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PAPER

Inhibition of peroxynitrite- and peroxidase-mediated protein tyrosine nitration by imidazole-based thiourea and selenourea derivatives†

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In the present study, the synthesis and characterization of a series of *N*-methylimidazole-based thiourea and selenourea derivatives are described. The new compounds were also studied for their ability to inhibit peroxynitrite (PN)- and peroxidase-mediated nitration of protein tyrosine residues. It has been observed that the selenourea derivatives are more efficient than the thiourea-based compounds in the inhibition of protein nitration. The higher activity of selenoureas as compared to that of the corresponding thioureas can be ascribed to the zwitterionic nature of the selenourea moiety. Single crystal X-ray diffraction studies on some of the thiourea and selenourea derivatives reveal that the C═S bonds in thioureas possess more of double bond character than the C═Se bonds in the corresponding selenoureas. Therefore, the selenium compounds can react with PN or hydrogen peroxide much faster than their sulfur analogues. The reactions of thiourea and selenourea derivatives with PN or hydrogen peroxide produce the corresponding sulfinic or seleninic acid derivatives, which upon elimination of sulfurous/selenous acids produce the corresponding *N*-methylimdazole derivatives.

Introduction

The nitration of tyrosine residues in proteins and enzymes leads to the formation of nitrotyrosine, which is known as the biomarker of oxidative as well as nitrosative stress in many inflammatory and allergic diseases.[1] The protein tyrosine nitration can take place by both of enzymatic or non-enzymatic pathways. The non-enzymatic nitration is generally mediated by peroxynitrite ($ONOO^-$, PN).[2] PN is produced in the biological system by diffusion-controlled reaction of superoxide anion radical ($O_2^{\bullet -}$) and nitric oxide ($NO^{\bullet}$).[3] In addition to the inactivation of a number of proteins and enzymes by nitration of tyrosine residues, PN can induce DNA damage[4] and initiate lipid peroxidation in biomembranes and low density lipoproteins.[5] On the other hand, the enzymatic nitration mainly occurs by peroxidases in the presence of hydrogen peroxide (H_2O_2) and nitrite (NO_2^-).[6] The enzymatic nitration catalyzed by peroxidases such as myeloperoxidase,[7] eosinophil peroxidase,[8] horseradish peroxidase,[7] lactoperoxidase[9] or heme proteins such as hemoglobin,[10] myoglobin[11] and cytochrome c[12] has been well-documented in the literature. Therefore, there is a considerable research interest in the development small-molecule sulfur/selenium compounds that can effectively prevent the enzymatic and non-enzymatic protein nitration.

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† Electronic supplementary information (ESI) available: The supporting information contains ^1H and ^{13}C NMR spectra of compounds **14–21**, and **23** and ^{77}Se NMR spectra of **15**, **17**, **19**, **21** and **23**. ESI-MS spectra of all these compounds are also available. CCDC reference numbers 800371–800373. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c1ob05773a

Recent evidences suggest that the thiourea-based antithyroid drugs (Fig. 1) show beneficial effects under oxidative stress conditions.[13] Recently, we have shown that the thiourea-based antithyroid drugs MMI (**1**), PTU (**3**), MTU (**4**) and their analogues (**2**, **5–6**) protect against PN-mediated nitration of bovine serum albumin (BSA) and cytochrome c.[14] We have also shown that the selenium analogue of MMI (MSeI, **2**) exhibits significant antioxidant activity by catalytically reducing H_2O_2 in the presence of glutathione (GSH).[15] These studies suggest that antithyroid drugs having antioxidant activity may be important in the treatment of hyperthyroidism as these compounds may protect the thyroid cells from oxidative damage.

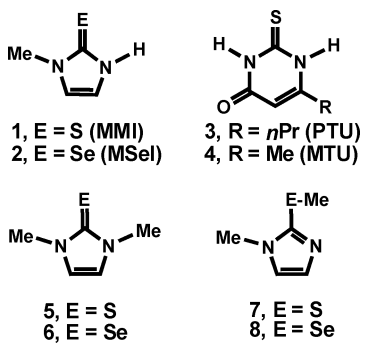


Fig. 1 Chemical structures of antithyroid drugs and related compounds **1–8**.

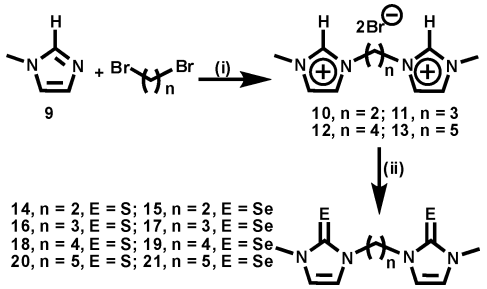
A detailed analysis of the protective effects of various thiourea- and selenourea-based compounds against peroxidase-catalyzed protein nitration indicated that these compounds effectively

inhibit the nitration of BSA and cytochrome c catalyzed by lactoperoxidase (LPO) and myeloperoxidase (MPO).[16] In addition to the inhibition of peroxidase-catalyzed reactions, several *N*-methylimidazole-based bidentate thiourea/selenourea ligands have been used in coordination chemistry by different research groups.[17] It has been shown that the coordination behaviour of these bidentate ligands depends on the nature of linker used to connect two thiourea/selenourea-containing imidazole moieties and the nature of metal ion.[17b,c] In this paper, we describe the synthesis and characterization of a series of *N*-methylimidazole-based thiourea and selenourea derivatives having two S/Se redox centres. We also describe the inhibition of peroxidase- and peroxynitrite-mediated nitration of tyrosine residues by these compounds.

