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Effect of thione–thiol tautomerism on the inhibition of lactoperoxidase by anti-thyroid drugs and their analogues

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Abstract. The keto-enol type tautomerism in anti-thyroid drugs and their selenium analogues are described. The commonly used anti-thyroid drug methimazole exists predominantly in its thione form, whereas its selenium analogue exists in a zwitterionic form. To understand the effect of thione/thiol and selone/selenol tautomerism on the inhibition of peroxidase-catalysed reactions, we have synthesized some thiones and selones in which the formation of thiol/selenol forms are blocked by different substituents. These compounds were synthesized by a carbene route utilizing an imidazolium salt. The crystal structures of these compounds reveal that the C=Se bonds in the selones are more polarized than the C=S bonds in the corresponding thiones. The structures of selones were studied in solution by NMR spectroscopy and the ^{77}Se NMR chemical shifts for the selones show large upfield shifts in the signals, confirming their zwitterionic structures in solution. The inhibition of lactoperoxidase by the synthetic thiones indicates that the presence of a free N-H moiety is essential for an efficient inhibition. In contrast, such moiety is not required for an inhibition by the selenium compounds.

Keywords. Anti-thyroid drugs; bioinorganic chemistry; enzyme inhibition; lactoperoxidase; methimazole.

1. Introduction

The heme peroxidase superfamily of 'mammalian peroxidases' include lactoperoxidase (LPO), myeloperoxidase (MPO), eosinophil peroxidase (EPO) and thyroid peroxidase (TPO), which catalyse several important biological reactions.[1] Thyroid peroxidase (TPO), in particular, involves in the biosynthesis of the thyroid hormone, thyroxine (**T4**). The synthetic routes for thyroxine generation in thyroid gland catalysed by TPO involve two distinct reactions: iodination of tyrosyl residues in tyroglobulin (Tg) and phenolic coupling of the resulting iodinated tyrosyl residues.[2] The first step in the thyroid hormone biosynthesis is the oxidation of the iron center in the native enzyme by hydrogen peroxide to form compound I that exist in two different forms: an oxoferryl protein radical and an oxoferryl porphyrin pi-cation radical. In the presence of reducing agents such as iodide and thyroglobulin, iodide is rapidly oxidized by π-cation radical to an iodinating species that is involved in the iodination of the tyrosyl residues on Tg. The coupling reaction occurs simultaneously with iodination by the π-cation radical (figure 1).

The prohormone **T4**, produced by the TPO/H_2O_2/I^- system, is then converted to its biologically active form **T3** by an outer ring de-iodination pathway. This particular reaction is catalysed by an iodothyronine deiodinase (ID-I), which is present in highest amounts in liver, kidney, thyroid and pituitary.[3] The thyroid gland also produces an inactive metabolite **rT3** by an inner ring deiodination pathway. The triiodo derivatives **T3** and **rT3** are further

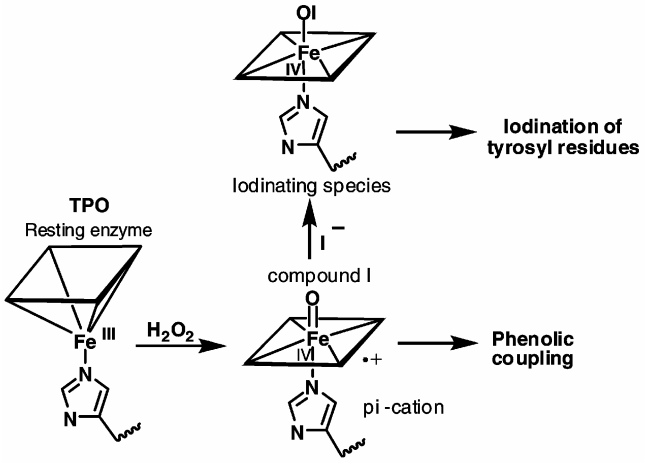

Figure 1. Synthesis of thyroid hormones by heme-containing thyroid peroxidase (TPO).

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metabolized by inner ring and outer ring de-iodination, respectively, by ID-I, and two other iodothyronine deiodinases called ID-II and ID-III to produce the inactive metaboilite **T2** (3,3′-**T2**, 3,5-**T2** and 3′,5′-**T2**). The outer ring 5'-de-iodination catalysed by the ID-I enzyme is considered to be the first step in thyroid hormone action, because this is the only de-iodination pathway that leads to the formation of an active thyroid hormone.[3]

Although the iodination and de-iodination reactions are essential for the function of thyroid gland, the activation of thyroid stimulating hormone (TSH) receptor by auto-antibodies leads to an overproduction of thyroid hormones. As these antibodies are not under pituitary feedback control system, there is no negative influence on the thyroid activity and, therefore, the uncontrolled production of thyroid hormones leads to a condition called 'hyperthyroidism'.[3] The overproduction of **T4** and **T3** can be controlled by blocking the thyroid hormone biosynthesis or reducing the conversion of **T4** to **T3**. The thiourea drugs such as methimazole (**1**, MMI), 6-*n*-propyl-2-thiouracil (**3**, PTU), and 6-methyl-2-thiouracil (**5**, MTU) are generally employed for reducing the thyroid hormone level (figure 2). Although the precise mechanism of their action is not known, these compounds have been shown to reduce the formation of thyroxine by inhibiting the TPO enzyme.[4] MMI has been found to be particularly effective and this imidazole-based compound normally leads to an irreversible inhibition of the enzyme. The thiourea-based compounds PTU (**3**) and MTU (**5**) also inhibit the TPO activity, but their action on the enzyme is not completely irreversible.

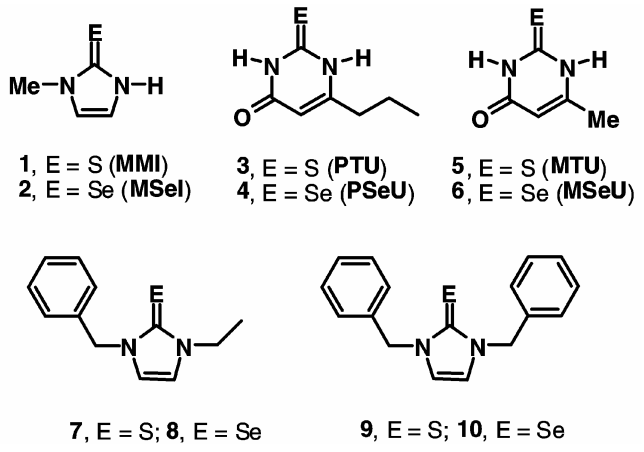


Figure 2. Chemical structures of some anti-thyroid drugs and their analogues.

