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PAPER

Synthesis, characterization and antioxidant activity of angiotensin converting enzyme inhibitors†

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Angiotensin converting enzyme (ACE) catalyzes the conversion of angiotensin I (Ang I) to angiotensin II (Ang II). ACE also cleaves the terminal dipeptide of vasodilating hormone bradykinin (a nonapeptide) to inactivate this hormone. Therefore, inhibition of ACE is generally used as one of the methods for the treatment of hypertension. 'Oxidative stress' is another disease state caused by an imbalance in the production of oxidants and antioxidants. A number of studies suggest that hypertension and oxidative stress are interdependent. Therefore, ACE inhibitors having antioxidant property are considered beneficial for the treatment of hypertension. As selenium compounds are known to exhibit better antioxidant behavior than their sulfur analogues, we have synthesized a number of selenium analogues of captopril, an ACE inhibitor used as an antihypertensive drug. The selenium analogues of captopril not only inhibit ACE activity but also effectively scavenge peroxynitrite, a strong oxidant found *in vivo*.

Introduction

Angiotensin II (Ang II), the peptide-based blood pressure regulating hormone in the renin-angiotensin system (RAS)[1] is produced *in vivo* from the decapeptide angiotensin I (Ang I) by cleavage of the C-terminal dipeptide His-Leu. The conversion of Ang I to Ang II is catalyzed by angiotensin converting enzyme (ACE), a metalloprotease having a zinc(II) ion at the active site (Fig. 1).[2] As the overproduction of Ang II leads to hypertension, ACE inhibitors are commonly used to treat cardiovascular diseases such as high blood pressure, heart failure, coronary artery disease and kidney failure.[1–3] Furthermore, ACE is also responsible for the elevation of blood pressure by cleaving the terminal dipeptide (Phe-Arg) of vasodilator hormone bradykinin (nonapeptide) to its inactive form (bradykinin 1-7).[4] Therefore, inhibition of ACE is important in the treatment of hypertension. Captopril (**1**),[5] the first pharmaceutical compound designed to inhibit a protease, prevents the conversion of Ang I to Ang II by inhibiting ACE. After the synthesis of captopril, several ACE inhibitory antihypertensive drugs such as enalaprilat (**2**),[6] zofenoprilat (**3**),[7] fosinoprilat (**4**)[8] *etc.* have been reported. It should be noted that most of these drugs contain a proline residue, which appears to be important for the ACE inhibition (Fig. 2).

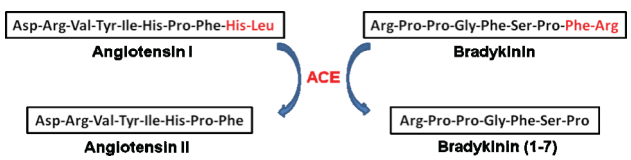

Fig. 1 ACE-catalyzed conversion of Ang I to Ang II and bradykinin to bradykinin (1-7). Elevation of Ang II and depletion of bradykinin concentrations result in the elevation of blood pressure.

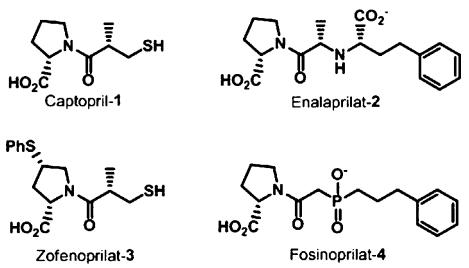

Fig. 2 Proline-based ACE inhibitors: captopril (**1**), enalaprilat (**2**), zofenoprilat (**3**) and fosinoprilat (**4**).

Recent evidence suggests that hypertension and oxidative stress are related to each other and that hypertension plays an important role in the pathogenesis of oxidative stress and inflammation.[9] It has been shown that hypertension induces oxidative stress by activating NADPH oxidase, leading to the overproduction of superoxide anion ($O_2^{\bullet -}$). These reactive superoxide anions interact with nitric oxide ($^{\bullet}NO$) to generate peroxynitrite ($ONOO^-$),[10] a highly reactive intermediate known to nitrate protein tyrosine residues and cause cellular damage.[11] Furthermore, Ang II-induced endothelial dysfunction has been associated with

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increased production of peroxynitrite (PN).[12] Therefore, antihypertensive drugs having antioxidant activity may play an important role in the treatment of hypertension. It is known that captopril (**1**) and zofenoprilat (**3**) have significant antioxidant activity that may contribute to their cardio- and endothelium-protective effects.[13] As selenium compounds are generally better antioxidants than their sulfur analogues,[14] it was thought worthwhile to synthesize the selenium analogues of captopril and a few cysteine- and selenocysteine-containing dipeptides (Fig. 3) and study their ACE inhibition properties. In this paper, we report that the selenium analogues of captopril and Cys-Pro and Sec-Pro peptides (**5–11**) not only inhibit ACE activity but also effectively scavenge peroxynitrite.

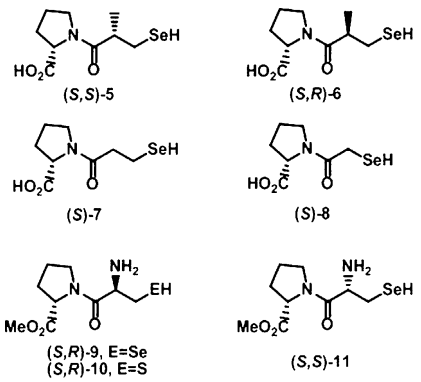
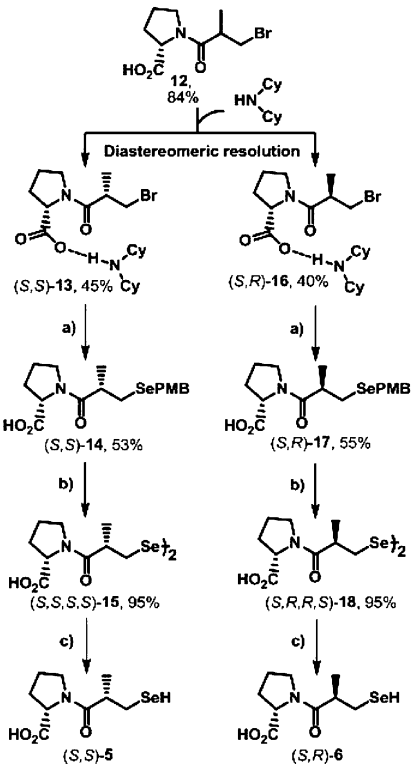


Fig. 3 Selenium analogues of captopril and related derivatives (**5–11**).



Scheme 1 Synthesis of compounds **5** and **6**. a) *p*-Methoxy benzyl diselenide, $NaBH_4$, anhyd. DMF, 36 h; b) I_2, 1 : 1 MeOH–water, 15 min; c) MeOH, $NaBH_4$,10 min; Cy = Cyclohexyl.

