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Antithyroid Drugs and their Analogues Protect Against Peroxynitrite-Mediated Protein Tyrosine Nitration—A Mechanistic Study

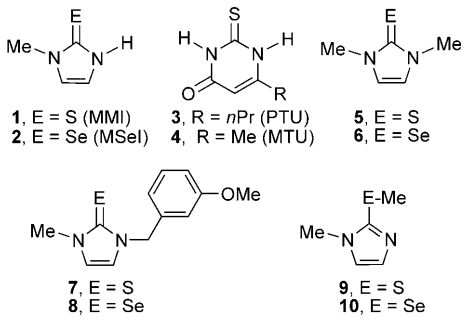
Krishna P. Bhabak and Govindasamy Mugesh*[a]

Abstract: In this paper, the effect of some commonly used antithyroid drugs and their analogues on peroxynitrite-mediated nitration of proteins is described. The nitration of tyrosine residues in bovine serum albumin (BSA) and cytochrome c was studied by Western blot analysis. These studies reveal that the antithyroid drugs methimazole (MMI), 6-*n*-propyl-2-thiouracil (PTU), and 6-methyl-2-thiouracil (MTU), which contain thione moieties, significantly reduce the tyrosine nitration of both BSA and cytochrome c. While MMI exhibits good peroxynitrite (PN) scavenging activity, the thiouracil compounds PTU and MTU are slightly less effective than MMI. The S- and Se-methylated compounds show a weak inhibitory effect in the nitration of tyrosine, indicating that the presence of a thione or selone moiety is important for an efficient inhibition. Similarly, the replacement of N–H moiety in MMI by *N*-methyl or *N*-*m*-methoxybenzyl substituents dramatically reduces the antioxidant activity of the parent compound. Theoretical studies indicate that the substitution of N–H moiety by N–Me significantly increases the energy required for the oxidation of sulfur center by PN. However, such substitution in the selenium analogue of MMI increases the activity of parent compound. This is due to the facile oxidation of the selone moiety to the corresponding selenenic and seleninic acids. Unlike *N,N′*-disubstituted thiones, the corresponding selones efficiently scavenge PN, as they predominantly exist in their zwitterionic forms in which the selenium atom carries a large negative charge.

Keywords: antioxidants · antithyroid drugs · peroxynitrite · protein nitration · selenium

Introduction

Thyroxine (T4), the main secretory product of the thyroid gland, is produced on thyroglobulin by thyroid peroxidase (TPO)/H_2O_2/I^- system.[1] Monodeiodination of T4 by iodothyronine deiodinase (ID-I) converts this prohormone to the biologically active hormone, 3,5,3′-triiodothyronine (T3).[2] However, an overproduction of T4 and T3 leads to hyperthyroidism, which is generally treated by antithyroid drugs; several examples of thiourea-based along antithyroid drugs and their analogues are shown here (**1**–**10**). Methimazole (**1**, MMI), 6-*n*-propyl-2-thiouracil (**3**, PTU) and 6-methyl-2-thiouracil (**4**, MTU) are the most commonly used antithyroid drugs, which either block the thyroid hormone biosynthesis or reduce the conversion of T4 to T3 by inhibiting the ID-I enzyme. The mechanisms by which antithyroid drugs block the thyroid hormone biosynthesis and inhibit the ID-I have been investigated by several research groups.[2–5]



It is known that thyroid hormones enhance the basal metabolic rate and oxidative metabolism by inducing certain mitochondrial enzymes.[6] It has been shown that the H_2O_2 level in thyroid gland is generally controlled by antioxidant enzymes such as the selenium-containing glutathione peroxidase (GPx).[7] However, the overproduction of thyroid hormones leads to an imbalance between oxidants and antioxi-

[a] K. P. Bhabak, Prof. Dr. G. Mugesh
Department of Inorganic and Physical Chemistry
Indian Institute of Science, Bangalore 560 012 (India)
Fax: (+91) 80-2360-1552/2360-0683
E-mail: mugesh@ipc.iisc.ernet.in

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dants status and thus enhances the oxidative stress.[8] Therefore, the antioxidant activity of antithyroid drugs attracts considerable attention. The degradation of intracellular oxidants by antithyroid drugs may be beneficial to the thyroid gland, as these compounds may protect the thyroid cells from oxidative damage. Sewerynek and co-workers reported that MMI can protect against oxidative stress induced by overproduction of thyroid hormones.[9] In agreement with this, Kim and co-workers have shown that MMI can scavenge H_2O_2 in the presence of thiols such as glutathione and thioredoxin.[10] Similarly, PTU has been shown to be highly efficient scavenger of hydroxyl radicals and an efficient inhibitor of lipid peroxidation at the free drug levels commonly attained in serum during such therapeutic use.[11] Recently, we have shown that the selenium analogue of MMI (**2**) exhibits significant GPx-like activity by catalytically reducing H_2O_2 in the presence of glutathione (GSH).[4] However, it is not clear whether antithyroid drugs can protect against reactive nitrogen species such as peroxynitrite ($ONOO^-$). In this paper, we show, for the first time, that the antithyroid drugs (**1**, **3** and **4**) and their analogues (**5**, **6**, **8** and **10**) effectively inhibit protein tyrosine nitration mediated by peroxynitrite. We also describe the mechanism by which these compounds inhibit the nitration reactions.

Results and Discussion

Inhibition of protein tyrosine nitration: Nitrotyrosine, generated by enzymatic or non-enzymatic nitration of tyrosyl residues in proteins, is a biomarker of oxidative and nitrative stess in inflammatory, allergic, and other diseases.[12] While the enzymatic nitration is catalyzed by a number of heme proteins, such as peroxidases (myeloperoxidase,[13] eosinophil peroxidase,[14] horseradish peroxidase,[13] lactoperoxidase[15]), hemoglobin,[16] myoglobin,[17] and cytochrome c,[18] the non-enzymatic or chemical nitration is caused extensively by peroxynitrite (PN). In biological medium, PN is produced by the diffusion-controlled reaction of superoxide anion radical ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$).[19] PN can induce DNA damage[20] as well as initiate lipid peoxidation in biomembranes or low density lipoproteins[21] and is known to inactivate a variety of enzymes by tyrosine nitration.[22] It has been shown that PN inactivates manganese superoxide dismutase (Mn-SOD) by nitration and oxidation of crucial tyrosine residues.[23]

To understand the protective effects of antithyroid drugs against protein tyrosine nitration, we have used the PN-mediated nitration of bovine serum albumin (BSA),[24] and cytochrome c.[25] The inhibition of tyrosine nitration was followed by SDS-PAGE and Western blotting methods using antibody against 3-nitro-L-tyrosine. Interestingly, antithyroid drugs MMI (**1**), PTU (**3**), MTU (**4**) and their analogues **2**, **5**, **6**, **8** and **10** significantly reduced the tyrosine nitration of both BSA and cytochrome c (Figure 1). MMI was particularly effective and a complete inhibition of nitration was observed for this compound. The dimethyl derivative **5** and its

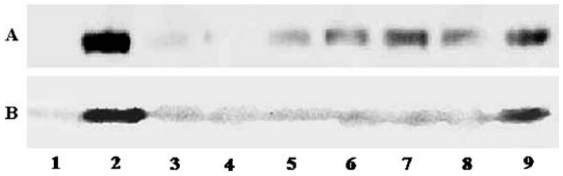
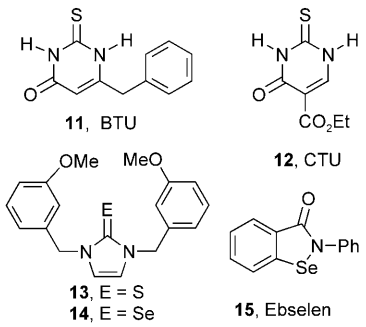


Figure 1. Western blots of the inhibition of PN-mediated tyrosine nitration of A) BSA and B) cytochrome c. Lanes: 1: pure protein; 2: protein+PN; 3: protein+PN+MMI (**1**); 4: protein+PN+**5**; 5: protein+PN+**6**; 6: protein+PN+**8**; 7: protein+PN+MTU (**4**); 8: protein+PN+PTU (**3**); 9: protein+PN+**10**. Proteins were incubated with PN (2.0 mM for BSA and 4.3 mM for cytochrome c) and inhibitors (85 μM for BSA and 200 μM for cytochrome c) at 20 °C for 20 min and then subjected to gel electrophoresis.

selenium analogue **6** also exhibited good activity against PN-mediated nitration. The thiouracil compounds PTU and MTU were found to be slightly less effective than MMI. On the other hand, the Se-methylated compound **10** showed weak inhibitory effect in both BSA and cytochrome c nitration. This indicates that the presence of thione or selone moiety is important for an efficient inhibition. It should be noted that the number of tyrosine residues in cytochrome c (4 tyrosines) is much less than that of BSA (36 tyrosines). Therefore, a higher concentration of cytochrome c as well as the inhibitors was required for the blotting experiments.

