounds to consume thiol groups was determined with *N*-acetyl-L-cysteine, which was exposed to the compounds and whose remaining thiol groups were subsequently quantified with Ellman's Reagent DTNB. A loss in thiol groups due to the presence of the selenium compounds would be noted by less TNB production assuming that the products of the reaction of *N*-acetyl-L-cysteine with the selenium compounds do not react with DTNB. As shown in Figure 3, the addition of the compounds (**1** and **6**) at concentrations as low as ≤50 μM to the *N*-acetyl-L-cysteine solution significantly decreased the levels of TNB, indicating that the compounds are able to modify cysteine, and hence may also be able to attack various proteins and enzymes of the cellular thiolstat of the bacteria. The cysteine-modifying activities of the compounds were comparable to or even higher than one of the reference molecules—hydrogen peroxide. Interestingly, the more pronounced effect was observed for compound **1**—which was also very active against bacteria—followed by ebselen and, to a lesser extent, by compound **6**.

2.5. Structure–Activity Relationship Discussion

Overall, the studies performed with selenazolinium salts **1**–**8** have identified this rather unusual class of selenium compounds as very reactive chemically and very active biologically. It is highly probable that the extraordinary electrophilic behaviour associated with the positively charged Se–N motif is responsible for the excellent growth inhibitory activities on all MDR *S. aureus* strains. Indeed, the selenazolinium salts **1**–**8** showed a comparable influence on *S. aureus*, often exceeding the one of ebselen, and in all MRSA strains were considerably more active when compared to the standard antibiotic oxacillin. Two compounds, the 3-bromo-2-(1-hydroxy-1-methylethyl)[1,2]selenazolo[2,3-*a*]pyridinium chloride (**1**) and the 3-bromo-2-(1-hydroxycyclohexyl)-7-(2-hydroxypropan-2-yl)[1,2]selenazolo[2,3-*a*]pyridinium chloride (**6**) displayed an even stronger antimicrobial effect than ebselen against two of the MRSA strains tested (LG-N017 and R45 CC22). It is worth emphasizing that their antistaphylococcal activities in form of MICs are superior to those recently reported for fluoroquinolones and their thiolated analogues [5]. Indeed, the activity of the selenazolinium salts is comparable to that of the most active agents produced by the latest lines of investigation, e.g., 9,13-disubstituted berberine derivatives [38] or polyhalogenated 2-phenylbenzimidazoles [39].

The studies on the possible mechanisms of action against MRSA strains identified a similar behaviour of the selenazolinium compounds (**1** and **6**) and ebselen in all three assays indicating that the mode of action of the compounds is related to an extensive modification of thiol groups, possibly in cysteine-containing cellular proteins that are crucial for bacterial survival and growth. Interestingly, in both cases, i.e., the 3-bromo-selenoazolinium compounds and ebselen, there was only a slight strain-related discrimination in the antibacterial effects observed, and an even more potent efficacy in the case of MDR bacteria than in the corresponding reference strains. In contrast to oxacillin, it is possible that such Se–N endocyclic selenocompounds can overcome certain MDR mechanisms. There may be various reasons for such behaviour. One may, for instance, consider a widespread and simultaneous attack of such reactive selenium compounds on various redox-sensitive cysteine proteins, hence avoiding the kind of resistance associated with "single target" drugs. Alternatively, a significant consumption of cellular thiols may also trigger unfavourable intracellular signalling processes which may eventually harm the bacterium affected. In any case, the considerably high activity, especially against MDR-strains, is of great interest for the current search of antimicrobial agents.

Here, an even more important finding of these studies is the high potency of the compounds **1**–**8** against Gram-negative ESKAPE bacteria, which clearly distinguishes these selenazolinium compounds from ebselen and places them on par with the best anti-ESKAPE agents found recently, such as isothiazolone [40] or bis-cyclic guanidine compounds [41]. The results obtained here with 14 different Gram-negative strains evidently confirm the promising properties of the entire group of both, the 3-bromo-selenazolopyridinium chlorides (**1**–**7**) and 3-bromo-2-(1-hydroxycyclohexyl)[1,2]selenazolo[2,3-*b*]thiazolinium chloride (**8**), against both types of ESKAPE strains, with special accent on the selenazolopyridinium compounds **1** and **3** (Figure 1, Tables 2 and 3).

Although it is difficult to discuss probable mechanisms of action, in this case, it is rather obvious that the charged endocyclic Se–N bond is beneficial for the action against Gram-negative pathogens. In contrast, ebselen is not active against the latter group of bacteria. Nonetheless, not all selenazolinium salts were equally active and some differences in activity have been observed within the group (**1**–**8**). Thus, decreased activities were observed for the hydroxycyclohexyl derivatives substituted at position 7 with formyl (**5**) or hydroxyl-alkyl (**6**) moieties. A very simple structure–activity relationship (SAR) analysis suggests that substituents at the pyridine part of the fused rings of compounds **1**–**7** may influence the action on Gram-negative pathogens. Thus, a substitution at position 7 could be responsible for a decrease of antibacterial action. The most active compounds **1** and **3** are not substituted within the pyridine part of the fused rings as well as they include 1-hydroxy-1-methylethyl- or 1-hydroxycyclohexyl terminal fragments, respectively (Figure 1, Tables 2 and 3). It is not entirely

clear yet, however, which of the structural properties are really responsible for the outstanding action observed for some of the compounds, since both the unsubstituted pyridine fragment and the 1-hydroxycyclohexyl one are also present in the less active compounds (**2**, **4**–**6** and **8**, respectively). In the case of activity against *S. aureus*, the most active compounds (**1** and **6**) are also the more hydrophilic. The first one (**1**) possesses the smallest acyclic alcohol moiety, whereas compound **6** includes two hydroxyl groups.

The excellent results obtained for compounds **1**–**8** in the assays against a panel of 25 ESKAPE bacterial strains demands further studies for this group of unique compounds in order to evaluate the pharmaceutical safety profile. In this context, we have already applied the Ames mutagenicity assays, a gold standard in the initial steps of drug R&D process. The results of low mutagenicity risk for compounds **1**–**8** obtained, in resemblance to ebselen, indicate that the new selenazolinium compounds can be considered as potential candidates for a new drug that is successful in the battle against the most problematic multidrug-resistant pathogens. Still, a high electrophilic reactivity may also imply a more random reactivity, for instance, also against human cells, and such issues related to selectivity should be considered in future studies.

3. Materials and Methods

3.1. Microbiological Assays

3.1.1. Chemical Compounds

The selenazolinium salts: 3-bromo-2-(1-hydroxy-1-methylethyl)[1,2]selenazolo[2,3-*a*]pyridinium chloride (**1**), 3-bromo-2-(1-hydroxy-1-phenylethyl)[1,2]selenazolo[2,3-*a*]pyridinium chloride (**2**), 3-bromo-2-(1-hydroxycyclohexyl)[1,2]selenazolo[2,3-*a*]pyridinium chloride (**3**), 3-bromo-2-(1-hydroxycyclohexyl)-5-methyl[1,2]selenazolo[2,3-*a*]pyridinium chloride (**4**), 3-bromo-7-formyl-2-(1-hydroxycyclohexyl)[1,2]selenazolo[2,3-*a*]pyridinium chloride (**5**), 3-bromo-2-(1-hydroxycyclohexyl)-7-(2-hydroxypropan-2-yl)[1,2]selenazolo[2,3-*a*]pyridinium chloride (**6**), 3-bromo-2-(1-hydroxycycloheptyl)[1,2]selenazolo[2,3-*a*]pyridinium chloride (**7**), and 3-bromo-2-(1-hydroxycyclohexyl)[1,2]selenazolo[2,3-*b*]thiazolinium chloride (**8**) were synthesized according to the procedures described previously [22].

Ebselen was purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solutions of the compounds tested were prepared in DMSO/H_2O and stored at −20 °C until used. Furthermore, the following solvents and chemical compounds were employed in our studies: 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and oxacillin (Sigma-Aldrich, St. Louis, MO, USA); 2-azobis(2-amidinopropane) dihydrochloride (AAPH; Sigma-Aldrich, Steinheim, Germany); H_2O_2 (Life Technologies, Eugene, OR, USA); 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Ellman reagent; Sigma-Aldrich, St. Louis, MO, USA); *N*-acetyl-L-cysteine (Alfa-Aesar, Karlsruhe, Germany); ampicillin (Polfa Tarchomin S.A., Warszawa, Poland); 4-nitroquinoline-*N*-oxide (NQNO; Sigma-Aldrich, Munich, Germany); DMSO and bromocresol purple (Sigma-Aldrich, Munich, Germany); Beef extract, L-histidine monochloride, and D-biotin (Bioshop, 5480 Mainway, Burlington, Ontario, Canada); peptone from casein (Merck, Darmstadt, Germany); KH_2PO_4, K_3PO_4, $(NH_4)_2SO_4$, $MgSO_4 \times 7H_2O$, NaCl, trisodium citrate dehydrate, and D-glucose (Chempur, Piekary Śląskie, Poland).

3.1.2. Bacterial Strains

Twenty-five bacterial strains used in this study are listed in Table 1. *S. aureus* ATCC®25923™ and MRSA HEMSA 5 were obtained from the Institute of Hygiene and Tropical Medicine, Universidade Nova de Lisboa, Lisbon, Portugal; and the *A. baumannii* isolate AB 4184/2/5 was obtained from Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Jagiellonian University Medical College, Cracow, Poland. The remaining bacterial strains were obtained from the stock collection of the Institute of Medical Microbiology and Hygiene, Saarland University, Homburg, Germany.

S. typhimurium TA100 strain (Xenometrix, Allschwil, Switzerland) with the base pair substitution (*hisG46* mutation, which target is GGG) was used for the Ames assays indicative of mutagenicity.

3.1.3. Antimicrobial Susceptibility Testing

The minimal inhibitory concentration tests were performed by the standard microdilution method in cation-adjusted Mueller Hinton II Broth (MHB II, Becton-Dickinson, Heidelberg, Germany) according to Clinical and Laboratory Standards Institute (CLSI) recommendations [42]. Antibacterial activities against *S. aureus* strains were evaluated in comparison with the β-lactam antibiotic oxacillin. The MICs of compounds **1**–**8** and ebselen were recorded after 20 h incubation at 37 °C. The antibacterial effect was determined in triplicate in at least three independent experiments.

3.1.4. Determination of Intracellular Oxidative Stress Levels via the DCFH-DA Assay

Reactive oxygen species (ROS) production in *S. aureus* strains ATCC 25923 and MRSA HEMSA 5 under exposure to a given selenium compound was measured by using the redox-sensitive fluorescent indicator dye DCFH-DA, according to the protocol published previously [43]. The concentrations of the compounds were used at levels ranging from 1/2 to 2× their MICs. AAPH at the final concentration of 50 mM was included as positive control in the assay. The fluorescence intensity was detected at 5 min intervals over a 60 min period using a microplate reader (EnSpire, PerkinElmer, Waltham, MA, USA), with an excitation wavelength of λ_{ex} = 480 nm and an emission wavelength of λ_{em} = 525 nm.

3.1.5. DTNB Assay

The assay was carried out following the procedure described earlier with a few modifications [44,45]. DTNB was used to quantify the concentration of free thiol groups in the sample. The method is based on the reaction of this aromatic disulfide with aliphatic thiol groups of a compound to form a mixed disulfide and 2-nitro-5-thiobenzoate (TNB), which ionizes to the TNB^{2-} dianion at neutral or alkaline pH. The reaction is rapid and stoichiometric, the addition of 1 mol of thiol-containing compound leads to the release of 1 mol of TNB. The latter gives an intense yellow color that can be quantified spectrophotometrically at the wavelength of 412 nm. The DTNB assay was initiated by mixing 180 µL of 100 µM *N*-acetyl-L-cysteine in 0.1 M phosphate buffer solution with either 10 µL of 200 µM, or 1 mM or 2 mM solution of ebselen or selenazolinium salts exhibiting the highest antibacterial activity in the previous studies (compound **1** and **6**), or hydrogen peroxide, which was used as a positive control in the assay. After 40 min incubation, 10 µL of DTNB (4 mM) solution was added and the decrease in absorbance was measured spectrophotometrically by using a microplate reader (EnSpire, PerkinElmer, Waltham, MA, USA) at a wavelength of 412 nm for 10 min at 25 °C. One should note that compared to *N*-acetyl-L-cysteine, the compounds were added in sub-stoichiometric amounts, as it is possible that such selenium agents react with more than one equivalent of thiols. It is therefore not expected that all thiols of *N*-acetyl-L-cysteine are eventually consumed in this assay.

3.1.6. Ames Test

The alternative Ames test adapted to the HTS microplate format (Xenometrix AG, Allschwil, Switzerland) was performed using the histidine-dependent *Salmonella typhimurium* strain TA100, according to the previously described method of Kamber et al. [25–29]. All the compounds tested and the reference (ebselen) were evaluated at final concentrations of 1 µM and 10 µM (in the well). NQNO was applied as a positive standard mutagen control (0.1 µM, 0.5 µM) [29]. Finally, the results were counted manually and by the use of microplate reader. The mutagenic index (MI) was calculated next, as the quotient of the number of revertant colonies induced in a test sample and the number of revertants in a negative control (media with 1% DMSO). A compound is considered mutagenic if its MI is above 2.0. The Binomial B-values were calculated according to the protocol provided by Xenometrix AG.

4. Conclusions

In the present study, a novel group of selenazolinium salts displaying excellent in vitro activity against ESKAPE pathogens has been described. Similar to ebselen, these reactive selenium species (RSeS) have demonstrated great potential against 11 strains of *S. aureus*, including multidrug-resistant MRSA and VISA clinical isolates. Yet in stark contrast to ebselen, the selenazolinium compounds also displayed a significant antibacterial action against various members of the Gram-negative ESKAPE family, including *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. coli*. Moreover, a beneficial pharmaceutical safety for all selenoazolinium compounds (**1**–**8**) has been confirmed in the Ames mutagenicity assays. These differences between ebselen on one side and the selenazolinium salts on the other may be explained by differences in reactivity and cellular target(s). Indeed, our preliminary mechanistic studies indicated that the mode of action for the Se-compounds is likely to be associated with an alteration of intracellular levels of glutathione and cysteine thiols of different proteins in the bacterial cells, hence supporting the idea that such compounds interact with the intracellular thiolstat. Future studies may reveal if this reaction is stoichiometric, or—as anticipated from the low amounts employed—catalytic with respect to pre-existing ROS and thiols. It is also possible that the selenazolinium salts react more than once, and that the active species is not the salt itself but possibly a reactive seleno-sulfide intermediate formed as part of the sequestration by and modification with GSH. Such investigations may also differentiate between ebselen, which is generally seen as an "antioxidant" promoting cell survival by reducing ROS in the presence of GSH, and the kind of oxidizing RSeS employed here, which, due to their high(er) reactivity, seem to attack thiol residues more randomly, hence modulating the level of GSH yet also affecting proteins and enzymes.

Taking into account such promising properties, we can conclude that the compounds with the selenazolinium scaffold represent a very promising chemical family in the ongoing search for new drug candidates that efficiently combat infections caused by highly resistant ESKAPE bacteria. Thus, the series is worth passing on for further stages of the drug research and development processes and also for more detailed investigations of the underlying mode(s) of action. At the same time, the Se–N motif may be refined and "tuned" further to enhance activity and selectivity.

Supplementary Materials: The following are available online. Table S1: Details of MIC values of the compounds **1**–**8** and ebselen against Gram-negative bacteria, Figure S1: Generation of intracellular ROS in the reference *S. aureus* ATCC 25923 strain and in the clinical MRSA HEMSA 5 isolate upon exposure to the different concentrations of the compound 1, Figure S2: Generation of intracellular ROS in the reference *S. aureus* ATCC 25923 strain and in the clinical MRSA HEMSA 5 isolate upon exposure to the different concentrations of compound 6, Figure S3: Generation of intracellular ROS in the reference *S. aureus* ATCC 25923 strain and in the clinical MRSA HEMSA 5 isolate upon exposure to the different concentrations of ebselen.

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Author Contributions: K.W., M.B., J.H., and C.J. conceived and designed the experiments; K.W., M.J.N., R.G., A.O., and M.A.M. performed the experiments and analyzed the data; P.A. and J.V. synthesized the compounds; G.L. and K.K.-K. helped to preform pharmaceutical safety experiments; K.W., C.J., and J.H. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are not available from the authors.

3.3 Publication 3

Aspects of a Distinct Cytotoxicity of Selenium Salts and Organic Selenides in Living Cells with Possible Implications for Drug Design.

Ethiene Castellucci Estevam, Karolina Witek, Lisa Faulstich, **Muhammad Jawad Nasim**, Gniewomir Latacz , Enrique Domínguez-Álvarez, Katarzyna Kieć-Kononowicz, Marilene Demasi, Jadwiga Handzlik and Claus Jacob.

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Article

Aspects of a Distinct Cytotoxicity of Selenium Salts and Organic Selenides in Living Cells with Possible Implications for Drug Design

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Abstract: Selenium is traditionally considered as an antioxidant element and selenium compounds are often discussed in the context of chemoprevention and therapy. Recent studies, however, have revealed a rather more colorful and diverse biological action of selenium-based compounds, including the modulation of the intracellular redox homeostasis and an often selective interference with regulatory cellular pathways. Our basic activity and mode of action studies with simple selenium and tellurium salts in different strains of *Staphylococcus aureus* (MRSA) and *Saccharomyces cerevisiae* indicate that such compounds are sometimes not particularly toxic on their own, yet enhance the antibacterial potential of

known antibiotics, possibly via the bioreductive formation of insoluble elemental deposits. Whilst the selenium and tellurium compounds tested do not necessarily act via the generation of Reactive Oxygen Species (ROS), they seem to interfere with various cellular pathways, including a possible inhibition of the proteasome and hindrance of DNA repair. Here, organic selenides are considerably more active compared to simple salts. The interference of selenium (and tellurium) compounds with multiple targets could provide new avenues for the development of effective antibiotic and anticancer agents which may go well beyond the traditional notion of selenium as a simple antioxidant.

Keywords: cellular thiolstat; MRSA; proteasome; redox modulation; resistant bacteria; ROS; selenium; tellurium; yeast

1. Introduction

Selenium as an element in general, and selenium salts and organic selenium compounds in particular, are traditionally considered as good antioxidants, as scavengers of free radicals and other Reactive Oxygen Species (ROS) [1,2]. Such compounds may be used in nutrition and therapy as chemopreventive and perhaps even as anticancer agents. Indeed, various selenium-based preparations, such as selenomethionine and sodium selenite (Na_2SeO_3), are sold freely in many pharmacies and supermarkets as food supplements, for the prevention of serious diseases, the stimulation of the immune system and also against more or less trivial medical problems, ranging from the common cold to loss of sexual performance and appetite. Recent studies performed by us and others have considered a range of such selenium preparations and uncovered a more sinister, darker side to this apparently antioxidant element [3]. In a yeast model, sodium selenite, for instance, seems to propagate DNA damage, possibly by a direct chemical action or, more indirectly, by inhibition of the relevant repair systems. Similarly, there are reports that an excess uptake of selenium may not prevent but actually promote the formation of certain diseases, possibly even including cancer [4].

Not surprisingly, therefore, a more differentiated view on the biological activity and role of selenium is required, a view which ultimately may open up new perspectives and avenues in drug design and development. Here, the more harmful actions of selenium salts just mentioned at first appear disappointing, yet may also be advantageous if their action could be focused on certain targets, such as bacteria, plasmodia, fungi or cancer cells [5]. Indeed, tellurium salts have long been considered as possible antibiotics, yet the interest in these compounds has declined with the advent of the penicillin era [6]. Nonetheless, with the emergence of multi-resistant bacteria, such as methicillin resistant *Staphylococcus aureus* (MRSA), such older remedies currently experience a certain renaissance in research and development.

The main aim of this study has therefore been to "revisit" of the antibiotic activity of certain selenium and tellurium salts and organic compounds, this time in particular against resistant strains of *S. aureus*, and in combination with known antibiotics. At the same time, the study has tried to uncover some basic aspects of the possible mode(s) of action underlying the (cyto-)toxic activities observed, with a focus on redox regulation and interference with key cellular events.

2. Results and Discussion

As part of this study, we have therefore turned our attention to redox active, often catalytic selenium compounds in order to explore their potential action against microorganisms, and also to investigate how such compounds may impact on a living cell. The chemical structures of the compounds used are shown in Figure 1, and these compounds are either available commercially or can be synthesized according to known literature methods [7].