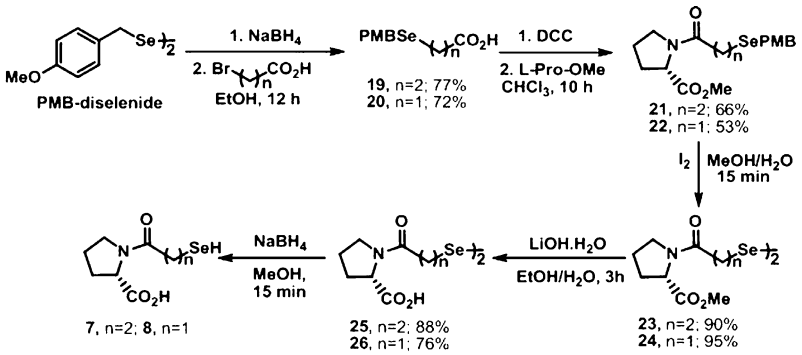
Synthesis

In contrast to the synthesis of captopril (**1**),[5] the synthesis of the selenium analogues is complicated due to a facile oxidation of the selenol to the corresponding diselenide. Therefore, we approached the synthesis of the diselenide **15** starting from the L-proline-based compound **12** (Scheme 1). Compound **12** can be obtained in pure form by coupling L-proline with (*R*,*S*)-3-bromo-2-methyl-propionic acid. Due to the unavailability of the optically pure form of (*R*,*S*)-3-bromo-2-methyl-propionic acid, the resolution of the diastereomers is the crucial step in the synthetic procedure. The diastereomeric mixture of compound **12** can be resolved by preferential crystallization of their dicyclo-hexyl amine (DCHA) salts (**13** and **16**).[15] When the mixture was treated with DCHA and kept at 4 °C for 12 h, one of the diastereomers (compound **13**) was crystallized preferentially, leaving the other diastereomer (compound **16**) in the solution. Removal of DCHA from compounds **13** and **16** by treatment with 1 M $KHSO_4$ solution followed by reactions with *p*-methoxy benzyl selenolate (PMB-selenolate) afforded the PMB-protected compounds **14** and **17**, respectively. The diastereomerically pure diselenides **15** and **18** were obtained by removal of the PMB-group with iodine. The selenols **5** and **6** required for the inhibition studies were prepared by reducing the corresponding diselenides with $NaBH_4$.

Compounds **13** and **15** were characterized by single crystal X-ray diffraction studies. The crystal structure of compound **13** shows that there is a hydrogen bond between the carboxylic group of proline residue and the amino group of DCHA. This also indicates that the (*S*,*S*)-diastereomer has crystallized preferentially over the (*S*,*R*)-diastereomer. The non-centrosymmetric space group (P1) and Flack parameter[16] (0.21) confirm the diastereomeric purity of the crystals. It should be noted that the bromine atom in compound **13** is disordered over two positions. The crystal structure of compound **15** indicates that there was no racemization during the conversion of **14** to **15**. Compound **15** crystallized in a $P2_1$ space group and all four chiral centers in this compound are present in the *S*-configuration. The absolute structure parameter for this compound (0.036) indicates that the crystals are optically pure (crystallographic details are given in the ESI†).

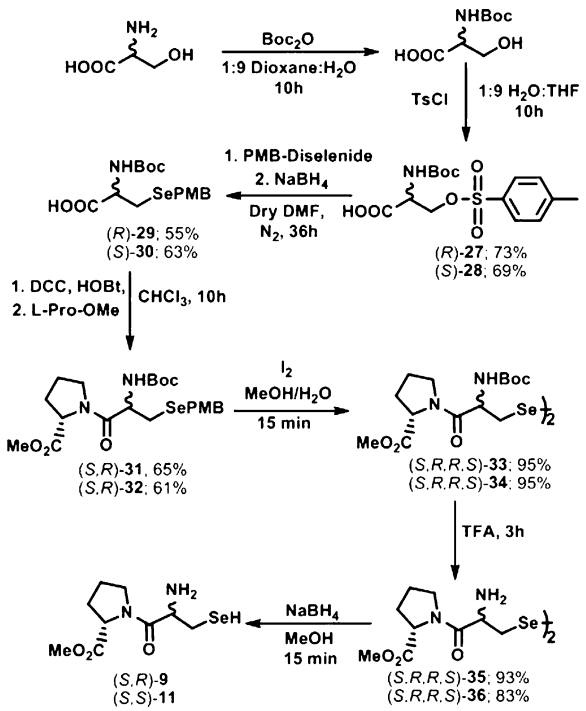
Compounds **7** and **8** were synthesized by following a different synthetic route (Scheme 2). In this method, the selenium moiety was introduced to bromo-propionic acid or bromo-acetic acid as PMB-protected selenolate to produce compounds **19** and **20**, respectively, in reasonably good yield. In addition to spectroscopic analysis, compound **20** was characterized by single crystal X-ray studies (ESI†). After coupling of the L-Pro moiety, the PMB-protecting group at the selenium center was removed by iodine from compounds **21** and **22** to obtain the corresponding diselenides (**23** and **24**). Hydrolysis of the ester group by LiOH followed by reduction with $NaBH_4$ produced the selenols **7** and **8**. The stability of **7** and **8** was found to be identical to that of Se-captopril.

Captopril (**1**) and Se-captopril (**5**) are structurally similar to D-Cys-L-Pro and D-Sec-L-Pro dipeptides, respectively. Harris *et al.* have shown that the replacement of the methyl group in captopril by an amino group decreases the inhibition activity of the parent compound.[17] The D-Sec-L-Pro dipeptide (**11**) resembles Se-captopril (**5**), whereas, L-Sec-L-Pro (**9**) resembles the



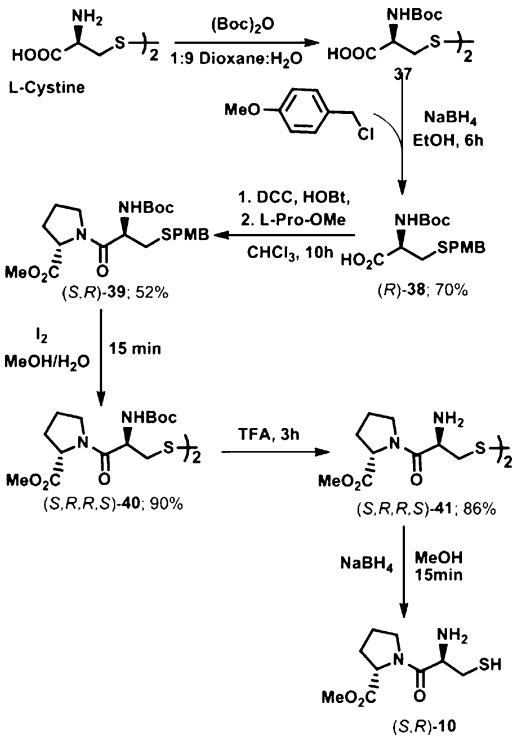
Scheme 2 Synthesis of compounds **7** and **8**.

R,*S*-diastereomer of Se-captopril (**6**). To understand the effect of the methyl group in captopril and its selenium analogues, we have synthesized Cys-Pro and Sec-Pro dipeptides (**9–11**) and studied their ACE inhibition activities. Dipeptides containing Sec-Pro and Cys-Pro were synthesized by following solution phase peptide synthetic routes.[18] Due to the presence of reactive thiol and selenol moieties, the protection of the side chain is important in the synthesis of Cys- and Sec-containing dipeptides.[19] Sec-Pro-containing dipeptides were synthesized by following the procedure given in Scheme 3.



Scheme 3 Synthesis of Sec-Pro dipeptides (compounds **9** and **11**).

