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PAPER

Effect of peptide-based captopril analogues on angiotensin converting enzyme activity and peroxynitrite-mediated tyrosine nitration†

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Angiotensin converting enzyme (ACE) regulates the blood pressure by converting angiotensin I to angiotensin II and bradykinin to bradykinin 1–7. These two reactions elevate the blood pressure as angiotensin II and bradykinin are vasoconstrictory and vasodilatory hormones, respectively. Therefore, inhibition of ACE is an important strategy for the treatment of hypertension. The natural substrates of ACE, *i.e.*, angiotensin II and bradykinin, contain a Pro-Phe motif near the site of hydrolysis. Therefore, there may be a Pro-Phe binding pocket at the active site of ACE, which may facilitate the substrate binding. In view of this, we have synthesized a series of thiol- and selenol-containing dipeptides and captopril analogues and studied their ACE inhibition activities. This study reveals that both the selenol or thiol moiety and proline residues are essential for ACE inhibition. Although the introduction of a Phe residue to captopril and its selenium analogue considerably reduces the inhibitory effect, there appears to be a Phe binding pocket at the active site of ACE.

Introduction

Angiotensin converting enzyme (ACE) catalyzes the production of angiotensin II (Ang II) from angiotensin I (Ang I)[1] and degradation of bradykinin to bradykinin 1–7,[2] which result in the elevation of blood pressure.[3–9] The conversions of both these substrates are domain dependent as the N-terminal domain of somatic ACE cleaves the terminal dipeptide of bradykinin and the C-terminal domain produces Ang II.[10,11] The active site of both these domains contains a zinc(II) ion coordinated by histidine and glutamate residues. The inhibition of ACE by synthetic compounds is an important strategy for the treatment of hypertension. Furthermore, it is known that hypertension and oxidative stress are interrelated. Therefore, antihypertensive drugs having antioxidant activity are considered to be beneficial for the treatment of hypertension.[12–16] Recently, we have shown that the selenium analogue of captopril (**2**) and dipeptides **3** and **4** (Fig. 1) not only inhibit ACE but also scavenge peroxynitrite (a strong oxidant found *in vivo*).[17] In this particular study, we have used the dipeptides having the terminal acid groups in their protected form. Therefore, it was thought worthwhile to synthesize the L-Sec-L-Pro- and L-Cys-L-Pro-based dipeptides (**5** and **6**, Fig. 2) having free terminal carboxylate groups. Furthermore, the structures of Ang I and bradykinin show the presence of a Pro-Phe motif near the site of hydrolysis, indicating that there may be a specific Phe binding pocket at the active site of ACE. In this paper, we report the synthesis and ACE inhibition activities of some captopril analogues having a Phe residue. We also describe the effect of these captopril analogues on peroxynitrite-mediated nitration of Ang II and bovine serum albumin.

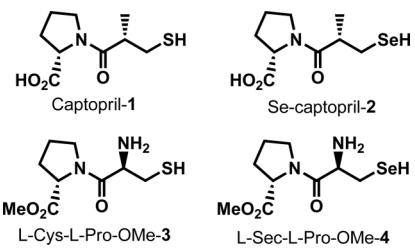

Fig. 1 Captopril (**1**) and its analogues studied as ACE inhibitors.[17]

Synthesis of dipeptides and captopril analogues

The Sec-Pro and Cys-Pro dipeptides **3–6** were synthesized by following a procedure reported by our group.[17] The captopril analogues having an additional amino group **7–14** (Fig. 2) were synthesized by following a similar procedure. Due to the presence of reactive thiol and selenol moieties, the protection of these groups was found to be important in the synthesis of Cys- and Sec-containing peptides. Particularly, the synthesis of Sec-containing peptides was difficult due to a facile oxidation of the selenol to the corresponding diselenide. Therefore, the deprotection of acid and amino groups was carried out in the diselenide or disulfide form (Scheme 1). The selenol **5** and thiol **6** required for the inhibition studies were obtained by reducing the corresponding diselenide and disulfide, respectively.