To understand the relative activities of antithyroid drugs and their analogues on tyrosine nitration, we have studied the effect of these compounds on PN-mediated nitration of free L-tyrosine. The conversion of L-tyrosine to 3-nitro-L-tyrosine was followed by reverse phase HPLC. The amount of 3-nitro-L-tyrosine formed in the reactions was determined from the chromatograms by comparing the peak areas of 3-nitro-L-tyrosine in the reaction mixture to that of authentic sample. The concentrations of test compounds required for the inhibition of 50% of the nitration are represented as IC_{50} values. The IC_{50} values obtained for different inhibitors (compounds **1**–**15**) are listed in Table 1 and the inhibition



curves obtained by plotting the percentage control activity against the concentration of test compounds are given in the Supporting Information.

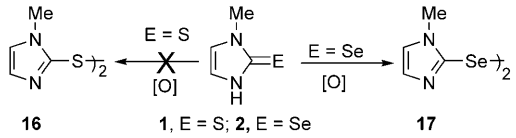
From Table 1, it is evident that most of the thiones and selones strongly inhibit the PN-mediated nitration. The anti-

Table 1. IC_{50} values of compounds **1**–**15** for the PN-mediated nitration of L-tyrosine.

	IC_{50} [μM][a]		IC_{50} [μM][a]
1, MMI	23.9±0.4	**9**	inactive[c]
2, MSeI	49.1±1.2	**10**	inactive[c]
3, PTU	95.0±0.3	**11**	90.7±0.8
4, MTU	66.7±1.9	**12**	52.0±0.2
5	–[b]	**13**	–[b]
6	12.2±0.1	**14**	42.2±0.1
7	–[b]	**15**	212.9±2.0
8	7.8±0.1		

[a] The IC_{50} values were determined from the inhibition plots, obtained by plotting the percentage control activity against the concentration of inhibitors. The test mixture with each concentration of inhibitor was kept at 22 °C for 7 min for the nitration assay. Assay Conditions: The reaction was carried out in sodium phosphate buffer (100 mM, pH: 7.5) at 22 °C. L-tyrosine (0.75 mM), peroxynitrite (0.3 mM), inhibitors (variable). [b] Percentage of inhibition at 200 μM concentration: **5**, 42%; **7**, 45%; **13**, 2%. [c] These compounds did not show any activity upto a concentration of 400 μM.

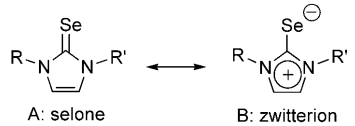
thyroid agent MMI inhibited the nitration with an IC_{50} value of 23.9±0.4 μM, which is about 2–3 times lower than that of other two antithyroid compounds PTU and MTU. The selenium analogue of MMI (**2**) was found to be almost two times less active than MMI. This is probably due to the instability of selone moiety, which leads to the spontaneous oxidation of compound **2** to the corresponding diselenide **17** (Scheme 1). This is in contrast to MMI, which does not undergo an aerial oxidation to produce the corresponding disulfide **16**. The formation of diselenide **17** decreases the nucleophilic reactivity of selenium moiety and hence reduces the PN scavenging activity of **2**. It should be noted that the formation of diselenide **17** was observed upon addition of PN to compound **2**. However, compounds **1**–**4** exhibited significantly higher activity than ebselen (**15**), a known organoselenium compound with antioxidant and anti-inflammatory activities.[26]



16 **1**, E = S; **2**, E = Se **17**

Scheme 1. Spontaneous oxidation of MMI (**1**) and its selenium analogue (**2**) to produce disulfide (**16**) and diselenide (**17**).

The substitution of free hydrogen atom (N–H) in MMI by methyl or *m*-methoxybenzyl substituents significantly reduced the activity. For example, IC_{50} values could not be obtained for compounds **5** and **7** upto a concentration of 200 μM. At this concentration, compounds **5** and **7** exhibited only 42 and 45% inhibition, respectively. Similarly, compound **13** with two *m*-methoxybenzyl groups did not show any noticeable activity. At 200 μM concentration, this compound exhibited only 2% inhibition. On the other hand, the corresponding selenium analogues **6** and **8** exhibited excellent activity. The IC_{50} values obtained for **6** (12.2±0.1 μM) and **8** (7.8±0.1 μM) were found to be much lower than that of MMI and other thiouracil-based compounds. In contrast to the sulfur analogue, the *m*-methoxybenzyl substituted selone **14** was found to be an efficient inhibitor, although the IC_{50} value for this compound (42.2±0.1 μM) is higher than that of MMI, **6** and **8**. This is probably due to the incorporation of two nonpolar substituents in the imidazole ring, which reduces the solubility of these compounds in the assay buffer. These results demonstrate that the selenium compounds are generally more potent than their sulfur analogues. These studies also reveal that the presence of free N–H group is important for the sulfur compounds to exhibit an efficient inhibition, whereas the selenium analogues do not require a free N–H moiety. The higher activities of the selenium compounds as compared to their sulfur analogues may arise from their zwitterionic nature. It has been shown that compounds **2** and **6** exist predominantly in their zwitterionic forms in which the selenium atom carries a large negative charge.[4b,5] In agreement with this, compounds **8** and **14** also exist as zwitterions as evidenced by their ^{77}Se NMR chemical shift values. The large upfield shift in the ^{77}Se NMR signals for compound **8** (−0.2 ppm) and **14** (1.6 ppm) suggest that the selone moiety in these compounds is polarized to give the corresponding zwitterion as shown in Figure 2. Owing to the zwitterionic nature of compounds **6**, **8** and **14**, the selenium moieties in these compounds are expected to scavenge PN more effectively than ebselen and the N,N′-disubstituted sulfur compounds.

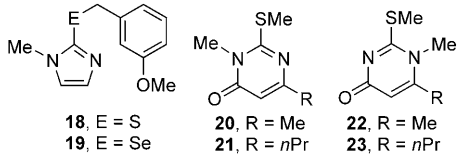


2, R = Me; R' = H; **6**, R, R' = Me
8, R = Me; R' = *m*-OMe-Bn
14, R, R' = *m*-OMe-Bn

Figure 2. The possible tautomeric structures of compounds **2**, **6**, **8** and **14**. These compounds exist predominantly in their zwitterionic forms B, which may only have a partial C–Se double bond character.

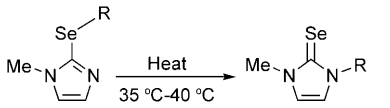
A comparison of the inhibitory activities of the thiouracil-based compounds **3**, **4**, **11** and **12** indicates that the introduction of a nonpolar substituent decreases the activity. For example, PTU (**3**) with an *n*Pr group at the 6-position is less active than MTU (**4**), which contains a methyl substituent. Similarly, the activity of compound **11** having a benzylic group is almost identical to that of PTU, but it is significantly lower than that of **12**, which contains a -CO_2Et substituent at the 5-position. These observations indicate that the thiouracil-based compounds are 2–4 times less active than the imidazole-based compound MMI. Furthermore, the PN scavenging activity of these compounds correlates well with their effect on peroxidase-catalyzed iodination reactions. It has been shown that MMI inhibits TPO more effectively than PTU and MTU.[3a]

In contrast to the *N,N′*-disubstituted thiones/selones **5**–**8**, the S- and Se-substituted compounds **9**, **10**, **18** and **19** did not show any inhibition upto a concentration of 400 μM.