In recent years, the selenium analogues of MMI (**2**, **MSeI**), PTU (**4**, **PSeU**) and MTU (**6**, **MSeU**) attracted considerable attention.[5,6] The selenium analogues may exhibit much higher inhibitory activity towards TPO as compared with their sulfur analogues due to their high nucleophilic character. In addition to their inhibitory action, the selenium analogues may have significant effect on the hydrogen peroxide and other reactive oxygen species. The degradation of the intracellular hydrogen peroxide by the selenium analogues of anti-thyroid drugs may be beneficial to the thyroid gland as these compounds may act as antioxidants and protect thyroid cells from oxidative damage. In this regard, our group is working on the mechanism of the inhibition of peroxidase-catalysed oxidation and iodination reactions by anti-thyroid drugs and their selenium analogues. Recently, we have shown that the selenium analogue of MMI (i.e. MSeI) is unstable and this compound oxidizes spontaneously to produce the corresponding diselenide.[6] In this paper, we describe the importance of the thione moiety in anti-thyroid agents and the effect of thiol/thione and selenol/selone tautomerism on peroxidase-catalysed oxidation reactions.

2. Experimental

2.1 *General procedure*

Lactoperoxidase from bovine milk and ABTS (2,2′-azino-bis 3-ethylbensthiazoline sulfonic acid) were purchased from Fluka Chemical Co. *n*-Butyllithium was purchased from Acros Chemical Co. (Belgium). Sodium borohydride ($NaBH_4$), benzyl chloride, suphfur and selenium powder were obtained from Aldrich. The anti-thyroid drugs 2-mercapto-1-methylimidazole **1**; 6-*n*-propyl-2-thiouracil **3**; 6-methyl-2-uracil **5** were obtained from TCI (Tokyo Kasei, Japan) company. All other chemicals were of the highest purity available. All experiments were carried out under dry and oxygen free argon/nitrogen using standard Schlenk techniques for the synthesis. Due to unpleasant odors of several of the reaction mixtures involved, most manipulations were carried out in a well-ventilated fume hood. Melting points were determined in open tubes on a Buchi melting point B-540 apparatus and are uncorrected. Mass spectral (MS) studies were carried out on a Q-TOF Micro mass spectrometer with electrospray ionization MS mode analysis. In the case of isotopic patterns, the

value given is for the most intense peak. Elemental analyses were performed on a ThermoFinigan FLASH EA 1112 CHNS analyser. Liquid state NMR spectra were recorded in $CDCl_3$ as a solvent. ^1H (400 MHz), ^{13}C (100 MHz) and ^{77}Se (76·3 MHz) NMR spectra were obtained on a Bruker Avance 400 NMR Spectrometer using the solvent as an internal standard for ^1H and ^{13}C. Chemical shifts (^1H, ^{13}C) are cited with respect to tetramethylsilane (TMS). The ^{77}Se NMR spectra were recorded using diphenyl diselenide as an external standard. The ^{77}Se NMR chemical shifts are reported relative to dimethyl selenide (0 ppm) by assuming that the resonance of the standard is at 461·0 ppm. In most of the cases, the ^{77}Se NMR experiments were run overnight to obtain good quality spectra. Thin-layer chromatography analyses were carried out on pre-coated silica gel plates (Merck) and spots were visualized by UV irradiation. Column chromatography was performed on glass columns loaded with silica gel or on automated flash chromatography system (Biotage) by using pre-loaded silica cartridges. High performance liquid chromatography (HPLC) experiments were carried out on a Waters Alliance System (Milford, MA) consisting of a 2690 separation module, a 2690 photodiode-array detector and a fraction collector. The assays were performed in 1·8 mL sample vials and a built-in autosampler was used for sample injection. The Alliance HPLC System was controlled with EMPOWER software (Waters Corporation, Milford, MA).

2.1a *Synthesis of 1-benzyl-1H-imidazole:* A mixture of imidazole (3·26 g, 0·048 mol), benzyl chloride (6·08 g, 0·048 mol) and four equivalents of sodium hydride (60%; 7·68 g, 0·19 mol) was refluxed overnight in dry THF (50 mL) under nitrogen atmosphere. The solvent was removed completely under vacuum. The resulting residue was treated with water (30 mL) and dichloromethane (30 mL), the organic layer was washed two more times with water to remove the excess sodium hydride, and then separated and dried it over anhydrous sodium sulfate. The solvent was removed completely under vacuum to give a pale yellow solid. This was washed with petroleum ether to remove the non-polar impurities, the residue was dried under vacuum to afford a pale yellow solid. Yield: 6·21 g, 82%· m.p. 71–73°C. ^1H NMR ($CDCl_3$, ppm): $\delta = 5{\cdot}12$ (*s*, 2H of $PhCH_2$), 6·90 (*s*, 1H, NCH of imidazole ring), 7·09 (*s*, IH, NCH of imidazole ring), 7·27 (*m*, 5H, CH of benzene ring), 7·56 (*s*, 1H, NCH); ^{13}C NMR ($CDCl_3$, ppm): $\delta = 50{\cdot}9$ (CH_2Ph), 119·4, 136·2 (C of imidazole), 127·3, 128·3, 129·0, 129·9, (C of benzene ring), 137·4 (NCN of imidazole ring).