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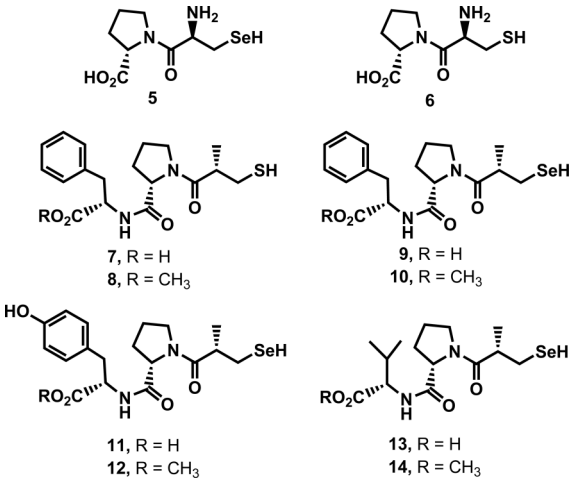
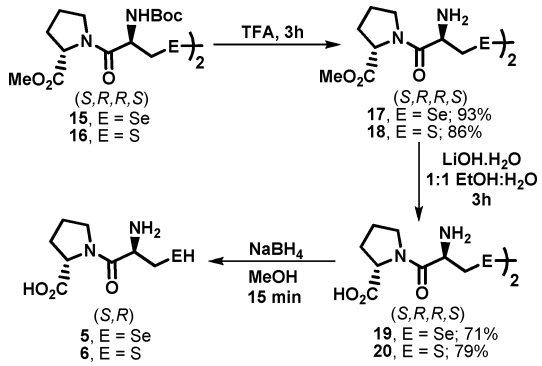
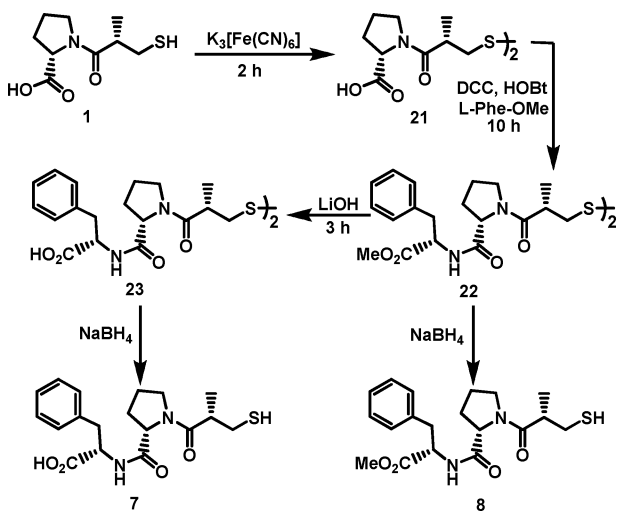


Fig. 2 Selenium analogues of captopril and related derivatives (**5–14**).



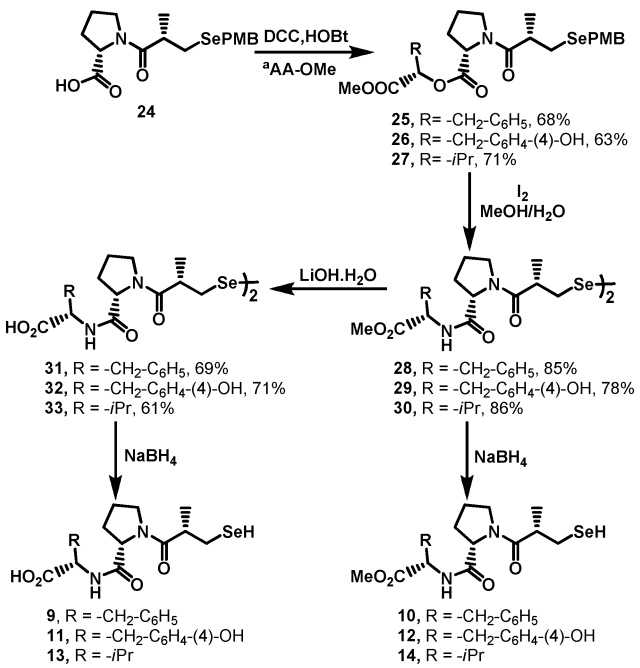
Scheme 1 Synthesis of Sec-Pro and Cys-Pro dipeptides.