18, E = S
19, E = Se
20, R = Me
21, R = *n*Pr
22, R = Me
23, R = *n*Pr

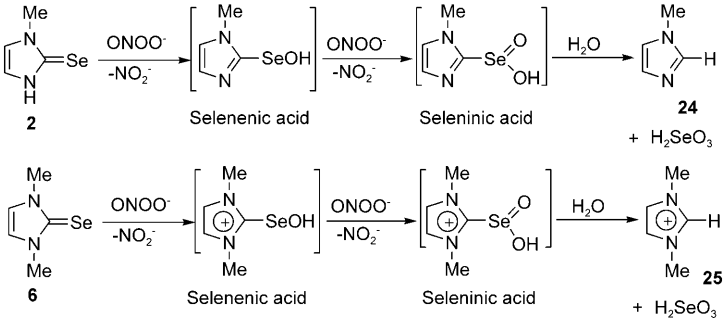
Similarly, the N- and S-substituted thiouracil derivatives **20**–**23** were found to be inactive. These observations suggest that the presence of thione or selone moiety is important for the inhibition of PN-mediated nitration. It should be noted that the S- and Se-methylated compounds **9** and **10** have been shown to be inactive in the inhibition of peroxidase-catalyzed iodination reactions.[5] Interestingly, when the methanolic solution of compounds **10** and **19** were heated to 35–40 °C before adding to the assay mixture, these compounds effectively inhibited tyrosine nitration. This is due to the migration of methyl[5] and *m*-methoxybenzyl groups from selenium to nitrogen in compounds **10** and **19** to produce the N,N′-disubstituted compounds **6** and **8**, respectively (Scheme 2). These observations suggest that compounds **10** and **19** may become effective inhibitors of PN-mediated nitration of proteins under physiological temperatures.



10, R = Me
19, R = *m*-OMe-Bn
6, R = Me
8, R = *m*-OMe-Bn

Scheme 2. Heat-induced isomerization of monoselenides **10** and **19** to the corresponding selones **6** and **8**.

Mechanistic study: To understand the mechanism by which antithyroid drugs and their analogues inhibit PN-mediated nitration, we have studied the reactivity of various thiones and selones toward PN. When the reaction of thiones and selones with PN was followed by UV/Vis spectroscopy, a rapid decrease in the PN absorption at 302 nm was observed upon addition of the test compounds. When the reaction was followed by HPLC, the peaks due to the thiones and selones gradually decreased upon the addition of PN, indicating that the sulfur and selenium moieties react with PN to produce the corresponding oxidized products. The products formed in the reactions were analyzed by ^{1}H, ^{13}C, and/or ^{77}Se NMR spectroscopy and mass spectral techniques. We have used mainly the selenium analogues of antithyroid drugs for this study, as the reactions can be easily monitored by ^{77}Se NMR spectroscopy. Although the selone **2** reacted rapidly with PN, all our attempts to detect the initial oxidation products were unsucessful. This is probably due to the instability of various possible oxidized products such as selenenic and seleninic acids (vide infra). However, a slow elimination of the selenium moiety was observed when the reaction was continued for longer time (≈12 h). The mass spectral analysis of the products inidicated the formation of *N*-methylimidazole as the major product. The ^{77}Se NMR showed a signal at 1272 ppm, which can be ascribed to Na_2SeO_3.[27] The elimination of selenium moiety may proceed via selenenic and seleninic acids intermediates as shown in Scheme 3. The reactions of other selones (**6** and **8**)

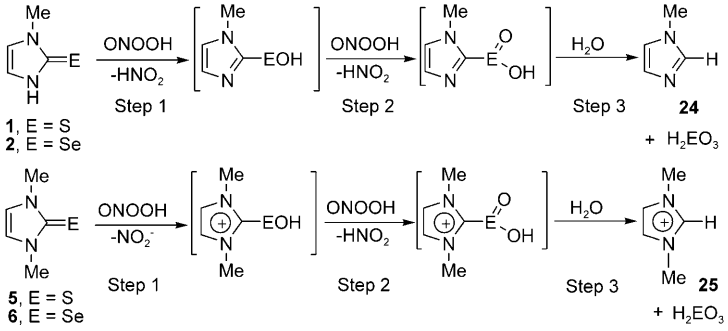


Scheme 3. Elimination of selenium moiety as selenous acid from compound **2** and **6** via the formation of selenenic and seleninic acid intermediates by the reaction with PN.

with PN also produced Na_2SeO_3 as evidenced by ^{77}Se NMR spectroscopy. This indicates that the elimination of selenium moiety takes place even when both the nitrogen atoms are substituted. Similarly, the ^{1}H NMR and mass spectral analysis indicate that the reactions of thiones with PN lead to the elimination of sulfur to produce the corresponding imidazole and uracil derivatives.

Theoretical calculations: To understand the relative activity of various sulfur and selenium compounds, we have carried out detailed density functional theory (DFT) calculations. The reactions of selones and thiones with peroxynitrous acid (ONOOH, PNA)[28] were studied by using B3LYP/6-31+G(d) level of theory. All the thiones/selones, intermediates, transition states (**TS**), and products were optimized in gas phase. A single imaginary frequency was obtained for each **TS** geometry and the nature of **TS** geometries were further confirmed by intrinsic reaction coordinates (IRC) calculations.[29] It has been shown that the reaction of ebselen (**15**) with PN produces the corresponding selenoxide, leading to the elimination of NO_2^-.[30] Our experimental results on thiones and selones show that the elimination of sulfur or selenium moiety in the presence of $ONOO^-$ takes place through an oxidative pathway. As these reactions produce H_2SO_3 or H_2SeO_3 as the final product, the sulfur or selenium moiety must undergo two consecutive oxidation steps. Therefore, we assumed that the reaction of thiones and selones with ONOOH produce the corresponding sulfenic or selenenic acids, which upon reaction with a second equivalent of ONOOH generate the corresponding sulfinic (RSO_2H) or

seleninic ($RSeO_2H$) acids. Hydrolysis of these sulfinic or seleninic acids would produce H_2SO_3 or H_2SeO_3 with the elimination of the corresponding imidazole derivatives (**24** and **25**) as shown in Scheme 4.



Scheme 4. Proposed mechanism for the elimination of sulfur and selenium moieties from thiones/selones via the formation of sulfenic/selenenic acids and sulfinic/seleninic acids intermediates followed by hydrolysis.

The first intermediate of the reaction between compound **1** and PNA was found to be the molecular complex **1a**. This complex is stabilized by two hydrogen bonds involving S···H–O and N–H···O interactions. Although PNA can exist both in *cis* and *trans* isomeric forms,[30a] the hydrogen bonding in **1a** stabilizes the *cis* form. In the next stage of the reaction, nucleophilic attack of the negatively charged sulfur at the oxygen center of PNA leads to the cleavage of O–O bond via transition state **1TSI**. This transformation is accompanied by an abstraction of N–H proton, which leads to the elimination of HNO_2. This results in the formation of the corresponding sulfenic acid **1b**, which is stabilized by the eliminated HNO_2 through hydrogen bonding. Further reaction of sulfenic acid with PNA produces the sulfenic acid-PNA complex (**1c**), which leads to the formation of the corresponding sulfinic acid (**1d**) via the transition state **1TSII**. Interestingly, the interaction between sulfur and -OH group of PNA leads to the cleavage of O–O bond. The NO_2^- ion is thus eliminated as HNO_2 by abstracting the proton from the -S–OH moiety. These changes result in the formation of sulfinic acid **1d**, which is stabilized by hydrogen bonding with HNO_2 as shown in Figure 3. In the final step, the nucleophilic attack of water at the sulfur center in **1e** leads to the cleavage of C–S bond via transition state **1TSIII**. The C–S bond cleavage generates an imidazole carbanion, which abstracts a proton from water to produce *N*-methylimidazole with an elimination of H_2SO_3.

The interactions between the selenium analogue of MMI (**2**) with PNA were found to be similar to that of MMI. The geometry of molecular complex **2a** indicates that this complex is stabilized by Se···H–O and N–H···O hydrogen-bonding interactions (Figure S12, Supporting Information). These interactions lead to the cleavage of O–O bond in PNA and proton transfer from nitrogen to oxygen to produce the corresponding selenenic acid **2c**. In contrast to MMI, the O–O bond cleavage produces intermediate **2b** via transition state