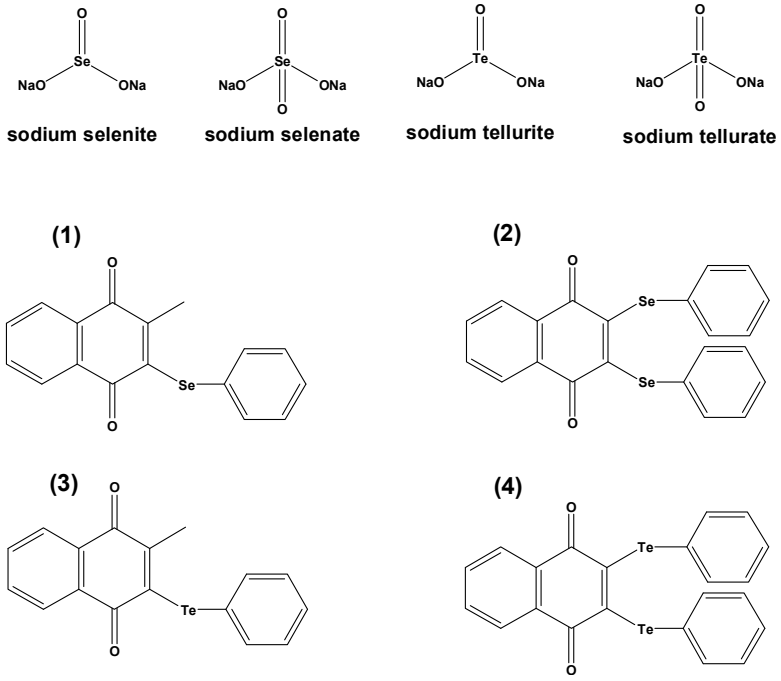

Figure 1. Chemical structures of the various selenium and tellurium compounds employed as part of this study. It should be noted that the salts (**top row**) used are highly polar and often charged, oxidizing compounds with a specific reactivity. Compounds **1**–**4**, in contrast, have been developed over the years as multicenter catalytic sensor/effector agents [8,9].

To our great surprise we have found that some simple selenium and tellurium salts act in concert with known antibiotics, hence apparently "sensitizing" such bacteria—which include strains of MRSA—against conventional antibiotics, possibly by inferring with the protease machinery/proteasome. Since such salts are not optimized for a particular biological activity and are also unlikely to penetrate the cell easily, the concentrations required for action were high. At the same time, the formation of red and black deposits could be observed in the case of certain selenium and tellurium salts, respectively. We have therefore subsequently turned our attention towards organic selenium (and tellurium) compounds, and as expected, these compounds show a higher activity. These findings will now be presented and discussed in more detail.

2.1. Antimicrobial Activity of Selenium Salts

Since selenium salts, such as Na_2SeO_3, nowadays are used widely in form of food supplements, we have first investigated the potential antimicrobial activity of such simple selenium salts and compared this activity to the one of their sulfur and tellurium analogues. This comparison is reasonable as tellurite

has long been considered as possible antibiotic and sulfite in fact is used extensively as antimicrobial agent to preserve fruits and vegetable nuts. At the high concentrations used, however, neither selenite or selenate, nor any of the other salts, showed any notable activity against *S. aureus* reference strain ATCC 25923 or multidrug resistant MRSA HEMSA 5 or MRSA HEMSA 5M strains. Indeed, it appears that the MIC values against those bacteria in most instances are well above 1 mM, which is much higher than those of conventional effective antibiotics (Table 1).

Table 1. MIC values (in μM) of selected chalcogen salts against three strains of *S. aureus*. Whilst these salts are clearly not active against any of the strains at pharmaceutically relevant concentrations, conventional antibiotics show some activity, which is reduced significantly in the case of the two resistant strains.

Compound		MIC Values (μM) in *S. aureus* Strains		
		ATCC 25923	**MRSA HEMSA 5**	**MRSA HEMSA 5M**
Salt	**Na_2TeO_3**	1000	>2000	>2000
	Na_2SeO_3	>2000	>2000	>2000
	Na_2SeO_4	>2000	>2000	>2000
	Na_2SO_3	>2000	>2000	>2000
	Na_2SO_4	>2000	>2000	>2000
Organic	**1**	31.25	125	500
	2	250	500	>1000
	3	62.5	62.5	>1000
	4	31.25	31.25	125
Antibiotic	**Oxacillin**	0.45	340	5400
	Cloxacillin	0.42	160	1300
	Ampicillin/sulbactam	0.54	200	200
	Ciprofloxacin	0.38	14	28
	Neomycin	1.0	240	240

This implies, of course, that neither the selenium nor the tellurium or sulfur salts used as part of this study on their own could be employed as antibiotics in practice. Similar results have been obtained against *Saccharomyces cerevisiae*, where neither selenite, selenate nor tellurite showed any significant activity against this type of fungus [3]. It should be noted that tellurate (Na_2TeO_4) was also considered, yet could not be tested reliably because of solubility issues in the buffers used which resulted in irreproducible results.

This rather disappointing finding is not entirely unexpected: such simple, highly polar salts are not optimized for antibiotic action and their ability to cross cell membranes is limited. Nonetheless, the interactions of such salts with biomolecules, especially cysteine containing proteins, are well documented *in vitro*, and one would be tempted to expect at least some impact on the bacterial, fungal or mammalian cell. Based on our previous studies in this field, one may speculate, for instance, that some of these compounds, particularly Na_2SeO_3, could "weaken" the cell by promoting oxidative stress and damage to DNA or by affecting the cellular thiolstat [10,11].

In the case of the organic selenium and tellurium compounds **1**–**4**, a slight antibacterial activity could be observed when these compounds were used in micromolar concentrations. While this activity often

was distinctively lower than that of the reference antibiotics used, it was much higher when compared to the inorganic salts—and was also rather competitive in the case of the drug resistant strains. The tellurium compounds **3** and **4**, in particular, showed some promising activity against the multidrug resistant strain HEMSA-5. The *bis*-phenyltellanyl derivative **4** was even able to inhibit the growth of both MDR MRSA strains (HEMSA 5 and extremely resistant HEMSA 5M) at a dose significantly lower than that of most antibiotics tested (excluding ciprofloxacin, see Table 1).

Since it appears that the selenium and tellurium compounds act differently, probably on different targets when compared to the conventional antibiotics, we have posed the question if they may not be able to also act synergistically, *i.e.*, in concert with conventional agents. We have therefore investigated both, the organic compounds **1**–**4** and some of the salts in combination with known antibiotic agents, such as oxacillin, cloxacillin, ampicillin/sulbactam, ciprofloxacin and neomycin, and indeed observed a significant enhancement in the antibacterial activity of such traditional antibiotics, even against some of the strains of MRSA (Table 2). Notably, whilst the inorganic salts often enhanced the efficiency of the antibiotics used, the organic compounds **1**–**4** were not particularly useful in those combination assays, bearing in mind, of course, that compounds **3** and **4** are fairly potent antibiotics on their own (see above). Particularly noteworthy, for instance, is the influence of tellurite (Na_2TeO_3), which at a concentration of just 500 μM enhances the toxicity of oxacillin and cloxacillin against MRSA HEMSA 5M by more than 1000-fold (Table 2). Notably, Na_2TeO_3 does not seem to exhibit any synergistic effects against the reference strain *S. aureus* ATTC 25923.

Table 2. Ability of the chalcogen compounds tested to enhance the antibacterial activity of selected antibiotics against *S. aureus* strains at a chalcogen compound concentration of 500 μM. Results are expressed as the quotient of the MIC value of antibiotics in the absence to that in the presence of the corresponding chalcogen compound.

Antibiotic	**Strain of *S. aureus***	**Antibiotic Efficacy Enhancement** $\left(\frac{MIC_{Ant}}{MIC_{Ant+Comp}}\right)$					
		1–4	**Na_2TeO_3**	**Na_2SeO_3**	**Na_2SeO_4**	**Na_2SO_3**	**Na_2SO_4**
Oxacillin	ATCC 25923	NE	NE *	4	4	NE	NE
	MRSA HEMSA 5	NE	16	NE	NE	NE	NE
	MRSA HEMSA 5M	NE	1024	NE	NE	NE	NE
Cloxacillin	ATCC 25923	NE	2 *	4	4	NE	NE
	MRSA HEMSA 5	NE	256	NE	NE	32	32
	MRSA HEMSA 5M	NE	≥1024	NE	NE	NE	NE
Ampicillin/ Sulbactam	ATCC 25923	NE	4 *	2	2	NE	NE
	MRSA HEMSA 5	NE	8	NE	NE	4	4
	MRSA HEMSA 5M	NE	8	NE	NE	NE	NE
Ciprofloxacin	ATCC 25923	NE	NE *	NE	NE	NE	NE
	MRSA HEMSA 5	NE	NE	NE	NE	NE	NE
	MRSA HEMSA 5M	NE	NE	NE	NE	NE	NE
Neomycin	ATCC 25923	NE	2 *	NE	NE	NE	NE
	MRSA HEMSA 5	NE	4–8	8	NE	NE	NE
	MRSA HEMSA 5M	NE	4	NE	NE	NE	NE

NE: No enhancement observed; * Tellurite was evaluated at a concentration of 250 μM (*i.e.*, at one quarter of direct MIC in ATCC25923).

These findings are rather interesting, as they indicate a certain "re-sensitization" of the drug resistant strains against classic antibiotics in the presence of certain selenium, and possibly also of tellurium or sulfur compounds. Tellurite, for instance promotes the activity of β-lactam antibiotics oxacillin and cloxacillin against both strains of MRSA studied, reducing the MIC values for these antibiotics from well over 100 μM in the case of HEMSA 5 and over 1000 μM in the case of HEMSA 5M to acceptable values in the sub- or low micromolar range, respectively. Furthermore, tellurite increases the action of ampicillin combined with sulbactam or of the aminoglycoside antibiotic neomycin against multidrug resistant MRSA strains (Table 2).

This finding in itself obviously is exceptionally attractive from the perspective of future drug combination therapies, which to a large extent will rely on this kind of re-sensitization of resistant strains. It also agrees with a very recent report in the literature, which shows that nanomolar concentrations of Na_2TeO_3 can enhance the toxic effects of the cephalosporin antibiotic cefotaxime against *Escherichia coli* [12]. In this study, the enhancement by Na_2TeO_3 appeared to be due to a tellurite-induced oxidative stress, and a widespread damage to DNA and to proteins, possible mode(s) of action which will be considered in more detail as part of the following sections.

Unfortunately, tellurite is also rather toxic in humans, and the two selenium salts are less active, yet also seem to be able to somewhat, up to 4- to 8-fold, promote the toxicity of different antibiotics. Na_2SeO_3, in particular, seems to enhance the action of aminoglycosides against multidrug resistant MRSA HEMSA 5, decreasing the effective dose of neomycin required from 240 μM to 30 μM. Bearing in mind that Na_2SeO_3 and Na_2SeO_4 are considerably less toxic towards humans when compared to their tellurium analogues, such an enhancement of antibiotic action in MRSA strains is rather interesting. The ultimate choice for a potential therapeutic use therefore may well consider selenite and selenate, rather than tellurite, and obviously at concentrations not damaging or even lethal to humans. Incidentally, an interesting activity of sulfite and sulfate—which at first were included in the study as controls—could be demonstrated. The toxicity of cloxacillin against MRSA HEMSA 5, for instance, is enhanced around 32-fold by 500 μM concentrations of either Na_2SO_3 or Na_2SO_4. Since sulfate, in particular, is non-toxic to humans even when administered at higher concentrations, this finding—and the possible usage of sulfur-based salts as potential "adjuvants" of this β-lactam antibiotic—should also be considered further in earnest.

2.2. Cellular Targets of Selenite and Selenate in Bacteria and Yeast

Whilst some of the selenium and tellurium salts studied seem to enhance the antibiotic activity of traditional antibiotics, such as β-lactams and aminoglycosides, none of chalcogen salts employed was able to increase the bactericidal activity of the fluoroquinolone ciprofloxacin. Ciprofloxacin is an inhibitor of bacterial gyrase and hence DNA replication. In contrast, the penicillins interfere with the bacterial cell wall synthesis. It is therefore possible that the synergistic effects observed in the case of the penicillins point toward a particular mode of action of salts such as Na_2TeO_3, Na_2SeO_3 or Na_2SeO_4. We have already mentioned in passing that based on the chemistry and *in vitro* studies of such redox active selenium and tellurium compounds, one would expect a "weakening" of cells exposed to higher concentrations of these agents. Although speculative at this time, it may be possible, for instance, that these salts interfere with the cell membrane or components thereof. They may hinder the cell wall

synthesis, perhaps inhibit (cysteine containing) β-lactamases or stress the cell more generally, for instance by the generation of Reactive Oxygen Species (ROS) [13,14]. Indeed, such actions have been associated with selenite and tellurite before [3,12].

To investigate this issue further, we have therefore considered the impact of these chalcogen salts on intracellular ROS levels in *S. aureus* ATCC 25923, HEMSA 5 and HEMSA 5M using 2′,7′-dichlorodihydrofluorescein diacetate (DCHFA) as redox-sensitive fluorescent indicator dye. The results of this crude ROS assay are presented for the different salts, and against different strains of *S. aureus* in Figures 2–4.

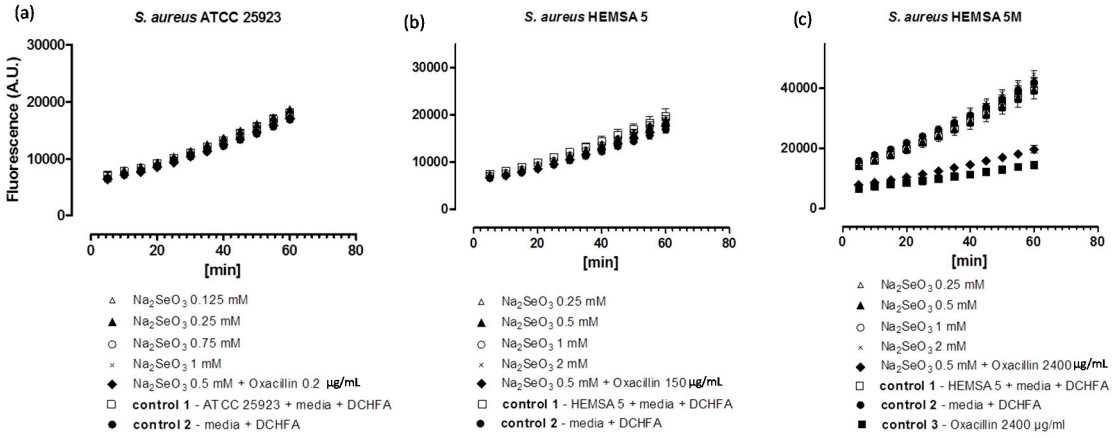

Figure 2. Generation of intracellular ROS by Na_2SeO_3 in different strains of *S. aureus*. ATCC 25923 (**a**); HEMSA 5 (**b**) and HEMSA 5M (**c**). Data is shown in terms of fluorescence emitted (in arbitrary units) by (oxidized) DCFA. The latter is generated by the reaction of certain ROS and Reactive Nitrogen Species (RNS) with DCHFA. Different concentrations of Na_2SeO_3 were assayed and the influence of the dual presence of the chalcogen salt and an antibiotic (oxacillin) on ROS generation was also evaluated. Values shown represent mean values with $n = 4$. The error bars represent the standard deviation (SD) and statistical significances were calculated using an one-way ANOVA followed by Bonferroni's multiple comparison test (Figure 2a,b: $p > 0.05$; 2c: $p < 0.05$).

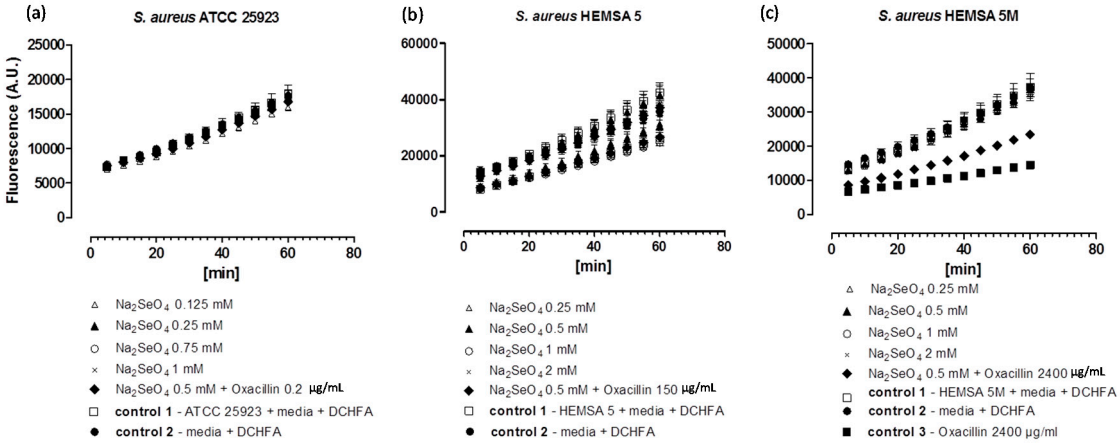

Figure 3. Generation of intracellular ROS by Na_2SeO_4 in different strains of *S. aureus*. ATCC 25923 (**a**); HEMSA 5 (**b**) and HEMSA 5M (**c**). See legend of Figure 2 for further details.

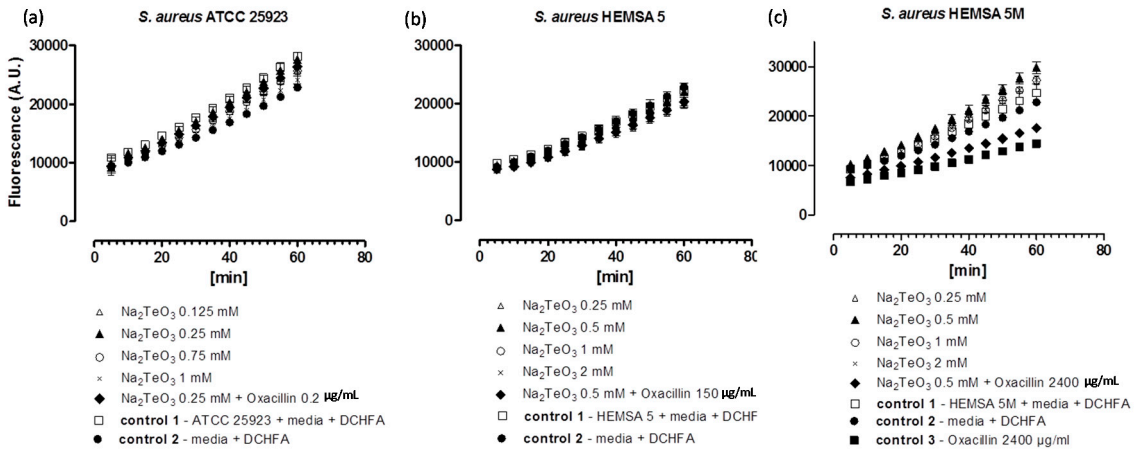

Figure 4. Generation of intracellular ROS by Na_2TeO_3 in different strains of *S. aureus*. ATCC 25923 (**a**); HEMSA 5 (**b**) and HEMSA 5M (**c**). See legend of Figure 2 for further details.

Despite the fact that some of the salts notably increased the efficiency of some of the antibiotics, none of the inorganic selenium or tellurium salts studied, either on its own or in combination with oxacillin, increased the intracellular levels of ROS significantly. This finding is truly unexpected, as compounds such as selenite are known to generate ROS *in vitro* [15–17], are able to modify the thiol redox state and cellular thiolstat, and also have the potential to inhibit enzymes involved in the reduction of oxidative stress. Selenite even promotes DNA strand breaks in yeast, possibly also via a redox mechanism [3,18–20]. As already mentioned above, there are also reports that K_2TeO_3 causes a rather strong increase in intracellular ROS levels in Gram-negative *E. coli* [21]. Hence, at least in theory, one may have expected an increase in ROS levels for Na_2TeO_3 and Na_2SeO_3, either by the direct chemical generation of additional ROS, by damaging cellular components subsequently leading to an indirect increase of ROS concentrations, or by the inhibition of ROS removal systems. Still, it seems that at the concentrations used, none of these redox events plays any major role in the bacteria studied here.

Noteworthy, there is also no consistent evidence of any "antioxidant" activity of Na_2TeO_3, Na_2SeO_3 and Na_2SeO_4 in the bacteria investigated. Generally, no significant differences in fluorescence in the presence of the compound and the controls were observed. Only in one case, *i.e.*, when the HEMSA 5M strain was treated with oxacillin (2400 µg/mL) (with or without the presence of the salts examined), could a significant decrease ($p < 0.005$) of fluorescence compared to the control be observed (Figures 2c, 3c and 4c). This observed decrease in fluorescence, however, could be due to the high concentration of oxacillin used in the ROS assay of this strain, which may have influenced the natural conversion of DCHFA into DCFA. Indeed, a specific control (*i.e.*, control 3) has been used in those cases to confirm that oxacillin, and not the selenium or tellurium salts, is probably responsible for this particular observation.

Eventually, one must also emphasize that the DCHFA-assay is widely used yet far from perfect: it does not capture all ROS and its outcome is also affected by the activity of certain cellular esterases (see also Section 3). Furthermore, Kalyanaraman has suggested that the intracellular oxidation of this dye may be mediated to some extent by iron or by cytochrome *c* [22]. Hence any results obtained by this fluorescent assay are rough estimates only and need to be treated with some caution. Whilst the DCHFA assay is still widely accepted as a good measure of intracellular levels of ROS, a more sensible, *i.e.*,

sensitive and selective method should be used in future studies to confirm the lack of pro-oxidant activity of the salts tested.