To understand the importance of chirality in dipeptides, we have used enantiomerically pure D- or L-serine. The tosylated compounds **27** and **28** were obtained from the corresponding Boc-protected serines. The PMB-Se moiety was then introduced to obtain enantiomerically pure **29** and **30**. DCC-mediated coupling of L-Pro-OMe to the PMB-protected Sec afforded the corresponding dipeptides **31** and **32**. The cleavage of the PMB group under mild conditions using iodine led to the formation of diselenides **33** and **34**. Removal of the Boc group by treatment with TFA afforded the Sec-Pro dipeptides. The selenols **9** and **11** could be conveniently prepared by reducing the diselenides **35** and **36**, respectively, by $NaBH_4$. The Cys-Pro dipeptide (**10**) was synthesized following a similar procedure (Scheme 4).



Scheme 4 Synthesis of Cys-Pro dipeptide (compound **10**).

Inhibition of angiotensin converting enzyme

The ACE inhibition activities of captopril and its selenium analogues (**5–11**) were studied by a HPLC method (Scheme 5). Ang I was used as the substrate for the enzyme and a decrease in the formation of Ang II with an increase in the concentration of inhibitors was followed at 215 nm wavelength. The peak area corresponding to Ang II was obtained for the initial 5–10% of the reaction and, wherever possible, the IC_{50} values (concentration of inhibitors required to inhibit 50% of the enzyme activity) were determined. The reaction mixture was incubated at 37 °C for

Scheme 5 Inhibition of ACE-catalyzed conversion of Ang I to Ang II by the selenium analogues of captopril.

30 min prior to analysis. The selenols (**5–8**) were freshly prepared by reducing the corresponding diselenides by $NaBH_4$ prior to use and were kept under N_2 atmosphere during the assay. The IC_{50} values obtained for the inhibition of ACE-catalyzed conversion of Ang I to Ang II by the captopril analogues are summarized in Fig. 4. In contrast to the thiol and selenols, the IC_{50} values could not be determined for the disulfide and diselenides up to a concentration of 50 μM.

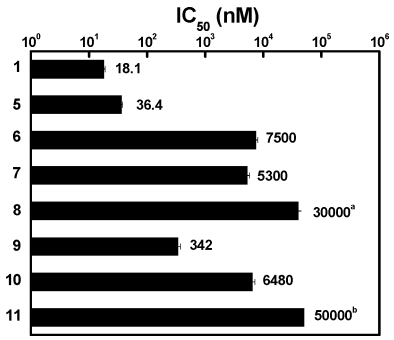


Fig. 4 IC_{50} values for ACE inhibition by **1**, **5–11**. [a] At this concentration, only 30% of the enzyme activity was inhibited. [b] Only 40% inhibition was observed. Assay conditions: the reaction was carried out in HEPES–HCl buffer (50 mM, pH 8.3) at 37 °C with a final concentration of 50 μM Ang I, 60mM NaCl and 2 milliunits of ACE in 400 μL reaction mixture.

It is clear from Fig. 4 that selenol **5** is an excellent inhibitor of ACE although the IC_{50} value (36.4 ± 1.5 nM) is almost two times higher than that of captopril (**1**, 18.1 ± 1.0 nM). Interestingly, the diastereomer **6** was found to be much less active than **5**. The IC_{50} value obtained for **6** (7500 ± 500 nM) is almost 200 times higher than that of **5**. This indicates that the correct stereochemistry at the side chain is important for an efficient inhibition. The IC_{50} value obtained for compound **7** (5300 ± 440 nM) indicates that the absence of the methyl group significantly reduces the inhibitory activity. However, this compound is moderately more active than compound **6**. The distance between the proline moiety and the selenol group appears to be important for the inhibition as compound **8**, having only one methylene group, exhibited very poor inhibition. At 30 μM concentration, this compound inhibited only 30% of the enzyme activity. Similar to the captopril analogues, the ACE inhibition activities by the dipeptides depends on the chirality. However, it was observed that the replacement of the methyl group of Se-captopril by an amino group in dipeptide **11** dramatically reduces the ACE inhibition potency. This compound inhibited only 40% of the enzyme activity up to 50 μM concentration. In contrast, the other diastereomer (L-Sec-L-Pro-OMe, compound **9**) exhibited very good activity (IC_{50}: 342 ± 33 nM). In the case of dipeptides, sulfur analogue (compound **10**, IC_{50}: 6480 ± 640 nM) showed about 20 times less activity than the selenium analogue. In contrast to the selenols and thiols, the diselenides and disulfides did not show any noticeable inhibition, indicating that the presence of the selenol moiety is important for ACE inhibition.

To understand the nature of ACE inhibition by the captopril analogues, we have carried out further kinetic experiments at different concentrations of inhibitors (**5** and **6**) by varying the concentrations of the substrate (Ang I) for each inhibitor concentration. The initial rates for the hydrolysis of Ang I by ACE in the presence of inhibitors were obtained by following the formation of Ang II. For each concentration of the inhibitor, a linear line was obtained when the reciprocal of the initial rate was plotted against the reciprocal of the concentration of Ang I. These linear lines (Lineweaver–Burk plots) for various concentrations of inhibitors intersect the ordinate with almost identical V_{max} values (Fig. 5), suggesting that inhibition of ACE activity by compounds **5** and **6** is competitive with respect to the substrate. It is known that the inhibition of ACE by captopril is competitive with respect to ACE substrates.[5] Therefore, the binding of compounds **5** and **6** to the active site of ACE is similar to that of captopril.

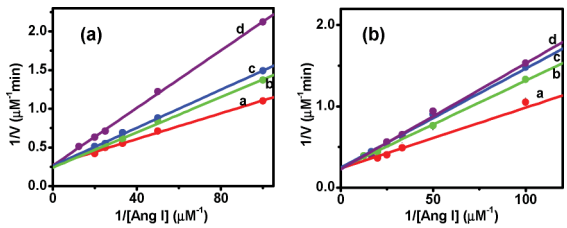


Fig. 5 Lineweaver–Burk plots obtained for (a) compound **5** and (b) compound **6** at various concentrations of Ang I and fixed concentration of the inhibitor (for more details, see the ESI†).

The crystal structure of the ACE-captopril complex reveals that the proline residue of captopril interacts with the S2′ subsite of the enzyme active site (Fig. 6).[20] This interaction is favored by two histidine residues at the enzyme active site. It has been shown that the thiol group of captopril interacts with the zinc(II) ion to form a zinc-thiolate complex. As compound **5** is structurally similar to that of captopril, we propose the formation of a zinc-selenolate complex. This is further supported by the observations that the PMB-protected compounds **14** and **17** and the diselenides **15** and **18** do not inhibit the enzyme activity. Although the formation of zinc-selenolate may be more favored than the formation of a

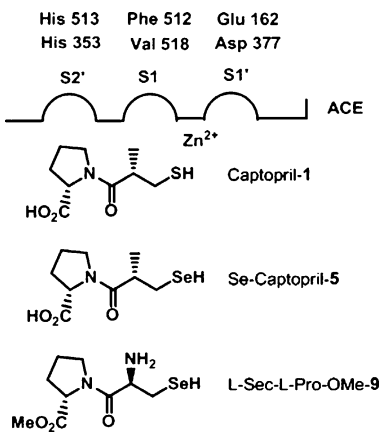
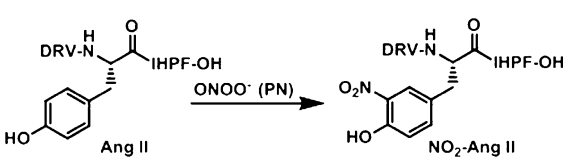