Results and discussion

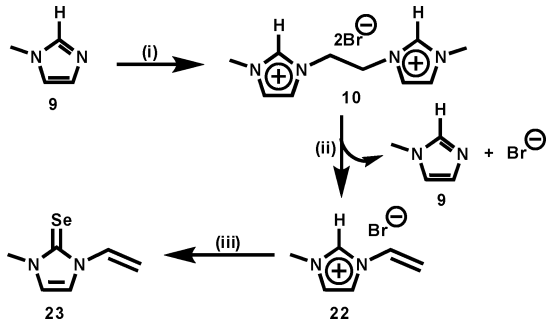
Synthesis of thiourea and selenourea derivatives

The *N*-methylimidazole-based thioureas and selenoureas **14–21** were synthesized by the treatment of *N*-methylimidazole (**9**) with appropriate dibromoalkanes followed by reaction with sulfur or selenium powder (Scheme 1). The treatment of two equivalents of *N*-methylimidazole with dibromoalkanes in THF afforded the corresponding *N*-methylimidazolium dibromide salts **10–13**. Subsequent addition of sulfur or selenium powder to the methanolic solution of dibromides **10–13** in the presence of K_2CO_3 produced the thioureas and selenoureas **14–21** in moderate yields. All the products were characterized by NMR spectroscopic and ESI-MS spectrometric analyses. Some of the compounds were further characterized by single crystal X-ray diffraction studies.



Scheme 1 Synthetic route to compounds **14–21**. (i) THF, reflux, 12 h; (ii) K_2CO_3, S or Se powder, MeOH, reflux, 12 h.

Interestingly, compound **23** was obtained as a by-product during the synthesis of compound **15**. The formation of **23** may involve a dehydrobromination reaction in the presence of K_2CO_3 as shown in Scheme 2. A deprotonation of the ethylene moiety in compound **10** by K_2CO_3 leads to the formation of compound **22** with the elimination of *N*-methylimidazole and hydrogen bromide. Subsequent addition of selenium powder to the methanolic solution of compound **22** produces the corresponding selenourea (**23**) having a vinyl side chain. Although a considerable amount of selenourea **23** was formed during the synthesis of compound **15**, only a trace amount of the corresponding thiourea analogue was obtained during the synthesis of compound **14**. Owing to the formation of **23**, the yield of the ethylene-substituted selenourea **15** was found to be much lower than that of other related compounds (**17**, **19** and **21**).

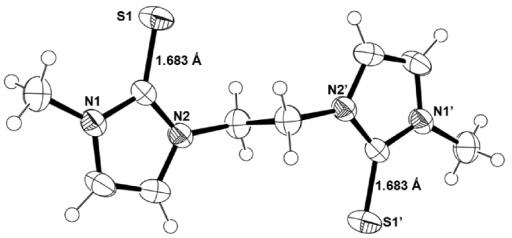


Scheme 2 Proposed pathway for the formation of compound **23**. (i) $BrCH_2CH_2Br$, THF, reflux, 12 h; (ii) K_2CO_3; (iii) K_2CO_3, Se powder, MeOH, reflux, 12 h.

Structural features of thiourea and selenourea derivatives

To understand the nature of thiourea/selenourea moieties, some of the compounds in this study were further characterized by single crystal X-ray diffraction analyses. The orientation of imidazole moieties and the packing of the molecular units depend on the length of linker connecting the two imidazole groups. For example, the two selenourea moieties in compounds **17** and **19** are oriented almost opposite to each other, whereas the selenoureas in compound **21** are aligned in the same direction. Similarly, the orientation of thiourea moieties in compounds **14** and **16** are also found to be opposite to each other. While the asymmetric units of **14** and **19** contain half of the molecular units, compounds **16–17** and **21** contain full molecules in the asymmetric units. This can be ascribed to the presence of a centre of symmetry within the molecular units in compounds **14** and **19**. This is possible due to the presence of an even number of –(CH_2)– units (n = 2, 4) in these molecules that leads the *N*-methylimidazole-2-thiourea/selenourea moieties in these molecules to adopt antiperiplaner geometry (Fig. 2). These observations suggest that the length of linker chain plays an important role in the occupancy of asymmetric units.

To understand the nature of C–S and C–Se bonds in these di-thiourea/di-selenourea derivatives, we compared the C–S/C–Se bond lengths in these compounds with that of the previously reported compounds **5–6** (Fig. 2, Table 1).[18] As determined by X-ray diffraction studies, the average C–S bond lengths in compounds **14** (1.683 Å) and **16** (1.687 Å) are much shorter than the C–S single bond (1.810 Å) and slightly longer than the C═S double bond (1.610 Å).[18a] In contrast, the C–Se bond lengths in the di-selenoureas are comparable to C–Se single bond length. For example, the average C–Se bond lengths in compounds **17** (1.842 Å), **19** (1.846 Å) and **21** (1.852 Å) are almost similar to the true C–Se single bond (1.940 Å). Notably, the C–S/C–Se bond lengths observed in these di-thioureas/di-selenoureas are comparable to that observed in *N*,*N*-dimethyl substituted thiourea **5** (1.698 Å) and selenourea **6** (1.843 Å) (Table 1).[18] These observations suggest that the thioureas have more of double bond character than the corresponding selenoureas, which is in agreement with our previous reports that thioureas exist predominantly in their thione forms having less zwitterionic characters.[14,15b,16,19] Owing to the significant difference in the polarizability of carbon and selenium atoms, the selenoureas predominantly exist in their zwitterionic forms having a large negative charge on the selenium centre