More or less by coincidence, however, we have observed a rather different, perhaps not entirely expected biological redox activity associated with some of these salts, which may well explain some of the findings shown in Table 2 and which may also provide a new angle to the discussion. Upon exposure to tellurite and selenite salts, the strains of bacteria investigated changed color, and in fact generated and then obviously accumulated solid red and black deposits, which based on previous reports appear to consist of elemental selenium and elemental tellurium, respectively. Indeed, a closer look at the literature on bacteria dealing with selenium and tellurium salts shows that this finding is not wholly unanticipated. Many bacteria, including *Halococcus salifodinae BK18*, *Azospirillum brasilense*, *Sulfurospirillum* and *Thauera selenatis* and also some more common ones such as *Pseudomonas*, *Lactobacillus* and *Bacillus cereus* are known to reduce selenite to elemental selenium (nano)particles [23–29]. *Thauera selenatis* is even able to reduce selenate to form elemental selenium deposits. In fact, different species of *Lactobacillus* are used as a natural source of selenium nanoparticles, for instance to enrich probiotics with selenium [30]. Similarly, bacteria such as *Bacillus beveridgei*, *Rhodobacter capsulatus* and even *E. coli* readily reduce Na_2TeO_3 to generate elemental tellurium, usually in form of nanoscopic or microscopic rods and needles [31–33]. Such a reactive cascade leading to elemental selenium or tellurium is shown in Figure 5. It should be noted that to the best of our knowledge, there have been no reports so far that *Staphylococcus* converts selenite or tellurite to elemental selenium or tellurium, respectively.

Indeed, the elemental precipitates formed not only stain the bacterial cells red or black, those particles also exert a massive mechanical stress on the bacterial cell which may ultimately cause cell death via a combination of mechanical stresses and unfavorable cellular responses toward them. Eventually, the bioreductive formation of these solid deposits in the bacterial cell may weaken the cell and hence may also explain—at least in part—the notable efficiency of traditional antibiotics in such compromised cells. This process may also contribute to the selective action of such simple chalcogen salts, as mammalian cells do not reduce selenite to elemental selenium. Here, selenite is mostly "sequestered" chemically by glutathione (GSH) in form of glutathione selenotrisulfide (GSSeSG). The latter is reduced further to hydrogen selenide (H_2Se) which either serves as a selenium source for the synthesis of selenocysteine or is methylated to dimethylselenide (CH_3SeCH_3) and ionic trimethylselenonium ($(CH_3)_3Se^+$) and excreted (Figure 5) [34,35]. In any case, mammalian cells normally do not form solid selenium or tellurium particles and hence are not affected by the mechanical stresses and toxicity caused by such particles [36].

Since the reduction of salts such as Na_2SeO_3 often proceeds spontaneously in the presence of thiols (such as GSH), another mode of (cytotoxic) action has been discussed for various selenium and tellurium compounds [37]. The underlying chemical mechanism includes the—probably spontaneous and more or less random—reaction of such compounds with thiol groups in proteins. Such reactions result in various posttranslational oxidative modifications which may also impact on the function and activity of the proteins and enzymes affected. Since many cysteine proteins and enzymes are involved in pivotal cellular processes (metabolism, signaling, maintenance of redox homeostasis *etc.*), and hence have recently been subsumed under the notion of the "cellular thiolstat", a modification of their cysteine residues and thus function and activity may have serious consequences for the cell [10].

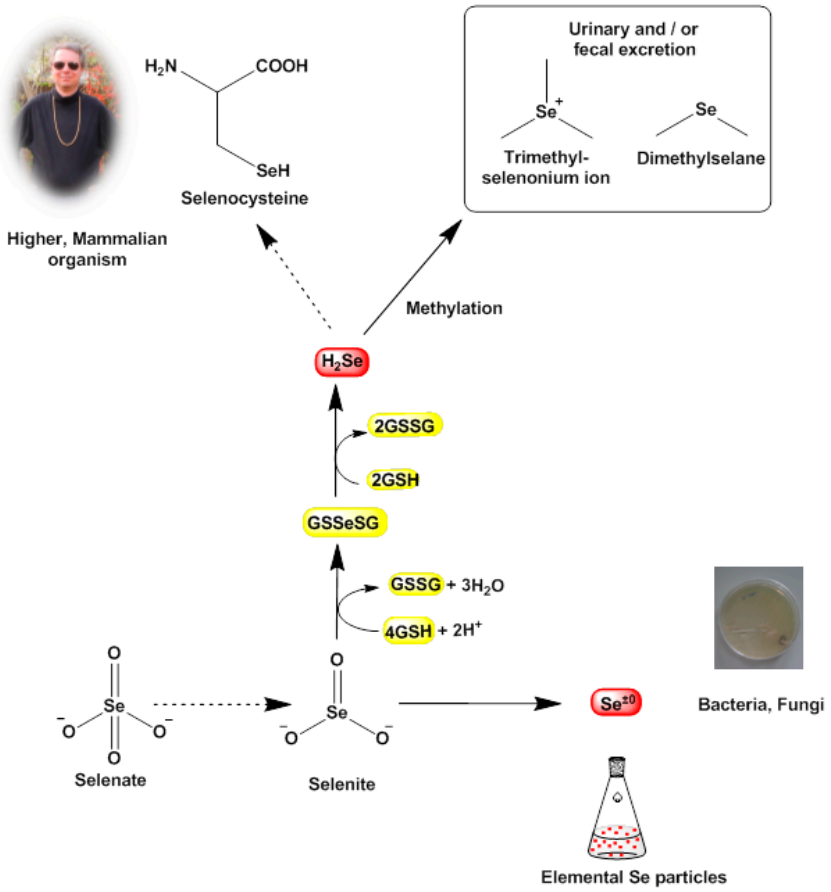

Figure 5. Known bioreductive pathways for SeO_3^{2-} (and TeO_3^{2-}) in certain bacterial cells. These pathways often terminate in the formation of (solid) elemental selenium and tellurium (nano)particles, which subsequently may cause damage to the cell. In our studies, it appears that selenite (SeO_3^{2-}) and tellurite (TeO_3^{2-}) are also reduced by *S. aureus*, as different reports have observed similar changes in color and the formation of deposits and have linked these physical changes to the formation of selenium and tellurium, respectively. In sharp contrast, mammalian cells tend to by-pass the "zero oxidation state" and form H_2Se as a valuable raw product for the synthesis of the amino acid selenocysteine, whilst excess selenium is excreted in form of methylated products. Hence the bioreductively generated toxicity of selenium and tellurium (nano)particles is probably more or less specific for lower organisms.

As part of our study, we have therefore considered if such compounds also interact with key cellular components or events, especially those which are related to the cellular thiolstat and whose inhibition may be highly detrimental to the cell. As part of this search for potential protein targets, we noticed a significant inhibition of the proteasome in yeast (Figure 6). It should be pointed out that *S. cerevisiae* was used as model as the yeast proteasome can be studied reliably in isolation as well as in the intact cell. Here, Na_2SeO_3 and especially Na_2SeO_4 were most active. The impact of Na_2SeO_4 on proteasome activity was observed at higher micromolar concentrations and, at a concentration of 1 mM, Na_2SeO_4 reduced the chymotrypsin-like activity of the yeast proteasome in intact cells significantly, to less than 25%.

Arguably, these concentrations of selenate are high and not relevant physiologically. Still, one needs to bear in mind that these experiments were performed with intact cells, and intracellular concentrations of Na_2SeO_4 may be lower. In fact, the known proteasome inhibitor bortezomib, which was used in this

part of the study as positive control, failed to inhibit proteasome activity in yeast cells altogether when used at its standard concentration of 1 micromolar. Whilst bortezomib and similar inhibitors penetrate human cells readily, they cannot penetrate wild-type yeast cells due to an inherent impermeability of the cell wall or membranes [38]. For this reason, yeast has been used simply as a model system, and not as a target for therapeutic intervention.

Notably, both selenium salts did not inhibit the trypsin-like activity and Na_2TeO_3, often considered as the most active of these chalcogen salts, had no inhibitory effect on the yeast proteasome under the conditions used. Indeed, at closer inspection, it even appears that Na_2SeO_3 and Na_2TeO_3 may actually stimulate the trypsin-like activity, although those increases are statistically not significant (Figure 6b).

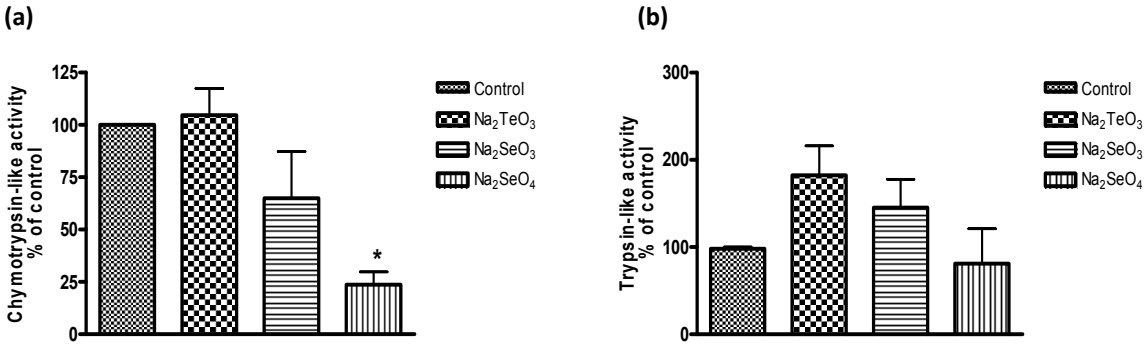

Figure 6. Inhibition of the proteasome in intact cells of *S. cerevisiae* (chymotrypsin-like activity (**a**) and trypsin-like activity (**b**)). Data was obtained from cell extracts after 3 h incubation in the presence of 1000 μM of tellurium and selenium salts. Values represent means with SD bars from at least three independent experiments, and statistical significances were calculated using a one-way ANOVA followed by Bonferroni's multiple comparison test. ***** $p < 0.05$.

Once more, one may speculate that the compounds employed are able to react with thiol groups in proteins, and therefore may also interfere with and inhibit a range of proteins and enzymes, including parts of the proteasome, either by a direct modification of cysteine residues in components of the proteasome itself or by a widespread modification of proteins which then end up and "overload" the proteasome in a detrimental manner. Indeed, the notion that thiol-selective yet otherwise unspecific (re-)agents may be able to modify a wide range of cellular, redox-sensitive cysteine-proteins, which subsequently not only lose their function and activity, but also end up as "garbage" and overload the proteasome, is highly stimulating and deserves a more detailed consideration in the future.

On the one side, these findings are rather instructive, as the proteasome represents an important target in modern therapy. In eukaryotes, the ubiquitin-proteasome system is the main proteolytic system, whilst in bacteria there are different proteolytic complexes that are directly linked to bacterial virulence [39,40]. Not surprisingly, the proteolytic machinery is being considered as a promising therapeutic target, especially in multi-resistant bacterial species [41]. Here, fellutamide B acts as inhibitor of the *Mycobacterium tuberculosis* proteasome, whilst an inhibitor of the protease ClpXP increases the sensitivity of methycillin-resistant *S. aureus* and *Bacillus anthracis* to antibiotics that act on the bacterial wall (synthesis) and membrane [42,43]. Indeed, such observations on the "re-sensitization" of the drug resistant strains against conventional antibiotics acting on the cell wall synthesis via an inhibition of

the proteasome mirror most of the still preliminary findings of our present study [39–43]. It is therefore a highly rewarding task to study the impact of selenium food supplements and organic selenium compounds on different proteolytic systems/proteasomes (e.g., bacteria, fungi, human cancer cells, normal cells) more extensively in the future.

On the other side, the proteasome represents probably just one target for salts such as Na_2SeO_3 and Na_2SeO_4. There may be many other—yet unidentified—targets, and some of them may also differ between bacterial, fungal and mammalian cells, depending on the specific components of the respective cellular thiolstat and the metabolic processes in action (such as specific bioreductive pathways).

In any case, the observation that simple salts, such as Na_2SeO_3, are able to compromise bacterial cells, even employing some redox driven "nanotechnology", underlines the considerable interest in redox active and catalytic selenium compounds—some of which form part of our daily nutrition—and their potential uses in medicine.

2.3. From Simple Selenium Salts to Redox Modulating Selenium Drugs

Whilst the results obtained so far for Na_2SeO_3 and Na_2SeO_4 (as well as for Na_2TeO_3) are interesting, such compounds are not particularly useful from a pharmaceutical point of view, not least because of the high concentrations required for a significant activity. As already mentioned, we have therefore investigated some of the most interesting findings obtained as part of this study further, this time employing some of our most potent redox modulating selenium and tellurium agents (compounds **1**–**4** in Figure 1) [5,7,44,45]. Since these compounds have been designed as potential therapeutic agents (for instance considering reactivity, stability, pharmacokinetics, Lipinski's "Rule of Five", *etc.*), it was hardly surprising to find a pronounced antibacterial activity for the selenium compound **1** against *S. aureus* ATCC25923 with MIC values as low as 31.25 μM. Interestingly, the two tellurium compounds **3** and **4** were even rather active against the MRSA strain HEMSA 5, with MIC values of 62.5 μM and 31.25 μM, respectively. These MIC values are rather low, also when compared to the MIC values listed for some of the conventional antibiotics in Table 1. Compound **4**, which contains two tellurium centers and a quinone moiety, therefore seems to be particularly promising as a potential antibacterial agent, and on its own [46].

When considered in combination with traditional antibiotics, such as oxacillin, these compounds did not enhance the activity of the antibiotics used. Yet again, this is hardly surprising. The special bioreductive "chemistry" witnessed in bacteria in the case of salts such as Na_2SeO_3, Na_2SeO_4 and Na_2TeO_3, for instance, is notably absent for compounds **1**–**4**. First and foremost, no color changes were observed when the organoselenium and organotellurium compounds were used. Secondly, there was also no evidence of any deposit formation when the cells were incubated with the organic selenium and tellurium compounds. Hence based on this evidence, it appears that no elemental selenium and tellurium particles similar to the ones observed for the salts were formed and that the kind of mechanical and (bio)chemical stresses discussed for compounds such as Na_2SeO_3 and Na_2TeO_3 was therefore almost certainly also absent.

Besides simple changes in color, these differences in "chemistry" between the inorganic and organic selenium and tellurium compounds obviously translate into further differences in biochemical activity. The organic compounds employed contain several redox active, catalytic centers and are known to

interfere with the intracellular redox homeostasis in human cells. As part of a related study, it has moreover become apparent that such compounds, especially compounds **2** and **4**, are able to interfere with proteasomes in intact cells as well as with isolated human proteasomes, where those two compounds at a concentration of just 1 μM reduce chymotrypsin-like activity by 80% and trypsin-like activity by 90%. We also know from our previous studies that these compounds inhibit mammalian thioredoxin reductase (TrxR) *in vitro*, and at rather low concentrations. The selenium compound **2**, at just 1 μM, reduces TrxR activity to less than 50%, whilst the tellurium compound **3** is even more active, with just 60% of the original TrxR activity remaining when used at 500 nM and less than 20% TrxR activity remaining when used at an 1 μM concentration [47]. There is also evidence that selenium compounds closely related chemically to **1** and **2** interfere with protein synthetic pathways and ultimately protein synthesis [47].

3. Experimental Section

3.1. Materials, Solvents and Bacteria

3.1.1. Materials

Chemicals, including the various sulfur, selenium and tellurium salts, educts and solvents for the chemical synthesis, and substances used for yeast cell culture were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium selenite (Na_2SeO_3) and sodium tellurite (Na_2TeO_3) were obtained from Merck KGaA (Darmstadt, Germany). Sodium sulfite (Na_2SO_3), sodium sulfate (Na_2SO_4), the antibiotics oxacillin, cloxacillin, ampicillin/sulbactam, ciprofloxacin and neomycin and the fluorescent redox indicator dye 2′,7′-dichlorodihydrofluorescein diacetate (DCHFA) were purchased from Sigma-Aldrich (Poznań, Poland). Chemicals were used without further purification unless stated otherwise. Compounds **1**–**4** were synthesized according to established literature procedures and purified. Their analytical data (^{1}H-NMR, ^{13}C-NMR, mass spectrometry) was in accordance with values reported in the literature [44]. The fluorescent substrates required for measurements of the proteasome activity (suc-LLVY-AMC and z-ARR-AMC) were purchased from Calbiochem (Merck Chemicals GmbH, Schwalbach, Germany).

3.1.2. Solvents

Distilled water (Telmed Destylator DE 8/70, Warsaw, Poland) was used as solvent for the preparation of Mueller-Hinton Broth (MHB) media and all antibiotic stock solutions. MHB medium was sterilized by autoclaving at 121 °C for 15 min. DCHFA stock solution (2 mM) was prepared in pure ethanol (≥99.8%, Avantor Performance Materials Poland, Gliwice, Poland). MilliQ water (resistance ≥ 18 MΩ·cm^{-1}) was used for the studies involving *S. cerevisiae*.

3.1.3. Bacterial Strains

Wild-type *S. aureus* American Type Culture Collection ATCC 25923 (oxacillin MIC 0.2 μg/mL) and clinical methicillin resistant MRSA strains HEMSA 5 (oxacillin MIC 150 μg/mL) and MRSA HEMSA 5M (oxacillin MIC 2400 μg/mL) were used as part of this study (Instituto de Higiene e Medicina Tropical, Universidade Nova, Lisbon, Portugal).

3.2. Microbiological Activity Assays

The impact of compounds **1**–**4** and inorganic salts was evaluated in three strains of *S. aureus*: the reference strain ATCC 25923 and the methicillin resistant strains MRSA HEMSA 5 and MRSA HEMSA 5M. Minimum inhibitory concentrations (MICs) of oxacillin, cloxacillin, ampicillin/sulbactam, ciprofloxacin and neomycin, as well as MICs of compounds **1**–**4**, Na_2TeO_3, Na_2SeO_3, Na_2SeO_4, Na_2SO_3, and Na_2SO_4, *i.e.*, the intrinsic antibacterial activity of the compounds, and the ability of the selenium and tellurium compounds to enhance the activity of the above-mentioned antibiotics were assessed by a broth microdilution method in Mueller-Hinton Broth (MHB) according to Clinical and Laboratory Standards Institute (CLSI) recommendations. The MICs were recorded as the lowest concentration of the compound or of the antibiotic inhibiting visible growth of bacteria after a 16 h incubation at 37 °C. In the first step, intrinsic antibacterial activity of each compound was examined. Subsequently, the MICs of oxacillin, cloxacillin, ampicillin/sulbactam, ciprofloxacin and neomycin were determined in the absence and presence of the compounds to investigate any synergistic effects. In order to avoid any significant toxicity of the selenium and tellurium compounds in this step, their concentrations were no greater than a quarter of their respective MIC values. All microbiological assays involving bacteria were performed in at least two repetitions, whilst the studies with yeast were performed in triplicate and on three different occasions.

3.3. Determination of Intracellular Levels of Oxidative Stress via the DCHFA Assay

Intracellular levels of ROS were estimated using the fluorescent dye DCHFA. The latter is a non-polar dye which can cross cell membranes and becomes trapped inside cells by deacetylation. The deacetylated form subsequently reacts with certain ROS to produce 2′,7′-dichlorofluorescein (DCFA), the oxidized, fluorescent form of DCHFA. It should be noted that certain ROS, such as the hydroxyl radical (•OH) or hydrogen peroxide (H_2O_2) react better with DCHFA in this assay compared to other ROS, and that oxidation can also be triggered by certain Reactive Nitrogen Species (RNS) [22,48].

As part of this assay, the reference strain *S. aureus* ATCC 25923 and the clinical strains *S. aureus* MRSA HEMSA 5 and MRSA HEMSA 5M were grown in Mueller-Hinton Broth (MHB) at 37 °C with shaking until reaching an optical density of 0.5 at 600 nm (OD_{600} = 0.5). At this point the bacterial cells were loaded in a 96-well plate and treated with different concentrations of Na_2TeO_3, Na_2SeO_3 or Na_2SeO_4 (0.125, 0.25, 0.75 and 1 mM for *S. aureus* ATCC 25923 and 0.25, 0.5, 1 and 2 mM for *S. aureus* MRSA HEMSA 5 and MRSA HEMSA 5M). ROS levels in bacterial cells were also investigated after adding both an inorganic salt (Na_2TeO_3, Na_2SeO_3 or Na_2SeO_4) and oxacillin at the concentrations employed in the microbiological assays (0.25 mM and 0.2 μg/mL for the *S. aureus* ATCC 25923 strain, 0.5 mM and 150 μg/mL for the *S. aureus* MRSA HEMSA 5 strain or 0.5 mM and 2400 μg/mL for the *S. aureus* MRSA HEMSA 5M strain, respectively).