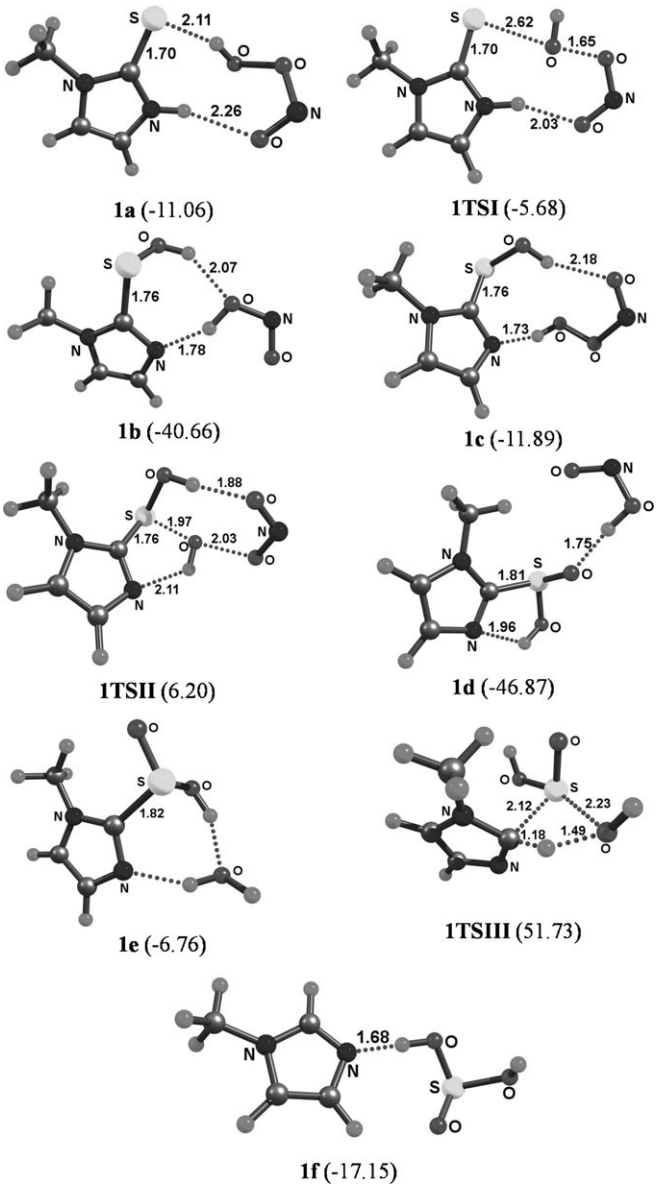


Figure 3. Energy-optimized geometries of the reactants, intermediates, transition states and products for the reaction of MMI with PNA. The values in parentheses correspond to the relative electronic energies (ΔE, kcal mol^{-1}) with zero-point energy corrections. The calculations were performed at B3LYP/6-31+G(d) level of theory. The relative energies are calculated with respect to the sum of energies of MMI and PNA.

2TSI and subsequent proton transfer from nitrogen to oxygen takes place via **2TSII** to produce the corresponding selenenic acid (**2c**). However, the overall energy required for the conversion of **2a** to **2c** via two transition states (5.51 kcal mol^{-1}) is comparable to that of **1a** to **1b** (5.38 kcal mol^{-1}), which takes place through a concerted pathway (Table 2). Further interactions of the selenenic acid **2c** with PNA produce the molecular complex **2d**. Similar to the oxidation of sulfenic acid, the oxidation of selenium center in complex **2d** occurs via transition state **2TSIII**, which involves transfer of a hydroxyl group from PNA to selenium and an abstraction of a proton by the eliminated NO_2^- to

Table 2. Energy barrier for each step (ΔE_{step1}, ΔE_{step2} and $\Delta E_{hydrolysis}$) and the overall energy required for oxidation ($\Delta E_{oxidation}$) in the reaction of thiones/selones with PNA. The calculation was performed using B3LYP/6-31+G(d) level of theory.

	Energy barriers $\Delta(E+ZPE)$ [kcal mol^{-1}]			
		Oxidation		Hydrolysis
	ΔE_{step1}	ΔE_{step2}	$\Delta E_{oxidation}$	$\Delta E_{hydrolysis}$
MMI (**1**)	5.38	18.09	23.47	58.49
MSeI (**2**)	5.51	9.13	14.64	42.33
5	7.40	25.84	33.24	44.36
6	5.79	9.64	15.43	42.80

produce the seleninic acid and HNO_2 complex **2e**. Hydrolysis of the seleninic acid via transition state **2TSIV** then leads to the formation of *N*-methylimidazole (**24**) and H_2SeO_3.

As shown in Table 2, the overall energy required for the reduction of PNA by **2** (14.64 kcal mol^{-1}) is much lower than that of MMI (**1**; 23.47 kcal mol^{-1}). However, the lower activity of compound **2** as compared to MMI (**1**; Table 1) is due to the spontaneous oxidation of **2** to produce the corresponding diselenide (**17**, Scheme 1). As the presence of free N–H moiety in **2** is responsible for the spontaneous oxidation, any substitution that prevents such oxidation would enhance the activity of the parent compound (**2**). It should be noted that the initial oxidation at the sulfur/selenium center in compounds **1** or **2** leads to an elongation of the C–S/Se bond. This bond is further elongated upon subsequent oxidation of the sulfenic/selenenic acid to sulfinic/seleninic acid. This helps in the hydrolytic cleavage of C–S/Se bond by water molecule. It has been proposed that the iodination of MMI (**1**) during its antithyroid action leads to the formation of the corresponding disulfide (**16**), which undergoes spontaneous degradation to produce *N*-methylimidazole (**24**) as a final metabolite.[31] The present study suggests that such metabolite can also be produced upon oxidation of sulfur moiety by PN or other related biological oxidants.

We have shown in the previous section that the replacement of N–H moiety in compound **1** by *N*-methyl or *N*-*m*-methoxybenzyl substituents dramatically reduces the antioxidant activity of the parent compound. For example, compound **5**, which has two N–Me substituents was found to be very less effective as compared to MMI (Table 1). Therefore, we carried out theoretical calculations to understand the importance of free N–H moiety in the antioxidant mechanism. These studies reveal that the initial oxidation of sulfur center in compound **5** is more difficult than that of **1**. This is due to a relatively weaker binding of thione **5** to PNA as compared to that of compound **1**. This difference may arise from the nature of N-substitution in the imidazole ring. For example, the presence of free N–H moiety in compound **1** provides an additional stabilization for the molecular complex **1a** by hydrogen bonding. This is reflected in the energy barriers for the initial oxidation. The energy required for the conversion of **1a** to **1b** (5.38 kcal mol^{-1}) is considerably lower than that of **5a** to **5b** (7.40 kcal mol^{-1}). Furthermore, the conversion of sulfenic acid **1c** to sulfinic acid **1d** by a second molecule of PNA is more favoured than the conversion of **5c** to **5d** (Table 2). This is probably due to the presence of a free nitrogen atom in complex **1c**, which brings PNA closer to the reaction center by N···H–O hydrogen-bonding interaction (Figure 3 and 4). In complex **5c**, the N–Me substituent does not allow any such hydrogen-bonding arrangement. Therefore, the energy required for the conversion of **5c** to **5d** (25.84 kcal mol^{-1}) is almost 7.75 kcal mol^{-1} higher than that of **1c** to **1d** (18.09 kcal mol^{-1}). In con-

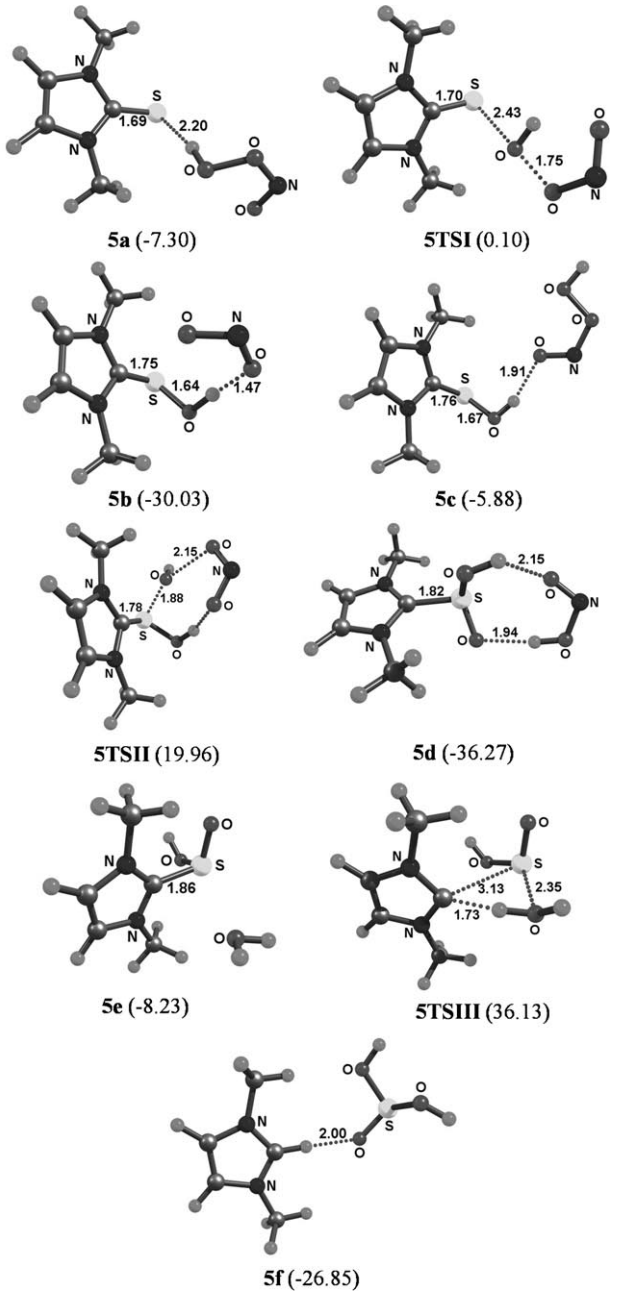


Figure 4. Energy-optimized geometries of the reactants, intermediates, transition states and products for the reaction of compound **5** with PNA. The values in parentheses correspond to the relative electronic energies (ΔE, kcal mol^{-1}) with zero-point energy corrections. The calculations were performed at B3LYP/6-31+G(d) level of theory. The relative energies are calculated with respect to the sum of energies of MMI and PNA.