2.1b *Synthesis of-1-benzyl-3-ethyl-1H-imidazole-2(3H)-thione (7):* 1-Benzylimidazole (0·2 g, 1·26 mmol) was dissolved in 10 mL of ethyl acetate in a 50 mL two-neck round bottom flask fitted with a reflux condenser. To the solution, ethyl bromide (0·1 mL, 1·26 mmol) was added drop-wise. This reaction mixture was refluxed for 24 h, and then the solvent was removed under vacuum to get a pale yellow solid. The solid obtained in the first step was taken in a 50 mL two-neck round bottom flask fitted with a reflux condenser and was added dry methanol (25 mL). The resulting solution was treated with sulfur powder (0·04 g, 1·26 mmol) and anhydrous potassium carbonate (0·16 g, 1·15 mmol). The reaction mixture was heated to reflux for 24 h. The solution was then filtered through a pad of Celite and washed two times with dry methanol. The small impurities in the sample can be removed by column chromatography (silica gel) by using 1 : 1 ethyl acetate: petroleum ether. Yield: 0·13 g (47%). m.p. 87–89°C. ^1H NMR ($CDCl_3$, ppm): $\delta = 1{\cdot}39$ (*t*, 3H, $J_{H-H} = 7{\cdot}2$ Hz, of NCH_2CH_3), 4·13 (*q*, 2H, $J_{H\text{-}H} = 7{\cdot}2$ Hz, of NCH_2), 5·26 (*s*, 2H, NCH_2), 6·56 (*d*, IH, $J_{H-H} = 2$ Hz, C–H of imidazole ring), 6·68 (*d*, IH, $J_{H-H} = 2{\cdot}4$ Hz, C–H of imidazole ring), 7·33 (*m*, 5H, CH of benzene ring); ^{13}C NMR ($CDCl_3$, ppm): $\delta = 13{\cdot}2$ (CH_3), 41·9 (CH_2 of ethyl group), 50·1 (CH_2Ph), 115·1, 115·5 (CH of imidazole ring), 127·1, 127·3, 127·8, 134·8 (CH of benzene ring), 161·1 (C=S of imidazole ring).

2.1c *Synthesis of-1-benzyl-3-ethyl-1H-imidazole-2(3H)-selone (**8**):* 1-Benzylimidazole (0·2 g, 1·26 mmol) was dissolved in 10 mL of ethyl acetate in a 50 mL two-neck round bottom flask fitted with a reflux condenser. To the solution, ethyl bromide (0·1 mL, 1·26 mmol) was added drop-wise. This solution was refluxed for 24 h, and then the solvent was removed under vacuum to give a pale yellow solid. The solid obtained in the first step was taken in a 50 mL two-neck round bottom flask fitted with a reflux condenser and was added dry methanol (25 mL). The resulting solution was treated with activated selenium powder (0·1 g, 1·26 mmol) and anhydrous potassium carbonate (0·16 g, 1·15 mmol). The reaction mixture was heated to reflux for 24 h. The solution was then filtered through a pad of celite to remove

the unreacted selenium and washed with methanol. The small impurities in the sample can be removed by column chromatography (silica gel) by using 1 : 1 ethyl acetate: petroleum ether. Yield: 0·16 g (47%). m.p. 92–94°C. ^{1}H NMR ($CDCl_3$, ppm): δ = 1·41 (*t*, 3H, $J_{H–H}$ = 7·2 Hz, of NCH_2CH_3), 4·23 (*q*, 2H, $J_{H–H}$ = 7·2 Hz, NCH_2), 5·36 (*s*, 2H, NCH_2), 6·73 (*d*, IH, $J_{H–H}$ = 2 Hz, C–H of imidazole ring), 6·86 (*d*, IH, $J_{H–H}$ = 2·4 Hz, C–H of imidazole ring), 7·34 (*m*, 5H, CH of benzene ring); ^{13}C NMR ($CDCl_3$, ppm): δ = 13·5 (CH_3), 43·9 (CH_2 of ethyl group), 51·9 (CH_2Ph), 117·3, 117·7, (CH of imidazole ring), 127·3, 127·4, 127·9, 134·5 (CH of benzene ring), 154·5 (C=Se of imidazole ring); ^{77}Se NMR ($CDCl_3$, ppm): δ = –9; Elemental analysis: Anal. Calcd. for $C_{12}H_{14}N_2Se$: C, 54·34; H, 5·32; N, 10·56 Found: C, 54·49; H, 5·51; N, 10·49.

2.1d *Synthesis of 1,3-dibenzyl-1H-imidazole-2(3H)-thione (**9**):* Benzyl chloride (0·8 mL, 6·96 mmol) was added to a two-neck flask containing sodium iodide (1·04 g, 6·96 mmol) and 1-benzylimidazole (1·0 g, 6·33 mmol) in 20 mL acetone. The reaction mixture was stirred for 6 h at room temperature. The solvent was removed under vacuum and the resulting residue was treated with dichloromethane. The mixture was stirred for 5 min and the precipitated sodium chloride was removed by filtration. The solvent dichloromethane was evaporated under reduced pressure to yield a yellow solid, which was used for the next step without any further purification. The yellow solid obtained in the first step was taken in a 100 mL two-neck round bottom flask fitted with a reflux condenser and was added dry methanol (30 mL). The resulting slurry was treated with sulfur powder (0·2 g, 6·33 mmol) and anhydrous potassium carbonate (0·8 g, 5·80 mmol). The reaction mixture was heated to reflux for 8 h. The solution was then filtered in hot condition through a pad of Celite and washed two times with dry methanol. The desired compound was obtained as a white crystalline solid. The small impurities in the sample can be removed by column chromatography (silica gel) using 1 : 5 ethyl acetate: petroleum ether. Yield: 0·80 g (45%). m.p.103–105°C. ^{1}H NMR ($CDCl_3$, ppm): δ = 5·30 (*s*, 4H of NCH_2Ph), 6·54 (*s*, 2H, NCH), 7·33 (*m*, 10H, CH of benzene ring); ^{13}C NMR ($CDCl_3$, ppm): δ = 51·4 (NCH_2Ph), 116·8 (CH of imidazole ring), 128·2, 128·4, 128·9, 135·9 (CH of benzene ring), 163·2 (C=S of imidazole ring); HRMS *m/z* (TOF) calcd. for $C_{17}H_{16}N_2S$ $[M + Na]^+$ 303·0932, found 303·0930; Elemental analysis: Anal. calcd. for $C_{17}H_{16}N_2S$: C, 72·82; H, 5·75; N, 9·99 Found: C, 72·74; H, 6·0; N, 10·09.