Next, 10 μM (final concentration) of DCHFA solubilized in ethanol (a 2 mM stock was prepared prior in ethanol and kept at −20 °C in the dark until further use) were added to the bacterial cultures and incubated at room temperature in the dark for 1 h. The samples were subjected to fluorescence spectrophotometric analysis. The fluorescence intensity was measured at 5 min intervals over a 60 min

period using a microplate reader (EnSpire, PerkinElmer, Waltham, MA, USA), with an excitation wavelength of λ_{ex} = 480 nm and an emission wavelength of λ_{em} = 525 nm.

3.4. Inhibition of the Proteasome of S. Cerevisiae

Treatment of yeast was carried out according to the method reported recently by Letavayová *et al.* [49]. Briefly, *S. cerevisiae* was placed on Yeast Extract-Peptone-Dextrose medium (YPD medium) at 37 °C overnight. Inocula were prepared by suspending colonies of these cultures in fresh YPD broth until the cell suspension reached a density of 2×10^7 cells/mL. Yeast cells were then incubated with 1000 μM of compounds at room temperature during 3 h under constant shaking [49]. By the end of the treatment, cells were collected by centrifugation and washed twice with phosphate buffered saline (PBS).

Cell pellets were resuspended in extraction buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 10% (*v*/*v*) glycerol, 0.5% (*v*/*v*) Nonidet P-40 substituent, and 1 mM $MgCl_2$ (pH 7.5). The cells were lysed by sonication followed by 25 min incubation on ice and centrifugation at 15,000 *g* for 30 min. The total protein content in the supernatant was then assessed by the Bradford assay.

Proteasomal activity was determined in cell extracts by incubating aliquots of total cellular protein (25–50 μg) at 37 ± 1 °C with the following fluorogenic substrates: suc-Leu-Leu-Val-Tyr-AMC (suc-LLVY-AMC; indicative of chymotrypsin-like activity) and z-Ala-Arg-Arg-AMC (z-ARR-AMC; indicative of trypsin-like activity). Fluorescence was recorded for 45 min (excitation wavelength of λ_{ex} = 380 nm and an emission wavelength of λ_{em} = 460 nm).

In each individual experiment, the control (untreated sample) was set at 100%. Final data represents the average of three independent experiments. Results were expressed as mean ± SD and the statistical significance was determined by one-way ANOVA followed by Bonferroni's multiple comparison test. A value of $p \leq 0.05$ was considered as statistically significant.

4. Conclusions

Eventually, we have been able to demonstrate that different selenium and tellurium compounds, ranging from selenite used as a selenium food supplement to multi-redox center organic molecules, are biologically active, also against some rather serious pathogens (such as two strains of MRSA). These compounds can either act on their own (such as compounds **1**–**4**) or in concert with traditional antibiotics. The role of selenite and other selenium supplements in fighting bacterial infections is therefore of considerable interest, especially since those compounds are also known to stimulate the human immune defense and to increase the antioxidant capacity—both highly beneficial processes during an infection.

Likewise, the biological chemistry of such compounds is truly fascinating and may combine different aspects of redox modulation and bioreductive formation of nanoparticles. No doubt, the relevant cellular targets, events, processes and subsequent biological activities certainly need to be studied in considerable more detail in the future. Here, the emerging selective fluorescent staining, quantification and microscopy techniques, together with classic activity assays and Western Blots will enable us to perform the kind of redox-related "intracellular diagnostics" which is now required to further illuminate the underlying cellular targets and processes [3]. Eventually, such knowledge may enable us to employ selenium, in its various chemical compositions and forms, effectively, in medicine and perhaps even in agriculture, and in the long run to enjoy fully probiotic drinks laced with natural selenium nanoparticles [50].

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Author Contributions

Ethiene Castellucci Estevam and Lisa Faulstich conducted the studies on the proteasome. Karolina Witek, Gniewomir Latacz and Enrique Domínguez-Álvarez performed the experimental studies on the antimicrobial activity of the selenium and tellurium salts and organic compounds. Muhammad Jawad Nasim was responsible for the synthesis of the organic compounds. Jadwiga Handzlik, Katarzyna Kieć-Kononowicz, Marilene Demasi and Claus Jacob coordinated the studies at their respective institutes and also drafted the manuscript. The latter includes work, text, tables and figures which were prepared jointly by all authors.

Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds **1**–**4** are available from the authors.

4. Discussion

Several Reactive Selenium Species (RSeS) demonstrated excellent activity against a broad spectrum of microorganisms and especially against multidrug resistant strains (MDR). The significant antimicrobial activity of these compounds supports the initial hypothesis that the concept of RSeS can be exploited to design effective lead structures for potential application in Medicine. The list of RSeS starts from elemental selenium nanoparticles and goes on to include several complex redox modulating organo-selenium compounds. The compounds employed as part of this thesis range in their chemical complexity from simple inorganic selenium salts, such as sodium selenite (Na_2SeO_3) and sodium selenate (Na_2SeO_4) to highly reactive organic selenazolinium salts and from simple aromatic selenocyanates to multi-component hybrid molecules.

4.1. Selenium Nanoparticles: The Spell of Red Moon

Selenium (elemental) nanoparticles represent the very first and foremost member in the list of RSeS which are very simple in nature yet extremely effective in biological systems. Although elemental (red) selenium is generally insoluble in aqueous solutions, nanotechnology could be employed to produce rather stable nano-suspensions of selenium nanoparticles. With the recent developments in the field of nanotechnology, such particles can be generated astoundingly easily and in remarkable yields and reasonable amounts following three different procedures: a) naturally (biologically) by employing certain bacteria, b) physically by applying shear forces with the help of grinding mills and high pressure homogenization, and c) by precise chemical reduction of inorganic salts of selenium, such as SeO_3^{2-}. Intriguingly, in all above mentioned procedures, the red allotropic form of elemental selenium predominantly prevails as compared to the grey form [70,107]. Besides the significance of

being ‘‘nano’’ in nature (and size), these particles provide additional benefits such as, they a) apparently foster the bioavailability of elemental selenium, b) can be generated not only naturally but also as part of green synthesis by a variety of conventional bacteria, including *Staphylococcus carnosus* (*S. carnosus*), *Staphylococcus aureus* (*S. aureus*) *Bacillus oryziterrae* (*B. oryziterrae*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Lactobacillus brevis* (*L. brevis*), c) are frequently coated with proteins, and d) serve as excellent multifunctional agents effective against plethora of targets, such as microorganisms and cancer cell lines [70,108-110].

Whilst the phenomenon of biologically produced particles of elemental selenium has been established during the last couple of decades, the characteristics, features and potential applications of such pure elemental selenium-based particles have very recently gained the attention of research and Industry. It is interesting to note that when certain bacteria, such as the ones mentioned above, are treated with excessive amounts of selenite, detoxification by simple ‘‘bioreduction’’ of inorganic selenite to elemental selenium is rendered inefficient. The target bacteria are eventually forced to employ the more aggressive approach of producing solid particles which, in turn, induce severe physical stress and trigger intense widespread mechanical damages within the target bacteria. The prokaryotic bacteria are generally incapable of digesting, dissolving, facilitating or excreting such very hard and solid particles themselves. Under these circumstances those microorganisms become “vulnerable” and immediately develop sensitivity towards antibiotics or even the particles which ultimately results in the death of these bacteria. Hence this very simple approach of nurturing the notorious methicillin-resistant strains of *S. aureus* on inorganic salts of selenium *i.e.* selenite and consequent generation of red elemental selenium nanoparticles can be utilized to re-sensitize these nasty, pathogenic and highly resistant members of ESKAPE family. In contrast, eukaryotic mammalian cells generally circumvent this rather dangerous ‘‘zero

oxidation state'' of elemental selenium by reducing it to H_2Se and a variety of consequent products which, subsequently culminate either in selenium-based amino acids or are expelled in the form of excretory products, such as methyl-2-acetamido-2-deoxy-1-seleno-*β*-d-galactopyranoside, dimethyl selenide and trimethyl selenonium salts [66,111-113].

These results undeniably raise the concerns regarding the inherent toxic nature of these particles and this notion is sturdily authenticated by the presence of a particular protein coating around the particle, distinctive of the relevant bacteria employed for the generation of particles [114,115]. Interestingly, these biologically produced environment friendly, green in nature and red in colour, selenium particles exhibit excellent antimicrobial activities against a plethora of microorganisms, including Gram-Positive bacteria (*Staphylococcus carnosus*), Gram-negative bacteria (*Escherichia coli*), yeast (*Saccharomyces cerevisiae*) and also multicellular round worms representative of agricultural pests (*Steinernema feltiae*). Moreover, *Lactobacillus* species were employed to produce similar particles in milk supplemented with selenite, and the resulting dairy product was employed as a fortified cocktail for promoting health [110,116].

Nonetheless, there are some remaining mysteries which need to be resolved. The first, and perhaps the most compelling query is related to the mode of action of these particles, for instance, whether these particles follow pro-oxidant or antioxidant pathways. It can be proposed that these particles follow the footsteps of elemental selenium which serves as antioxidant at sub-nutritional and pro-oxidant at supra-nutritional doses [117]. Secondly, it remains to be demonstrated whether the selenium component, or the protein coating, is mainly involved in their most prominent biological activities. A comparative study reveals that biogenic SeNPs exhibited more antimicrobial activity compared to synthetic SeNPs which divulges the notion that proteins associated with the SeNPs contribute to the overall biological activities of biogenic SeNPs [118]. From a more pragmatic perspective, what would be the

practical applications of such particles? Pharmaceutical preparations in the form of anti-infective creams or lotions for dermal application provide a vast ground for the possible applications for such particles. Moreover, from the perspective of application in agriculture, selenium nanoparticles may be employed as (a) ‘‘green’’ phyto-protectants efficiently shielding against the attacks of certain pests, molds and other pathogens, (b) an essential trace element enhancing the strength and intrinsic resistance of crops against pathogenic attacks and, finally, (c) nutritional enrichment of crops with selenium which adds special value to the long-lasting food chain and subsequently to consumers *i.e.* most frequently human beings.

4.2. Selenocyanates: Inspired by Nature

Selenocyanates are selenium analogues of naturally occurring thio and isothiocynates in cruciferous vegetables [119]. Isothiocyanates and thiocyanates belong to the group of Reactive Sulfur Species (RSS), which are generally very reactive and electrophilic in nature. Naturally occurring (iso)thiocyanates are prominent for their interactions with the cysteine residues of proteins and enzymes of the cellular thiolstat [87]. Such an extensive “oxidative onslaught” in target microorganisms often causes a substantial increase in toxicity, even in otherwise resistant organisms [87,119-122]. A significant increase in chemical and biological (re)activity is observed for the selenium analogues of naturally occurring RSS. The idea of isosteric replacement of sulfur with selenium in naturally occurring RSS is quite fascinating from the perspective of small molecule drug design [123]. Intriguingly, literature reveals that isosteric replacement of sulfur with selenium in aromatic isothiocyanate significantly increases the sensitivity and activity of compounds towards thiols in proteins and thereby inducing higher levels of apoptosis [124].

Selenocyanates represent a very interesting class of organo-selenium compounds like its *iso* neighbour (*i.e.* isoselenocyanates) which is highly reactive. One of the most interesting

feature of selenocyanates is their ability to serve as multifunctional agent as these compounds are not only active on their own, they are also frequently metabolized to a wide range of several other RSeS, such as selenols, diselenides and seleninic acids [98,99,125-127]. Selenocyanates are not "outlandish" and - either directly or as metabolites - perform several important roles in Biology [128]. Nonetheless, there is a lack of detailed investigations.

In line with other organo-selenium compounds and RSeS, selenocyanates, prepared as a part of this thesis, demonstrated excellent antimicrobial, nematicidal and cytotoxic activities. The detailed studies disseminated in the previous sections stipulated innovative insights into the chemistry, pharmacokinetic behaviour and biological activities of these compounds, which may be valuable in the hunt for novel antimicrobial agents. Interestingly, most of the compounds employed, especially benzyl selenocyanate (**1**), demonstrated considerable activity against both Gram-positive and Gram-negative bacteria at significantly lower concentrations (*i.e.* 0.76 μg mL^{-1}). The antibacterial activity of benzyl selenocyanate (**1**) was comparable to or even higher than the ones of conventional antibiotics, such as piperacillin, oxacillin and ampicillin. The activity of some other compounds even surpassed the antibacterial effect of oxacillin, especially against the most dangerous, aggressive and drug-resistant pathogenic strains of *S. aureus*. Likewise, a significant activity was observed against rather challenging Gram-negative bacteria, such as *A. baumannii* and *P. aeruginosa*. In order to broaden the scope of antimicrobial activity, the compounds were evaluated for antifungal activity revealing certain levels of selectivity; some selenocyanates exhibited a relatively high fungicidal activity against infectious *C. albicans* and a low activity against baker's yeast *S. cerevisiae* which is good news for medical and culinary applications.

Detailed investigations are required to explicate the underlying mechanism(s) of action and to improve the chemical and biological activity, and hence, selectivity of selenocyanates. It can be primarily postulated that selenocyanates and their respective metabolites, *i.e.* selenides, diselenides and seleninic acids randomly interact with the cysteine proteins and enzymes and, thereby, disturb the cellular thiolstat of the target organism [87]. Such an arbitrary interaction is rather common within chalcogen redox chemistry and may also illuminate the capability of such electrophilic compounds to overpower different modes and mechanisms of resistance in the target organisms.

The scope of natural RSeS extends from simple elemental selenium nanoparticles produced naturally from microorganisms to the plethora of synthetic compounds inspired by nature, such as seleno-flavonoids, seleno-resveratrol and seleno-coumarines [129-133]. Selenocyanates and isoselenocyanates could now be considered as fascinating addition to the "*carte du jour*" of RSeS which are capable of breaching the mechanism(s) of resistance in harmful pathogens [134]. These compounds may spice up the future hunt for effective antibiotics inspired by natural chemistry and biology.

4.3. Selenazolinium salts: Simple yet effective

Moving from the selenium analogues of natural products to pure synthetic organo-selenium compounds, we come across a huge number of selenium-containing organic molecules. Despite an ever growing scientific interest in the development of organo-selenium compounds, which has resulted in such a massive number of selenium-containing compounds, there is only one selenium agent which has successfully crossed the milestone and entered clinical trials *i.e.* ebselen [27,135]. Ebselen chemically belongs to the class of selenazoles and has been reported to serve as multifunctional agent due to the broad range of applications

associated with ebselen ranging from simple detoxification to its efficacy in treatment of various diseases associated with vital organs, such as the nervous system and cardiovascular system [28]. Ebselen, therefore, serves as an excellent lead structure from the perspective of drug design and one of the many journeys for the hunt of ebselen-like selenium compounds paves the way towards the development of selenazolinium salts [73].

In line with other RSeS, the innovative class of selenazolinium salts demonstrated outstanding *in vitro* antibacterial activity against notorious ESKAPE pathogens. The compounds exhibited excellent potential against eleven strains of *S. aureus* and especially against the multidrug-resistant *Staphylococcus aureus* (MRSA) and vancomycin-intermediate *Staphylococcus aureus* (VISA) clinical isolates. Interestingly, and in contrast to ebselen, the selenazolinium salts demonstrated a significant antibacterial activity against Gram-negative ESKAPE bacteria, such as *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. coli*. Furthermore, safety of the compounds with regard to pharmaceutical applications was confirmed employing Ames mutagenicity assays. The difference in the rate and extent of (re)activity of different compounds towards specific or general cellular target(s) may explain the difference in the antimicrobial activities of ebselen and the selenazolinium salts. Generally, as mentioned earlier (section 4.1.), RSeS interact with the thiol groups of the cysteine residues of proteins and interrupt the cellular thiolstat by disturbing the intracellular levels of thiols and glutathione in the target microorganisms [87]. It would be interesting to investigate if these interactions are stoichiometric, or — as may also be expected from the activities even at lower concentrations — catalytic with respect to intracellular levels of ROS and thiols. It may also be postulated that the selenazolinium salts interact more than once with cellular proteins and enzymes, and that the most active species are not the salts themselves, and rather a highly reactive seleno-sulfide intermediate generated as a consequence of interactions of these salts with GSH.

Detailed future investigations focussed on exploring the mechanisms of actions may reveal further differences between ebselen, which is most frequently considered as an "antioxidant" promoting cell survival by decreasing intracellular ROS levels together with GSH, and these unique RSeS, which, owing to their relatively high(er) (re)activity, apparently interact with the thiol residues of cysteine proteins rather randomly and therefore modulate the level of GSH and also interfere with proteins and enzymes. In view of such remarkable properties, it can be concluded that the selenazolinium scaffold provides a prominent chemical moiety in the pursuit of novel selenium-containing drug molecules

4.4. Selenium based Multifunctional Redox catalysts

Oxidatively stressed cells are generally characterised by elevated levels of intracellular ROS when compared to normal cells [136]. In normal healthy cells, the intracellular levels of ROS are generally lower and any unusual upsurge in the levels of ROS even in low micromolar concentrations is indicative of diseases associated with OS [137,138]. This difference of intracellular ROS levels could be exploited to design agents with sensor and effector characteristics to serve as redox catalysts for selective targeting of oxidatively stressed cells [139-141]. Within the realm of redox catalysts, some members of RSeS, selenides and diselenides for instance, serve as excellent catalytic agents which can mimic the most prominent family of redox enzymes, such as glutathione peroxidase (GPx). Moreover, these selenium-based agents are not only extremely selective for their substrate(s) *i.e.* ROS, they are also highly effective, *i.e.* catalytic at low concentrations [139-141].

Several studies reinforce the notion of utilizing redox catalysts as drug molecules capable of sensing a specific (bio)chemical cellular ‘‘signature’’ as a substrate and subsequently unleashing their potential antioxidant or cytotoxic activity in response. Amongst these redox catalysts, selenium-based compounds which respond to a wide spectrum of ROS are of

particular significance, since these agents are able to sense the complicated intracellular redox status of the cell during OS. Another promising approach to tackle such a complex intracellular redox situation involves the introduction of multiple redox centres in the same molecule enabling these agents to interact with the broad range of intracellular ROS. The gamut of redox centres is not confined to the chalcogen family alone, it also contains benzoquinones, naphthoquinones, lapachones and some other ‘‘redox rogues’’ [142-144]. Then again, chalcogens enjoy special status in the list of redox catalysts as other ‘‘redox rogues’’ generally interact with cellular components and oxidize different targets, for example, membranes, metalloproteins and a variety of redox systems. Chalcogen-based catalysts, in contrast, demonstrate significant selectivity for thiols, thereby interacting specifically with the cellular thiolstat of the target organism by activating highly specific thiol related signalling or cascade pathways [87].

Several of these chalcogen-based multifunctional hybrid molecules comprising of two redox centres, *i.e.* chalcogen and redox modulating quinone were designed, synthesized and biologically evaluated by the research group of Prof. Jacob. Interestingly, these compounds served as excellent redox catalysts and initial studies revealed that the compounds were able to selectively target oxidatively stressed cells. The mechanistic studies subsequently unveiled the mode of action of these compounds and it was observed that these catalysts increase the level of OS in the target cells. These multifunctional redox catalysts were evaluated against a broad spectrum of microorganisms to extend the scope of applications of these compounds from cytotoxicity to antimicrobial activities. In line with the previous studies, the compounds exhibited excellent antimicrobial activities at concentrations even lower than established conventional antibiotics.

The concept of introducing multiple redox centres is not limited to the seleno-quinones but also includes compounds in which selenium is combined with other redox centres, such as

lapachones and products of Click Chemistry. From the perspective of medicinal chemistry and drug design, these compounds usher in an innovative generation of RSeS which provides plenty of impetus for an astounding synthetic chemistry especially in this era of selenium chemistry.

5. Conclusions

The present study revolves around demonstrating the synthesis and biological significance of selected organo-selenium compounds to emphasise the implication of the emerging concept of Reactive Selenium Species (RSeS). Indeed, the results obtained as part of this thesis confirm the significance of this concept and its applications in various fields, such as synthetic and medicinal chemistry, redox biology, pharmacology, biochemistry and medicine. The concept of RSeS is currently in its primitive form and is expected to manifest itself fully in the near future. This is, of course, possible not only by following the footsteps of Reactive Sulfur Species (RSS) due to the inherent analogy of sulfur with selenium, but may also move a few steps further due to the highly nucleophilic nature of selenium when compared to sulfur.

Although there is growing interest in the hunt for the hidden treasures of naturally occurring selenium-based organo-molecules, the expedition is challenged by certain factors, such as lower or even trace amounts of such species, crude detection methods, limitation of the available analytical techniques or merely due to the presence of these species in areas which no “fortune hunter” has reached so far. At the same time, there is also an increasing interest in the synthesis of organo-selenium compounds for potential applications in Medicine.