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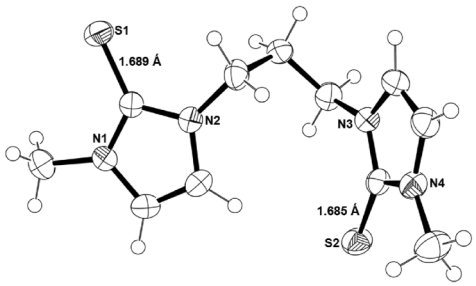

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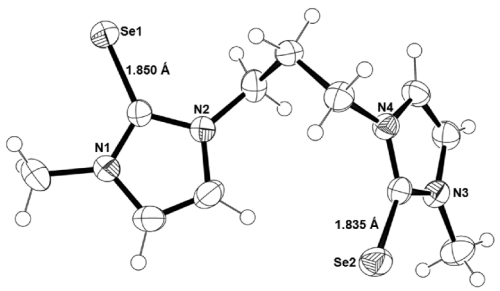

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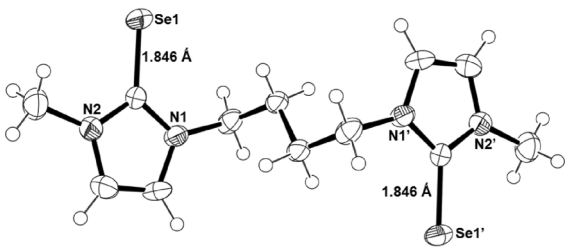

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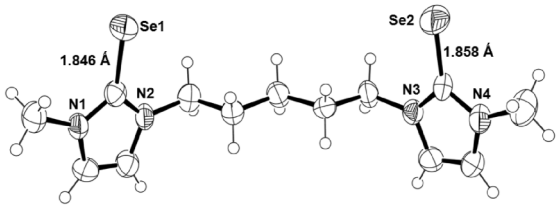

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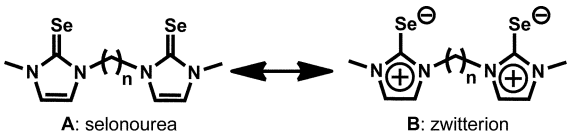
Fig. 2 Single crystal X-ray structures of compounds **14**, **16**, **17**, **19** and **21**. Displacement ellipsoids are drawn at 50% probability level and hydrogen atoms are shown as small spheres of arbitrary radii.

(Scheme 3). In addition to the elongation of C–Se bonds as evidenced by X-ray diffraction studies, the zwitterionic nature of selenoureas is also reflected in their chemical shift values in ^{77}Se NMR spectroscopic experiments. A significant upfield shift of the ^{77}Se NMR signals is indicative of their existence as zwitterions in solution due to the accumulation of significant negative charge on the selenium centres as shown in Scheme 3. It should be noted that

Table 1 A comparison of C–S and C–Se bond lengths in thioureas/selenoureas **5–6**, **14**, **16–17**, **19** and **21**

Compound	$d_{C\text{-}S1/C\text{-}Se1}$ (Å)	$d_{C\text{-}S2/C\text{-}Se2}$ (Å)	Mean $d_{C\text{-}S/C\text{-}Se}$ (Å)
5	1.698	—	1.698
6	1.843	—	1.843
14	1.683	1.683	1.683
16	1.689	1.685	1.687
17	1.850	1.835	1.842
19	1.846	1.846	1.846
21	1.846	1.858	1.852



Scheme 3 Tautomeric structures of selenoureas **15**, **17**, **19** and **21**. Existence of these compounds in zwitterionic form (B) leads to an accumulation of significant negative charge on selenium centers.

the zwitterionic nature of selenoureas leads to an enhancement in their nucleophilic reactivity.

Inhibition of protein tyrosine nitration

To investigate the antioxidant properties of thioureas and selenoureas, we have studied the effect of these compounds on the PN-mediated nitration of BSA. The inhibition of tyrosine nitration was followed by SDS-PAGE and immunoblotting methods using antibody against 3-nitro-L-tyrosine as reported earlier.[14,16,20] As shown in Fig. 3, all the selenium compounds (**2**, **6**, **15**, **17**, **19**, **21** and **23**) exhibited significant inhibition of the PN-mediated nitration of BSA. As expected, compounds **15**, **17**, **19** and **21** having two selenourea moieties exhibited better activities than the mono-substituted compounds **2**, **6** and **23** under identical

