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Binding motif of ebselen in solution: chalcogen and hydrogen bonds team up†

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Ebselen (2-phenyl-1,2-benzoselenazol-3(2*H*)one), a glutathione peroxidase mimic, is active against several RNA viruses, among others the retrovirus responsible for the COVID-19 pandemic. In this paper ^{77}Se and ^{1}H NMR studies of ebselen are reported and they identify the chalcogen bond (ChB) and hydrogen bond (HB) that are central in the landscape of interactions formed by the compound in solution. The selenium atom and the hydrogen atom at the C7 carbon act as ChB and HB donors and the O and N atoms of neutral molecules function as acceptors. The ChB and HB give rise to a bifurcated supramolecular synthon, which fastens the interaction acceptor opposite to the N–Se covalent bond of the selenazole ring. It is known that the biologically important reaction of ebselen with cysteine thiol groups is favoured when selenium acts as a chalcogen bond donor.

1. Introduction

Selenium has long been considered to be a toxic element, but in the 1950s its reputation began to change dramatically as evidence emerged[1] that it is an essential trace element in many organisms, humans included.[2] The initial source of selenium for natural organoselenium compounds are the selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) anions, two abundant forms of selenium in the soil. Plants reduce these anions to selenide ions that then afford selenocysteine (Sec) through a cascade of reactions. Sec, the 21st proteinogenic amino acid,[3] is present in all three domains of life and is a major form of selenium in cells. In living organisms it is reversibly transformed into selenomethionine and Se-methylselenocysteine, two other naturally occurring amino acids. Another milestone in changing the hostile reputation of selenium was the discovery that glutathione peroxidase (GPx), a widespread enzyme that protects organisms from reactive oxygen species (ROS), is a selenoprotein that contains, at its active site, multiple Sec units that directly participate in ROS deactivation.[4] Many other selenoproteins are now known, approximately 25 of them in humans;[5] of those *thioredoxin reductases* (TRxRs) and *iodothyronine deiodinases* (DIOs) are the most extensively studied.[6]

Even after these milestones, a number of decades passed before selenium derivatives entered the toolbox of medicinal chemists.[7] Two events that definitively manumitted selenium's bad reputation occurred in the 1980s when the antiviral activity of selenazofurin[8] (2-β-D-ribofuranosyl-selenazole-4-carboxamide, a selenium analogue of the broad spectrum antiviral drug ribavirin) was first reported and when ebselen[9] (2-phenyl-1,2-benzoselenazol-3(2*H*)one) was described as a GPx-mimic. Currently Se is considered a particularly relevant element for the redox homeostasis in living organisms[10] and researches targeting Se derivatives with GPx-like activity are systematic.[11] Ebselen has an extremely low cytotoxicity (LD_{50} in rats >4600 mg kg^{-1}, per os)[12] and its safety to humans has been thoroughly evaluated.[13] It has been investigated as a possible treatment of reperfusion injury stroke, bipolar disorder, hearing loss and tinnitus.[14] It shows anti-inflammatory, antiatherosclerotic, antibacterial, and cytoprotective properties and these activities have all been related to its GPx-mimicking ability.[15]

Ebselen is active against several RNA pathogens, among them hepatitis C virus (HCV) and human immunodeficiency virus (HIV). The anti-HIV activity[16] of ebselen as well as its activity against other pathogens[17] and its GPx-mimicking action[18] are all mediated by the formation of a covalent selenylsulfide (–Se–S–) linkage between the selenium of ebselen and the thiol group of a cysteine in the target protein (Table S.11, ESI†). The analogous formation of a selenylsulfide linkage was proposed for the mechanism of action against HCV[15b] and has been documented for the activity against SARS-CoV-2,[19] the retrovirus responsible for the