2.1e *Synthesis of 1,3-dibenzyl-1H-imidazole-2(3H)-selone (**10**):* Benzyl chloride (0·8 mL, 6·96 mmol) was added to a two-neck flask containing sodium iodide (1·04 g, 6·96 mmol) and 1-benzylimidazole (1·0 g, 6·33 mmol) in 20 mL acetone. The reaction mixture was stirred for 6 h at room temperature. The solvent was removed under vacuum and the resulting residue was treated with dichloromethane. The mixture was stirred for 5 min and the precipitated sodium chloride was removed by filtration. The solvent dichloromethane was evaporated under reduced pressure to yield a yellow solid, which was used for the next step without any further purification. The yellow solid obtained in the first step was taken in a 100 mL two-neck round bottom flask fitted with a reflux condenser and was added dry methanol (30 mL). The resulting slurry was treated with activated selenium powder (0·5 g, 6·33 mmol) and anhydrous potassium carbonate (0·8 g, 5·80 mmol). The reaction mixture was heated to reflux for 8 h. The solution was then filtered in hot condition through a pad of Celite and washed two times with dry methanol. The desired compound was obtained as a white crystalline solid. The small impurities in the sample can be removed by column chromatography (silica gel) by using 1 : 5 ethyl acetate: petroleum ether. Yield: 0·85 g (40%). m.p. 112–114°C. ^{1}H NMR ($CDCl_3$, ppm): δ = 5·38 (*s*, 4H of NCH_2Ph), 6·87 (*s*, 2H, NCH), 7·33 (*m*, 10H, CH of benzene ring); ^{13}C NMR ($CDCl_3$, ppm): δ = 53·4 (NCH_2Ph), 118·9 (CH of imidazole ring), 128·4, 128·5, 129·0, 135·5 (CH of benzene ring), 157·1 (C=Se of imidazole ring); ^{77}Se NMR ($CDCl_3$, ppm): δ = 0. HRMS *m/z* (TOF) calcd for $C_{17}H_{16}N_2Se$ $[M + Na]^+$ 351·0376, found 351·0374; Elemental analysis: Anal. calcd. C, 62·39; H, 4·93; N, 8·56 Found: C, 62·31; H, 4·85; N, 8·63.

2.1f *LPO-catalysed oxidation:* The LPO inhibition experiments were performed in phosphate buffer (pH 7·0) at 25°C. The spectral measurements were carried out on a UV-Vis spectrophotometer and the assay of LPO enzyme activity was followed by catalysis of the oxidation of ABTS. The initial rate for the oxidation reaction was calculated by following UV absorption increase at 411 nm. Enzyme activity after the addition of various inhibitors was expressed

as the percentage of that observed in the absence of inhibitors. The peroxide concentration was always present in excess with respect to enzyme. The inhibition plots were obtained by using Origin 6.1 software and these plots were used for the calculation of the IC_{50} values. In a typical experiment, 100 mM ABTS (used as diammonium salt) and 30 mM hydrogen peroxide (from 30% *w*/*w* solution) solutions were freshly prepared in deionized water. Lactoperoxidase enzyme solution containing 0·15–0.25 unit/mL was prepared in cold deionized water and used immediately for the assay. In a 1 mL reaction mixture, the final concentrations are 12·9 nM LPO, 28·7 μM H_2O_2, 1·4 mM ABTS and 1–200 mM inhibitor. For the compounds that are not soluble in buffer, a minimum amount of DMSO (~10 μL) and diluted with the test buffer. Likewise the control reactions were carried out with the same amount of DMSO.

2.1g *Single crystal X-ray crystallography:* X-ray crystallographic studies were carried out on a Bruker CCD diffractometer with graphite-monochromatized Mo-Kα radiation (λ = 0·71073 Å) controlled by a Pentium-based PC running on the SMART software package. (Bruker AXS: Madison, WI). Single crystals were mounted at room temperature on the ends of glass fibres and data were collected at room temperature. The structures were solved by direct methods and refined using the SHELXTL software package (Siemens Industrial Automation Inc.: Madison, WI).[24] In general, all non-hydrogen atoms were refined anisotropically. Hydrogen atoms were assigned idealized locations. Empirical absorption corrections were applied to all structures using SADABS. (Siemens Industrial Automation Inc.: Madison, WI).[25,26]

3. Results and discussion

3.1 *Thiol/thione and selenol/selone tautomerism*

The keto-enol type tautomerism in the anti-thyroid drugs and their selenium analogues has been a focus of several investigations.[7] It is known that the anti-thyroid agent MMI (**1**) exists almost exclusively as the thione tautomer, which is important for the inhibition of thyroid hormone synthesis. The other sulfur-containing drugs, PTU (**4**) and MTU (**6**) have also been shown to exist as the thione tautomers.[7] The stability of the thione tautomer may prevent these compounds from being oxidized spontaneously to their corresponding disulfides, which may account for their high anti-thyroidal activity. Laurence *et al* have shown that the thione tautomer of MMI is responsible for its complexation with diiodine and the iodine complex of the thione tautomer **1a** is favoured by 13·2 kJ mol^{-1} compared to that of the thiol tautomer **1b**.[7g] Therefore, the facile oxidation of **2** to the corresponding diselenide requires the compound to be in its selenol form (**2b**) and not in the selone form (**2a**) (figure 3). Although compound **2** can exist in both selenol and selone forms in solution, the ^{77}Se NMR spectrum recorded immediately after the workup of the reaction showed a signal at 4 ppm, which cannot be ascribed to the true selone (**2a**) or undissociated selenol (**2b**) tautomer, but it can be ascribed to a species with more negative charge on selenium (**2c**). In the presence of air, this species slowly oxidizes to the corresponding diselenide, which shifts the equilibrium from a selone to a dissociated selenol, and this process continues until all the selone–selenolate mixture is converted to diselenide.[6b] In contrast, the sulfur analogue, which exists predominantly in its thione tautomer form (**1a**), was found to be stable under normal atmosphere.

The synthesis of the selenium analogue of methimazole (**2**, MSeI) was first described by Guziec *et al*.[5d] In this case, the authors have mentioned that their initial attempts to synthesize MSeI by an alkylation-selenation sequence, commonly employed for the preparation of acyclic selenoureas, did not afford the desired selone. However, an alternative approach involving low temperature metallation of 1-methylimidazole, followed by selenium insertion afforded the desired compound in moderate yield.[5d] This methodology was recently employed by Parkin *et al* to synthesize 1-mesityl-1,3-dihydro-imidazole-2-selone.[8] It should be mentioned that the replacement of N–H moiety in MMI with an N–Me group has been shown to abolish the inhibitory activity of MMI.[9] Therefore, it would be interesting to know whether the replacement of the free hydrogen atom