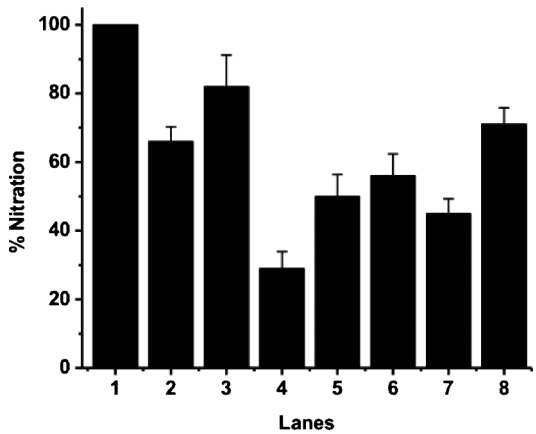


Fig. 3 Immunoblots for the inhibition of PN-mediated nitration of BSA by the selenium analogues of antithyroid drugs. 0.1 mM of BSA was incubated with 130 μM of inhibitor and 2.0 mM of PN for 30 min at 20 °C and then denatured by loading dye and subjected to gel electrophoresis. Lanes: 1, protein + PN; 2, protein + PN + **2**; 3, protein + PN + **6**; 4, protein + PN + **15**; 5, protein + PN + **17**; 6, protein + PN + **19**; 7, protein + PN + **21**; 8, protein + PN + **23**. Each bar diagram represents the mean ± SD of at least three independent experiments.

experimental conditions. Compound **15** having a -CH_2CH_2- linker exhibited the highest activity among all the inhibitors used in the study. Although mono-selenoureas **2**, **6** and **23** significantly inhibited the nitration reaction, compound **2** having a free N–H group in the imidazole ring exhibited slightly higher potency than that of compounds **6** and **23**. These observations suggest that the enhancement of antioxidant activities of imidazole-based di-selenoureas probably due to the incorporation of more than one selenourea moieties in a single molecule. Furthermore, this study supports our previous observations that the selenourea moieties in the imidazole-based compounds are mainly responsible for their PN-scavenging activities.[14] The high activity of compounds **2**, **6**, **15**, **17**, **19**, **21** and **23** can be ascribed to the existence of selenoureas in their zwitterionic forms (Scheme 3). Owing to the higher polarizability of selenium as compared to carbon, the C═Se bonds have only partial double bond character, which leads to the formation of zwitterionic structures with significant negative charge on the selenium atoms. Therefore, the highly nucleophilic selenolates act as effective scavengers of PN. The zwitterionic nature of C═Se bonds are also reflected in their X-ray crystal structures (Fig. 2) and ^{77}Se NMR chemical shifts.

To understand the PN-scavenging activities of the di-thioureas in comparison to the mono-thioureas, the inhibition of PN-mediated nitration of BSA was carried out in the presence of compounds **1**, **5**, **14**, **16**, **18** and **20**. Interestingly, the antithyroid drug MMI (**1**) exhibited the highest PN-scavenging activity among all the thioureas used in the present study (Fig. 4). This is probably due to the presence of a free N–H group, which enhances the nucleophilicity of thiourea by generating a highly reactive thiolate anion. Substitution of the hydrogen atom by a methyl group (compound **5**) significantly reduced its scavenging activity. Similarly, all the *N*,*N*-disubstituted di-thioureas exhibited much lower activity as compared to MMI. The relatively weaker PN-scavenging activity of *N*,*N*-disubstitued thioureas is probably due to their existence in thione forms. This is in agreement with our previous observation that the presence of a free N–H group is necessary for the sulfur analogues to exhibit their antioxidant activities.[14,16] The PN-scavenging activity of di-thioureas **14**, **16**, **18** and **20** was comparable to that of the mono-thiourea **5**, indicating that the inhibition of PN-mediated protein nitration could not be enhanced by increasing the number of thiourea moieties in a single molecule. This is in contrast to the *N*,*N*-disubstituted di-selenoureas, which are more effective scavengers of PN as compared to the mono-substituted selenoureas. The lower PN-scavenging activity of the di-thioureas as compared to that of compound **1** can be ascribed to the absence of any free N–H group in the imidazole ring.

As the enzymatic nitration of protein tyrosine residues are generally catalyzed by peroxidases,[7–9] we have also studied the inhibition of peroxidase-catalyzed nitration of BSA. The nitration of BSA was carried out using lactoperoxidase (LPO), which is readily available in pure form and also has been shown to effectively catalyze tyrosine nitration in proteins in the presence of H_2O_2 and NO_2^-.[9,16] Similar to the PN-mediated nitration, the LPO-catalyzed nitration of BSA was followed by SDS-PAGE and immunoblotting methods using antibody against 3-nitro-L-tyrosine. Interestingly, all the selenium compounds **2**, **6**, **15**, **17**, **19**, **21** and **23** exhibited significant inhibition of LPO-catalyzed nitration of BSA (Fig. 5). The selenium analogue of MMI (**2**, MSeI) exhibited the highest activity among the selenoureas. It has been shown previously that the selenium analogues of antithyroid drugs prevent the peroxidase-catalyzed nitration reactions mainly by reacting with H_2O_2 that is necessary for the nitration.[16] The higher activity of MSeI is due to the facile oxidation of this compound by H_2O_2 to produce the corresponding diselenide. The *N*,*N*-disubstituted selenoureas do not undergo such oxidation. The substitution of hydrogen atom in MSeI (**2**) by a methyl group (compound **6**) reduced its inhibition potency. This is in agreement