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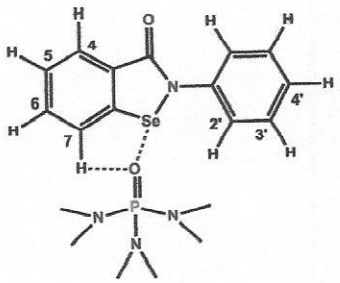
COVID-19 pandemic.[20] The –Se–S– bond formation that produces the cysteine conjugates requires the cleavage of the selenazolone Se–N bond. It has been proposed that this cleavage is crucial for the biological activity of ebselen and related compounds.[10a,21]

Noncovalent interactions are of pivotal importance in controlling molecular recognition phenomena and in determining the kinetics of reactions in chemical and biological systems. Calculation and solid state studies[22] on ebselen showed that when selenium forms interactions with donors of electron density, *viz.* when forming chalcogen bonds (ChBs),[23] the Se–N bond is lengthened and its cleavage is favoured. Importantly, ebselen analogues, which form intramolecular ChBs are up to be 10^3 times more reactive in the –Se–S– bond formation than the parent compound[10c] and arylselenium derivatives, which form intramolecular Se···O/N ChBs are particularly effective GPx-mimics.[24] In general, the formation/activation of selenosulfide bonds mediates several important biological functions, viral entry into cells included.[25]

We reasoned that an assessment of the ability of ebselen to act as a ChB donor in solution might afford indications on the interactions driving the binding of ebselen to biomolecular targets and might give useful information for tuning the rate of Se–N bond activation and/or –Se–S– bond formation. In other words, new instruments are made available for the rational design of ebselen analogues with optimized affinity for the target-active molecule, for instance M^{pro}, a key SARS-CoV-2 enzyme, which interacts with ebselen and mediates viral replication and transcription.[19]

Here we describe the results of ^{1}H and ^{77}Se NMR studies on ebselen solutions. It is shown that ChB and hydrogen bond (HB) cooperate in the binding phenomena involving ebselen. The Se atom and hydrogen atom at C7 carbon (C7–H) act as ChB and HB donor sites and the O and N atoms of neutral molecules function as acceptors. The titration experiments using ^{1}H and ^{77}Se NMR prove that the formed ChB and HB give rise to a bifurcated supramolecular synthon (Scheme 1), which fastens the ChB/HB bond acceptor opposite to the N–Se covalent bond.[22b] The apparent association constants of some of the formed complexes are established. ChB has thus to be considered as one of the interactions, which may contribute to the *in vivo* binding of ebselen to the target biomolecules where oxygen and nitrogen atoms are present.[15b]



Scheme 1 Structural formula of the ebselen/HMPA adduct with carbon atom numbering. ChB (brown dotted line) and HB (black dotted line) cooperate in pinning the oxygen atom opposite to the Se–N bond.

2. Experimental section

2.1. Materials and methods

Ebselen, diphenyl diselenide, acetone-d_6 (DMK-d_6), dimethylsulfoxide-d_6 (DMSO-d_6), tetrahydrofuran-d_8 (THF-d_8), hexamethylphosphoramide (HMPA) and other non-deuterated solvents were reagent grade compounds purchased from TCI and Sigma Aldrich and were used without further purification.

^{1}H and ^{77}Se NMR spectra were acquired on a Bruker Avance 300 at 7.05 T or a Bruker NEO 500 at 11.7 T. The Larmor frequency for ^{1}H and ^{77}Se on those instruments were 300.13 and 57.22 MHz or 500.13 and 95.37 MHz, respectively. The NEO instrument was equipped with a directly observing BBFO iProbe with a fully automatized tuning routine. The ^{1}H chemical shifts are reported in parts per million (δ) relative to tetramethylsilane (TMS). The ^{77}Se chemical shifts are reported in parts per million (δ) referenced to 0.25 M diphenyl diselenide in DMSO-d_6 at 448.00 ppm as the external standard contained in a coaxial capillary inserted in a 5 mm NMR tube. Selenous acid (1 M solution in D_2O) was used for the experimental setup and calibrations; the chemical shift of the Se resonance was set to 1300.1 ppm.[26] All spectra were registered at 30.0 °C.