Fig. 6 Possible binding of captopril (**1**), Se-captopril (**5**) and L-Sec-L-Pro-OMe (**9**) to the enzyme active site.

zinc-thiolate complex,[21] the partial oxidation of the selenol to the corresponding diselenide is probably responsible for the relatively higher IC_{50} value for Se-captopril as compared to that of captopril. The poor inhibition by compound **6** indicates that the binding of captopril and its analogues to the ACE active site is stereospecific. Interestingly, the higher activity of compound **7** as compared to **6** indicates that the side chain lacking any substituent is probably a better inhibitor than the one having incorrect stereochemistry. As the distance between the selenol group and proline moiety plays an important role in the inhibitor binding, shortening of this distance by removing one methylene group may reduce the binding ability. In agreement with this, compound **8**, having one $–CH_2–$ group, shows very weak inhibition. In the case of dipeptides, replacement of the non-polar methyl group by the polar amino group in the same stereochemistry hinders the binding of compound **11** to the enzyme active site. However, when the orientation of the amino group is reversed, compounds **9** and **10** can probably interact with the polar amino acid residues present at the active site. The higher activity of the Sec-containing peptide as compared to the Cys-containing peptide may be attributed to the stabilization of the reactive selenol of compound **9** by the electron-withdrawing nature of the amino functional group to form a strongly bound zinc(II)-selenolate complex.

Inhibition of PN-mediated nitration reactions

To evaluate the antioxidant activity of the sulfur and selenium compounds, we have studied the effect of these compounds on the peroxynitrite-mediated nitration[22] of Ang II. It has been demonstrated that nitration of the tyrosine residue in Ang II completely inhibits its vasoconstrictive properties, which may correlate with the endothelial dysfunction observed at the early stage of Ang II action.[12c,23] As the formation of 3,5-dinitro-Ang II was also observed in the reaction, only the initial 5–10% of the conversion was followed, for which the mononitro compound was produced as the major product (Scheme 6). In order to compare the PN-scavenging activity of the diselenides with that of a disulfide, we have synthesized captopril-disulfide (compound **42**) from captopril using $K_3[Fe(CN)_6]$ as oxidizing agent.[24] The IC_{50} values obtained for the inhibition of PN-mediated nitration of Ang II are summarized in Fig. 7. Interestingly, all the selenium compounds strongly inhibited the nitration reaction. The inhibitory activity of the selenium analogue of captopril (**5**) was found to be almost 10 times higher than that of captopril (**1**). The Sec-containing dipeptides were observed to be as potent inhibitors of PN-mediated nitration reactions as the other selenium-containing compounds. The PN-scavenging ability of Cys-containing peptides was lower than that of the Sec-containing dipeptides. In contrast to the ACE inhibition activities, the PN-scavenging activity does not depend on the chirality, but it depends on the presence of a sulfur or selenium center. This



Scheme 6 Peroxynitrite-mediated nitration of Angiotensin II to mono-nitro angiotensin II.

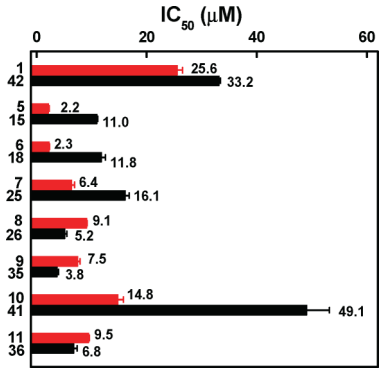


Fig. 7 Peroxynitrite-scavenging activity of various captopril analogues. Assay conditions: reactions were carried out in sodium phosphate buffer (100 mM, pH 7.5) at 22 °C with a final concentration of 20 μM Ang II and 300 μM PN.

is, however, not surprising since the selenium compounds exhibit their inhibitory effects mainly by scavenging the peroxynitrite.

In addition to the inhibition of Ang II nitration, the disulfides and diselenides effectively protect against PN-mediated nitration of bovine serum albumin (BSA). At 60 μM concentration of these compounds, ~40–60% inhibition of tyrosine nitration was observed (Fig. 8). Similar to the effect of these compounds on the nitration of Ang II, the diselenides were found to be more effective than the corresponding disulfide derivatives (**41** and **42**). Although compounds **25** and **26** were found to be more active than the other disulfides and diselenides in the Ang II assay, these compounds were slightly less potent than the captopril analogues **15** and **18** in the inhibition of the nitration of BSA.

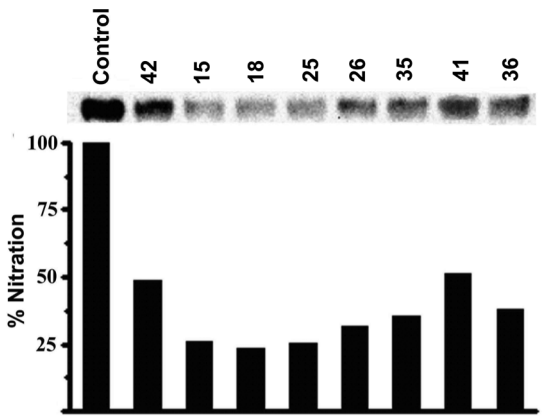


Fig. 8 Immunoblots for the inhibition of PN-mediated nitration of BSA. 100 μM of BSA was incubated with 60 μM of inhibitor and 1.2 mM of PN for 30 min at 20 °C and then subjected to gel electrophoresis.

Conclusions

In conclusion, we provided the first experimental evidence that the selenium analogues of captopril not only inhibit angiotensin converting enzyme but also effectively protect against peroxynitrite-mediated nitration of tyrosyl residues in peptides and proteins. The inhibition of PN-mediated nitration of Ang II is particularly important as such nitration has been implicated in cardiovascular diseases. This study reveals that the ACE inhibition is

highly stereospecific to the inhibitor conformation, whereas, PN-scavengening activity depends on the presence of the selenium or sulfur center. Selenium-containing compounds are better scavengers of peroxynitrite. Although the selenols are oxidized to the corresponding diselenides in air, biological thiols such as glutathione (GSH) can prevent the oxidation.

Experimental section

General procedure. Angiotensin converting enzyme (ACE) and captopril (**1**) were purchased from Sigma-Aldrich Chemical Co. All experiments involving selenols and thiols were carried out under dry and oxygen free nitrogen using standard Schlenk techniques. Column chromatography was performed on glass columns loaded with silica gel or on an automated flash chromatography system (Biotage) using pre-loaded silica cartridges. ^{1}H (400 MHz), ^{13}C (100.56 MHz), and ^{77}Se (76.29 MHz) NMR spectra were obtained on a Bruker 400 MHz NMR spectrometer. Chemical shifts are cited with respect to $SiMe_4$ 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 6000 plus mass spectrometer with ESI-MS mode analysis. The purities of the compounds were greater than 95% as determined by reversed-phase HPLC.