trast to the oxidation, the elimination of sulfur moiety from **5e** to produce the corresponding *N,N′*-dimethylimidazolium cation (**25**) is more favored as compared to such elimination in **1e** (Table 2, Figure 5). However, this high-energy process

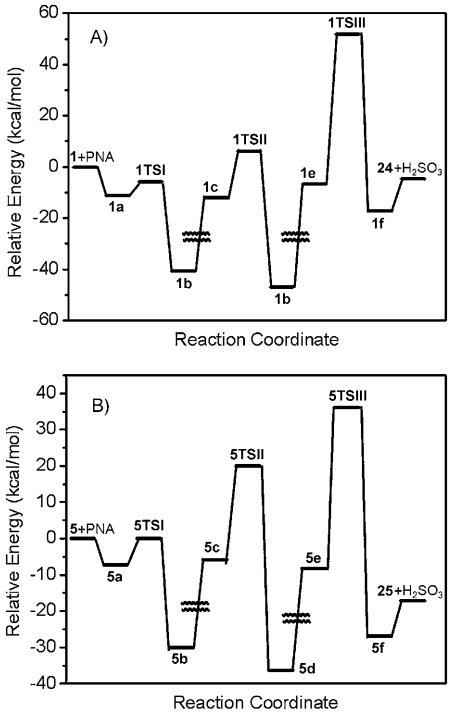


Figure 5. Schematic presentation of the potential energy surface (PES) of the reaction of MMI (**1**) (A) and compound **5** (B) with PNA. The energy values are scaled for the $\Delta(E+ZPE)$ values of all the species.

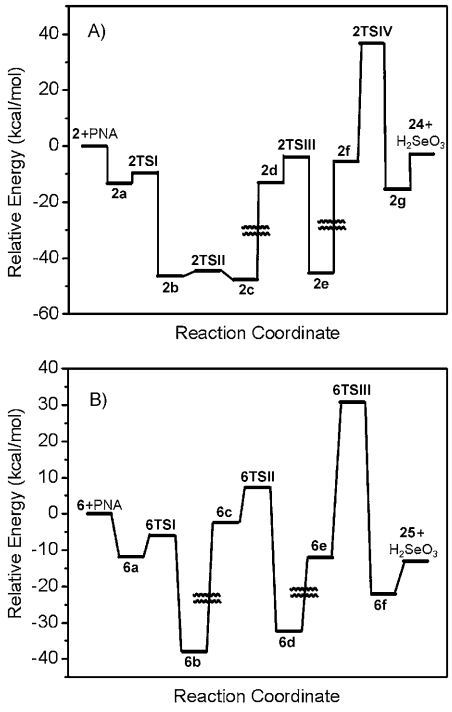


Figure 6. Schematic presentation of the potential energy surface (PES) of the reaction of MSeI (**2**) and compound **6** with PNA. The energy values are scaled for the $\Delta(E+ZPE)$ values of all the species.

may not have any significance in the activity of thiones as the PN scavenging activity of these compounds depends on their reactivity toward PN and not on the rate of elimination. Although the formation of small amounts of imidazole derivatives **24** and **25** during the inhibition experiments cannot be completely ruled out, there was no noticeable inhibition of the nitration reaction in the presence of **24** or **25** and the eliminated SO_3^{2-}/SeO_3^{2-}. This suggests that the high-energy third step (elimination of sulfur/selenium moieties) is not important for the PN scavenging activities of thiones and selones.

In contrast to MMI, the replacement of free N–H moiety in the selenium analogue (**2**) by *N*-methyl or *N*-*m*-methoxybenzyl substituents (**6** and **8**) significantly enhanced the PN scavenging activity. The IC_{50} values obtained for compound **6** (12.1 ± 0.1 μM) and **8** (7.8 ± 0.1 μM) are much lower than that of **2** (49.1 ± 1.2 μM) (Table 1). The theoretical calculations suggest that the difference in reactivity between the thione (**5**) and selone (**6**) is mainly due to the difference in energy required for the oxidation (Table 2). The reaction pathway indicates that the intermediates and transition states derived from *N,N′*-disubstituted selone **6** are similar to that of the thione **5** (Figure 4, and Figure S13, Supporting Information). However, the energy required for the conversion of selone **6** to the corresponding seleninic acid ($15.43\ kcal\,mol^{-1}$) is almost two times lower than the energy required for the conversion of **5** to the corresponding sulfinic acid ($33.24\ kcal\,mol^{-1}$) (Table 2, Figures 5 and 6). This is in agreement with the report by Musaev and co-workers that the oxidation of dimethylselenide (Me_2Se) to dimethylselenoxide (Me_2SeO) mediated by PN or PNA is much faster than the oxidation of dimethylsulfide (Me_2S) to the corresponding sulfoxide (Me_2SO).[32] The main reason for this difference in the energy is that the oxidation of Se^{II} to Se^{IV} by PNA is much more facile than the oxidation of S^{II} to S^{IV}. Therefore, the lower redox potential of selenium as compared to sulfur makes the selenium compounds better scavengers of PN as compared to their sulfur analogues.

Conclusions

This study shows that the commonly used antithyroid drugs, methimazole, 6-*n*-propyl-2-thiouracil and 6-methyl-2-thiouracil, possess strong antioxidant activity. These compounds and some of their sulfur and selenium analogues efficiently prevent the peroxynitrite-mediated nitration of tyrosine residues in proteins. A structure–activity correlation in these compounds reveals that the presence of thione or selone moiety is important for the inhibition. While the thiones require a free N–H moiety adjacent to the C=S bond for the inhibition, the selenium analogues effectively inhibit the ni-

tration reactions even in the N,N′-disubstituted forms. As the reduction of PN takes place through the oxidation of the sulfur or selenium center, the facile oxidation of the selone moiety to selenenic and seleninic acids is responsible for the higher activity of selones as compared to that of thiones. Theoretical calculations suggest that the difference in reactivity between thiones and selones is mainly due to the difference in energy required for the oxidation. The degradation of PN by antithyroid drugs and their analogues having thione or selone moieties suggest that the presence of such moiety is important not only for their antithyroid activity, but also for their antioxidant activity. Furthermore, antithyroid agents having PN scavenging activity may be beneficial in the treatment of hyperthyroidism as these compounds may protect the thyroid gland from nitrative and nitrosative stress.

Experimental Section

Materials and methods: L-Tyrosine, 3-nitro-L-tyrosine, sodium borohydride, and selenium powder were obtained from Aldrich. The sulfur analogues of antithyroid drugs 2-mercapto-1-methylimidazole (MMI) **1**; 6-*n*-propyl-2-thiouracil (PTU), **3**; 6-methyl-2-uracil (MTU) **4**; 6-benzyl-2-thiouracil (BTU) **11** and 5-carboethoxy-2-thiouracil (CTU) **12** were obtained from TCI (Tokyo Kasei, Japan) company. Methanol was obtained from Merck and dried before use by following standard procedure. Dry acetone and dichloromethane were used for the reaction as received from the local suppliers. All other chemicals were of the highest purity available. All experiments were carried out under dry and oxygen free nitrogen using standard Schlenk techniques for the synthesis. Due to unpleasant odour of several of the reaction mixtures involved, most manipulations were carried out in a well-ventilated fume hood. 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. ^{1}H (400 MHz), ^{13}C (100 MHz), and ^{77}Se (76 MHz) NMR spectra were obtained on a Bruker 400 MHz NMR spectrometer. Chemical shifts are cited with respect to Me_4Si as internal (^{1}H and ^{13}C), and Me_2Se as external (^{77}Se) standards. The UV/Vis experiments were carried out on a Varian CARY 300 Bio spectrophotometer. Mass spectral studies were carried out on a Q-TOF micro mass spectrometer or on a Bruker Daltonics 6000plus mass spectrometer with ESI-MS mode analysis. Compound **2** was synthesized by reducing the corresponding diselenide **17** with sodium borohydride in deoxygenated water and the product was extracted with deoxygenated dichloromethane. Stock solution of the compound was kept under N_2 atmosphere throughout the experiment. Compounds **5**,[5] **6**,[5] **9**,[4b] and **10**[4b] were synthesized following the literature procedures.