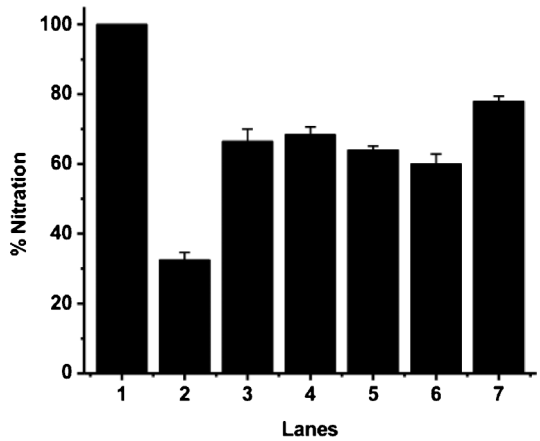


Fig. 4 Immunoblots for the inhibition of PN-mediated nitration of BSA by the sulfur analogues of antithyroid drugs. 0.1 mM of BSA was incubated with 130 μM of inhibitor and 2.0 mM of PN for 30 min at 20 °C and then denatured by loading dye and subjected to gel electrophoresis. Lanes: 1, protein + PN; 2, protein + PN + **1**; 3, protein + PN + **5**; 4, protein + PN + **14**; 5, protein + PN + **16**; 6, protein + PN + **18**; 7, protein + PN + **20**. Each bar diagram represents the mean ± SD of at least three independent experiments.

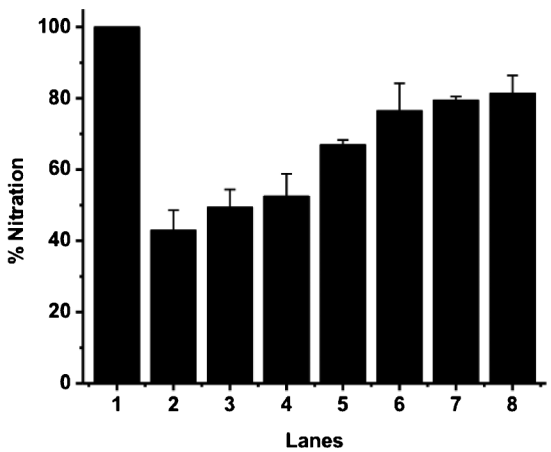
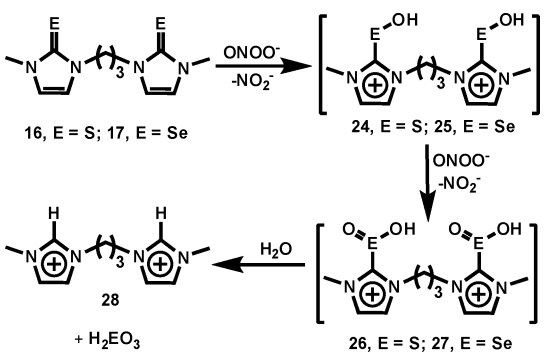


Fig. 5 Immunoblot of the inhibition of LPO-catalyzed tyrosine nitration in BSA by the selenium analogues of antithyroid drugs. Lanes: (1), protein + LPO + H_2O_2 + NO_2^-; (2), protein + LPO + H_2O_2 + NO_2^- + **2**; (3), protein + LPO + H_2O_2 + NO_2^- + **6**; (4), protein + LPO + H_2O_2 + NO_2^- + **15**; (5), protein + LPO + H_2O_2 + NO_2^- + **17**; (6), protein + LPO + H_2O_2 + NO_2^- + **19**; (7), protein + LPO + H_2O_2 + NO_2^- + **21**; (8), protein + LPO + H_2O_2 + NO_2^- + **23**. Assay condition: BSA (50 μM), LPO (50 nM), NO_2^- (2 mM) and H_2O_2 (1 mM) with or without inhibitors (63 μM). Protein was incubated with nitrating mixture and inhibitors for 30 min at 20 °C and then denatured with loading dye and subjected to gel electrophoresis. Each bar diagram represents the mean ± SD of at least three independent experiments.

with our observation on the PN-mediated nitration of BSA (Fig. 3). However, in contrast to the PN-mediated nitration, the di-selenoureas exhibited lower activity than the mono-selenoureas towards inhibition of the LPO-catalyzed nitration. A gradual decrease in the inhibitory potency of di-selenoureas with an increase in the linker -CH_2- chain length was observed as shown in Fig. 5. On the other hand, the olefin-substituted selenourea **23** exhibited a weaker inhibition towards LPO-catalyzed nitration, which is similar to the PN-mediated nitration. The difference in activities of mono- and di-selenoureas towards PN-mediated and LPO-catalyzed nitration reactions can be ascribed to the difference in their reactivity towards PN and H_2O_2. Although the PN-scavenging activities of selenium compounds can be enhanced by increasing the number of selenourea moieties in a single molecule, such strategy does not work well for the inhibition of peroxidase-catalyzed protein tyrosine nitration.