Synthesis of compound 12. To a solution of 3-bromo-2-methyl propionic acid (0.75 g, 4.5 mmol) in benzene (20 mL), $SOCl_2$ (1 mL, 12.5 mmol) was added dropwise at 0 °C. To this mixture, DMF (0.034 mL, 0.4 mmol) was added as catalyst. The reaction mixture was allowed to attain the room temperature and the stirring was continued for 3 h. Solvent and excess $SOCl_2$ were evaporated under reduced pressure to yield the acyl chloride, which was dissolved in chloroform and cooled to 0 °C. L-proline (0.78 g, 6.7 mmol) was added to the above solution, followed by the addition of Et_3N (0.9 mL, 6.5 mmol). The reaction mixture was allowed to attain the room temperature and stirred for 10 h. The mixture was acidified by adding 1 M aqueous $KHSO_4$ solution and the compound was extracted with chloroform. The organic layer was washed two times with brine. The solvent was evaporated and the compound was purified by column chromatography (hexane–ethyl acetate; 1 : 1). Yield 1.0 g (84%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.24–1.29 (d, 3H), 2.05–2.11 (m, 3H), 2.34–2.35 (m, 1H), 3.05–3.10 (m, 1H), 3.31–3.35 (m, 1H), 3.60–3.68 (m, 3H), 4.15–4.67(dd, 1H); ^{13}C NMR ($CDCl_3$) δ (ppm): 17.3, 25.2, 29.1, 34.4, 41.8, 47.9, 59.6, 174.3, 175.3; HRMS (ESI mode) calcd. for $C_9H_{14}BrNO_3$ $[M+Na]^+$ 264.0235, found 264.0240.

Synthesis of 13. Compound **12** (1.01 g, 3.7 mmol) was dissolved in 10 mL chloroform and dicyclohexyl amine (DCHA) (0.71 g, 3.8 mmol) was added. To this, 10 mL of acetonitrile was added and the mixture was kept at 4 °C for 12 h. After this period, dicyclohexyl amine salt of compound **13** crystallizes out. The solvent was removed and the crystals were washed with acetonitrile. The crystals were dissolved in chloroform and washed several times with a saturated solution of $KHSO_4$. The organic layer was dried with anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. Yield 0.45 g (45%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.24–1.29 (d, 3H), 2.05–2.11 (m, 3H), 2.34–2.35 (m, 1H), 3.05–3.10 (m, 1H), 3.31–3.35 (m, 1H), 3.60–3.68 (m, 3H), 4.15–4.67 (d, 1H); ^{13}C NMR ($CDCl_3$) δ (ppm): 17.3, 25.2, 29.1, 34.4, 41.8, 47.9, 59.6, 174.3, 175.3; HRMS (ESI mode) calcd. for $C_9H_{14}BrNO_3$ $[M+Na]^+$ 264.0235, found 264.0229.

Synthesis of 16. This compound was obtained from the filtrate from the above reaction. The excess DCHA was removed by acidification. Yield 0.41 g (40%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.24–1.28 (d, 3H), 2.06–2.10 (m, 3H), 2.35–2.36 (m, 1H), 3.07–3.11 (m, 1H), 3.37–3.41 (m, 1H), 3.60–3.68 (m, 3H), 4.15–4.67(dd, 1H); ^{13}C NMR ($CDCl_3$) δ (ppm): 17.4, 25.3, 29.1, 34.7, 41.3, 48.0, 59.2, 174.1, 176.0; HRMS (ESI mode) calcd. for $C_9H_{14}BrNO_3$ $[M+Na]^+$ 264.0235, found 264.0245.

Synthesis of 14. To a solution of 4-methoxy benzyl diselenide (0.24 g, 0.6 mmol) in dry DMF, $NaBH_4$ was added in small portions (~0.09 g, 2.4 mmol). The mixture was stirred for 15 min to obtain the corresponding selenol. A solution of **13** (0.26 g, 1.0 mmol in 15 mL ethanol) in 10 mL DMF was added to the selenol over 15 min at 0 °C. The reaction mixture was stirred for 36 h at room temperature under nitrogen atmosphere. To this, 100 mL of 1 M $KHSO_4$ was added and the compound was extracted with ethyl acetate. The organic layer was washed three times with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure and the compound was purified by flash chromatography (hexane–ethyl acetate; 1 : 2) to afford a pale yellow liquid. Yield 0.21 g (53%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.15–1.17 (d, 3H), 1.97–2.00 (m, 4H), 2.49–2.54 (m, 2H), 2.83–2.89 (m, 1H), 3.38–3.44 (m, 1H), 3.76–3.79 (m, 6H), 4.61–4.63 (dd, 1H), 6.82–6.84 (dd, 2H), 7.20–7.22 (dd, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 18.7, 25.2, 27.2, 28.1, 28.6, 40.1, 47.9, 55.8, 59.9, 114.4, 130.4, 131.9, 158.9, 174.5, 176.7; ^{77}Se NMR ($CDCl_3$, Me_2Se) δ (ppm): 247; HRMS (ESI mode) calcd. for $C_{17}H_{23}NO_4Se$ $[M+Na]^+$ 408.0690, found 408.0693.

Synthesis of 17. Compound **17** was synthesized following a similar procedure to that given for compound **14**. Yield 0.22 g (55%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.14–1.16 (d, 3H), 1.99–2.05 (m, 3H), 2.51–2.55 (m, 1H), 2.68–2.75 (m, 1H), 2.82–2.87 (m, 1H), 3.34–3.36 (m, 1H), 3.45–3.48 (m, 1H), 3.76–3.79 (m, 6H), 4.54–4.56 (d, 1H), 6.81–6.84 (dd, 2H), 7.21–7.23 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 18.2, 25.1, 27.3, 28.2, 40.3, 48.1, 55.7, 60.5, 114.4, 130.5, 131.8, 158.9, 173.5, 177.5; ^{77}Se NMR ($CDCl_3$, Me_2Se) δ (ppm): 245; HRMS (ESI mode) calcd. for $C_{17}H_{23}NO_4Se$ $[M+Na]^+$ 408.0690, found 408.0702.

Synthesis of 15. To a 10 mL methanolic solution of **14** (0.20 g, 0.5 mmol), iodine (0.15 g, 0.6 mmol) was added followed by 10 mL of water. The reaction mixture was stirred for 15 min. To this, 0.5 mL of hydrazine hydrate was added to decompose the excess iodine. Methanol was removed under reduced pressure and 20 mL of 1 M $KHSO_4$ solution was added. The compound was extracted three times with ethyl acetate and purified by flash chromatography (hexane–ethyl acetate; 1 : 3). Yield 0.13 g (95%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.25–1.27 (d, 6H), 2.02–2.10 (m, 6H), 2.46–2.50 (m, 2H), 3.08–3.12 (m, 2H), 3.33–3.37 (m, 2H), 3.60–3.62 (m, 2H), 3.65–3.69 (m, 4H), 4.67–4.70 (dd, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 18.3, 25.3, 28.8, 32.67, 39.9, 48.0, 59.8, 174.9, 175.9; ^{77}Se NMR ($CDCl_3$, Me_2Se) δ (ppm): 305; HRMS (ESI mode) calcd. for $C_{18}H_{28}N_2O_6Se_2$ $[M+Na]^+$ 551.0175, found 551.0173.

Synthesis of 18. Compound **18** was synthesized following a similar procedure to that given for compound **15** using compound

17 as the starting material. Yield 0.13 g (95%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.22–1.24 (d, 6H), 1.96–1.38 (m, 6H), 2.90–3.02 (m, 6H), 3.21–3.23 (m, 2H), 3.53–3.56 (m, 2H), 3.76–3.79 (m, 2H), 4.49–4.52 (dd, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 17.6, 25.2, 28.9, 30.1, 39.8, 48.0, 60.1, 174.4, 176.1; ^{77}Se NMR ($CDCl_3$, Me_2Se) δ (ppm): 306; HRMS (ESI mode) calcd. for $C_{18}H_{28}N_2O_6Se_2$ $[M+H]^+$ 529.0356, found 529.0341.