The Phe-substituted captopril analogues **7** and **8** were synthesized from captopril as shown in Scheme 2. Although the coupling of Phe to captopril can be achieved after the protection of the thiol group of captopril, we found it convenient to oxidize captopril to the corresponding disulfide (**21**) before coupling with Phe. The DCC-mediated coupling of **21** with 3 equiv. of L-Phe-OMe afforded compound **22**. The cleavage of ester group



Scheme 2 Synthesis of captopril having an additional amino acid residue attached to the proline moiety.

in compound **22** by LiOH afforded **23**, which upon reduction by $NaBH_4$ generated the thiol **7**. Similarly, the reduction of compound **22** by $NaBH_4$ produced compound **8**. As the captopril-based diselenides are generally less stable than the disulfides, a modified procedure was followed for the synthesis of compounds **9–14** (Scheme 3). According to this procedure, the PMB-protected selenium compound **24** was coupled with L-Phe-OMe in the presence of DCC to produce compound **25**. Cleavage of the PMB group by iodine then produced the corresponding diselenide (**28**), which can be converted to the corresponding selenols **9** and **10** either after or before the hydrolysis of the methyl ester, respectively. Compounds **11–14** having L-Tyr and L-Val residues were synthesized by following a similar method. It should be noted that the nature of the amino acid residue does not appear to affect the stability of the final compounds. The ^{77}Se NMR spectra of the selenium compounds show that the nature of the amino acid residue does not have any significant effect on the chemical shift values. However, the introduction of Tyr residue enhances the solubility of the diselenides and selenols in buffer.



Scheme 3 Synthesis of Se-captopril having an additional amino acid residue to the proline moiety. [a]amino acid: L-Phe, L-Tyr and L-Val.

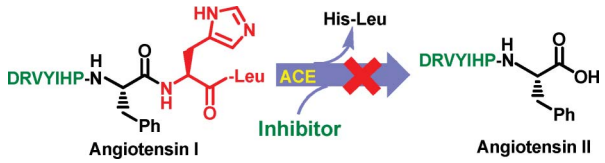
Angiotensin converting enzyme assay

The conversion of Ang I to Ang II by ACE was studied in the presence of various inhibitors (Scheme 4). The decrease in the concentration of Ang II with an increase in the concentration of inhibitors was followed by HPLC. The peak area corresponding to Ang II was obtained 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 30 min prior to analysis. The thiols (**6**, **7** and **8**) and selenols (**5**, **9–14**) were freshly prepared by reducing the corresponding disulfides and diselenides by $NaBH_4$ prior to use and were kept under N_2 atmosphere during the assay. The IC_{50}

Table 1 IC_{50} values for the inhibition of ACE by compounds **1–14**

Compound	IC_{50}/nM	Compound	IC_{50}/nM
1	18.1 ± 1.0	**2**	36.4 ± 1.5
3	342 ± 33	**4**	6480 ± 640
5	207.1 ± 2.3	**6**	2290 ± 160
7	696.2 ± 12.8	**8**	40 000[a]
9	76.2 ± 1.0	**10**	4100 ± 330
11	5350 ± 435	**12**	40 000[b]
13	5150 ± 330	**14**	40 000[a]

[a] 35% enzyme activity was inhibited at 40 000 nM inhibitor concentration. [b] 40% enzyme activity was inhibited at 40 000 nM inhibitor concentration. 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, 60 mM NaCl and 2 milliunits of ACE in 400 μL reaction mixture.