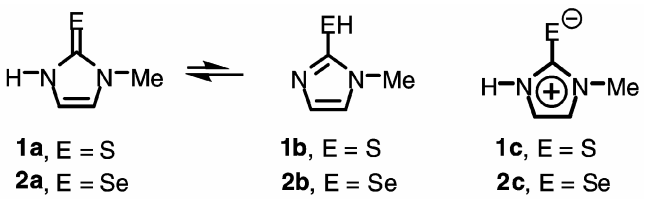
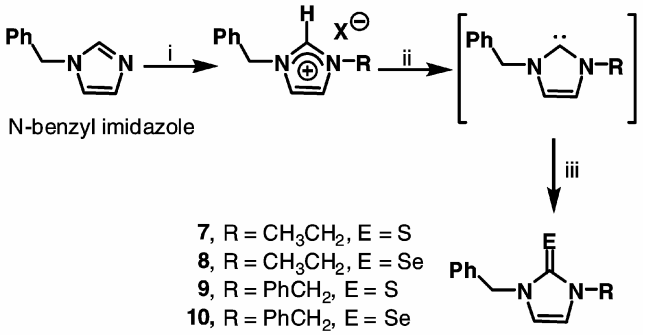


Figure 3. Tautomeric structures of MMI (**1**) and MSeI (**2**).[6b]

in MMI and MSeI by other substituents would lead to inactive substances. In view of this, we have synthesized a variety of thiones and selones having different substituents on both the nitrogen atoms present in the 5-membered ring.

In the present study, the required starting material, 1-benzylimidazole, was synthesized conveniently from imidazole and benzylchloride. The treatment of imidazole with sodium hydride generated the corresponding anion, which upon reaction with benzyl chloride afforded the expected compound. The abstraction of the proton from the imidazole nitrogen was found to be a clean reaction due to the stabilization of the resulting anion by resonance contribution from the imidazole ring. The N,N-disubstituted derivatives (**7–10**) are not accessible via the low-temperature lithiation route. Therefore, the thiones (**7**, **9**) and selones (**8**, **10**) were synthesized by a carbene route utilizing an imidazolium salt (scheme 1). The N,N-disubstituted derivatives were synthesized by treating 1-benzylimidazole with appropriate halides to produce the corresponding imidazolium salts, followed by reactions with elemental sulfur or selenium to afford the corresponding thiones or selones. In the first step of these reactions, the halides were added into the carbon–nitrogen double bond to give the corresponding N,N-substituted imidazolium salts. These salts were isolated and treated with a base (anhydrous potassium carbonate) and selenium or sulphur powder in dry methanol to give the corresponding selones and thiones. In these reactions, the deprotonation of imidazolium salts by the base leads to an *in situ* generation of reactive carbenes, which in turn react with elemental sulfur or selenium to afford the corresponding thiones or selones. These



Scheme 1. Synthetic routes for compounds **7**–**10** via imidazolium salt/carbene. Reagents and conditions: (i) RX = CH_3CH_2Br for compounds **7** and **8**; RX = $PhCH_2Cl$ for compounds **9** and **10**; (ii) dry methanol, K_2CO_3, reflux; (iii) S or Se powder.

compounds are readily soluble in dichloromethane and are thus easily separated from salt-like components of the reaction mixture. In contrast to the selenium analogue of MMI, the selenium derivatives synthesized in the present study are found to be stable in the presence of air and we did not observe any oxidation leading to the formation of diselenides.

We have shown in our previous studies that the replacement of the N-H moiety in MSeI by an N–Me or N–Bz group does not affect the nature of C–Se bond or the electron density around the selenium center.[6g] The computational and ^{77}Se NMR spectroscopic studies on these compounds suggested that the molecules exist in their zwitterionic forms. The X-ray structure of 1,3-dimethyl-2(3*H*)-imidazolethione (figure 4, R = Me) was first reported by Ansel *et al*[10] and based on the C–N and C–C bond lengths [C(thionyl)–N: 1·350 Å; C(ethylenic)–N: 1·41 Å and C=C: 1·31 Å] in the imidazole ring, they have proposed that the electronic structure of this compound would best be represented by a resonance hybrid of structures **I** and **IV** (figure 4). Tomlin *et al*[11] on the other hand, re-evaluated the crystal structure of the same compound and proposed a more delocalized zwitterionic structure **V** (figure 4). We have shown in our previous studies that the replacement of sulfur with selenium leads to an elongation of the C–E (E = S or Se) bond and generation of a more delocalized structure.[6b,g] The crystal structure of **2** clearly showed that the two C–N bond lengths are almost identical (N1–C1: 1·350; N2–C1: 1·346).[6g] Therefore, the N,N-disubstituted thiones and selones that we synthesized in the present work can be considered as zwitterions having large negative charge on selenium and delocalized positive charge in the 5-membered ring.

The crystal structures of compounds **7**, **9** and **10** (figure 5, table 1) reveal that these compounds do not have a true C=S or C=Se double bond. The C–S bond lengths in the thiones (**7**, **9**) are found to be in the range of 1·674 to 1·683 Å, indicating much shorter bond lengths as compared with a C–S single bond (1·81 Å), but longer bond lengths when compared with a C=S double bond (1·61 Å).[12] This suggests that the C–S bonds in the thiones may be treated as partial double bonds. Similarly, the C–Se bond in **10** is found to be much weaker than a typical C–Se double bond and slightly stronger than a C–Se single bond. The shortening of the adjacent C–N bonds, a considerable elongation in the olefinic double bond (C=C bond) from a true C–C double bond and a sig-

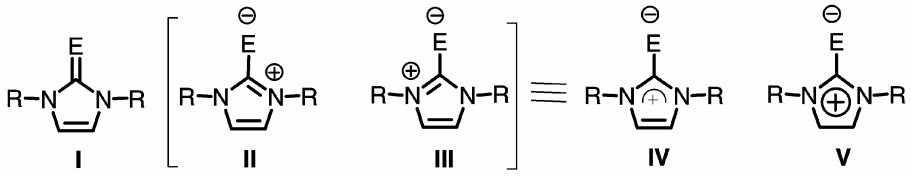


Figure 4. Possible tautomeric structures of N-N-disubstituted thiones (E = S) and selones (E = Se).

Table 1. Crystallographic data for compounds **7**, **9** and **10**.