Synthesis of 19. To a solution of 4-methoxy benzyl diselenide (0.93 g, 2.3 mmol) in ethanol under N_2 atmosphere, $NaBH_4$ was added in small portions (~0.37 g, 10.0 mmol). The mixture was stirred for 1 h to obtain the corresponding selenol. To this, 15 mL ethanolic solution of 3-bromo acetic acid (0.83 g, 6.0 mmol) was added over a period of 15 min at 0 °C. The reaction mixture was stirred for 12 h at room temperature under nitrogen atmosphere. The solvent was evaporated under reduced pressure and acidified with 1 M $KHSO_4$ solution. The compound was extracted from the aqueous mixture with $CHCl_3$ and washed three times with brine. The organic layer was dried over anhydrous Na_2SO_4. Removal of solvent afforded a pale yellow liquid. Pure compound was obtained by column chromatography (hexane–ethyl acetate; 2 : 1). Yield 1.21 g (77%). ^{1}H NMR ($CDCl_3$) δ (ppm): 3.04 (s, 2H), 3.80 (s, 3H), 3.97 (s, 2H), 6.84–6.87 (d, 2H), 7.27–7.29 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 21.5, 28.1, 55.3 114.0, 129.9, 130.4, 158.7, 178.2; ^{77}Se NMR ($CDCl_3$, Me_2Se) δ (ppm): 312; HRMS (ESI mode) calcd. for $C_{10}H_{12}O_3Se$ $[M+Na]^+$ 282.9849, found 282.9857.

Synthesis of 21. To a solution of **19** (0.35 g, 1.3 mmol) in 50 mL chloroform, dicyclohexyl carbodiimide (DCC) (0.29 g, 1.4 mmol) and 1-hydroxy benzotriazole (HOBt) (0.21 g, 1.4 mmol) were added at 0 °C successively and the resulting mixture was stirred for 20 min. To this, L-proline-OMe (0.19 g, 1.5 mmol) was added. The reaction mixture was allowed to attain room temperature in 1 h and the stirring was continued for a further 10 h. The solvent was evaporated and the residue was dissolved in ethyl acetate. The organic layer was washed three times with 1 M solution of $KHSO_4$ and brine. The organic layer was dried over anhydrous Na_2SO_4. Removal of solvent afforded a pale yellow liquid. The product was purified by flash chromatography (hexane–ethyl acetate; 1 : 1). Yield 0.37 g (66%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.95–2.09 (m, 3H), 2.14–2.22 (m, 1H), 3.13 (s, 2H), 3.46–3.52 (m, 1H), 3.60–3.65 (m, 1H), 3.73 (s, 2H), 3.77 (s, 3H), 3.86–3.95 (q, 2H), 6.80–6.82 (d, 2H), 7.29–7.31 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 22.9, 24.9, 27.0, 29.4, 47.5, 52.3, 55.3, 59.0, 113.9, 130.4, 130.7, 158.5, 167.0, 172.8; ^{77}Se NMR ($CDCl_3$, Me_2Se) δ (ppm): 275; ESI-MS: *m/z* calcd. for $C_{16}H_{21}NO_4Se$ $[M+H]^+$ 372.0714, found 371.9364.

Synthesis of 23. Compound **23** was synthesized following a similar procedure to that given for compound **15** using compound **21** as the starting material. Yield 90%. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.91–2.02 (m, 8H), 2.14–2.16 (m, 2H), 3.55–3.58 (m, 2H), 3.65 (s, 6H), 3.84 (s, 4H), 4.40–4.44 (dd, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 23.2, 25.3, 27.8, 28.8, 48.4, 55.8, 60.2, 170.7, 174.1; ^{77}Se NMR ($CDCl_3$, Me_2Se) δ (ppm): 335; ESI-MS: *m/z* calcd. for $C_{16}H_{24}N_2O_6Se_2$ $[M+Na]^+$ 501.0043, found 500.8780.

Synthesis of 25. 0.12 g (0.3 mmol) of compound **23** was dissolved in 10 mL ethanol and 20 mg (0.5 mmol) of $LiOH{\cdot}H_2O$ was added to it followed by 10 mL of water. The reaction mixture was stirred for 3 h. Ethanol was evaporated under reduced pressure and the aqueous layer was acidified with $KHSO_4$. The compound was extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and the compound was purified by flash chromatography (hexane–ethyl acetate; 1 : 1). Yield 0.10 g (88%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.91–2.02 (m, 8H), 2.15–2.16 (d, 2H), 3.64–3.67 (m, 2H), 3.84 (s, 2H) 4.40–4.44 (dd, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 23.2, 25.7, 28.1, 48.7, 55.0, 171.7, 174.8; ^{77}Se NMR ($CDCl_3$, Me_2Se) δ (ppm): 335; HRMS (ESI mode) calcd. for $C_{13}H_{18}N_2O_6Se_2$ $[M+Na]^+$ 494.9549, found 494.9621.

Synthesis of 20. This compound was synthesized by following a similar procedureto that given for compound **19**. Yield 72%. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.68 (s, 4H), 3.80 (s, 5H), 6.82–6.84 (d, 2H), 7.20–7.22 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 17.2, 27.2, 35.5, 55.6, 114.3, 131.1, 158.8, 178.0; ^{77}Se NMR ($CDCl_3$, Me_2Se) δ (ppm): 270; HRMS (ESI mode) calcd. for $C_{11}H_{14}O_3Se$ $[M+Na]^+$ 297.0006, found 296.9972.

Synthesis of 22. Compound **22** was synthesized by following a similar procedure to that given for compound **21**. Yield 0.27 g (53%); ^{1}H NMR ($CDCl_3$) δ (ppm): 1.94–2.04 (m, 3H), 2.13–2.16 (m, 2H), 2.53–2.63 (m, 2H), 2.76–2.80 (m, 1H), 3.38–3.41 (m, 1H), 3.51–3.54 (m, 1H), 3.70 (s, 3H), 3.81 (s, 5H), 4.46–4.48 (dd, 1H), 6.80–6.82 (d, 2H), 7.19–7.21 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 16.5, 21.4, 24.3, 28.1, 34.6, 45.8, 51.5, 54.2, 57.5, 112.8, 128.8, 129.9, 157.3, 169.6, 171.4; ^{77}Se NMR ($CDCl_3$, Me_2Se) δ (ppm): 267; HRMS (ESI mode) calcd. for $C_{17}H_{23}NO_4Se$ $[M+H]^+$ 386.0871, found 385.9808.

Synthesis of 24. Compound **24** was synthesized by following a similar procedure to that given for compound **15** using compound **22** as starting material. Yield 95%. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.97–2.02 (m, 4H), 2.15–2.19 (m, 2H), 2.81–2.86 (m, 4H), 3.07–3.16 (m, 2H), 3.34–3.79 (m, 18H), 4.37–4.48 (dd, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 23.6, 24.7, 29.2, 36.1, 47.0, 52.3, 58.7, 170.4, 172.8; ^{77}Se NMR ($CDCl_3$, Me_2Se) δ (ppm): 325; HRMS (ESI mode) calcd. for $C_{18}H_{28}N_2O_6Se_2$ $[M+Na]^+$ 551.0175, found 551.0163.