Synthesis of compound 7: A solution of 3-methoxybenzyl chloride (1.14 g, 7.30 mmol) in acetone (20 mL) was added to a mixture containing *N*-methylimidazole (0.50 g, 6.09 mmol) and sodium iodide (1.00 g, 6.70 mmol), and the mixture was stirred for 12 h at room temperature. The solvent was removed under vacuum to obtain yellow solid, which was used for the next step without any further purification. Sulfur powder (0.19 g, 6.09 mmol) and anhydrous potassium carbonate (0.76 g, 5.48 mmol) was added to the yellow solid, and the mixture was heated to reflux in dry methanol (25 mL) for 4 h. The brown solution was then filtered hot through a pad of celite and washed two times with dry methanol. The desired compound was obtained as yellow oil and the small impurity in the sample was removed by silica gel column chromatography using ethyl acetate and petroleum ether as eluent. Yield: 0.9 g (57%); ^{1}H NMR ($CDCl_3$): $\delta = 3.60$ (s, 3H), 3.74 (s, 3H), 5.18 (s, 2H), 6.55–6.56 (d, $J = 2.4$ Hz, 1H), 6.64–6.65 (d, $J = 2.4$ Hz, 1H), 6.80–6.86 (m, 3H), 7.20–7.24 ppm (t, $J = 8.0$ Hz, 1H); ^{13}C NMR ($CDCl_3$): $\delta = 35.3$, 51.3, 55.3, 113.6, 113.9, 116.4, 118.0, 120.5, 130.0, 137.4, 159.9, 162.9 ppm; ESI-MS (HRMS): *m/z* calcd for $C_{12}H_{14}N_2OS$ $[M+Na]^+$: 257.0725; found: 257.0726.

Synthesis of compound 8: A solution of 3-methoxybenzyl chloride (1.14 g, 7.30 mmol) in acetone (20 mL) was added to a mixture containing *N*-methylimidazole (0.50 g, 6.09 mmol) and sodium iodide (1.00 g, 6.70 mmol), and the mixture was stirred for 12 h at room temperature. The solvent was removed under vacuum to obtain yellow solid which was used for the next step without any further purification. Selenium powder (0.48 g, 6.09 mmol) and anhydrous potassium carbonate (0.76 g, 5.48 mmol) was added to the yellow solid, and the mixture was heated to reflux in dry methanol (25 mL) for 4 h. The brown solution was then filtered hot through a pad of celite and washed two times with dry methanol. The desired compound was obtained as yellow oil and the small impurity in the sample was removed by silica gel column chromatography using ethyl acetate and petroleum ether as eluent. Yield: 1.1 g (64%); ^{1}H NMR ($CDCl_3$): $\delta = 3.73$ (s, 3H), 3.79 (s, 3H), 5.30 (s, 2H), 6.72–6.73 (d, $J = 2.4$ Hz, 1H), 6.83–6.84 (d, $J = 2.4$ Hz, 1H), 6.84–6.91 (m, 3H), 7.24–7.28 ppm (t, $J = 8.0$ Hz, 1H); ^{13}C NMR ($CDCl_3$): $\delta = 37.3$, 51.3, 55.3, 113.7, 114.0, 118.5, 120.1, 120.5, 130.0, 137.0, 156.4, 159.9 ppm; ^{77}Se NMR ($CDCl_3$): $\delta = -0.23$ ppm; ESI-MS (HRMS): *m/z* calcd for $C_{12}H_{14}N_2OSe$ $[M+H]^+$: 283.0349; found: 283.0325.

Synthesis of compound 13: A solution of 3-methoxybenzyl chloride (0.92 g, 5.85 mmol) in acetone (30 mL) was added to a mixture containing *N*-(3-*m*ethoxybenzyl) imidazole[33] (1.00 g, 5.32 mmol) and sodium iodide (0.87 g, 5.85 mmol), and the reaction mixture was stirred for 12 h at room temperature. The solvent was removed under vacuum to obtain a light yellow solid which was used for the next step without any further purification. Sulfur powder (0.17 g, 5.32 mmol) and anhydrous potassium carbonate (0.66 g, 4.78 mmol) was added to the light yellow solid, and the mixture was heated to reflux in dry methanol (35 mL) for 3 h. The brown solution was then filtered hot through a pad of celite and washed two times with dry methanol. The desired compound was obtained as yellow oil and the small impurity in the sample was removed by silica gel column chromatography using ethyl acetate and petroleum ether as eluent to obtain the product as yellow solid. Yield: 0.9 g (49%); ^{1}H NMR ($CDCl_3$): $\delta = 3.77$ (s, 6H), 5.25 (s, 4H), 6.55 (s, 2H), 6.82–6.90 (m, 6H), 7.23–7.27 ppm (t, $J = 8.0$ Hz, 2H); ^{13}C NMR ($CDCl_3$): $\delta = 50.2$, 54.2, 112.3, 115.7, 119.4, 128.9, 136.3, 158.9, 162.2 ppm; ESI-MS (HRMS): *m/z* calcd for $C_{19}H_{20}N_2O_2S$ $[M+H]^+$: 341.1323; found: 341.1310.

Synthesis of compound 14: A solution of 3-methoxybenzyl chloride (0.45 g, 2.92 mmol) in acetone (30 mL) was added to a mixture containing *N*-(3-*m*ethoxybenzyl) imidazole[33] (0.50 g, 2.66 mmol) and sodium iodide (0.44 g, 2.92 mmol), and the reaction mixture was stirred for 12 h at room temperature. The solvent was removed under vacuum to obtain a light yellow solid which was used for the next step without any further purification. Finely ground selenium powder (0.21 g, 2.66 mmol) and anhydrous potassium carbonate (0.33 g, 2.40 mmol) was added to the light yellow solid, and the mixture was heated to reflux in dry methanol (25 mL) for 3 h. The brown solution was then filtered hot through a pad of celite and washed two times with dry methanol. The desired compound was obtained as yellow oil and the small impurity in the sample was removed by silica gel column chromatography using ethyl acetate and petroleum ether as eluent to obtain the product as yellow solid. Yield: 0.65 g (62%); ^{1}H NMR ($CDCl_3$): $\delta = 3.76$ (s, 6H), 5.34 (s, 4H), 6.70 (s, 2H), 6.83–6.90 (m, 6H), 7.23–7.27 ppm (t, $J = 8.0$ Hz, 2H); ^{13}C NMR ($CDCl_3$): $\delta = 53.3$, 55.3, 113.9, 118.9, 120.6, 130.0, 137.0, 157.2, 160.0 ppm; ^{77}Se NMR ($CDCl_3$): $\delta = 1.64$ ppm; ESI-MS (HRMS): *m/z* calcd for $C_{19}H_{20}N_2O_2Se$ $[M+H]^+$: 389.0768; found: 389.0751.

Synthesis of compound 18: A solution of *n*BuLi (3.80 mL, 1.6 M in hexane) was added by syringe to a cooled (−78 °C) solution of *N*-methylimidazole (0.48 mL, 6.09 mmol) in freshly distilled THF (50 mL). The reaction mixture was stirred at this temperature for 30 min and then allowed to warm to room temperature at which time finely powdered elemental sulfur (0.27 g, 8.52 mmol) was added under a brisk flow of dry nitrogen gas, and the resulting mixture was stirred for 5 h. Then 3-methoxy-

benzyl chloride (0.82 mL, 6.09 mmol) was added dropwise and the mixture was stirred overnight. The solution was filtered through a celite pad and solvent was evaporated to obtain brown colored oil, which was purified by flash chromatography with pre-loaded silica gel cartridges using petroleum ether and ethyl acetate as eluent. Yield: 0.87 g (61%); ^{1}H NMR ($CDCl_3$): δ = 3.21 (s, 3H), 3.68 (s, 3H), 4.07 (s, 2H), 6.57 (s, 1H), 6.68–6.70 (d, J = 7.6 Hz, 1H), 6.73–6.75 (d, J = 8.4 Hz, 1H), 6.83 (s, 1H), 7.09–7.14 ppm (m, 2H); ^{13}C NMR ($CDCl_3$): δ = 33.1, 40.2, 55.2, 113.4, 113.7, 121.1, 122.5, 129.5, 129.6, 139.3, 140.3, 159.6 ppm; ESI-MS (HRMS): m/z calcd for $C_{12}H_{14}N_2OS$ $[M+Na]^+$: 257.0725; found: 257.0724.