2.2. ^{77}Se spectra in different solvents

The spectra in Table 1 were recorded by using 0.25 M solutions of ebselen or a saturated solution of the compound when its solubility in the used solvent was lower. The spectra were registered with a spectral width of 900 ppm, a recycling time of 5 s, and enough scans to obtain a signal of both ebselen and diphenyl diselenide at a signal/noise ratio higher than 5.

2.3. Titration experiments

Titrations were performed as follows: 50 mM solutions of ebselen have been prepared using the three different deuterated solvents (DMK-d_6, THF-d_8, and DMSO-d_6). Spectra were acquired with a spectral width of 500 ppm, a recycling time of 5 s, and 128 scans. For each titration, solutions containing up to 15 equivalents of HMPA have been prepared using the 50 mM ebselen solution for the HMPA dissolution. ^{1}H and ^{77}Se NMR spectra of solutions containing different amounts of HMPA have been recorded in order to obtain 12–13 points for the binding fit in DMK-d_6 and THF-d_8. Less points were acquired for DMSO-d_6 solutions. The K_c binding constants have been calculated using the data fitting offered by the online

Table 1 ^{77}Se chemical shifts (δ, ppm) of ebselen in different solvents[a]

	$CHCl_3$	CH_2Cl_2	CH_3CN	$(CH_3)_3CN$	Dioxane	THF
Ebselen	960.95	957.57	948.41	935.92	945.90	936.50
	DMK	DMF[b]	DMA[c]	DMSO	Pyridine	HMPA
Ebselen	941.41	929.55	925.38	914.17	912.11	905.67

[a] ^{77}Se NMR spectra of ebselen solutions in simple amines (*e.g.*, Et_3N and n-Bu_3N) could not be obtained as a reaction occurred after dissolution. [b] DMF = dimethylformamide. [c] DMA = dimethylacetamide.

tools for supramolecular chemistry research and analysis with the option of 1 : 1 data fit for host–guest equilibria.[27]

3. Results and discussion

Electrophile–nucleophile interactions have been investigated in solution using a variety of spectroscopic techniques and nuclear magnetic resonance (NMR) proved to be a particularly useful tool.[28] For instance, interactions where the electrophile belongs to group 17 have been studied by using ^{19}F,[29] ^{13}C,[30] and ^{15}N[31] NMR; ^{17}O,[32] ^{77}Se,[33] and ^{125}Te[34] NMR were employed when the electrophile belongs to group 16 and ^{13}C[35] NMR to group 14.

3.1. Analyses *via* ^{77}Se NMR

The natural abundance of ^{77}Se nucleus (7.6%), its sensitivity (2.98 times that of ^{13}C), and its spin (I = 1/2) that enables for narrow resonance signals make ^{77}Se a valuable candidate as an NMR reporter nucleus.[36] Importantly, the ^{77}Se has a large chemical shift range (~3400 ppm) and is extremely sensitive to the chemical and electronic environments[6,37] (*e.g.*, the sensitivity of nucleus shielding to variations in electron distribution is several times greater than that of phosphorus)[38] so that ^{77}Se is particularly suitable for studying aggregation phenomena.[39]

The ^{77}Se NMR spectra of ebselen in different solvents proved the ability of the compound to function as a ChB donor towards different acceptor atoms. The use of the ChB acceptor as a solvent was expected to maximize the possible ChB formation by that acceptor. Indeed, the observed ^{77}Se chemical shifts span over the fifty ppm range (Table 1) on solvent change. Oxygen and nitrogen atoms belonging to a variety of functionalities work as effective ChB acceptor sites. The heteroatoms can be sp, sp^2, or sp^3 hybridized and some of the employed solvents were chosen as containing functionalities that mimic electron donor moieties commonly present in biological systems (*e.g.*, DMF and DMA were used as mimics of the carbonyl units of peptides and HMPA as a mimic of the P=O unit of phosphorylated compounds).