The scope of RSeS extends from simple elemental selenium nanoparticles (SeNPs) to rather complex multifunctional selenium-based redox catalysts equipped with sensor / effector properties capable of targeting cells with redox imbalances selectively and effectively. Irrespective of the method of generation (*i.e.* chemically, biologically or mechanically), SeNPs have been employed for a broad range of biological and medical

applications as antimicrobial, antiviral, antioxidant, immuno-modulatory, cytoprotective and cytotoxic agents [118,145-147]. The range of applications for SeNPs is not restricted to medicine only, yet it also involves nutrition and agriculture [70,148-150]. Naturally or biologically produced SeNPs, which are generated by employing certain microorganisms, are generally coated with proteins which may not only interfere with the biological activities of these particles, they may also serve as stabilizers, and as surfactants to avoid agglomeration of these particles, which is important from the perspective of dosage form development. The nature, type, biochemical composition and biological implications of these coating proteins provide further grounds for detailed investigations.

Moving from naturally produced SeNPs to the selenium analogues of natural products, selenocyanates represent a class of very simple and often overlooked species. Selenocyanates are highly effective selenium-based organic compounds which are closely related to the isothiocyanates found in cruciferous species. Selenocyanates synthesized as a part of this thesis demonstrated excellent antimicrobial, nematicidal and cytotoxic activities especially against the resistant strains. Moreover, the compounds successfully endured various assays concerning the safety and stability. Detailed investigations are required to unveil the underlying mode of action of these highly reactive RSeS. The interesting biological effects of these organo-selenium compounds could be explained by interactions of these species themselves or their metabolites, such as diselenides or seleninic acids, or combinations of both, with the components of the cellular thiostat of the target (micro)organisms. There are several other examples where selenium analogues of certain natural products have been prepared and biologically evaluated, such as resveratrol, coumarins and flavonoids. Efficiency of these RSeS against a broad spectrum of biological targets confirms the

significance of the notion of RSeS and provides opportunities for the addition of selenium-based spices, such as red pepper, cumins or curcuma to the existing menu of RSeS.

Apart from selenium analogues of natural products, the growing interest expands to include the synthesis of organo-selenium compounds from the perspectives of pure synthetic and medicinal chemistry. This has already resulted in a plethora of such agents and some of them, such as ebselen, have been successful enough to enter clinical trials. Ebselen, therefore, serves as an excellent lead structure for the design and synthesis of selenium-based agents. Selenazolinium salts represent one of the most prominent class of compounds with ebselen-like chemical structures. These members of RSeS are characterized by the presence of selenazole ring and a positive charge on the selenium attributing more nucleophilic character to these compounds, also when compared to ebselen. Selenazolinium salts demonstrated excellent antimicrobial activity especially against the resistant strains of bacteria. As expected, significant differences remain in some of the biological activities of selenzolinium salts and ebselen, which beg the question of scientific exploration in greater detail employing certain tools of intracellular diagnostics. An overall remarkable biological activity associated with selenazolinium salts clearly indicates that selenazolinium motif, in the near future, could be employed in the design of efficient RSeS. Moreover, these RSeS may also be considered for the next steps of drug development. A thorough and comprehensive understanding of the modes and mechanisms of action related to selenazolinium salts is required to expand the scope of applications of these agents. Furthermore, the Se–N moiety may be more tuned and “sharpened” to increase the (bio)chemical (re)activity and selectivity of these compounds.

Another interesting approach for enhancing the selectivity and biological activity of the organo-selenium compounds involves the introduction of another redox centre, besides selenium, in the same molecules. The last couple of decades has witnessed a significant interest in the design, synthesis and biological evaluation of catalytic RSeS, especially the ones equipped with ‘‘sensor/effector’’ features. Synthetic medicinal chemistry of organo-selenium compounds continues to move forward and provides advances towards the synthesis of multifunctional redox catalysts, which were primarily synthesized following very simple and rather straightforward nucleophilic displacement reactions of diphenyl diselenide with halogenated quinones. Other sophisticated approaches involve the coupling of two or three redox modulating components in linear and/or sequential fashion, multi-component reactions, such as Passerini and Ugi reactions, and last but not the least, Click reactions to merge the most appropriate redox motifs to produce RSeS with desired characteristics.

The interest in the emerging concept of RSeS is fuelled by novel discoveries which unveil the significant and facet-rich medicinal and biological applications of these species especially against targets which are difficult to tackle, such as multi-drug resistant bacteria and cancer cells.

Although the concept of RSeS is rather vague now, it provides guidance and direction for both the current and future “pedigree” of selenium researchers and scientists or even for some “Selenists”. Moreover, the concept of RSeS also paves the way for research on the very special “red carpet” of selenium-based naturally occurring and chemically synthesized, organic and inorganic compounds with significant biological relevance. Finally, the natural or naturally inspired RSeS constitute a very peculiar niche of redox biology, whilst the synthetic RSeS furnish leads for unique and novel drug molecules, yet the pursuit kind of lingers to eternity and the scientific toil should never stop.

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7. Supplementary Material :

7.1. Supplementary material for Publication 1: Pronounced activity of aromatic selenocyanates against multidrug resistant ESKAPE bacteria.

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Electronic Supplementary Information

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General procedure for the synthesis of selenocyanates

Selenocyantes were synthesized using the general protocol described by Wheeler and Merriam with some modifications.[1] According to the procedure, alkyl halides (10-20 mmol) were treated with KSeCN (12-25 mmols) in the presence of ethanol (10-20 mL). The reaction mixture was refluxed for 6 h and the progress of the reaction was monitored periodically by Thin Layer Chromatography (TLC). After the completion of the reaction, the inorganic salt was separated by filtration and the filtrate was heated with charcoal. The reaction mixture was filtered hot and the filtrate was left for cooling. On cooling, the solution yielded crystals which were separated by filtration. TLC was performed to evaluate the purity of the compound. Once purified, the samples were analysed using Mass Spectroscopy (MS) and Nuclear Magnetic Resonance (NMR) for structural confirmations as well as purity. Synthesis and chemical characteristics of compounds **1**, **3**-**7** and **10**-**12** have been described before and our values are in agreement with the reported values [2-4].

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Synthesis of Benzyl selenocyanate (1)

Benzyl bromide (1.71 g, 10 mmol), KSeCN (1.73 g. 12 mmol) and ethanol (10 mL) were employed. The compound (**1**) was obtained as light crystals after purification by recrystallization with ethanol. Yield 72.5 % (1.43 g, 7.25 mmol). m.p.= 71-72 °C, TLC R*f* (DCM, 100 %): 0.64, ^{1}H NMR (DMSO-d_6, ppm): δ 7.36(m, 3H, 3C-H), 7.35 (m, 2H, 2 C-H), 4.30 (t, *J*=9.15 Hz, 2H, CH_2). ^{13}C NMR (DMSO-d_6, ppm): δ 138.79, 129.32 (2C), 129.04 (2C), 128.26,105.36(Se-CN), 33.08. LC–MS: purity 100 %, t_R = 5.52, (ESI) m/z: calculated for C_8H_7NSe $[M+H]^+$. 91.05, found: 91.00.

Synthesis of 3-Methylbenzyl selenocyanate (3)

3-Methylbenzyl chloride (2.812 g , 20 mmol) , KSeCN (3.6 g. 25 mmol) and ethanol (20 mL) were employed. The compound (**3**) was obtained as light crystals after purification by recrystallization with ethanol. Yield 83.5 % (3.51 g, 16.7 mmol). m.p.= 55.5-56.5 °C, TLC R*f* (DCM, 100 %): 0.65, ^{1}H NMR (DMSO-d_6, ppm): δ 7.23(m, 1H, CH), 7.16 (m, 2H, 2CH), 7.11 (m, 1H, CH), 4.26 (t, *J*=9.10 Hz, 2H, CH_2), 3.24 (s, 3H, CH_3). ^{13}C NMR (DMSO-d_6, ppm): δ 138.60, 138.15, 129.82 (2C), 128.93 (2C), 126.42, 105.34 (Se-CN), 33.09, 21.42. LC–MS: purity 98.57 %, t_R = 6.24, (ESI) m/z: calculated for C_9H_9NSe $[M+H]^+$: 105.07, found: 105.02.

Synthesis of 4-trifluoromethylbenzyl selenocyanate (4)

4-Trifluoromethylbenzyl bromide (4.73 g, 20 mmol), KSeCN (3.6 g. 25 mmol) and ethanol (20 mL) were employed. The compound (**4**) was obtained as light crystals after purification by recrystallization with ethanol. Yield 81.75% (4.32 g, 16.35 mmol). m.p.= 54-55 °C, TLC R*f* (DCM, 100%): 0.94, ^{1}H NMR (DMSO-d_6, ppm): δ 7.64 (d, *J*=8.21 Hz, 2H, 2 C-H), 7.42(d, *J*=8.21 Hz, 2H, 2 C-H), 4.02 (t, *J*=8.21 Hz, 2H, CH_2). ^{13}C NMR (DMSO-d_6, ppm): δ 144.67, 130.06, 128.07, 127.64, 125.56, 110.00, 30.87. LC–MS: purity 95.66 %, t_R = 9.82, (ESI) m/z: calculated for $C_9H_6F_3NSe$ $[M+H]^+$: 159.04, found: 159.04.

Synthesis of 4-fluorobenzyl selenocyanate (5)

4-Fluorobenzyl chloride (2.89 g, 20 mmol), KSeCN (3.6 g. 25 mmol) and ethanol (20 mL) were employed. The compound (**5**) was obtained as light crystals after purification by recrystallization with ethanol. Yield 83.45% (3.57 3g, 16.69 mmol). m.p.= 64-65 °C, TLC R*f* (PE:EA; 4:1) : 0.60, ^{1}H NMR (DMSO-d_6, ppm): δ 7.25 (m, 2H, 2 CH), 7.12 (m, 2H, 2 C-H), 3.92 (t, *J*=7.62 Hz, 2H, CH_2). ^{13}C NMR (DMSO-d_6, ppm): δ 163.30, 160.07, 135.94, 131.29, 115.43, 30.93. LC–MS: purity 96.30 %, t_R = 9.06, (ESI) m/z: calculated for C_8H_6FNSe $[M+H]^+$: 109.05, found: 109.00.

Synthesis of 2-fluorobenzyl selenocyanate (6)

2-Fluorobenzyl chloride (2.892g, 20 mmol), KSeCN (3.6g. 25 mmol) and ethanol (20 mL) were employed. The compound (**6**) was obtained as light crystals after purification by recrystallization. Yield 75.45% (3.231g, 15.09 mmol). m.p.= 48-50 °C, TLC R*f* (DCM, 100%): 0.53, ^{1}H NMR (DMSO-d_6, ppm): δ 7.44 (m, 1H, 1 CH), 7.36 (m, 1H, 1 CH), 7.21(m, 2H, 2 CH), 4.33 (t, *J*=9.14 Hz, 2H, CH_2). ^{13}C NMR (DMSO-d_6, ppm): δ 161.13, 159.16, 131.56, 130.16, 124.51, 115.59, 104.39 (Se-CN), 25.37. LC–MS: purity 96.30 %, t_R = 9.06, (ESI) m/z: calculated for C_8H_6FNSe $[M+H]^+$: 109.05, found: 109.00.

Synthesis of 4-chlorobenzyl selenocyanate (7)

4-Chlorobenzyl bromide (2.673g, 16.6 mmol), KSeCN (2.8 g, 19.4 mmol) and ethanol (20 mL) were employed. The compound (**7**) was obtained as light crystals after purification by recrystallization with ethanol. Yield 85.24% (3.263g, 14.15 mmol). m.p.= 58-59 °C, TLC R*f* (DCM, 100%): 0.75, ^{1}H NMR (DMSO-d_6, ppm): δ 7.34 (d, *J*=8.79 Hz, 2H, 2 CH), 7.25 (d, *J*=8.21 Hz, 2H, 2 CH), 3.93 (t def., 2H, CH_2). ^{13}C NMR (DMSO-d_6, ppm): δ 138.78, 131.96, 131.15, 128.72, 30.87. LC–MS: purity 99.35 %, t_R = 9.99, (ESI) m/z: calculated for C_8H_6ClNSe $[M+H]^+$: 125.02, found: 125.02.

Synthesis of 4-bromobenzyl selenocyanate (10)

4-Bromobenzyl bromide (5 g, 20 mmol), KSeCN (3.6 g. 25 mmol) and ethanol (20 mL) were employed. The compound (**10**) was obtained as light crystals after purification by recrystallization with ethanol. Yield 62.15% (3.417 g, 12.43 mmol). m.p.= 102-103 °C, TLC R*f* (DCM, 100%): 0.86, ^{1}H NMR (DMSO-d_6, ppm): δ 7.47 (d, *J*=8.21 Hz, 2H, CH), 7.18 (d, *J*=8.21 Hz, 2H, CH), 3.91 (t, *J*=7.62 Hz , 2H, CH_2). ^{13}C NMR (DMSO-d_6, ppm): δ 139.18, 131.65, 120.46, 30.93. LC–MS: purity 100 %, t_R = 10.23, (ESI) m/z: calculated for C_8H_6BrNSe $[M+H]^+$: 168.97, found: 168.94.

Synthesis of 4-nitrobenzyl selenocyanate (11)

4-Nitrobenzyl chloride (3.432 g, 20 mmol), KSeCN (3.6 g. 25 mmol) and ethanol (20 mL) were employed. The compound (**11**) was obtained as light crystals after purification by recrystallization with ethanol. Yield 72% (3.471g, 14.4 mmol). m.p.= 99-100 °C, TLC R*f* (DCM, 100%): 0.50, ^{1}H NMR (DMSO-d_6, ppm): δ 8.21 (m, 2H, CH), 7.63 (m, 2H, CH), 4.39 (t def, 2H, CH_2). ^{13}C NMR (DMSO-d_6, ppm): δ 146.97, 130.57, 124.26, 105.25, 31.61. LC–MS: purity 97.77%, t_R = 5.30, (ESI) m/z: calculated for $C_8H_6N_3O_2Se$[M+H]$^+$: 136.04, found: 135.98.

Synthesis of 2-(selenocyanatomethyl) naphthalene (12)

2- Chloromethyl naphthalene (2.51g, 14.2 mmol), KSeCN (2.6 g. 18 mmol) and ethanol (14 mL) were employed. The compound (**12**) was obtained as light crystals after purification by recrystallization wit ethanol. The compound was obtained in 81.27 % yield (2.85 g, 11.54 mmol). m.p.= 119-120 °C, TLC R*f* (DCM, 100%): 0.77, ^{1}H NMR (DMSO-d_6, ppm): δ 7.92 (m, 4H, CH), 7.53 (m, 3H, CH), 4.49 (t, *J*=9.14 Hz, 2H, CH_2). ^{13}C NMR (DMSO-d_6, ppm): δ 135.76, 132.65, 132.36, 128.40, 127.76, 127.60, 127.38, 126.89, 126.49, 126.33, 104.94 (SeCN), 33.12. LC–MS: purity 97.56%, t_R = 6.73, (ESI) m/z: calculated for $C_{12}H_9NSe$ $[M+H]^+$: 141.07, found: 141.03

Crystallographic Studies

The crystallographic studies have been performed to provide evidence about the stability of the arylmethylselenocyanates when exposed to different solvents and temperature, considering a potential reactivity of the selenocyanate moiety. The molecular structures and atomic-numbering schemes of **1** and **12** are presented and described in main article (Figure 2). Parameters of intermolecular C-H.···N interactions for **1** and **12** are shown in Table S1. The geometries of the methyleneselenocyanate groups in both compounds differ slightly. The responsible angles have values C1-Se1-C2 = 95.2° and 95.5°, Se1-C2-C3 = 113.7° and 114.3°, C1-Se1-C2-C3 = −62.4° and -66.5°, Se1-C2-C3-C4 = 98.6° and 98.8°, whereas the torsion angle N1-C1-Se1-C2 exhibits a significant difference of −164.4° and 67.3° for compound **1** and **12**, respectively. Methylselenocyanate fragments were also searched for in the Cambridge Structural Database (CSD, Version 5.37), which resulted in five crystal structures, in one case two independent molecules.[5] In these crystal structures, the values of torsion angles N1-C1-Se1-C2 were 66.8°, −170.9°, −139.5°, 29.4°, −131.7° and −173.1°.

Table S1. Parameters of intermolecular C-H.···N interactions for **1** and **12**.

Cpd	D-H.···A	H···A (Å)	D. ···A (Å)	D-H···A (°)	Symmetry Codes
1	C2-H2A···N1	2.59	3.559(3)	174	−x, −y, −z
	C2-H2B···N1	2.73	3.328(3)	119	x + 1, y, z
	C6-H6···N1	2.84	3.513(3)	128	−x, y − 1/2, −z + 1/2
12	C2-H2A···N1	2.58	3.487(2)	155	x + 1/2, −y + 1, −z
	C2-H2B···N1	2.76	3.333(1)	118	x, y − 1, z
	C8-H8···N1	2.75	3.520(2)	141	x − 1/2, y − 1/2, z − 1/2

Ames fluctuation assay

Compounds **1**, **2** and **13** were also non-mutagenic at the higher concentration of 10 μM which was employed in most of the assays. Only compound **4**, at the higher concentration (10 μM), exhibited an increased binomial B–value (B = 1.0), which may point towards a probable mutagenic potential. The mutagenicity of this derivative is rather ambiguous, as the value of the second parameter indicative of mutagenicity (MI = 1.75) was still below the threshold of 2.0, and substantially lower compared to the MI value for the mutagenic reference NQNO with a MI = 6.91 calculated at a concentration of 0.5 μM (Figure 1).

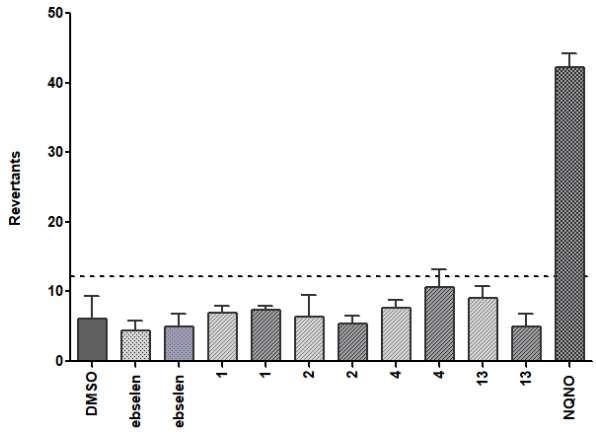


Figure 1. Results of the Ames liquid microtitre test indicative of mutagenic potential; DMSO (1 % in growth medium)-negative control, ebselen (reference compound) at the concentration 1 µM and 10 µM, NQNO (4-nitroquinoline-*N*-oxide, benchmark mutagenic agent) at concentration 0.5 µM; **1, 2**, **4** and **13** — selenocyanates at concentrations 1 µM and 10 µM, ----- baseline defining the mutagenicity threshold (significant mutagenicity above this line).

***In Vitro* PAMPA Permeability.**

The results calculated for compound **13** may be ambiguous due to the instability of the compound in phosphate buffered saline (pH 7.4), as around 50 % decomposition was determined by controlled LC-MS analysis (data not shown).

Table 2. ADMET properties of the most active compounds (**1**, **2**, **4**, **13**).

ADME–Tox Properties					
	Mutagenic Potential				PAMPA-Permeability
Compounds	MI (1 µM)	B	MI (10 µM)	B	Kp (cm s^{-1})
1	1.15	0.74	1.20	0.81	2.69×10^{-6}
2	1.04	0.56	0.87	0.28	3.17×10^{-6}
4	1.25	0.87	1.75	1.0	2.57×10^{-6}
13	1.47	0.96	0.82	0.14	2.25×10^{-6}
Ebselen	0.69	0.26	0.80	0.46	nd
Caffeine	nd	nd	Nd	nd	3.61×10^{-6}
Norfloxacin	nd	nd	Nd	nd	0.95×10^{-6}

Ebselen - selenium reference compound in Ames test. Reference compounds in PAMPA: high-permeable drug, caffeine; low-permeable drug, norfloxacin. MI - mutagenic index (the quotient of the number of revertant colonies induced in a test sample and the number of revertants in a negative control). B - Binomial B value, nd not determined; *K*p - permeability coefficient.

Notes and references

1 1. H. L. Wheeler and H. F. Merriam, *Journal of the American Chemical Society*, 1901, **23**, 283-299.

2 2. D. Plano, Y. Baquedano, D. Moreno-Mateos, M. Font, A. Jimenez-Ruiz, J. A. Palop and C. Sanmartin, *Eur J Med Chem*, 2011, **46**, 3315-3323.