Synthesis of compound 19: A solution of *n*BuLi (3.80 mL, 1.6 M in hexane) was added by syringe to a cooled (−78 °C) solution of *N*-methylimidazole (0.48 mL, 6.09 mmol) in freshly distilled THF (50 mL). The reaction mixture was stirred at this temperature for 30 min and then allowed to warm to room temperature at which time finely powdered elemental selenium (0.72 g, 9.12 mmol) was added under a brisk flow of dry nitrogen gas and stirred for 5 h. Then 3-methoxybenzyl chloride (0.82 mL, 6.09 mmol) was added dropwise and the mixture was stirred overnight. The solution was filtered through a celite pad and solvent was evaporated to obtain brown colored oil, which was purified by flash chromatography with pre-loaded silica gel cartridges using petroleum ether and ethyl acetate as eluent. Yield: 0.94 g (55%); ^{1}H NMR ($CDCl_3$): δ = 3.11 (s, 3H), 3.60 (s, 3H), 4.01 (s, 2H), 6.43 (s, 1H), 6.58–6.60 (d, J = 7.6 Hz, 1H), 6.64–6.66 (d, J = 8.4 Hz, 1H), 6.82 (s, 1H), 7.02–7.06 ppm (m, 2H); ^{13}C NMR ($CDCl_3$): δ = 33.2, 34.2, 55.2, 113.2, 113.5, 120.9, 123.0, 129.5, 130.6, 134.6, 140.3, 159.5 ppm; ^{77}Se NMR ($CDCl_3$): δ = 282 ppm. ESI-MS (HRMS): m/z calcd. for $C_{12}H_{14}N_2OSe$ $[M+Na]^+$: 305.0169; found: 305.0124.

Synthesis of compounds 20 and 22: Powdered potassium hydroxide (1.37 g, 24.61 mmol) was added to a stirred suspension of **4** (0.70 g, 4.92 mmol) in acetone (25 mL). After 15 min, methyl iodide (1.23 mL, 19.69 mmol) was added to the reaction mixture, which was then stirred vigorously for 1 h. The acetone solution was then transferred to a separating funnel with excess benzene. The organic layer was washed with water (2×50 mL) and dried over sodium sulfate. The solvent was evaporated to obtain white semi-solid compound which was purified by flash chromatography with pre-loaded silica gel cartridges using ethyl acetate and petroleum ether as eluent. The expected compounds eluted with 30 and 45% ethyl acetate, respectively. For the first fraction, the solvent was evaporated to obtain **20** as colorless oil. Yield: 0.17 g (21%); ^{1}H NMR ($CDCl_3$): δ = 2.35 (s, 3H), 2.54 (s, 3H), 3.94 (s, 3H), 6.22 ppm (s, 1H); ^{13}C NMR ($CDCl_3$): δ = 13.0, 22.7, 52.5, 100.8, 166.5, 168.5, 170.3 ppm; ESI-MS (HRMS): m/z calcd for $C_7H_{10}N_2OS$ $[M+Na]^+$: 193.0412; found: 193.0408. For the second fraction, the solvent was evaporated to obtain **22** as a white solid. Yield: 0.27 g (32%); ^{1}H NMR ($CDCl_3$): δ = 2.23 (s, 3H), 2.57 (s, 3H), 3.49 (s, 3H), 6.05 ppm (s, 1H); ^{13}C NMR ($CDCl_3$): δ = 15.2, 24.0, 30.1, 107.5, 162.2, 162.6, 162.7 ppm; ESI-MS: m/z calcd for $C_7H_{10}N_2OS$ $[M+H]^+$; 171.05; found: 170.84.

Synthesis of compounds 21 and 23: Powdered potassium hydroxide (0.50 g, 8.81 mmol) was added to a stirred suspension of **3** (0.30 g, 1.76 mmol) in acetone (20 mL). After 15 min, methyl iodide (0.44 mL, 7.05 mmol) was added and the reaction mixture was stirred vigorously for 1 h. The solution was then transferred to a separating funnel with excess benzene. The organic layer was washed with water (2×50 mL) and dried over sodium sulfate. The solvent was evaporated to obtain colorless oil which was purified by flash chromatography with pre-loaded silica gel cartridges using ethyl acetate and petroleum ether as eluent. The expected compounds eluted with 25 and 40% ethyl acetate, respectively. For the first fraction, the solvent was evaporated to obtain **21** as colorless oil. Yield: 91 mg (26%); ^{1}H NMR ($CDCl_3$): δ = 0.92–0.96 (t, J = 8.0 Hz, 3H), 1.65–1.74 (m, 2H), 2.53 (s, 3H), 2.54–2.58 (t, J = 8.0 Hz, 2H), 3.94 ppm (s, 3H), 6.20 (s, 1H); ^{13}C NMR ($CDCl_3$): δ = 12.7, 13.0, 20.8, 38.8, 52.5, 100.3, 168.5, 170.2, 170.3 ppm; ESI-MS: m/z calcd for $C_9H_{14}N_2OS$ $[M+H]^+$: 199.08; found: 198.86. For the second fraction, the solvent was evaporated to obtain **23** as a faint yellow solid. Yield: 0.13 g (36%); ^{1}H NMR ($CDCl_3$): δ = 0.92–0.96 (t, J = 8.0 Hz, 3H), 1.63–1.74 (m, 2H), 2.40–2.44 (t, J = 8.0 Hz, 2H), 2.55 (s, 3H), 3.47 (s, 3H), 6.02 ppm (s, 1H); ^{13}C NMR ($CDCl_3$): δ = 13.7, 15.0, 20.9, 39.2, 52.5, 106.8, 161.9, 162.7, 165.7 ppm; ESI-MS: m/z calcd for $C_9H_{14}N_2OS$ $[M+H]^+$: 199.08; found: 199.33.

General procedure for the reaction of inhibitors with PN: To the ice-cooled solution of the inhibitor in water was added alkaline solution of excess peroxynitrite (PN) in portions (three times with 3–4 h intervals) and stirred vigorously for 12 h at room temperature. The reaction mixture was extracted with dichloromethane 3–4 times to remove the unreacted starting material and the nonpolar impurities. The aqueous solution was neutralized with 1 N HCl and the solvent was evaporated by lyophilization. The solid obtained was dissolved in minimum amount of dry methanol to remove the excess NaCl. The solution was filtered and the solvent was evaporated to obtain the product in a reasonably pure form.

*Compound **1** with excess PN*: 2-Mercapto-1-methylimidazole (**1**; 0.25 g, 2.19 mmol); product: colorless oil; yield: 0.10 g (57%); ^{1}H NMR (D_2O): δ = 3.66 (s, 3H), 7.17 (s, 2H), 8.39 ppm (s, 1H); ^{13}C NMR (D_2O): δ = 35.2, 119.3, 122.7, 134.8 ppm; ESI-MS: m/z calcd for $C_4H_6N_2$ $[M+H]^+$: 83.06; found: 82.99.

*Compound **2** with excess PN*: 1-Methylimidazoleselone (**2**; 0.10 g, 0.62 mmol); product: colorless oil; yield: 28.5 mg (56%); ^{1}H NMR (D_2O): δ = 3.65 (s, 3H), 7.17 (s, 2H), 8.40 ppm (s, 1H); ^{13}C NMR (D_2O): δ = 35.3, 119.3, 122.8, 134.8 ppm; ^{77}Se NMR (D_2O): δ = 1314 ppm; ESI-MS: m/z calcd for $C_4H_6N_2$ $[M+H]^+$: 83.06; found: 83.00.