While the observed ^{77}Se chemical shift changes represent the combined result of all interactions between ebselen and the solvent, it seems plausible that the single most important contribution to the $\Delta(\delta Se)$ comes from the Se···solvent ChB formation. This hypothesis is supported by the solubility profile of the compound. Ebselen solubility is very low in non-coordinating aprotic hydrocarbon solvents (*e.g.*, *n*-hexane, and *n*-pentane) but becomes intermediate in solvents containing N and O atoms, suggesting that ebselen dissolution is driven by the formation of Se···N/O ChBs, which override the Se···O ChBs observed in the crystal structure of pure ebselen.[22b] Moreover, the solvent-induced $\Delta(\delta Se)$ values shown by ebselen are much larger than those shown by other selenium functional groups, *e.g.*, the selenium chemical shifts of ebselen, diphenyldiselenide (Table S.1, ESI†), or a selenocarbonyl derivative[40] in solution of THF, an intermediate strength ChB acceptor, which are 24, 7, and 1 ppm upfield with respect to the corresponding solutions in chloroform (a poorly coordinating solvent, which was assumed as the reference solvent). This proves that the tendency of ebselen to act as a ChB donor in solution is remarkable and that the interaction may play a major role in the binding pattern of the compound.

Analyses of the Cambridge Structural Database (CSD) show that ChB systematically controls or affects the crystal packing of ebselen and its derivatives, this being a further indication that the compound may act as a robust ChB donor in solution. The by-far shortest contact in crystalline ebselen is the intermolecular Se···O ChB,[22b] the corresponding normalized contact‡ (N_c) being 0.74. Molecules are so tightly packed in the crystal that its density is 1.647 g cm^{-3} (at room temperature). The N_c value for the intermolecular Se···N ChB in the ebselen/4-dimethylaminopyridine cocrystal is as small as 0.69.[22a] A CSD survey reveals that 29 structures contain the 2-phenyl-1,2-benzoselenazol-3(2*H*)one moiety and 23 of them (~79%) show the presence of a Se···O/N ChB§ (Table S.8, ESI†). CSD analysis also indicates that in ebselen the electron withdrawing ability of the –NC(=O) residue bound to selenium plays a major role in determining the strong ChB donor ability of the compound. In fact, when weaker electron withdrawing residues are covalently bonded to the selenium atom, the occurrence of Se···O/N ChBs decreases dramatically, *e.g.*, it is 14% in compounds containing the C–Se–C moiety (Table S.9, ESI†). X-Ray crystallography charge density studies and atom-in-molecules theoretical analysis further prove that the Se atom in ebselen can function as a particularly good ChB donor.[22b]

Consistent with the similar nature of the ChB and the halogen bond (HaB),[23b] close analogies exist between the solvent-induced chemical shift changes of the ^{77}Se signal in ebselen and the solvent-induced changes of the ^{19}F signal in iodoperfluorocarbons.[30] For both ChB and HaB, the electron donor site can be a lone pair on an sp^3, sp^2, or sp atom. The greater the ability of the solvent to donate electron density to the electrophilic selenium or halogen atom, the larger the observed upfield changes of chemical shift. For instance, the ^{77}Se signal of ebselen and the ^{19}F signals of –CF$_2$I groups (*e.g.*, in ICF$_2$CF$_2$I) occur at higher fields in pyridine and DMSO than in acetonitrile and THF. The greater donation of electron density by the two former solvents to the ChB and HaB donor atoms causes increased electron density and stronger shielding effect at selenium and fluorine and corresponding NMR signals are at higher fields. The ^{77}Se signal of ebselen and ^{19}F signal of –CF$_2$I groups are at higher fields when the solvent is DMSO

‡ The "normalized contact" Nc for an interaction between atoms i and j is the ratio $D_{ij}/(rvdW_i + rvdW_j)$ where D_{ij} is the experimental distance separating atoms i and j and rvdW$_i$ and rvdW$_j$ are the van der Waals radii[41] of atoms i and j. If the electron donor j is an anionic atom, rvdW$_j$ is substituted by the Pauling ionic radius[42] of the anion atom j. Nc is a useful indicator, allowing for a more rigorous comparison of separations between different interacting atoms than absolute values of interaction lengths.