Synthesis of 26. This compound was synthesized by following a similar procedure to that given for **25** using compound **24** as the starting material. Yield 76%. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.97–2.02 (m 4H), 2.15–2.19 (m, 2H), 2.81–2.86 (m, 4H), 3.07–3.16 (m, 2H), 3.34–3.79 (m, 12H), 4.37–4.48 (dd, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 23.6, 24.7, 29.3, 36.1, 47.0, 52.3, 170.4, 172.8; ^{77}Se NMR ($CDCl_3$, Me_2Se) δ (ppm): 324; HRMS (ESI mode) calcd. for $C_{16}H_{24}N_2O_6Se_2$ $[M+Na]^+$ 522.9862, found 522.9901.

Synthesis of 29. In a two-necked 250 mL round-bottom flask, *p*-methoxy benzyl diselenide (3.02 g, 7.5 mmol) and $NaBH_4$ (1.10 g, 30.0 mmol) were mixed under nitrogen atmosphere. To this, 100 mL of dry DMF was added at 0 °C and the reaction mixture was stirred for 10 min. **29** (2.50 g, 7.0 mmol) was dissolved in 25 mL dry DMF and was dropwise added to the reaction mixture. The reaction mixture was allowed to attain 25 °C and the stirring was continued for 36 h. After this time, 200 mL 1 M $KHSO_4$ was added to the reaction mixture and the compound was extracted with ethyl acetate. The ethyl acetate layer was washed three times with brine. Solvent was evaporated and the compound was purified by column chromatography (hexane–ethyl acetate; 1 : 1). Yield 1.49 g (55%); ^{1}H NMR ($CDCl_3$) δ (ppm): 1.43 (s, 9H), 2.61–2.69 (m, 2H), 3.76–3.77 (d, 5H), 4.32–4.35 (m, 1H), 6.78–6.79 (d, 2H), 7.19–7.20

(d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 22.1, 28.7, 35.7, 54.0, 61.3, 81.6, 128.3, 130.3, 132.6, 145.8, 155.3, 171.8; ^{77}Se NMR ($CDCl_3$) δ (ppm): 213; HRMS (ESI mode) calcd. for $C_{16}H_{23}NO_5Se$ $[M+Na]^+$ 412.0639, found 412.0653.

Synthesis of 31. To a solution of **29** (0.80 g, 2.0 mmol) in 120 mL chloroform, DCC (0.62 g, 3.0 mmol) and HOBt (0.46 g, 3.0 mmol) were added at 0 °C and stirred for 20 min. L-Pro-OMe (0.39 g, 3 mmol) was added to this and the mixture was stirred at 25 °C for 10 h. The precipitate was filtered and the filtrate was washed three times each with 1 M $KHSO_4$ solution, 1 M Na_2CO_3 solution and brine. The solvent was evaporated and the compound was purified by column chromatography (hexane–ethyl acetate; 1 : 2). Yield 0.68 g (65%); ^{1}H NMR ($CDCl_3$) δ (ppm): 1.44 (s, 9H), 1.96–2.04 (m, 3H), 2.18–2.21 (m, 1H), 2.65–2.70 (m, 1H), 2.81–2.86 (m, 1H), 3.51–3.59 (m, 2H), 3.70 (s, 3H), 3.78 (s, 3H), 3.81 (s, 2H), 4.48–4.51 (dd, 1H), 4.60–4.64 (m, 1H), 6.81–6.83 (d, 2H), 7.26–7.28 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 25.2, 25.7, 27.7, 28.8, 29.5, 47.5, 52.3, 52.7, 55.7, 59.4, 80.3, 114.4, 130.6, 131.4, 155.7, 158.9, 170.5, 172.7; ^{77}Se NMR ($CDCl_3$) δ (ppm): 222; ESI-MS: *m/z* calcd. for $C_{22}H_{32}N_2O_6Se$ $[M+Na]^+$ 523.1323, found 523.0989.

Synthesis of 33. This compound was synthesized by following a similar procedure to that reported for compound **15** by using compound **31**. Yield 0.18 g (95%); ^{1}H NMR ($CDCl_3$) δ (ppm): 1.38 (s, 18H), 1.97–1.99 (m, 6H), 2.15–2.17 (m, 2H), 3.10–3.14 (m, 2H), 3.25–3.28 (m, 2H), 3.67 (s, 6H), 3.70–3.73 (m, 4H), 4.46–4.47 (d, 2H), 4.71–4.72 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 25.2, 28.7, 29.3, 32.5, 47.5, 52.7, 52.9, 59.3, 80.3, 155.7, 170.2, 172.5; ^{77}Se NMR ($CDCl_3$) δ (ppm): 309; ESI-MS: *m/z* calcd. for $C_{28}H_{46}N_4O_{10}Se_2$ $[M+Na]^+$ 781.1442, found 781.2930.

Synthesis of 35. Compound **33** (0.38 g, 0.5 mmol) was taken in a round-bottom flask and 2 mL of trifluoroacetic acid (TFA) was added. The flask was sealed and stirred for 3 h. TFA was evaporated under reduced pressure to obtain the Boc free dipeptide **35**. Yield 0.26 g (93%); ^{1}H NMR ($CDCl_3$) δ (ppm): 1.94–2.00 (m, 6H), 2.15–2.19 (m, 2H), 2.63–2.68 (m, 2H), 2.79–2.84 (m, 2H), 3.73–3.76 (dd, 4H), 3.80 (s, 6H), 4.46–4.49 (dd, 2H), 4.58–4.60 (m, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 25.6, 27.7, 29.4, 47.4, 52.7, 55.6, 59.3, 158.8, 172.6; ^{77}Se NMR ($CDCl_3$) δ (ppm): 298; HRMS (ESI mode) calcd. for $C_{18}H_{30}N_4O_6Se_2$ $[M+Na]^+$ 581.0393, found 581.0291.

Synthesis of 30. This compound was synthesized following a method similar to the synthesis of compound **29**. Yield 63%. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.45 (s, 9H), 2.93–2.94 (d, 2H), 4.62–4.64 (dd, 1H), 6.81–6.84 (dd, 2H), 7.19–7.22 (dd, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 14.7, 28.8, 48.0, 55.7, 59.8, 80.5, 114.4, 130.6, 131.4, 155.7, 158.9, 171.2, 174.6; ^{77}Se NMR ($CDCl_3$) δ (ppm) 223.

Synthesis of 32. This compound was synthesized following a similar method to that given for compound **31**. Yield 61%. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.42 (s, 9H), 1.89–1.99 (m, 3H), 2.16–2.19 (m, 1H), 2.61–2.66 (m, 1H), 2.75–2.80 (m, 1H), 3.49–3.52 (m, 2H), 3.69 (s, 3H), 3.74 (s, 2H), 3.75 (s, 3H), 4.39–4.42 (dd, 1H), 4.63–4.65 (m, 1H), 6.77–6.80 (d, 2H), 7.20–7.22 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 25.1, 25.8, 27.7, 28.8, 29.6, 47.6, 52.3, 52.8, 55.7, 59.4, 80.3, 114.3, 130.6, 131.4, 155.5, 158.9, 170.3, 172.8; ^{77}Se NMR ($CDCl_3$) δ (ppm) 223; ESI-MS: *m/z* calcd. for $C_{22}H_{32}N_2O_6Se$ $[M+Na]^+$ 523.1323, found 523.1168.