Reaction of thioureas/selenoureas with PN

To understand the mechanism of inhibition of PN-mediated nitration reactions, we have treated the thioureas/selenoureas with PN and the products were analyzed by NMR spectroscopic and ESI-MS spectrometric techniques. Interestingly, the reaction mixture of compound **17** with an excess amount of PN exhibited a signal at 1275 ppm in ^{77}Se NMR spectrum. A downfield shift to 1317 ppm was observed upon acidification of the solution, suggesting the formation of selenous acid (H_2SeO_3) as the major product. The oxidation of selenium centers in compound **17** by PN produces the corresponding seleninic acids that undergo subsequent hydrolysis leading to the formation bis-(*N*-methylimidazolium) salt **28** with the elimination of selenium as selenous acid (H_2SeO_3). Although the reaction of thiourea **16** with PN was found to be relatively slow, it also produced the *N*-methylimidazolium salt (**28**) and sulfurous acid (H_2SO_3) as the final products. The formation of imidazolium derivative was further confirmed by ESI-MS analyses. This is in agreement with our previous observations in the reactions of compounds **1–6** with PN.[14] In the previous report, the removal of thiourea/selenourea moieties from compounds **1–6** was experimentally confirmed and it was further supported by theoretical calculations using density functional theory (DFT). Based on these observations, we can propose the reaction sequence as shown in Scheme 4. The di-thiourea/di-selenourea derivatives react with two equivalents of PN to generate the corresponding sulfenic acid (**24**) or selenenic acid (**25**) intermediates. Subsequent reactions of **24–25** with second equivalents of PN produce the corresponding sulfinic acid (**26**) or seleninic acid (**27**), which undergoes a facile hydrolysis leading to the formation of *N*-methylimidazole derivative **28** with the elimination of sulfurous acid (H_2SO_3) or selenous acid (H_2SeO_3). These observations suggest that the *N*-methylimidazole-based di-thioureas/di-selenoureas inhibit the PN-mediated protein tyrosine nitration mainly by scavenging the PN present in the assay mixture. Furthermore, it has been reported previously that the selenium analogues of antithyroid drugs inhibit the peroxidase-catalyzed oxidation, iodination and nitration reactions mainly by reacting with H_2O_2.[15,16,19] Therefore, we have carried out the reaction of the di-selenourea **17** with an excess amount of H_2O_2. The analysis of final products in the reaction by NMR spectroscopy and ESI-MS spectrometry indicated the formation of H_2SeO_3 and the imidazole derivative **28**. These studies indicate that the reactions of the selenium analogues of antithyroid drugs with PN are similar to that observed with H_2O_2.



Scheme 4 Reaction of di-thiourea/di-selenourea derivatives **16–17** with PN and subsequent hydrolysis leading to the formation of *N*-methylimidazole derivative **28** with the elimination of sulfurous or selenous acid. These compounds produce the same products in the reaction with H_2O_2.

Conclusions

Synthesis and structural analyses of a series of *N*-methylimidazole-based di-thiourea/di-selenourea derivatives (**14–21**) linked by a –$(CH_2)_n$– chain indicates that the selenium-based compounds are more zwitterionic than the corresponding di-thioureas. The inhibition of PN-mediated nitration of bovine serum albumin (BSA) by these di-thioureas and di-selenoureas indicate that all the selenoureas effectively prevent the PN-mediated nitration of BSA. The inhibition of PN-mediated nitration by di-selenoureas was found to much higher than that of the mono-selenoureas indicating that the inhibitory potency of selenium analogues of antithyroid drugs can be enhanced by incorporating more than one selenourea moieties in a single molecule. However, further experiments with animal models are required to prove the antioxidant activity of these compounds *in vivo*. In contrast to the selenoureas, the activities of thioureas could not be enhanced by introducing additional thiourea moieties. The di-selenoureas also exhibited significant inhibition of lactoperoxidase (LPO)-catalyzed nitration of BSA. The reactions of di-thioureas/di-selenoureas with PN and H_2O_2 produce *N*-methylimdazole derivatives with the elimination of sulfurous/selenous acids.

Experimental section

General procedure

Synthesis of all the inhibitors were carried out under dry inert atmosphere using standard Schlenk techniques 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. 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 with sodium borohydride in deoxygenated water and the product was extracted with deoxygenated dichloromethane.[15] Stock solution of the compound was kept under N_2 atmosphere throughout the experiment. Compounds **5**,[19] **6**,[19] **7**[15b] and **8**[15b] were synthesized following the literature procedures. PN was synthesized in the laboratory following the literature procedure.[14,16,21]

General synthesis of selenoureas and thioureas

Step I: A mixture of *N*-methylimidazole (1.0 g, 12.18 mmol) and appropriate ω-dibromo-alkane (7.30 mmol) was stirred at room temperature for 30 min without any solvent. Dry THF (15 mL) was added to the reaction mixture which was then allowed to reflux overnight to obtain the white precipitate of the corresponding *N*-methylimidazole dibromide salts **10–13**. The solvent was evaporated under high vacuum and the crude product obtained was used for the next step without any further purification.

Step II: To a methanolic solution (20 mL) of the crude dibromide was added the finely ground selenium or sulfur powder (13.39 mmol) followed by K_2CO_3 (10.96 mmol). The reaction mixture was refluxed for 12 h and the solution was filtered hot through a pad of celite to remove the unreacted selenium or sulfur powder. The solvent was evaporated to obtain the crude solid. The product was then purified by silica gel column chromatography using petroleum ether and ethyl acetate as eluent.

Compound 14: Yield: 0.76 g (41%); m.p. 200–202 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 3.61 (s, 3H), 4.49 (s, 2H), 6.59 (s, 1H), 6.62 (s, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 35.1, 45.5, 117.7, 117.8, 162.3. ESI-MS: *m/z* calcd for $C_{10}H_{14}N_4S_2$: 277.0558 $[M+Na]^+$; found: 277.0558.