§ It was assumed that a ChB is present in the crystal when the interatomic Se···O/N distance is below the sum of van der Waals radii[41] and the N–Se···O/N and C–Se···O/N angles span the range of 155–180°.

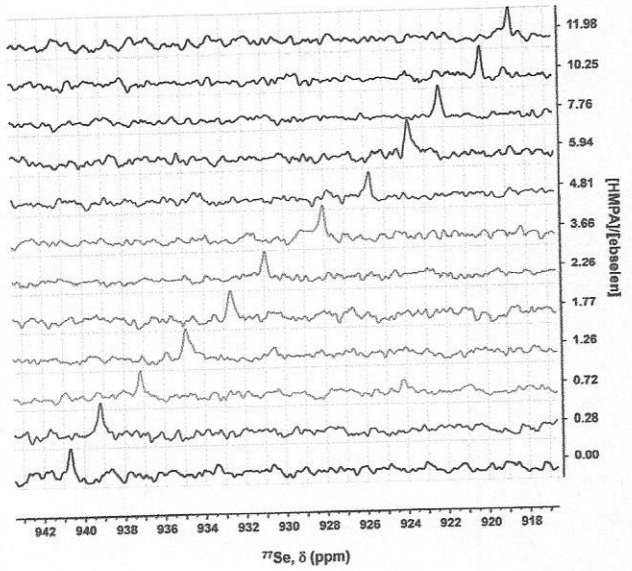


Fig. 1 ^{77}Se NMR spectra of ebselen solutions in acetone-d_6 on increasing [HMPA]/[ebselen] ratio (ordinate on the right).

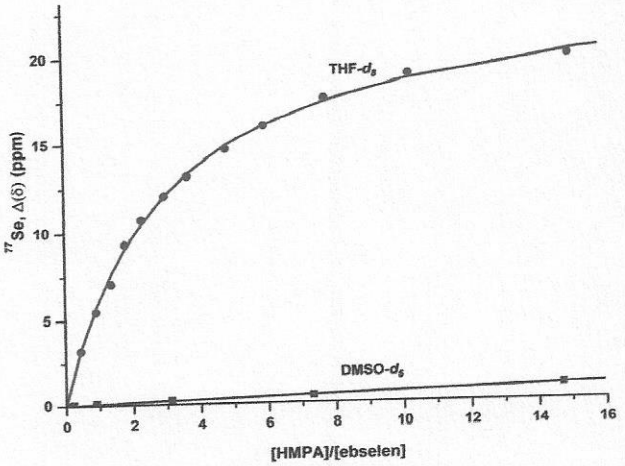


Fig. 2 Plotted $\Delta\delta$ of ^{77}Se of ebselen in DMK-d_6 and DMSO-d_6 on addition of incremental amounts of HMPA ($\Delta\delta = \delta_0 - \delta_i$, where δ_0 and δ_i are the chemical shifts of a solution of pure ebselen and of a solution containing the i-th aliquot of HMPA).

than when it is acetone, suggesting that in both ChB and HaB the donation of electron density by a given atom increases with its negative charge.

These results indicate that it is possible to evaluate the intermolecular ChB formation between ebselen and a neutral ChB acceptor in solution by ^{77}Se NMR. The most upfield shifted ^{77}Se signal for ebselen was obtained when HMPA was used as the solvent. The formation of the ebselen/HMPA complex was monitored through ^{77}Se and ^{1}H NMR titrations of solutions where the solvents were ChB acceptors weaker than HMPA.

The ^{77}Se NMR spectra of ebselen in acetone-d_6 and in the presence of trace amounts of HMPA (*e.g.*, <0.05 eq.) can hardly be distinguished from those in solutions of pure acetone. ^{77}Se signals progressively move upfield on addition of incremental amounts of HMPA (Fig. 1 and Table S.2, ESI†). A single peak was observed in all spectra, no signal splitting was noticed independent of the ebselen:HMPA ratio, showing that the formation of ebselen/HMPA complexes is rapid at room temperature on the NMR time scale. The increased shielding of selenium in the presence of incremental amounts of HMPA indicates that the ChB-driven formation of an ebselen/HMPA complex is due to the fact that the more negative oxygen of HMPA functions as the ChB acceptor with preference over the less negative oxygen of acetone. By fitting the observed upfield shifts (1 : 1 pairing of ebselen and HMPA), an association constant K_c of 4.63 M^{-1} was calculated (Fig. 2).