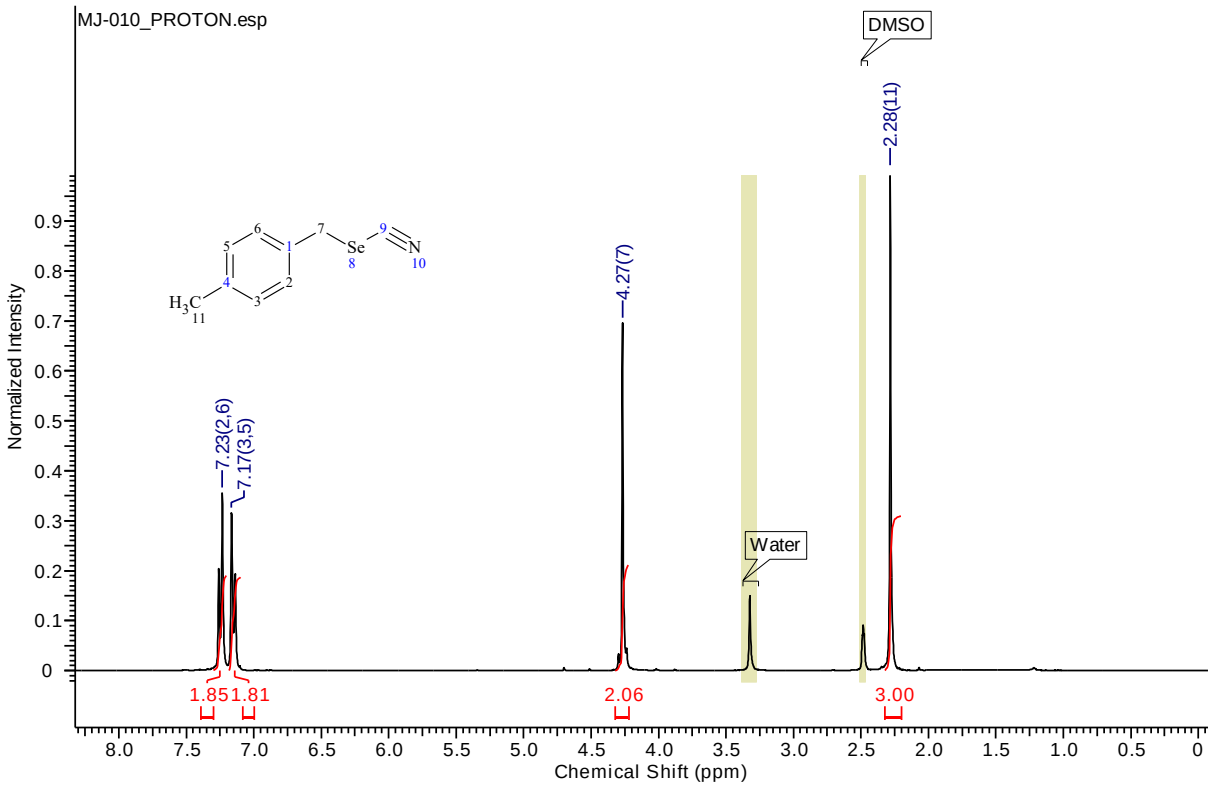
3 3. H. Suzuki, M. Usuki and T. Hanafusa, *Synthesis*, 1979, **1979**, 705-707.

4 4. L. A. Jacob, B. Matos, C. Mostafa, J. Rodriguez and J. K. Tillotson, *Molecules*, 2004, **9**, 622-626.

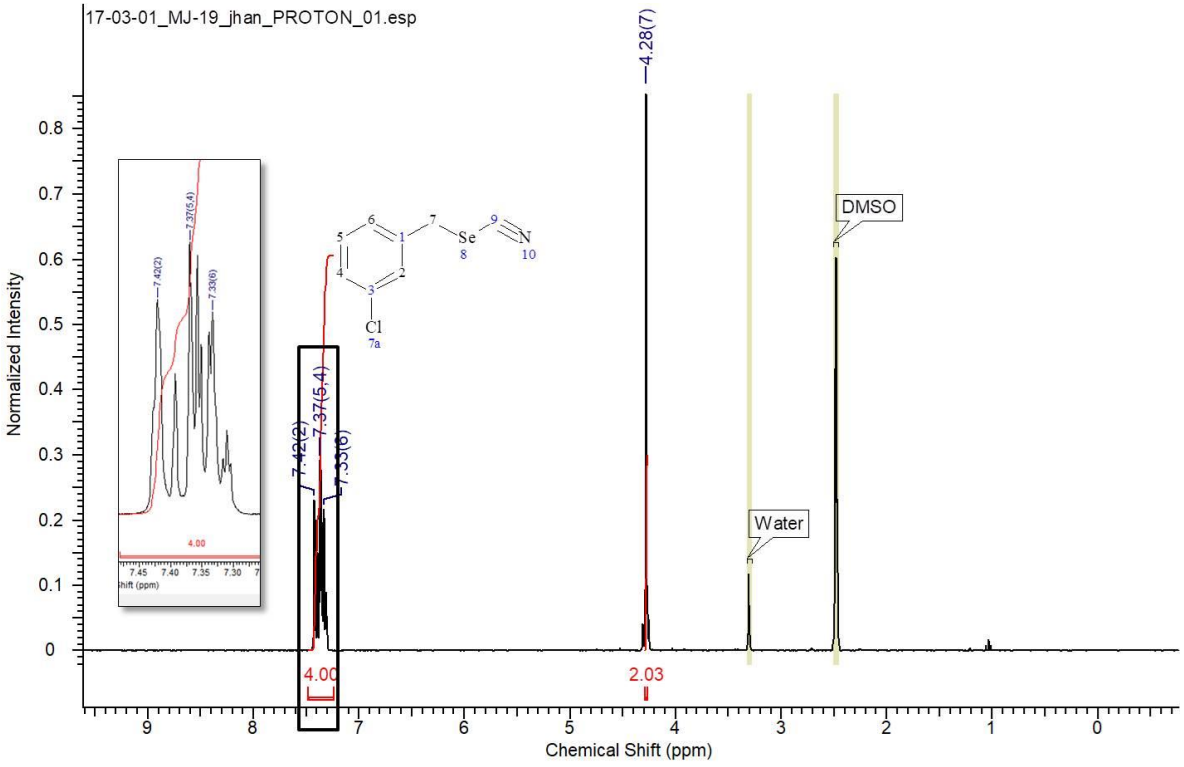
5 5. C. R. Groom, I. J. Bruno, M. P. Lightfoot and S. C. Ward, *Acta Crystallographica Section B-Structural Science Crystal Engineering and Materials*, 2016, **72**, 171-179.