*Compound **3** with excess PN*: 6-*n*-Propyl-2-thiouracil (**3**; 0.25 g, 1.46 mmol); product: colorless oil; yield: 83 mg (41%); ^{1}H NMR (D_2O): δ = 0.66–0.70 (t, J = 8.0 Hz, 3H), 1.40–1.45 (m, 2H), 2.40–2.43 (t, J = 8.0 Hz, 2H), 6.33 (s, 1H), 8.89 ppm (s, 1H); ^{13}C NMR (D_2O): δ = 12.2, 20.0, 33.5, 113.7, 152.0, 158.0, 160.7 ppm; ESI-MS: m/z calcd for $C_7H_{10}N_2O$ $[M+H]^+$: 139.07; found: 138.88.

*Compound **4** with excess PN*: 6-Methyl-2-thiouracil (**4**; 0.25 g, 1.77 mmol); product: colorless oil; yield: 88 mg (46%); ^{1}H NMR (D_2O): δ = 2.15 (s, 3H), 6.31 (s, 1H), 8.88 ppm (s, 1H); ^{13}C NMR (D_2O): δ = 17.9, 99.1, 114.4, 150.8, 154.6, 160.6 ppm; ESI-MS: m/z calcd for $C_5H_6N_2O$ $[M+H]^+$: 111.04; found: 110.91.

*Compound **5** with excess PN*: 1,3-Dimethylimidazolethione (**5**; 0.25 g, 1.95 mmol); product: colorless oil; yield: 0.10 g (56%); ^{1}H NMR (D_2O): δ = 3.64 (s, 6H), 7.16 (s, 2H), 8.39 ppm (s, 1H); ^{13}C NMR (D_2O): δ = 35.4, 123.2, 136.4 ppm; ESI-MS: m/z calcd for $C_5H_9N_2^+$ $[M]^+$: 97.07; found: 96.95.

*Compound **6** with excess PN*: 1,3-Dimethylimidazoleselone (**6**; 0.25 g, 1.42 mmol); product: colorless oil; yield: 80 mg (58%); ^{1}H NMR (D_2O): δ = 3.66 (s, 6H), 7.18 (s, 2H), 8.44 ppm (s, 1H); ^{13}C NMR (D_2O): δ = 35.5, 123.2, 136.4 ppm; ^{77}Se NMR (D_2O): δ = 1314 ppm; ESI-MS (HRMS): m/z calcd for $C_5H_9N_2^+$ $[M]^+$: 97.0760; found: 97.0771.

*Compound **8** with excess PN*: Compound **8** (0.25 g, 0.88 mmol); product: colorless oil; yield: 93 mg (52%); ^{1}H NMR (D_2O): δ = 3.69 (s, 3H), 3.74 (s, 3H), 5.21 (s, 2H), 6.85–6.91 (m, 3H), 7.25–7.32 ppm (m, 3H); ^{13}C NMR (D_2O): δ = 35.7, 52.6, 55.4, 114.1, 114.7, 121.1, 122.2, 123.8, 130.6, 135.1, 136.0, 159.4 ppm; ^{77}Se NMR (D_2O): δ = 1314 ppm; ESI-MS: m/z calcd for $C_{12}H_{15}N_2^+$ $[M]^+$: 203.11; found: 202.85.

Synthesis of peroxynitrite (PN): Peroxynitrite was synthesized by following the literature method with minor modifications.[34] A solution of 30% (≈8.8 M) H_2O_2 (5.7 mL) was diluted to 50 mL with water, chilled to about 4 °C in an ice/water mixture, added to NaOH (5 N, 30 mL) and DTPA (0.04 M 5 mL) in NaOH (0.05 N) with gentle mixing, and then diluted to a total volume of 100 mL. The concentration of H_2O_2 in the final solution was 0.5 M with the pH ranging from 12.5 to 13.0. The buffered H_2O_2 was stirred vigorously with an equimolar amount of isoamyl nitrite (0.05 M or 6.7 mL) for 3–4 h at room temperature. The reaction was monitored by withdrawing aliquots at an interval of 15 or 30 min, and assaying for peroxynitrite at 302 nm by UV/Vis spectrophotometer. When the yield of peroxynitrite reached a maximum, the aqueous phase was washed with (3×100) volume of dichloromethane, chloroform, and hexane in a separatory funnel to remove the contaminating isoamyl alcohol and isoamyl nitrite. The unreacted H_2O_2 was removed by passing the aqueous phase through a column filled with 25 g of granular MnO_2. The concentration of the stock solution of peroxynitrite was measured after

500 times dilution with 0.1 N NaOH solution and then assaying for peroxynitrite at 302 nm ($\varepsilon = 1670\,M^{-1}cm^{-1}$) by UV/Vis spectrophotometric method.

PN-mediated nitration assay—nitration of L-tyrosine: High performance liquid chromatography (HPLC) experiments were carried out on a Waters Alliance System (Milford, MA) consisting of a 2695 separation module, a 2996 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). The nitration assay of L-tyrosine was analyzed by reverse phase HPLC method (Vydac column, 4.6×250 mm, 5 μm) using gradient elution (100% water with 0.1% TFA to 60% water with 0.1% TFA and 40% acetonitrile with 0.1% TFA over 16 min). In the PN-mediated nitration of L-tyrosine assay, we employed a mixture containing L-tyrosine (0.75 mM) and peroxynitrite (0.30 mM) in sodium phosphate buffer (100 mM) of pH 7.5 without and with increasing concentration of the inhibitor was added to the assay mixture. The reaction mixture was incubated for 7 min before injection. The formation of 3-nitro-L-tyrosine was monitored at the wavelength of 275 nm. The inhibition plots were obtained by using Origin 6.1 software utilizing sigmoidal curve fitting and these plots were used for the calculation of IC_{50} values.

Inhibition of protein nitration

Nitration of BSA: For bovine serum albumin (BSA), the nitration was performed by the addition of PN (2.0 mM) to BSA (0.1 mM) in 0.5 M phosphate buffer of pH 7.0 with DTPA (0.1 mM) at 20 °C. After the addition of PN, the final pH was maintained below 7.5. The reaction mixture was incubated for 20 min at 20 °C. Similarly, the reactions of BSA with PN were performed in the presence of different antithyroid drugs and analogues (85 μM) as inhibitors. Upon performing the reactions, the mixture was denatured by boiling at 100 °C for 5 min in the presence of sample loading dye and subjected to polyacrylamide gel electrophoresis and Western blot analyses.

Nitration of cytochrome c: For cytochrome c, the nitration was performed by the addition of PN (4.3 mM) to cytochrome c (0.5 mM) in 0.5 M phosphate buffer of pH 7.0 with DTPA (0.1 mM) at 20 °C. After the addition of PN, the final pH was found to be below 7.5. The reaction mixture was incubated for 20 min at 20 °C. Similarly, the reactions of cytochrome c with PN were performed in the presence of different antithyroid drugs and analogues (200 μM) as inhibitors. Upon performing the reactions, the reaction mixture was denatured by boiling at 100 °C for 5 min in the presence of sample loading dye and subjected to polyacrylamide gel electrophoresis and Western blot analyses.

Electrophoretic analysis: Gel was prepared with 10% and 15% polyacrylamide with 6% stacking gel for BSA and cytochrome c respectively. The gel was run in the running buffer of pH 8.3 with glycine and SDS. After separating the proteins, the gel was analyzed by Western blot experiments. The proteins were transferred to a PVDF membrane and the nonspecific binding sites were blocked by 5% nonfat skimmed milk in PBST (blocking solution) for 2 h. Then the membrane was probed with rabbit polyclonal primary antibody against nitrotyrosine (1:20000 dilutions) in blocking solution for 2 h followed by incubation with horseradish peroxidase-conjugated donkey polyclonal anti-rabbit IgG (1:20 000 dilutions) for another 2 h. The probed membrane was then washed three times with blocking solution with 0.1% Tween 20 and the immunoreactive protein was detected by luminol-enhanced chemiluminiscence (ECL, Amersham).

Computational methods: All calculations were performed by using the Gaussian 98 suite[35] of quantum chemical programs. The hybrid Becke 3-Lee–Yang–Parr (B3LYP) exchange correlation functional was applied for DFT calculations.[36] Geometries were fully optimized at B3LYP level of theory using 6–31+G(d) basis sets. Transition states were located using Schlegel's synchronous transit-guided quasi-Newton (STQN) method.[29] Transition states were searched by using the QST3 keyword and the resultant conformation was optimized using the TS keyword. Furthermore, the transition state and the stable conformers were characterized by the presence or absence of a single imaginary mode respectively. The activation energies are the difference in the zero-point vibrational energy corrected electronic energy between the transition state and the stable conformations.

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