	Compd **7**	Compd **9**	Compd **10**
Formula	$C_{12}H_{14}N_2S$	$C_{17}H_{16}N_2S$	$C_{17}H_{16}N_2Se$
F_w	218·3	276·4	326·3
Crystal system	Orthorhombic	Monoclinic	Monoclinic
Space group	*P*222	*P*2(1)/*n*	*P*21
a (Å)	6·7558(11)	7·9600(11)	7·4223(8)
b (Å)	7·9881(13)	11·2967(17)	17·4649(18)
c (Å)	21·9223(37)	16·3814(24)	11·6597(13)
α (°)	90·00	90·00	90·00
β (°)	90·00	92·636(3)	92·432(2)
γ (°)	90·00	90·00	90·00
V (Å^3)	1183·06(3)	1471·49(4)	1510·08(3)
D_{calc} (mg m^{-3})	1·23	1·25	1·43
Z	4	4	4
μ (mm^{-1})	0·243	0·210	2·478
Reflections collected/unique	9231/2325	12623/3478	4438/4150
Parameters	137	181	361
R_{int}	0·025	0·042	0·014
R^a	0·043	0·061	0·041
$R_w{}^b$	0·097	0·162	0·087
Goodness-of-fit on F^2	1·071	1·112	0·987
$\Delta\rho_{min}$ and $\Delta\rho_{max}$ (e.Å^{-3})	–0·121 and 0·262	–0·304 and 0·551	–0·345 and 0·411

nificant shortening of the C(ethylenic)–N bonds in the imidazole ring in the selone **10** point to a delocalized zwitterionic structure as shown for MSeI. When we compare the structure of the thione **9** with that of its selenium analogue **10**, it appears that C–Se bond in compound **10** is more polarized than the C–S bond in compound **9**, indicating a weak C-Se pi-overlap in the selenium compound.

3.2 *Inhibition of lactoperoxidase-catalysed oxidation*

The inhibition of peroxidase-catalysed oxidation reactions by thiourea compounds has been routinely used not only to determine the potency of clinically useful anti-thyroid drugs, but also to understand the mechanism by which the drugs exert their anti-thyroidal activity. Although the inhibition of thyroid peroxidase (TPO) and a related enzyme, lactoperoxidase (LPO) by anti-thyroid agents has been studied extensively in recent years, the mechanism of the inhibition of heme-peroxidases or the inhibition of peroxidase-catalysed oxidation and iodination reactions is still not clear. Magnusson *et al* suggested that the inhibition of TPO or LPO by the thiourea drugs may occur through competition with hydrogen peroxide for a common form of oxidized iodine.[13] Davidson *et al* proposed that anti-thyroid drugs block the iodination *in vivo* by reducing the oxidized iodide generated by the TPO, thus diverting it from the natural substrate tyrosyl residues.[14] In contrast, Engler *et al* suggested that MMI and PTU exert their activity by reacting with the oxidized TPO heme group and thus inactivating the enzyme.[15] It is also possible that the thiourea drugs are oxidized by the TPO/H_2O_2 system and the drugs in their oxidized forms may bind to the heme group of the enzyme. Taurog and Dorris, on the other hand, suggested that

the inhibition of iodination by compounds such as PTU involves a competition reaction between the drugs and tyrosine residues of thyroglobulin (Tg) for oxidized iodine.[14,16]

In the present study, we have carried out the enzyme inhibition experiments with lactoperoxidase (LPO, figure 6) since it is readily available in purified form.[17] Furthermore, LPO has been shown to behave similarly to TPO with respect to iodination of thyroglobulin, the natural substrate, and other iodide acceptors.[18] Edelhock *et al* have reported the inactivation of LPO by thiourea-based drugs using LPO-N-acetyltyrosylamide assay.[19] We have employed 2,2′-azio-bis 3-ethyl-benthiazoline-6-sulphonic acid (ABTS) and H_2O_2 as substrates to determine the half-maximal inhibitory concentration (IC_{50}) of test compounds.[6,20,21] The LPO activities at different concentration of MMI and MSeI are summarized in figure 7 and the corresponding IC_{50} values are given in table 2, respectively.

To obtain reliable IC_{50} values for compound **2** and to make a direct comparison with the sulfur analogue, it is important to carry out the inhibition experiments with the completely reduced species. We have observed that the selenium compound obtained directly from the reaction does not give any reproducible results because it contains a mixture of MSeI and the corresponding diselenide. Therefore, we carried out the experiment with the reduced species (**2**), which was obtained by reducing the diselenide with $NaBH_4$ in an aqueous solution and extracted in dichloromethane. However, compound **2** generated by this method is reasonably stable to obtain reliable IC_{50} values.

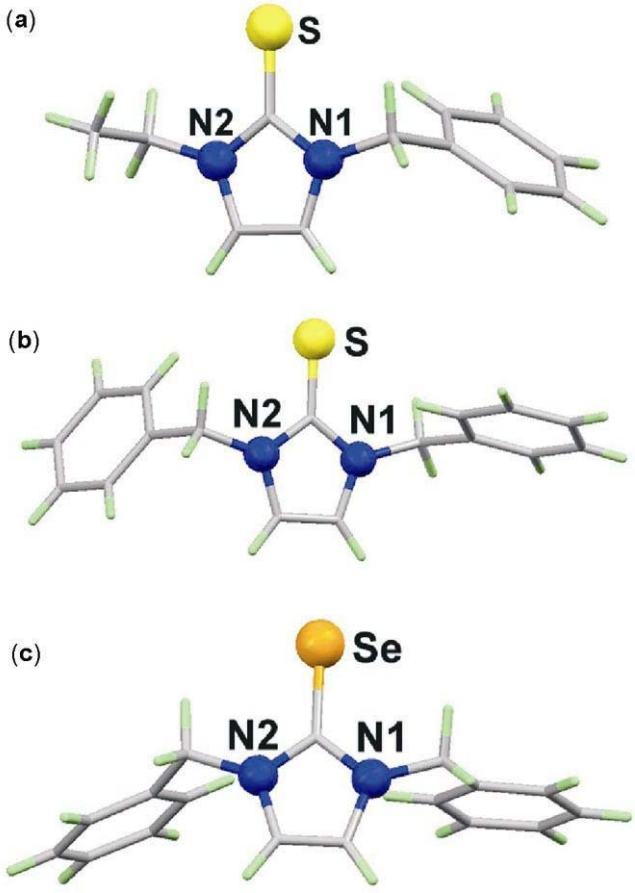


Figure 5. X-ray crystal structures of compounds **7** (**a**), **9** (**b**) and **10** (**c**).