^{1}H NMRs of Novel Compounds

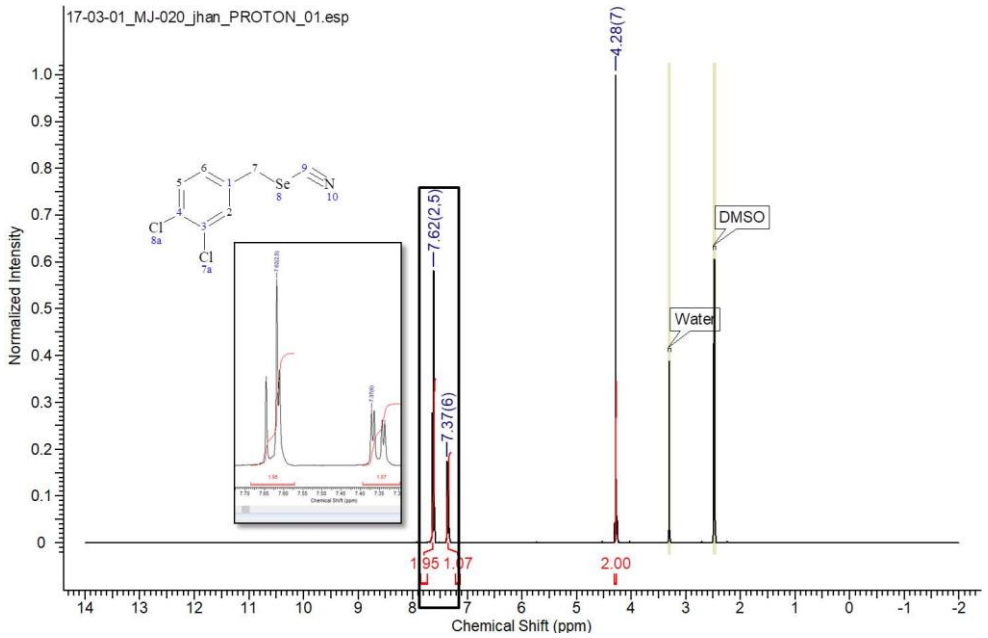
Compound 2



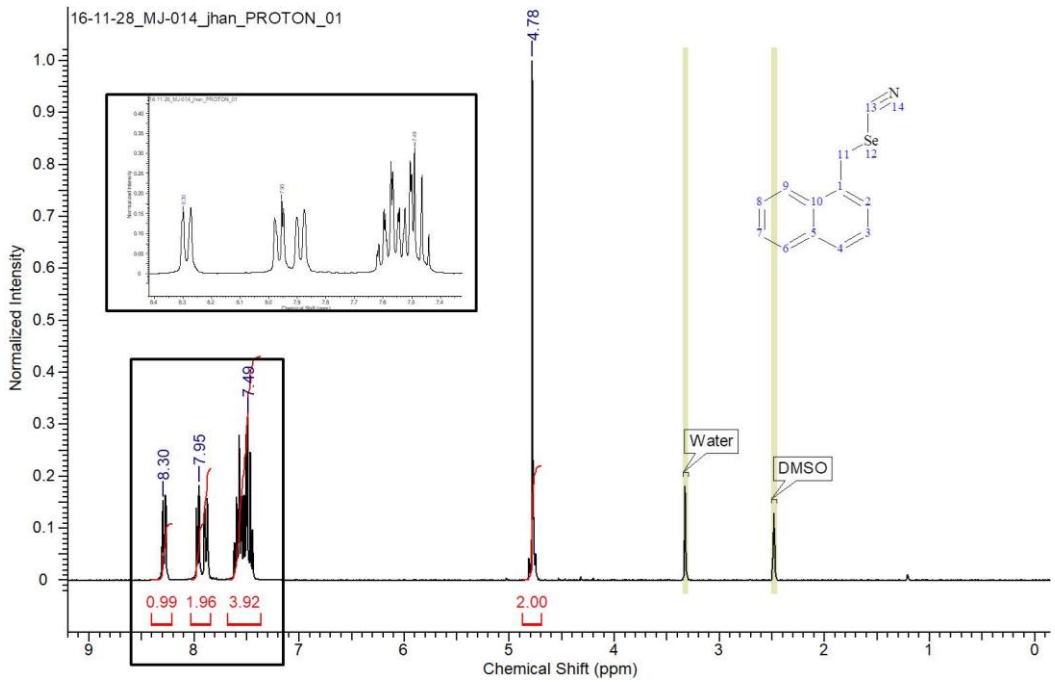
Compound 8



Compound 9

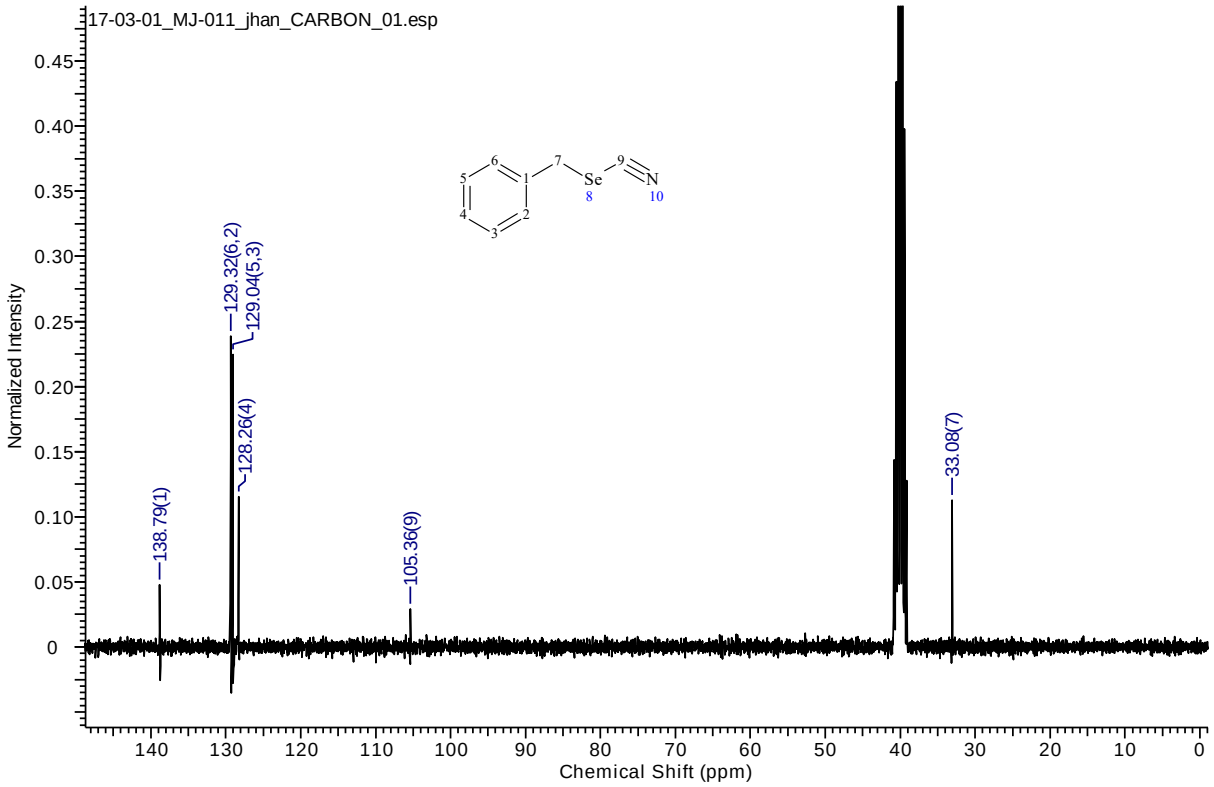


Compound 13

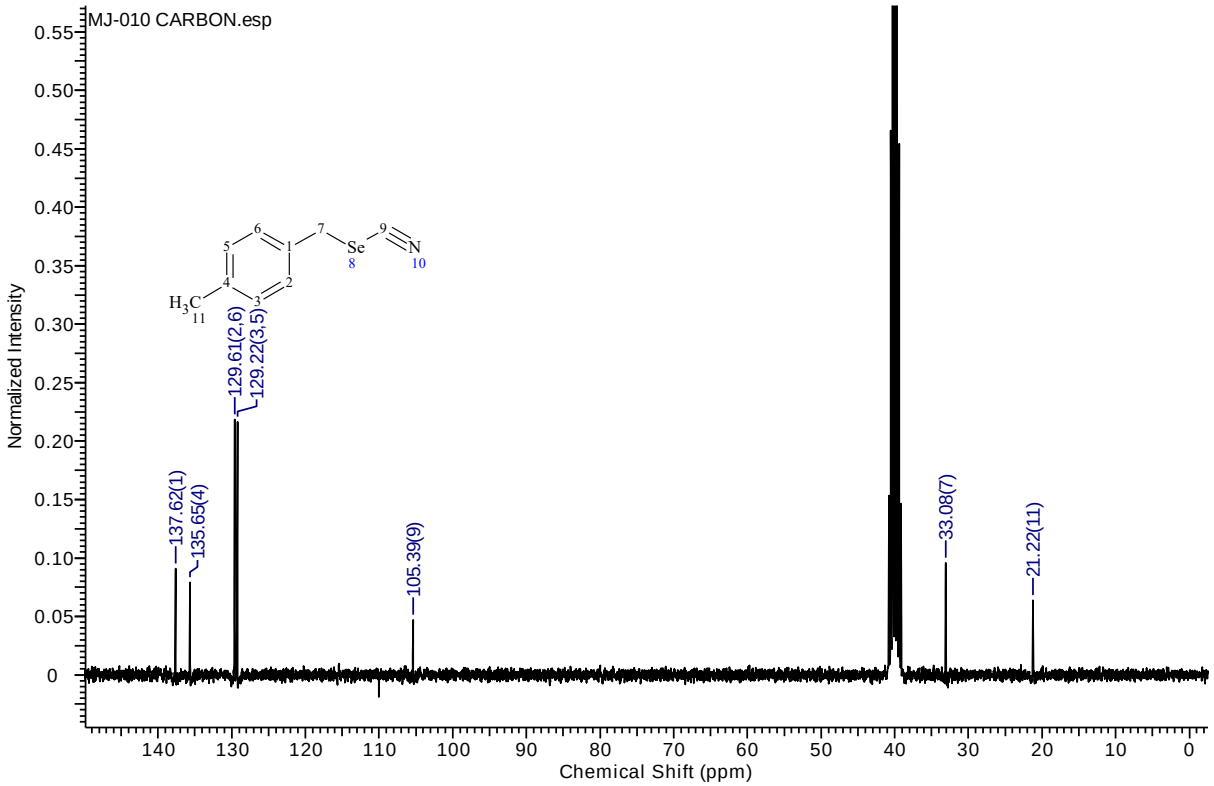


13 C NMRs of synthesized compounds

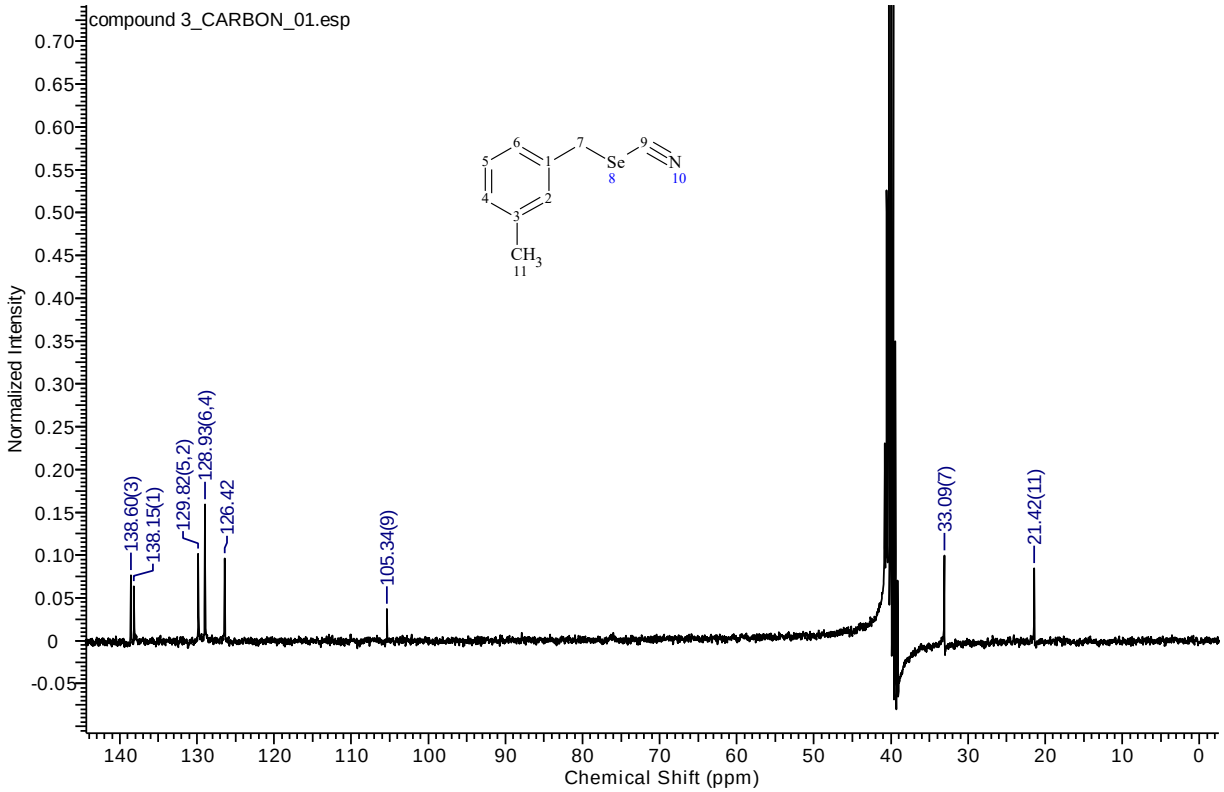
Compound 1



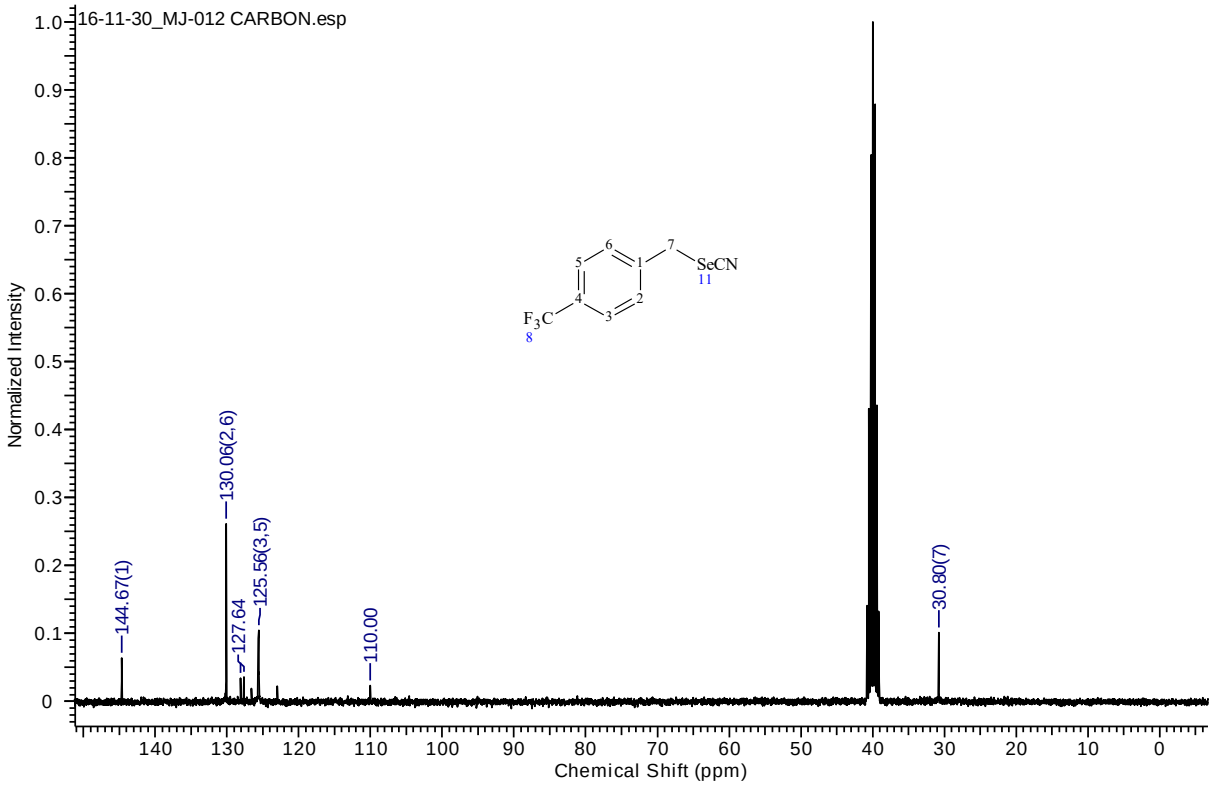
Compound 2



Compound 3



Compound 4



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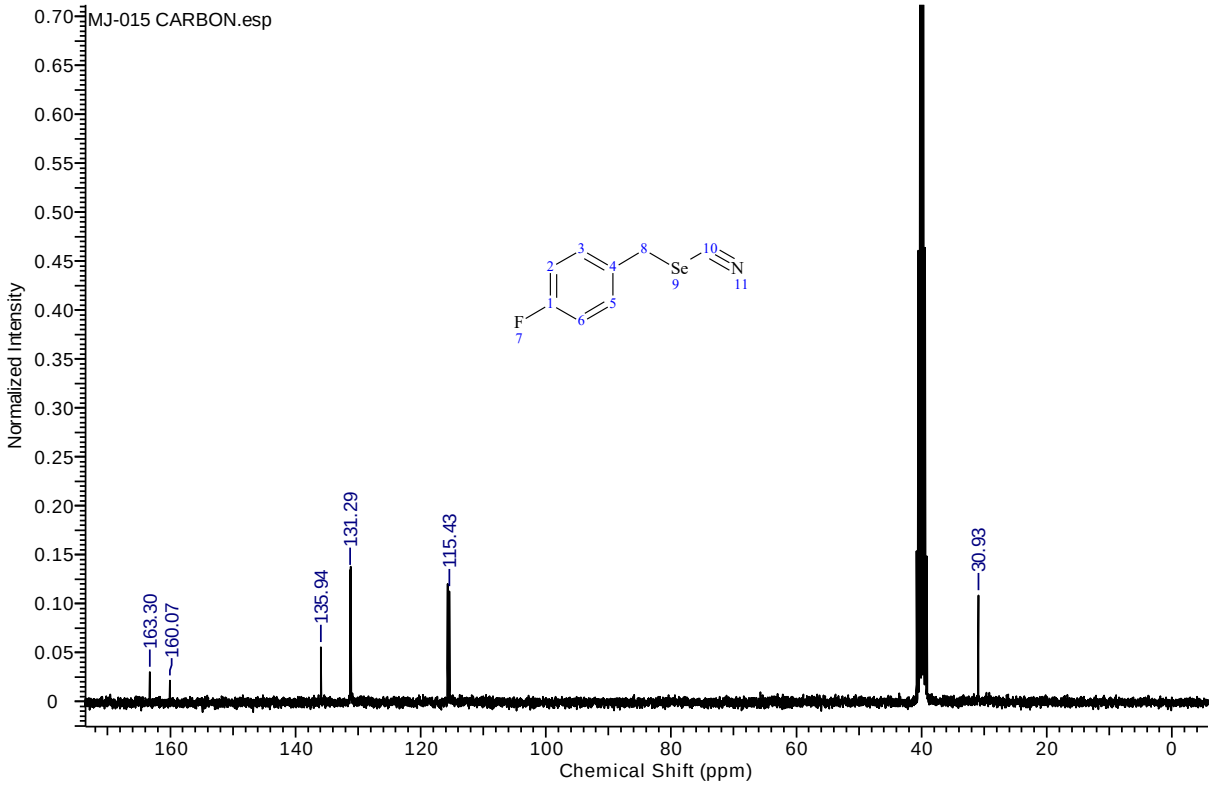
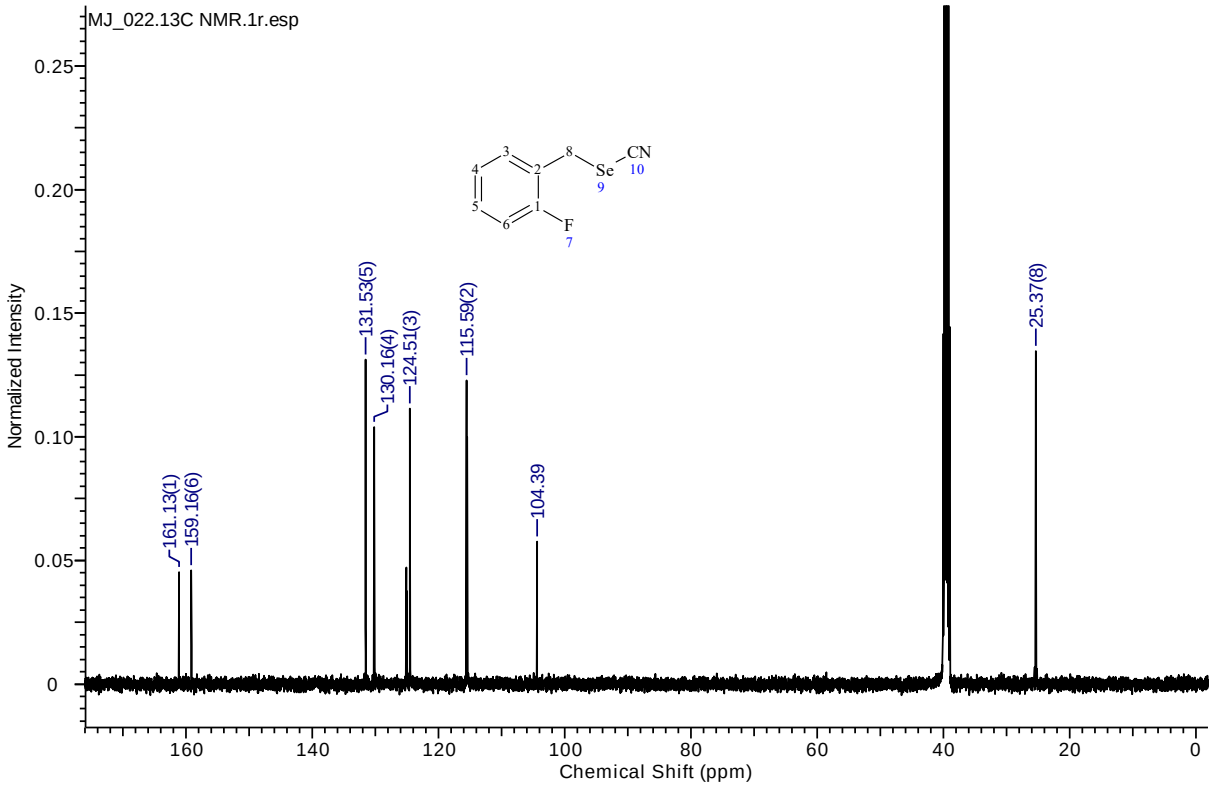


Compound 6



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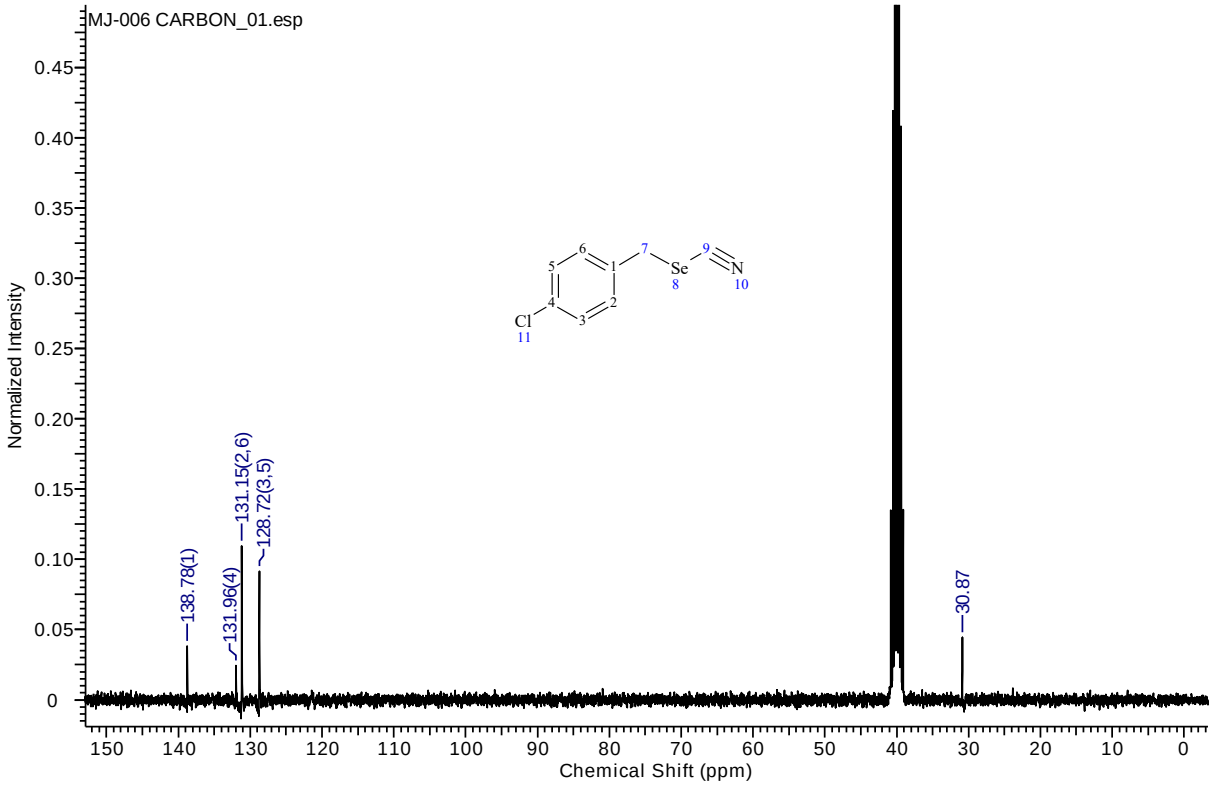
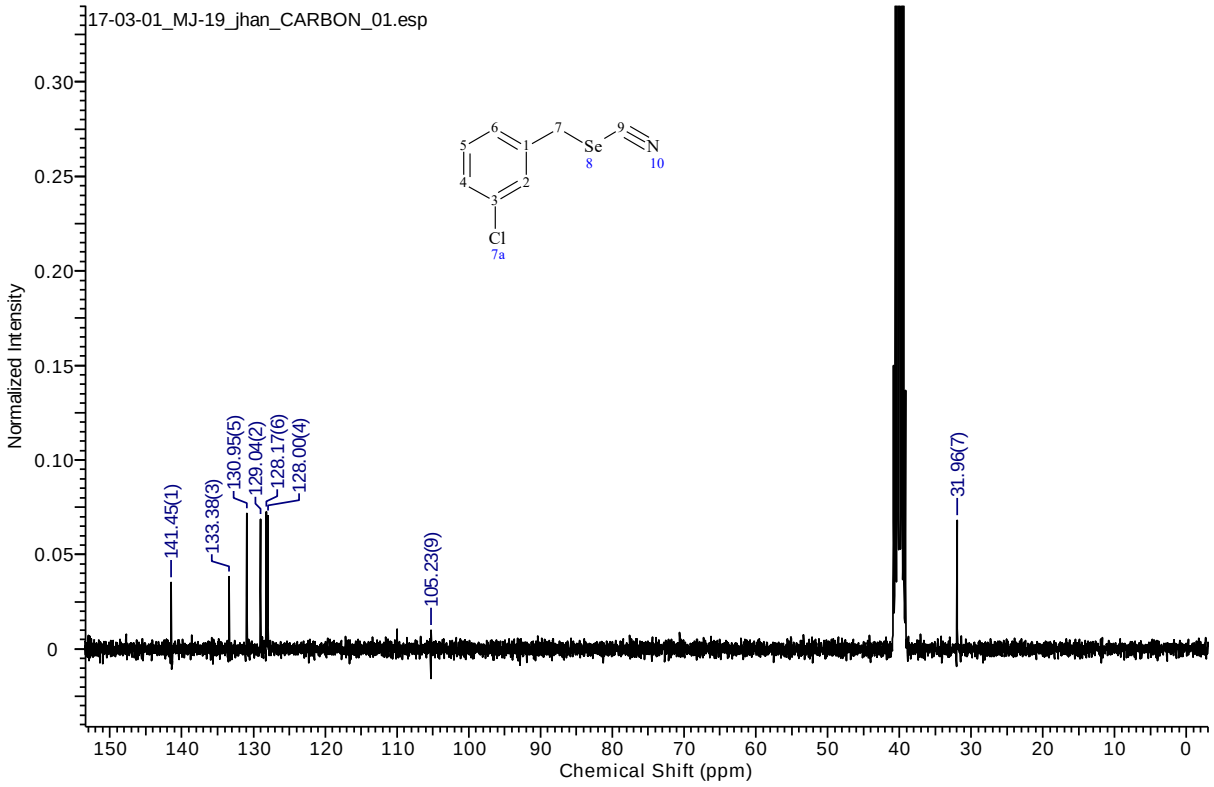
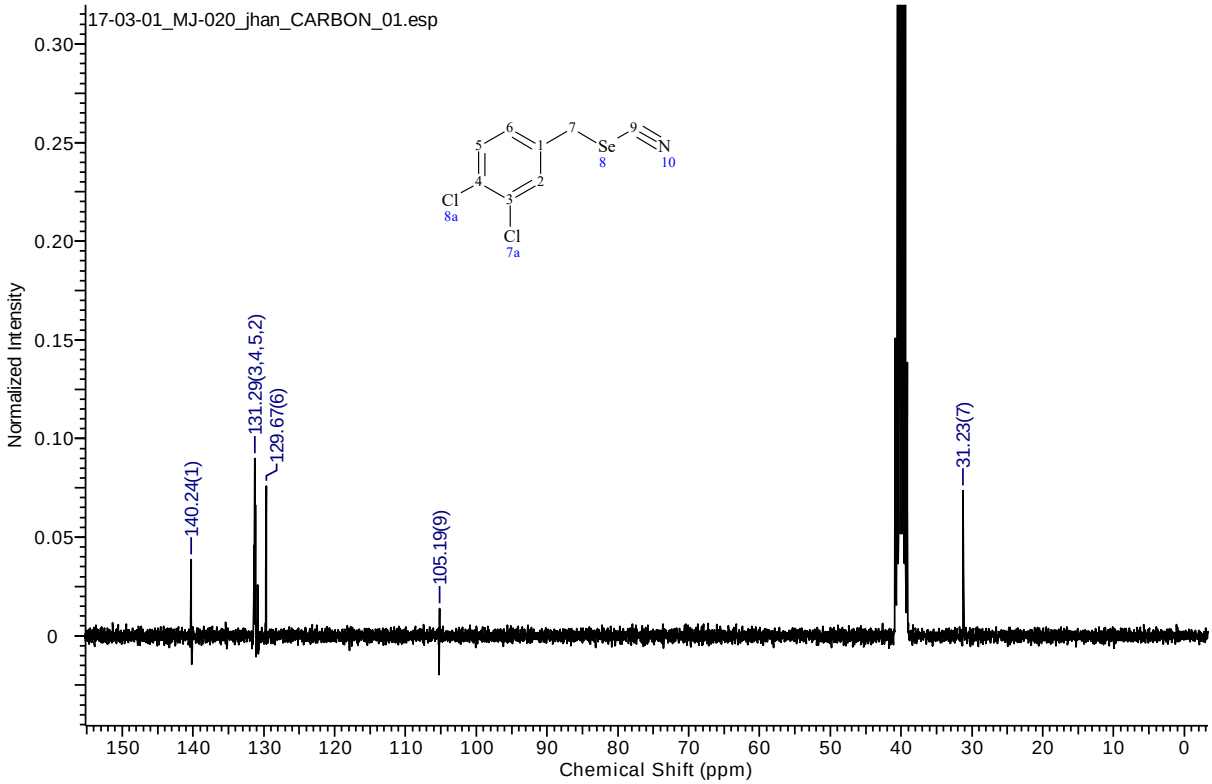


Compound 8



Compound 9



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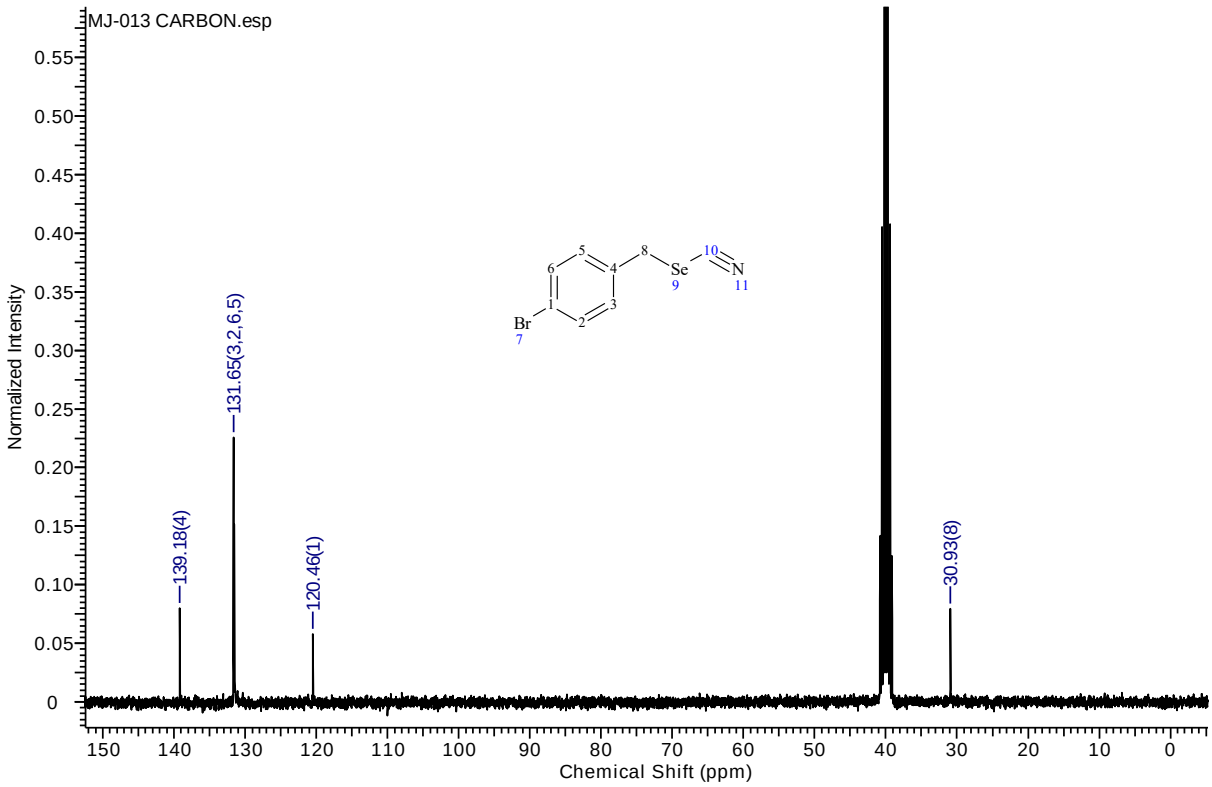