A similar behaviour was observed when THF-d_8 was used as the solvent. Also with this solvent, no signal splitting or broadening has ever been observed and, at the same ebselen:HMPA ratios, upfield shifts were slightly larger than in acetone (Fig. S.1 and Table S.3, ESI†). The apparent association constant of the ebselen/HMPA complex in tetrahydrofuran is 8.43 M^{-1}, marginally greater than in acetone. Upfield shifts of ^{77}Se signal were observed also when incremental amounts of HMPA were added to solutions of the ebselen in dimethylsulfoxide-d_6, but they were very minor even at the highest HMPA:ebselen ratios (Table S.4 and Fig. S.2, ESI†) and a reliable association constant of the ebselen/HMPA complex could not be calculated in this solvent. DMSO, a stronger ChB acceptor than DMK and THF (Table 1), competes with HMPA in serving as an acceptor more effectively than the two other solvents.

3.2. Analyses *via* ^{1}H NMR

^{1}H NMR spectra of the same solutions studied *via* ^{77}Se NMR revealed that an HB, involving C7–H as a donor site, contributes to the complex formation. This adds structural information on the ebselen/HMPA complex formed in solution. All ebselen protons show chemical shift changes on HMPA addition; they are all quite small (Tables S.5–S7, ESI†), apart from those of C7–H. On incremental addition of HMPA this proton moves downfield, while all other protons, but C2′–H, move upfield. This behaviour is shown in both acetone (Fig. 3 and 4) and THF and DMSO (Fig. S.3 and S.4, ESI†) suggesting that, in the three solvents, an HB is formed between C7–H and HMPA oxygen. The N–Se···O ChB and the C7–H···O HB mutually favour their formation and cooperate in pinning the HMPA oxygen opposite to the N–Se covalent bond, where calculations show that there is the most positive σ-hole at selenium.[22b]

Changes of C7–H chemical shift are similar when DMK and THF are used as solvents and they are much greater than when DMSO is used. Parallel to what was suggested by ^{77}Se NMR analyses, this indicates that DMSO competes with HMPA, in serving as a HB acceptor, more successfully than DMK and THF.

The K_c values for the ebselen/HMPA adduct, calculated by fitting C7–H chemical shift changes for a 1 : 1 complex, are 5.79 M^{-1} in DMK-d_6 solution and 9.79 M^{-1} in THF-d_8 solution. When DMSO was used as the solvent, a reliable calculation of K_c was prevented by the too small changes of C7–H chemical

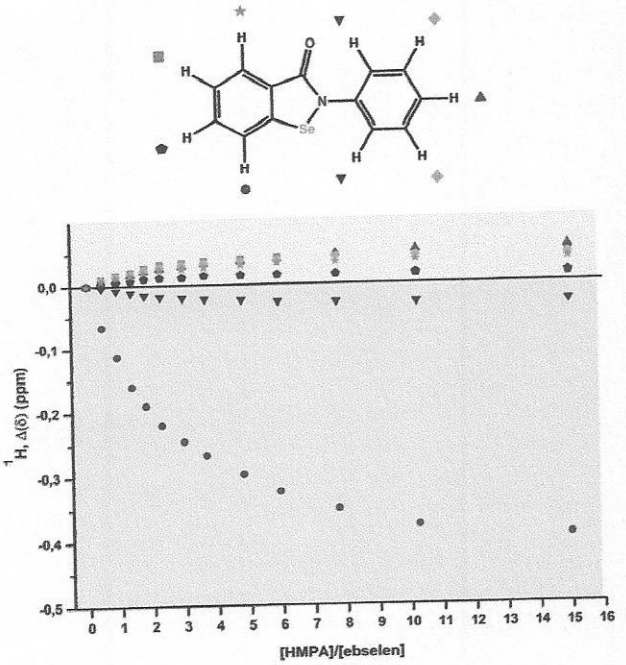


Fig. 3 Plotted $\Delta\delta$ of proton signals of ebselen in DMK-d_6 in the presence of incremental amounts of HMPA.