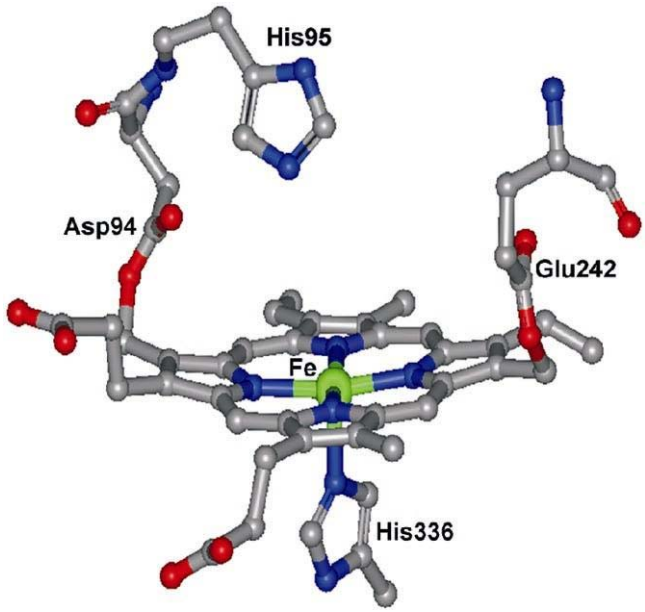


Figure 6. The active site structure of lactoperoxidase (PDB Code: 2GJ1).[17]

Table 2. Inhibition of LPO activity by compounds **1–3**, **5**, and **7–10**.

No.	Compound	IC_{50} (μM)[a]
1	**MMI** (**1**)	7·8
2	**MSeI** (**2**)	16·4
3	**PTU** (**3**)	45.0
4	**MTU** (**5**)	47·8
5	**7**	Inactive[b]
6	**8**	12·3
7	**9**	Inactive[b]
8	**10**	9·6

[a]Concentration of the compound causing 50% inhibition. [b]The compounds were inactive up to 100 μM and some solubility problems in the test buffer were observed above this concentration

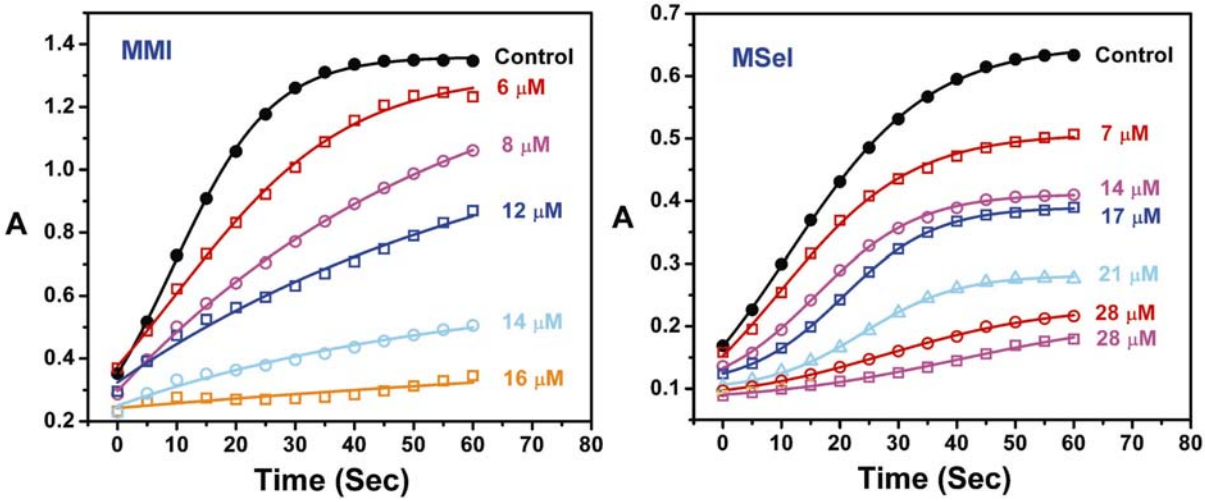


Figure 7. Inhibition of LPO-catalysed oxidation of ABTS by **MMI** and **MSeI** at pH 7 and 30°C. The reaction mixture contained 0·5 μg/ml LPO, 1·4 mM ABTS, 0·067 M phosphate buffer (pH 7) and 28·67 μM H_2O_2. The reaction was initiated by addition of H_2O_2. The initial formation of ABTS radical cation was monitored by an UV-vis spectrophotometer at 411 nm.

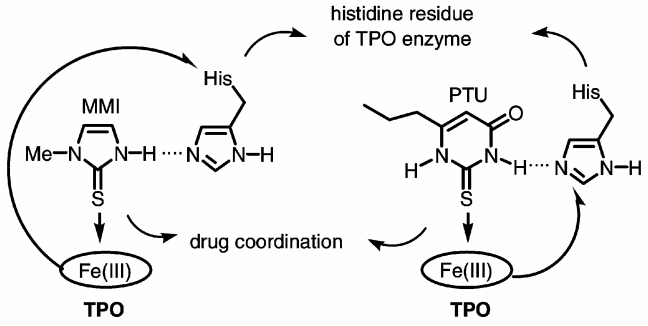


Figure 8. A hypothetical model for the coordination of thiourea drugs to the Fe-center of TPO.

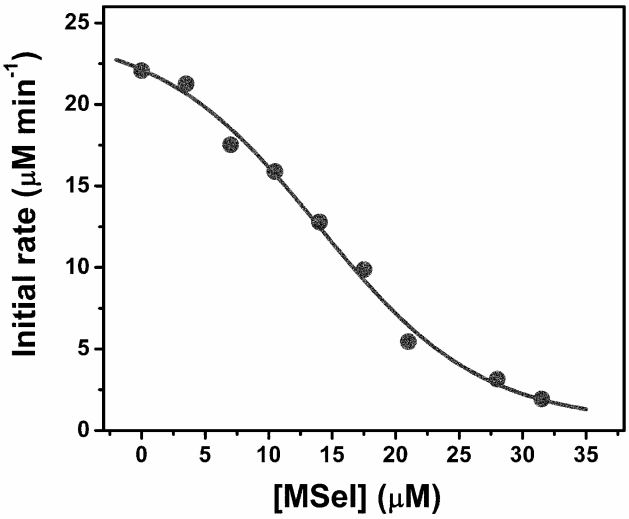


Figure 9. Plot of initial rates (v_o) for the LPO-catalysed oxidation of ABTS vs concentration of MSeI (**2**). The reaction mixture contained 0·5 μg/ml LPO, 1·4 mM ABTS, 0·067 M phosphate buffer (pH 7) and 28·67 μM H_2O_2. The reaction was initiated with H_2O_2. The formation of ABTS radical cation was monitored at 411 nm.