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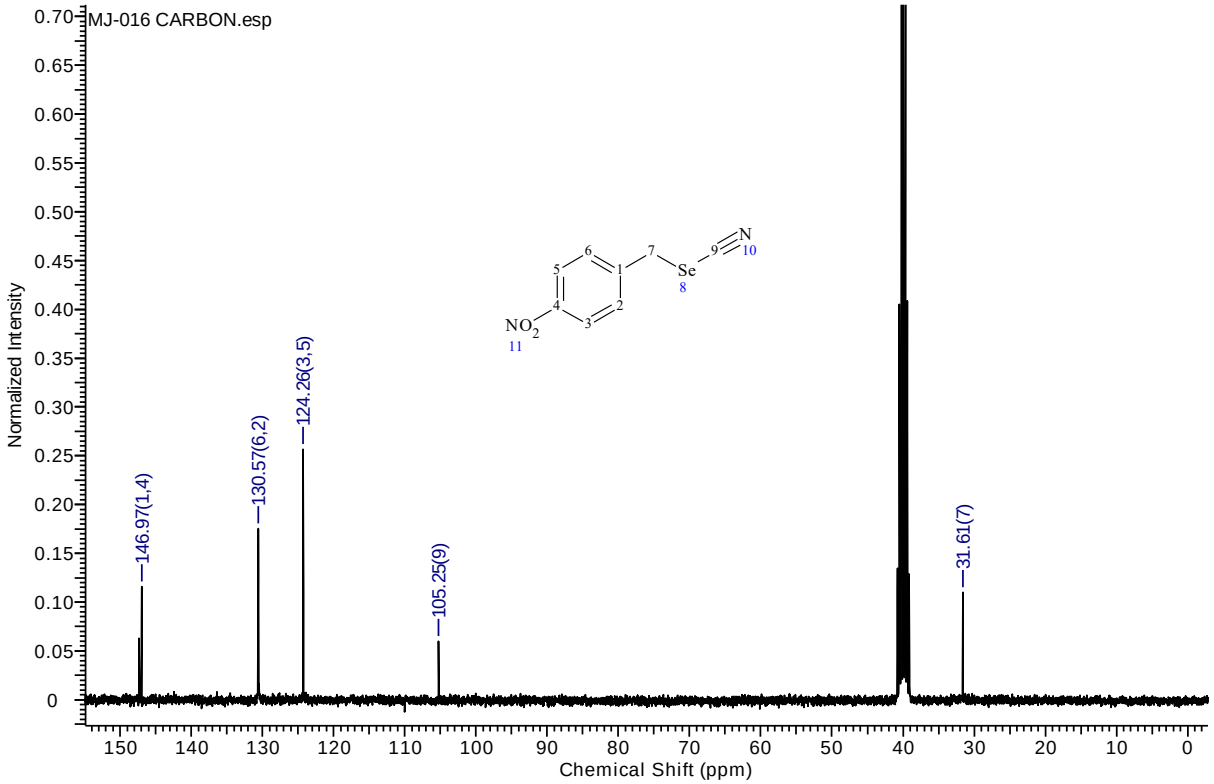

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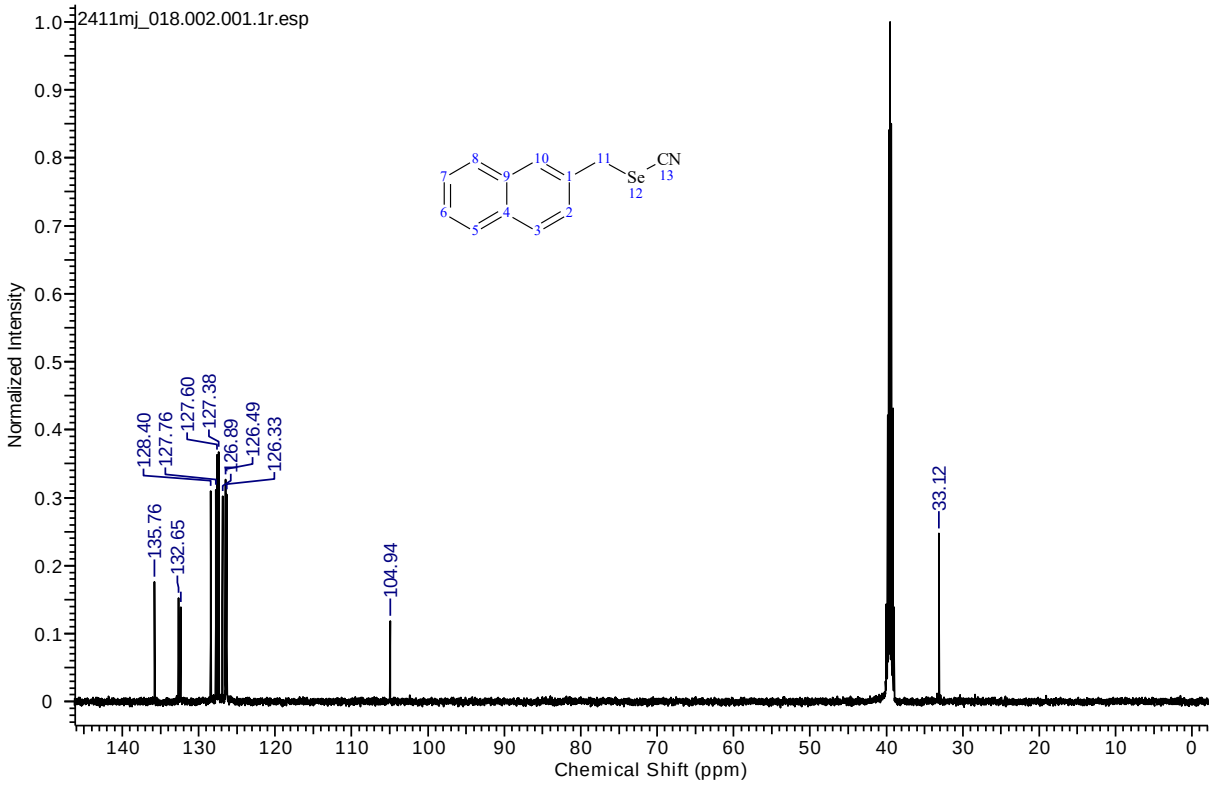
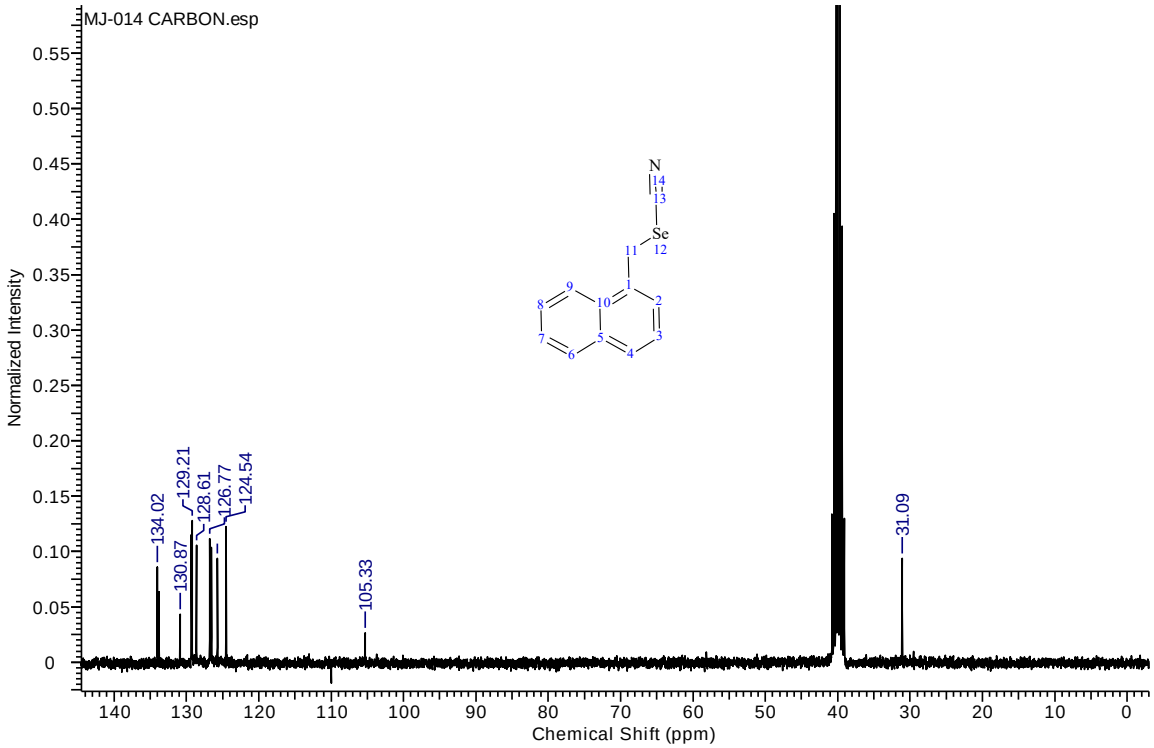


Compound 13



7.2. Supplementary material for Publication 2: Selenazolinium Salts as "Small Molecule Catalysts" with High Potency against ESKAPE Bacterial Pathogens.

Supplementary

Table S1. Details of MIC values of the compounds **1**-**8** and ebselen against Gram-negative bacteria.

	Bacteria Strains	MIC* of the tested compounds (**1**-**8**) [μg/ml]								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Ebselen**
K. pneumoniae	**NRZ-00103**	1.24[1]	13.76	.4-2.8	2.88-5.76	46.08	46.08	2.88	5.76	≥ 143.35
	KP 2151307	0.62-1.24	6.88-13.76	1.4	2.88	23.04-46.08	11.52-23.04	2.88	5.76	71.68-143.36
	KP 1963584	0.62	6.88	1.4	2.88	11.52-23.04	11.52-23.04	1.44-2.88	2.88	71.68
Acinetobacter	**AC 2151300**	0.31-0.62	0.86	0.35-0.7	0.36-0.72	2.88	1.44	0.36-0.72	1.44	17.92
	AB 1995594	0.62	1.72-3.44	1.4	0.72	2.88-5.76	2.88	0.72	1.44-2.88	17.92-35.84
	AB 4184/2/5	0.31	0.86	0.35	0.36	2.88	0.72-1.44	0.36	1.44	17.92
P. aeruginosa	**ATCC 27853**	2.48	110.08	5.60-11	5.76-11.52	46.08-92.16	92.16-184.32	11.52-23.04	23.04	71.68
	PA T18	0.31	1.72	0.7	1.44	11.52	11.52	0.72	1.44-2.88	17.92
	PA54	0.62-1.24	13.76-27.52	1.4-2.8	5.76	11.52	46.08-92.16	5.76	5.76-11.52	71.68-143.36
	PA58	0.62	6.88-13.76	1.4	2.88	11.52-23.04	23.04	2.88	5.76	17.92-35.84
E. coli	**NCTC 13351**	1.24-2.48	6.88-13.76	1.4	2.88	11.52-23.04	11.52	2.88	5.76	71.68-143.36
	EC 2151612	1.24-2.48	6.88	1.4	2.88	11.52-23.04	11.52	1.4-2.88	5.76	71.68
	EC 1995591	2.48	6.88-13.76	1.4-2.8	2.88	23.04	11.52	2.88	5.76	71.68
	EC 1227107	1.24-2.48	13.76	2.8	5.76	23.04	11.52-23.04	2.88	5.76-11.52	35.84-71.68

* Particularly potent antibacterial activities (MIC < 5 μg/ml) are underlined.

3.3. Evaluation of ROS formation

To analyze the effect of the selenazolinium salts tested on intracellular oxidative stress production in *S. aureus* DCHFA assay was performed. For this purpose, the impact of the most active compounds which were identified in the previous studies (the compounds **1** and **6**) and ebselen on ROS release was determined in the reference *S. aureus* ATCC 25923 strain and the clinical isolate MRSA HEMSA 5 (**Figure S1-S3**)

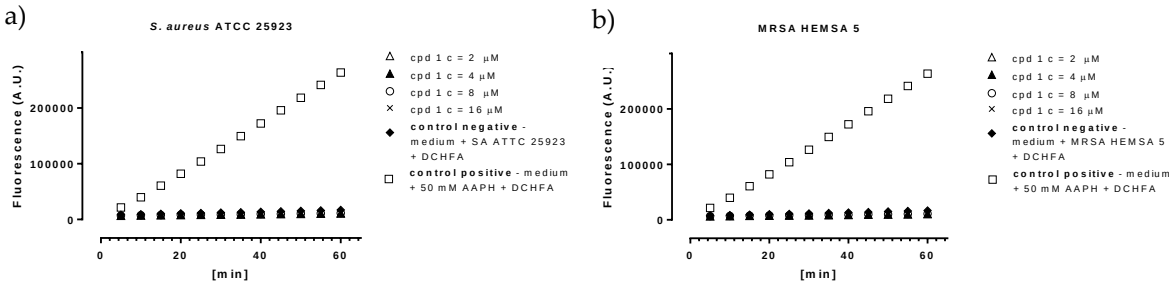


Figure S1. Generation of intracellular ROS in the reference *S. aureus* ATCC 25923 strain (a) and the clinical MRSA HEMSA 5 isolate (b) upon exposure to the different concentrations of the compound **1**. 2, 2′-azobis (2-amidinopropane) dihydrochloride (AAPH) was included as a positive control in the assay. The level of oxidative stress was detected by the use of fluorogenic dye 2', 7'-dichlorodihydrofluorescein diacetate (DCHFA) which in the presence of cellular esterases and ROS is converted to highly fluorescent 2', 7'-dichlorofluorescein (DCFA). Values represent means with standard deviation (SD) bars from at least four repeats. Statistical significances were calculated using a one-way ANOVA followed by Bonferroni's multiple comparison test (**Figure S1 a**, **b**: $p > 0.05$).

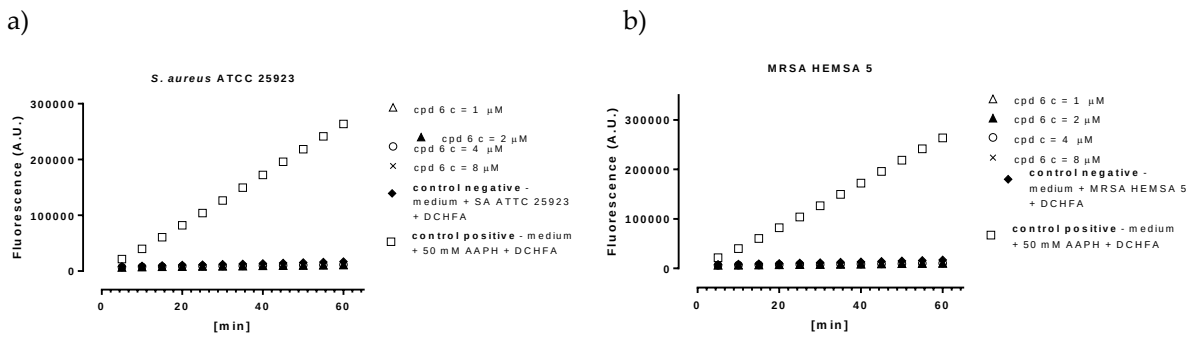


Figure S2. Generation of intracellular ROS in the reference *S. aureus* ATCC 25923 strain (a) and the clinical MRSA HEMSA 5 isolate (b) upon exposure to the different concentrations of compound **6**. For further details refer to the **Figure S1**.

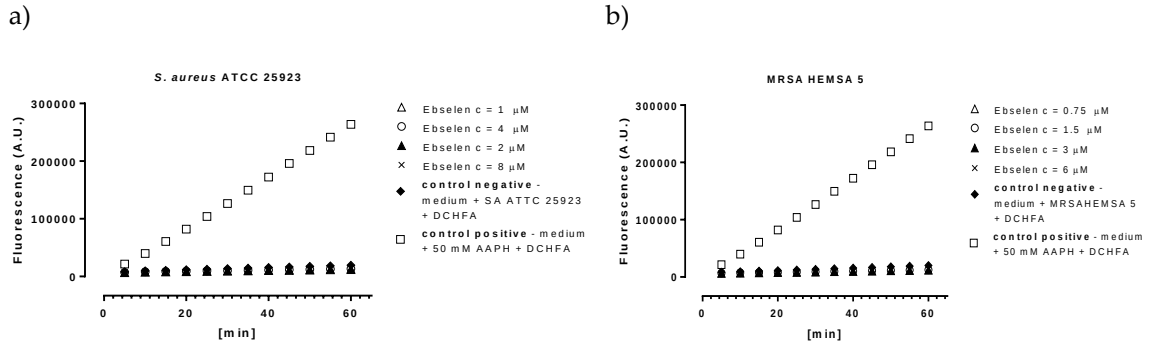


Figure S3. Generation of intracellular ROS in the reference *S. aureus* ATCC 25923 strain (a) and the clinical MRSA HEMSA 5 isolate (b) upon exposure to the different concentrations of ebselen. For further details, refer to the **Figure S1**.

8. List of publications

1. R. Alhasan, **M. J. Nasim**, C. Jacob, C. Gaucher. Selenoneine: a Unique Reactive Selenium Species From the Blood of Tuna With Implications for Human Diseases. *Curr Pharmacol Rep*, 5 (3), pp 163–173. (2019)
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3. **M. J. Nasim**, K. Witek, A. Kincses, A. Y. Abdin, E. Żesławska, M. A. Marć, M. Gajdács, G. Spengler, W. Nitek, G. Latacz, E. Karczewska, K. Kieć-Kononowicz, J. Handzlik, C. Jacob. Pronounced activity of aromatic selenocyanates against multidrug resistant ESKAPE bacteria. *New J. Chem.,* 43, 6021-6031. (2019)
4. **M.J. Nasim**, R. Alhasan, A. Y. Abdin, F. Alnahas, C. Jacob. Inspired by Nature: Redox Modulators and Natural Nanoparticles. *Proceedings*, 11(1), 24. (2019)
5. R. Alhasan, A. Kharma, **M. J. Nasim**, A. Y. Abdin, J. Bonetti, P. Giummelli, C. E.C.C. Ejike, P. Leroy, C. Gaucher, C. Jacob, Flush with a flash: Natural three-component antimicrobial combinations based on *S*-nitrosothiols, controlled superoxide formation and “domino” reactions leading to peroxynitrite. *Med. Chem. Commun.,* 9(12), 1994-1999, (2018)
6. **M. J. Nasim**, P. Denezhkin, M. Sarfraz, R. Leontiev, Y. Ney, A. Kharma, S. Griffin, M. I. Masood, C. Jacob, The Small Matter of a Red Ox, a Particularly Sensitive Pink Cat, and the Quest for the Yellow Stone of Wisdom. *Curr. Pharmacol. Rep*. 4:380–396 (2018)
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8. M. Sarfraz,. S. Griffin, T. Gabour Sad, R. Alhasan, **M. J. Nasim**, M. Irfan Masood, K. H.

Schäfer,C. E. Ejike, C. M. Keck, C. Jacob, A. P. Ebokaiwe. Milling the Mistletoe: Nanotechnological Conversion of African Mistletoe (*Loranthus micranthus*) Into antimicrobial Materials. *Antioxidants* 7, 60. (2018)

9. S. Griffin, M. Sarfraz, S. F. Hartmann, S. R. Pinnapireddy, **M. J. Nasim**, U. Bakowsky, M. C. M. Keck, C. Jacob, Resuspendable Powders of Lyophilized Chalcogen Particles with Activity against Microorganisms. *Antioxidants*;7(2), 23. (2018)

10. S. Griffin, M. Sarfraz, V. Farida, **M: J. Nasim**, A. P. Ebokaiwe,C. M. Keck, C. Jacob, No time to waste organic waste: Nanosizing converts remains of food processing into refined materials, *J Environ Manage*, 210, 114-121 (2018)

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12. **M. J. Nasim**, W. Ali, ,E. Dominguez-Álvarez, E. N. da Silva Júnior, R. S. Z. Saleem, C. Jacob. Reactive Selenium Species: Redox Modulation, Antioxidant, Antimicrobial and Anticancer Activities. In book: Organoselenium Compounds in Biology and Medicine: Synthesis, Biological and Therapeutic Treatments. *Royal Society of Chemistry*, 277-302 (2018)

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14. E. Y. Efe A. Mazumder, J. Lee, A. Gaigneaux F. Radogna, **M. J. Nasim**, Christo Christov , Claus Jacob, K. Kim, M. Dicato, P. Chaimbault, C. Cerella, M. Diederich. Tubulin-binding anticancer polysulfides induce cell death via mitotic arrest and autophagic interference in colorectal cancer. *Cancer Letters*, 410, 139-157 (2017)

15. G.I. Giles, **M.J. Nasim**, W. Ali, C. Jacob, The Reactive Sulfur Species Concept: 15 Years

On. *Antioxidants*, 6(2):38, 1-29 (2017)

16. J. Rendeková, D. Vlasáková, P. Arsenyan, J. Vasiljeva, **M. J. Nasim**, K. Witek, E. Domínguez-Álvarez, E. Żesławska, D. Mániková, W. Tejchman, R. S. Z. Saleem, K. Rory, J. Handzlik, M. Chovanec, The Selenium-Nitrogen Bond as Basis for Reactive Selenium Species with Pronounced Antimicrobial Activity, *Curr Org Synth*, 14, (2017)

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