Compound 15: Yield: 0.86 g (34%); m.p. 215–217 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 3.70 (s, 3H), 4.62 (s, 2H), 6.80 (s, 1H), 6.83 (s, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 37.6, 47.7, 120.2, 120.4, 156.2. ^{77}Se NMR ($CDCl_3$) δ (ppm): 3.6. ESI-MS: *m/z* calcd for $C_{10}H_{14}N_4Se_2$: 372.9447 $[M+Na]^+$; found: 372.9445.

Compound 16: Yield: 1.06 g (54%); m.p. 155–157 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 2.32 (quin, J = 8.0 Hz, 1H), 3.61 (s, 3H), 4.11 (t, J = 8.0 Hz, 2H), 6.70 (s, 1H), 6.92 (s, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 28.2, 35.2, 45.2, 117.0, 118.0, 162.2. ESI-MS: *m/z* calcd for $C_{11}H_{16}N_4S_2$: 291.0714 $[M+Na]^+$; found: 291.0711.

Compound 17: Yield: 1.50 g (57%); m.p. 159–161 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 2.40 (quin, J = 8.0 Hz, 1H), 3.71 (s, 3H), 4.22 (t, J = 6.0 Hz, 2H), 6.87 (d, J = 4.0 Hz, 1H), 7.11 (d, J = 4.0 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 28.5, 37.2, 47.3, 119.1, 120.0, 155.7. ^{77}Se NMR ($CDCl_3$) δ (ppm): –1.1. ESI-MS: *m/z* calcd for $C_{11}H_{16}N_4Se_2$: 386.9603 $[M+Na]^+$; found: 386.9637.

Compound 18: Yield: 1.07 g (52%); m.p. 188–190 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.84 (br, 2H), 3.62 (s, 3H), 4.12 (br, 2H), 6.70 (s, 1H), 6.80 (s,1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 25.8, 35.1, 47.1, 116.9, 117.9, 162.0. ESI-MS: *m/z* calcd for $C_{12}H_{18}N_4S_2$: 305.0851 $[M+Na]^+$; found: 305.0852.

Compound 19: Yield: 1.45 g (53%); m.p. 135–137 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.88 (br, 2H), 3.70 (s, 3H), 4.22 (br, 2H), 6.86 (s, 1H), 7.01 (s, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 25.9, 37.1, 48.9, 119.1, 119.9, 155.5. ^{77}Se NMR ($CDCl_3$) δ (ppm): –7.0. ESI-MS: *m/z* calcd for $C_{12}H_{18}N_4Se_2$: 400.9760 $[M+Na]^+$; found: 400.9752.

Compound 20: Yield: 1.06 g (49%); m.p. 95–97 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.40 (quin, J = 6.0 Hz, 1H), 1.85 (quin, J = 8.0 Hz, 2H), 3.62 (s, 3H), 4.04 (t, J = 8.0 Hz, 2H), 6.70 (s, 1H), 6.72 (s, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 21.1, 23.1, 28.3, 35.1, 47.6, 116.7, 117.8, 162.0. ESI-MS: *m/z* calcd for $C_{13}H_{20}N_4S_2$: 319.1027 $[M+Na]^+$; found: 319.1029.

Compound 21: Yield: 1.56 g (55%); m.p. 104–106 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.41 (quin, J = 6.0 Hz, 1H), 1.88 (quin, J = 8.0 Hz, 2H), 3.71 (s, 3H), 4.14 (t, J = 8.0 Hz, 2H), 6.88 (d, J = 4.0 Hz, 1H), 6.93 (d, J = 4.0 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 23.0, 28.4, 37.1, 49.4, 118.9, 119.9, 155.3. ^{77}Se NMR ($CDCl_3$) δ (ppm): –8.6. ESI-MS: *m/z* calcd for $C_{13}H_{20}N_4Se_2$: 414.9916 $[M+Na]^+$; found: 414.9923.

Compound 23: Yield: 0.22 g (16%); m.p. 82–84 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 3.71 (s, 3H), 5.00 (d, J = 8.0 Hz, 1H), 5.27 (d, J = 16.0 Hz, 1H), 7.00 (s, 1H), 7.22 (s, 1H), 7.60 (q, J = 8.0 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 36.8, 102.2, 114.8, 121.2, 131.8, 157.0. ^{77}Se NMR ($CDCl_3$) δ (ppm): 22.2. ESI-MS: *m/z* calcd for $C_6H_8N_2Se$: 188.9931 $[M+H]^+$; found: 188.9911.

General procedure for the reaction of inhibitors with PN or H_2O_2

The reactions of thioureas/selenoureas with PN were carried out following the literature method with minor modifications.[14] To an ice-cooled solution of the inhibitor in water was added the alkaline solution of an excess amount of peroxynitrite (PN) in portions (three times with 3–4 h intervals) and the mixture was 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 non-polar 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.