Similarly to the inhibition of LPO by MMI, the LPO activity decreases with an increase in the concentration of **MSeI** (figure 7). As expected, **MMI** exhibited high inhibitory activity towards LPO with an IC_{50} value of 7·8 μM, which is much lower than that observed with **PTU** and **MTU**. The selenium analogue (**2**) also inhibited LPO, and the IC_{50} value was found to be almost three times lower than that of **PTU** and **MTU**. The higher activity of **MMI** as compared with **PTU** and **MTU** is in agreement with the previous studies on the inhibition of TPO.[15a,22] Since the activation of the iron center in TPO must proceed through an interaction of Fe(III) with H_2O_2, the inhibition of TPO may occur through a competitive coordination of the drug to iron, assisted by hydrogen bonding with a histidine residue of the TPO enzyme (figure 8).[23] Under these conditions, **MMI** might compete more successfully than **PTU** with H_2O_2, because the hydrogen-bond (hard) basicity pk_{HB} value of **MMI** (2·11) is much higher than that of PTU (~ 1·32).[7g] Similar to **PTU**, the methyl derivative **6** (**MTU**) is also expected to be a weak inhibitor of TPO. On the other hand, the nucleophilicity of the selenium moiety in compound **2** that exists predominantly in its zwitterionic form is expected

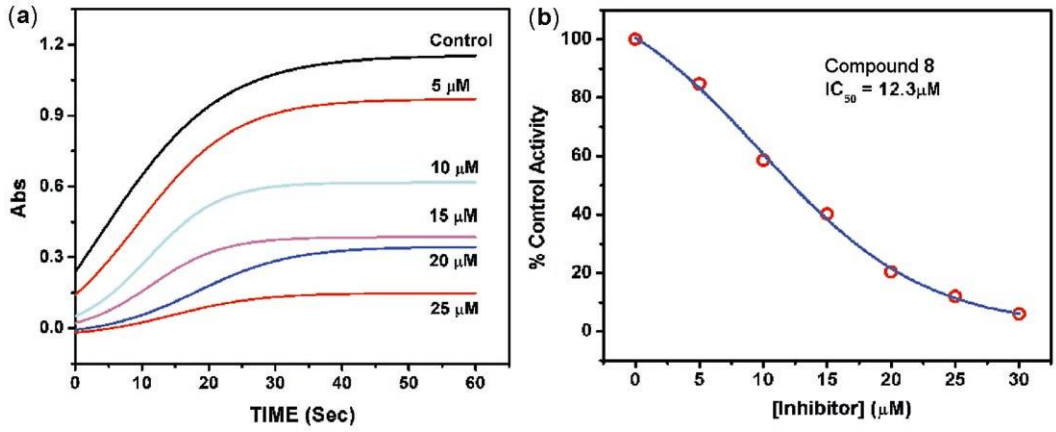


Figure 10. (**a**) Effect of compound **8** on the LPO-catalysed oxidation of ABTS. (**b**) Inhibition plots obtained by plotting the % control activity against the concentrations of compound **8**.

to be much higher than that of the sulfur analogue. However, the lower activity of **MSeI** as compared with **MMI** indicates that the selenium analogue of **MMI** may inhibit LPO by a different mechanism.

As expected, the plot of initial rates (v_0) vs concentration of MSeI shows that the rate of the reaction decreases with increasing concentration of MSeI (figure 9). In all these cases, the LPO activity could be recovered by increasing the hydrogen peroxide concentration. These experimental observations support the conclusions made by Taurog *et al* that MSeI, unlike MMI, cannot act as an irreversible inhibitor of TPO. These observations also support the *in vivo* experiments, which showed that MMI is at least 50 times more potent than MSeI as an inhibitor of organic iodine formation in the thyroid.[5] Crucially, the treatment of **2** with the selenolate specific reagent, iodoacetic acid, abolished the inhibitory potency of **2**, confirming that the oxidation of the selenolate group by H_2O_2 is responsible for the inhibition. In contrast, the sulfur analogue MMI was found to be less sensitive to the iodoacetic acid treatment, and this also confirms that the thiol (or thiolate) form of MMI is not only less predominant in solution but also less reactive as compared with the thione form. The decrease in the inhibitory activity of **2** upon iodoacetic acid treatment can be ascribed to the formation of a protected selenide as a dead-end product.

In contrast to MMI, the thiones **7** and **9**, which do not have any free N–H group do not inhibit the LPO-catalysed oxidation. Surprisingly, the replacement of sulfur atoms in compounds **7** and **9** with selenium (i.e. compounds **8** and **10**) led to almost a complete inhibition of LPO activity with IC_{50} values of 12.3 μM and 9·6 μM, respectively, which are lower than that of MSeI (16·4 μM). Also, these two selenium compounds are much more active than the 2-thiouracil derivatives. The effect of **8** on the LPO-catalysed oxidation of ABTS is summarized in figure 10a and the inhibition plot for **8** is shown in figure 10b. Although compounds **8** and **10** lack the essential N–H group, the higher inhibitory activity of these compounds as compared with their sulfur analogues can be ascribed to the existence of **8** and **10** in zwitterionic structures where the selenium moiety acts as a selenolate rather than a selone. Similarly to MSeI (**2**), the negatively charged selenium moiety may scavenge the H_2O_2 substrate effectively present in the model system (ABTS assay) or this compound may interfere with the oxidized LPO species, leading to a reversible inactivation.

4. Conclusion

In this paper, we have described the keto-enol type tautomerism in anti-thyroid drugs and their selenium analogues. The present study supports our previous observations that the anti-thyroid agent methimazole (MMI) exists predominantly in its thione form, whereas its selenium analogue exists in a zwitterionic form with a large negative charge on selenium. We have studied the importance of thione/thiol and selone/selenol tautomerism on the inhibition of peroxidase-catalysed reactions by using N,N-disubstituted thiones and selones. These studies reveal that the presence of a free N-H moiety in the sulfur-based

compounds is essential for an efficient inhibition. In contrast to the thiones, the N,N-disubsituted selones were found to be efficient inhibitors of LPO-catalysed oxidation, indicating that the free N-H moiety is not required for the inhibition.

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