Synthesis of 34. Compound **34** was synthesized following a similar method to that given for compound **15**. Yield 95%. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.36 (s, 18H), 1.94–1.96 (m, 6H), 2.15–2.17 (m, 2H), 3.09–3.13 (m, 2H), 3.22–3.25 (m, 2H), 3.49 (s, 6H), 3.70–3.73 (m, 4H), 4.35–4.37 (d, 2H), 4.70–4.73 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 25.1, 28.7, 29.5, 32.8, 47.6, 52.6, 52.9, 59.5, 80.3, 155.4, 169.7, 172.6; ^{77}Se NMR ($CDCl_3$) δ (ppm) 303; HRMS (ESI mode) calcd. for $C_{28}H_{46}N_4O_{10}Se_2$ $[M+Na]^+$ 781.1442, found 781.1450.

Synthesis of 36. This compound was synthesized following a similar method to that given for compound **35** by using compound **34** as the starting material. Yield 83%. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.92–2.04 (m, 4H), 2.15–2.21 (m, 2H), 2.63–2.67 (m, 2H), 2.76–2.81 (m, 2H), 3.51–3.53 (m, 2H), 3.71–3.72 (d, 4H), 3.76 (s, 6H), 4.41–4.44 (dd, 2H), 4.64–4.66 (m, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 25.7, 27.6, 29.5, 47.6, 52.7, 55.7, 59.4, 158.8, 172.7; ^{77}Se NMR ($CDCl_3$) δ (ppm) 295; HRMS (ESI mode) calcd. for $C_{18}H_{30}N_4O_6Se_2$ $[M+Na]^+$ 581.0393, found 581.0350.

Synthesis of 38. 1.35 g (3.0 mmol) Boc-L-cystine was dissolved in 75 mL ethanol. To it $NaBH_4$ (0.37 g, 10.0 mmol) was added. The mixture was stirred for 15 min under nitrogen atmosphere. To it an ethanolic solution of *p*-methoxy benzyl chloride (1 mL, 7.5 mmol) was added dropwise. The reaction mixture was further stirred for 6 h. The ethanol was evaporated under reduced pressure and the compound was dissolved in chloroform. The organic layer was washed 2 times with $KHSO_4$ solution. The organic layer was dried over anhydrous Na_2SO_4 and compound was purified by column chromatography (hexane–ethyl acetate; 1 : 1). Yield 1.48 g (70%); ^{1}H NMR ($CDCl_3$) δ (ppm): 1.45 (s, 9H), 2.83–2.89 (m, 2H), 3.69 (s, 2H), 3.79 (s, 3 H), 4.50–4.51 (d, 1H), 6.82–6.85 (d, 2H), 7.20–7.23 (d, 2H); 22.2, 29.6, 34.9, 54.5, 60.1, 80.4, 129.0, 131.1, 132.9, 146.8, 154.9, 171.8; ESI-MS: *m/z* calcd. for $C_{16}H_{23}NO_5S$ $[M+Na]^+$ 364.1195, found 363.8573.

Synthesis of 39. This compound was synthesized following a similar procedure to that given for compound **31** using the PMB-protected L-Cys derivative (**38**). Yield 52%. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.35 (s, 9H), 1.84–1.86 (m, 3H), 2.05–2.08 (m, 1H), 2.49–2.52 (m, 1H), 2.68–2.73 (m, 1H), 3.39–3.42 (m, 2H), 3.57 (s, 3H), 3.65 (s, 2H), 3.67 (s, 3H), 4.37–4.40 (dd, 1H), 4.47–4.51 (m, 1H), 6.72–6.75 (d, 2H), 7.16–7.19 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 25.2, 28.7, 29.4, 33.7, 36.1, 47.4, 51.9, 52.6, 55.6, 59.3, 80.2, 114.3, 130.3, 130.6, 155.8, 159.1, 170.4, 172.6; ESI-MS: *m/z* calcd. for $C_{22}H_{32}N_2O_6S$ $[M+Na]^+$ 475.1879, found 475.0789.

Synthesis of 40. This compound was synthesized from compound **39** by using a similar method to that given for compound **15**. Yield 90%. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.38 (s, 18H), 1.98–2.00 (m, 6H), 2.17–2.19 (m, 2H), 2.85–2.87 (m, 2H), 3.04–3.06 (m, 2H), 3.67 (s, 6H), 3.70–3.76 (m, 4H), 4.48–4.50 (m, 2H), 4.74–4.76 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 25.3, 28.7, 29.4, 41.4, 47.6, 51.8, 52.7, 59.4, 80.4, 155.93, 170.4, 172.6; HRMS (ESI mode) calcd. for $C_{28}H_{46}N_4O_{10}S_2$ $[M+Na]^+$ 685.2553, found 685.2550.

Synthesis of 41. This compound was synthesized from compound **40** by using a similar method to that given for compound **35**. Yield 86%. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.93–1.96 (m, 4H),

2.15–2.18 (m, 2H), 2.56–2.61 (m, 2H), 2.78–2.83 (m, 2H), 3.49–3.52 (m, 2H), 3.66–3.67 (d, 4H), 3.76 (s, 6H), 4.46–4.49 (dd, 2H), 4.57–4.59 (m, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 24.9, 33.5, 35.8, 47.1, 52.3, 55.3, 59.0, 158.8, 170.1; HRMS (ESI mode) calcd. for $C_{18}H_{30}N_4O_6S_2$ $[M+Na]^+$ 485.1504, found 485.1537.

Synthesis of peroxynitrite (PN). Peroxynitrite was synthesized by following the literature method with minor modifications.[25] 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 30 mL of 5 N NaOH and 5 mL of 0.04 M DTPA in 0.05 N NaOH 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 (6.7 mL, 50 mmol) for 3–4 h at room temperature. The reaction was monitored by withdrawing aliquots at intervals 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 mL 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.

ACE assay. The assay was performed in 400 μL sample vials and an autosampler was used for sample injection. Ang I and AngII were analyzed by reverse-phase HPLC method (Princeton C18 column, 4.6 × 150 mm, 5 μm) with isocratic elution of 50 : 50 MeOH–0.1% TFA in water. In the ACE inhibition assay, we employed a mixture of 50 μM Ang I, 60 mM sodium chloride and 2 milliunits of ACE in 50 mM HEPES-HCl buffer at pH 8.3 with various concentrations of the inhibitors. The reaction mixture was incubated at 37 °C for 30 min prior to injection. Selenols and thiols were freshly prepared by reducing the diselenides and disulfides by $NaBH_4$ prior to use and were kept under N_2 atmosphere during the assay. The decrease in the formation of Ang II with an increase in the concentration of inhibitor was monitored at 215 nm and the % inhibition was calculated by comparing the peak areas. 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.

Nitration of angiotensin II. 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 Ang II was analyzed by reverse phase HPLC method (Princeton C18 column, 4.6 × 150 mm, 5 μm) with isocratic elution of 45 : 55 MeOH–0.1% TFA in water. In the PN-mediated nitration of Ang II, we employed a mixture containing Ang II (20 μM) and peroxynitrite (300 μM) in sodium phosphate buffer (100 mM) of pH 7.5, without and with increasing concentration of the inhibitor added to the assay mixture. The reaction mixture was incubated for 5 min before injection. The formation of nitro-Ang II was monitored at the wavelength of 215 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 nitration of BSA. For bovine serum albumin (BSA), the nitration was performed by the addition of PN (1.2 mM) to BSA (0.1 mM) in 0.5 M phosphate buffer of pH 7.0 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. The reactions of BSA with PN were performed in the presence of various inhibitors at 60 μM final concentration. 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.

Electrophoretic analysis. Gel was prepared with 10% polyacrylamide with 6% stacking gel. 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 blotting. 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).

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