Similarly, in the reaction with H_2O_2, the 30% solution of H_2O_2 was added in portion to the aqueous solution of the inhibitor at 0 °C and the mixture was stirred at room temperature for 12h. The reaction mixture was extracted with dichloromethane to remove the unreacted starting materials. The solvent from the combined aqueous layer was evaporated by lyophilization. The product was characterized by ^{77}Se NMR spectroscopic and ESI-MS spectrometric analyses. ^{77}Se NMR (D_2O) δ (ppm) for H_2SeO_3: 1317.7; ESI-MS: *m/z* calcd for $C_{11}H_{18}N_4$ (compound **28**): 103.0760 $[M]^{2+}$; found 103.0828.

Inhibition of protein nitration

PN-mediated 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 0.1 mM DTPA at 20 °C. After the addition of PN, the final pH was maintained below 7.5. The reaction mixture was incubated for 30 min at 20 °C. Similarly, the reactions of BSA with PN were performed in the presence of different antithyroid drugs and analogues (130 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 immunoblot analyses.

LPO-catalyzed nitration of BSA. Bovine serum albumin (BSA) (50 μM) in 50 mM phosphate buffer (pH 7.5) was incubated for 30 min at 20 °C with LPO (50 nM), sodium nitrite (2 mM) and hydrogen peroxide (1 mM) in the absence and presence of different antithyroid drugs and analogues (63 μM). 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 immunoblot analyses.

Electrophoretic analysis. Gel was prepared with 10% polyacrylamide with 6% stacking gel for BSA. 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 immunoblotting experiments. The proteins were transferred to a PVDF membrane and the non-specific binding sites were blocked by 5% non-fat skimmed milk in PBST (blocking solution) for 1 h. Then the membrane was probed with rabbit polyclonal primary antibody against nitro-tyrosine (1 : 20000 dilutions) in blocking solution for 1 h followed by incubation with horseradish peroxidase-conjugated donkey polyclonal anti-rabbit IgG (1 : 20000 dilutions) for another 1 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). Each bar diagram in Fig. 3–5 represents the mean±SD of at least three independent experiments.

X-ray crystallography

Single crystal X-ray diffraction data was collected on a Bruker AXS SMART APEX CCD diffractometer. The X-ray generator was operated at 50 kV and 35 mA using Mo-Kα radiation ($\lambda = 0.71073$ Å). The data was collected using SMART software package.[22] The data were reduced by SAINTPLUS,[22] an empirical absorption correction was applied using the package SADABS[23] and XPREP[22] was used to determine the space group. The crystal structure was solved by direct methods using SIR92[24] and refined by full-matrix least-squares method using SHELXL97.[25] All non-hydrogen atoms were refined anisotropically and hydrogen atoms were assigned at idealized locations.

Crystal data for **16**:[26] $C_{11}H_{16}N_4S_2$ $M_r = 268.40$, monoclinic, space group $P21/c$, $a = 6.893(2)$, $b = 20.222(6)$, $c = 9.776(3)$ Å, $\alpha = 90°\beta = 106.617(5)°\gamma = 90°$; $V = 1305.7(7)$ Å^3, $Z = 4$, $\rho_{calcd} = 1.365$ g cm^{-3}, GOF = 1.036, $R_1 = 0.0594$, w$R_2 = 0.1131$ [$I > 2\sigma(I)$]; $R_1 = 0.0407$, w$R_2 = 0.1011$ (all data).

Crystal data for **17**:[26] $C_{11}H_{16}N_4Se_2$ $M_r = 362.20$, monoclinic, space group $P21/c$, $a = 6.9814(2)$, $b = 20.5732(6)$, $c = 9.8023(2)$ Å, $\alpha = 90°\beta = 105.6430(10)°\gamma = 90°$; $V = 1355.75(6)$ Å^3, $Z = 4$, $\rho_{calcd} = 1.774$ g cm^{-3}, GOF = 1.646, $R_1 = 0.0436$, w$R_2 = 0.0872$ [$I > 2\sigma(I)$]; $R_1 = 0.0293$, w$R_2 = 0.0663$ (all data).

Crystal data for **19**:[26] $C_{12}H_{18}N_4Se_2$ $M_r = 376.22$, monoclinic, space group $C2/c$, $a = 11.5510(6)$, $b = 8.8676(4)$, $c = 14.669(9)$ Å, $\alpha = 90°\beta = 107.566(3)°\gamma = 90°$; $V = 1432.48(13)$ Å^3, $Z = 4$, $\rho_{calcd} = 1.744$ g cm^{-3}, GOF = 1.047, $R_1 = 0.0576$, w$R_2 = 0.1100$ [$I > 2\sigma(I)$]; $R_1 = 0.0404$, w$R_2 = 0.0982$ (all data).

Crystal data for **21**:[26] $C_{13}H_{20}N_4Se_2$ $M_r = 390.25$, orthorhombic, space group $Pbca$, $a = 11.7252(9)$, $b = 8.7848(7)$, $c = 33.465(3)$ Å, $\alpha = 90°\beta = 90°\gamma = 90°$; $V = 3447.1(5)$ Å^3, $Z = 8$, $\rho_{calcd} = 1.504$g cm^{-3}, GOF = 0.837, $R_1 = 0.1313$, w$R_2 = 0.1419$ [$I > 2\sigma(I)$]; $R_1 = 0.0601$, w$R_2 = 0.1270$ (all data).

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26 CCDC-800371 (**16**); CCDC-800370 (**17**); CCDC-800372 (**19**); CCDC-800373 (**21**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre *via* www.ccdc.cam.ac.uk/data_request/cif.