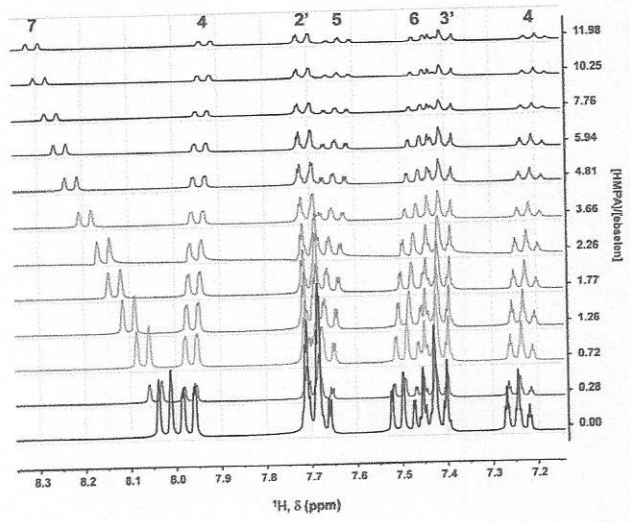


Fig. 4 ^{1}H NMR spectra of ebselen solutions in DMK-d_6 on increasing [HMPA]/[ebselen] ratio (ordinate on the right). Numbers at the top give peak assignment according to the atom numbering in Scheme 1.

shifts observed on HMPA addition. Titrations *via* ^{1}H and ^{77}Se NMR measurements give consistent relative tendencies for the complex formation, which vary in the order THF ≥ DMK ≫ DMSO. K_c values established *via* ^{1}H NMR are slightly greater than those established *via* ^{77}Se NMR in both DMK and THF. This might suggest that the existence in solution of the ebselen/HMPA complex is associated with the presence of the bifurcated C7–H···O···Se–N supramolecular synthon and, to a lesser extent, of the monofurcated C7–H···O synthon. These two synthons cause different changes of Se and H chemical shifts of ebselen and the observed $\Delta(\delta Se)$ and $\Delta(\delta H)$ values are the combined result of these different contributions. A CSD analysis confirms that the bifurcated supramolecular synthon is the favoured pattern in the interaction landscape of ebselen in the solid.[43] The presence of the monofurcated C7–H···O synthon in solution might be enabled by the dynamic character of association processes in the liquid.

4. Conclusions

In summary, ebselen is a compound, which displayed a quite promising pharmacological profile. In particular, it showed, out of ~10 000 known pharmacologically active compounds, the strongest inhibition of M^{pro}, a key enzyme which mediates replication of the SARS-CoV-2 virus.[19] ^{77}Se and ^{1}H NMR studies reported in this paper prove that ebselen forms ChB and HB in solution with O and N atoms. The main binding mode in various solvents is a bifurcated supramolecular synthon where the ChB and HB cooperate in fastening the lone-pair donor atom on the elongation of the N–Se covalent bond. ChB has thus to be considered as one of the interactions, which may mediate the binding of ebselen to biomolecular targets where oxygen and nitrogen atoms are typically present. The interaction may also help in designing ebselen analogues with optimized pharmacological activity. This specific ability complements the numerous, important, and general roles that ChB has in several fields,[44] *e.g.* medicinal chemistry,[45] catalyses,[46] and anion binding and transport.[47] Finally, ^{77}Se NMR is shown to be a powerful tool for studying association equilibria in solution, specifically for improving the current knowledge of ChB also in the presence of cooperating/competing interactions.

Conflicts of interest

There are no conflicts to declare.

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