Scheme 4 Inhibition of ACE-catalyzed conversion of Ang I to Ang II by the selenium analogues of captopril.

values obtained for the inhibition of ACE-catalyzed conversion of Ang I to Ang II by the captopril analogues are summarized in Table 1. The IC_{50} values for ACE inhibition by compounds **1–4** are included for comparison.[17]

From the IC_{50} values, it is clear that the Sec-Pro dipeptides are better inhibitors of ACE than the Cys-Pro dipeptides. The free carboxylate moiety at the C-terminal appears to be important as the Sec-Pro dipeptide **5** having a free carboxylate moiety inhibits ACE with an IC_{50} value of 207.1 ± 2.3 nM, which is about 1.5 times less than that observed for compound **3** (IC_{50}: 342 ± 33 nM) having the terminal carboxylate moiety in its ester form. Similarly, the IC_{50} value for compound **6** (IC_{50}: 2290 ± 160 nM) is about three times less than that of compound **4** (IC_{50}: 6480 ± 640 nM). The ACE inhibitory potency of compound **7** (IC_{50}: 696.2 ± 12.8 nM) having an L-Phe residue is much lower than that of captopril (IC_{50}: 18.1 ± 1.0 nM). However, only a marginal decrease in the activity was observed when a phenylalanine residue was added to the proline moiety of Se-captopril. Interestingly, the decrease in the activity upon introduction of L-Phe is less pronounced in Se-captopril (**2**) as compared to that of captopril (**1**). This is probably due to the higher reactivity of selenol toward zinc(II) ion as compared to thiol.[18] The IC_{50} value observed for compound **9** (IC_{50}: 76.2 ± 1.0 nM) is only two times higher than that of Se-captopril (IC_{50}: 36.4 ± 1.5 nM). In contrast, the introduction of other amino acids such as L-Tyr or L-Val instead of L-Phe drastically reduced the inhibition activity. For example, the IC_{50} values for compounds **11** and **13** were found to be ~150 times higher than that of Se-captopril. These observations indicate that the presence of a free carboxylic acid at the terminal amino acid is important for ACE inhibition as compounds **8**, **10**, **12** and **14** having a methyl ester group exhibited very weak inhibition. This is similar to the inhibition properties of Cys-Pro and Sec-Pro peptides for which stronger inhibition was observed for compounds having a free carboxylic group. Introduction of an amino acid residue decreases the ACE inhibition potency of the captopril analogues. However, the presence of an L-Phe residue at the C-terminal of the L-Pro moiety slightly stabilizes the enzyme–inhibitor complex, which may be due to the presence of a phenylalanine binding pocket at the enzyme active site. In contrast to the thiols and selenols, the corresponding disulfides and diselenides exhibited very weak inhibition. In all the cases, the IC_{50} values could not be determined up to 50 μM concentration of the inhibitors. The maximum inhibition observed at this concentration for the disulfides and diselenides is given in Table S1 (ESI†). In these experiments, compound **28** having Phe residues exhibited the highest activity. These observations indicate that a thiol or selenol moiety in addition to the Phe-Pro motif is important for the inhibition of ACE.

From the X-ray crystal structure, it is known that captopril binds to the active site of ACE through the thiol moiety. The ACE–captopril complex is stabilized by additional hydrogen bonding interactions with the carbonyl group of captopril through His353 (2.54 Å) and His 513 (2.69 Å) residues. The phenolic –OH group of Tyr520 interacts with one of the carboxylate oxygens of the proline moiety.[9,19] Furthermore, it is observed that the phenolic –OH of Tyr520 and τ-nitrogen of His353 are positioned in close proximity to the carboxylate residue of the proline moiety (2.66 Å and 3.74 Å, respectively, Fig. 3). The weak inhibition of captopril analogues having an additional amino acid residue may be due to the steric hindrance at the active site. The activity of the Se-captopril derivative having an L-Phe residue (compound **9**) is about 150 times higher than that of compounds **11** and **13** having L-Tyr and L-Val residues, respectively. On the other hand, only about two times decrease in the ACE inhibition activity was observed when an L-Phe residue was introduced to Se-captopril (**2**). These observations suggest that there is a Phe binding pocket at the active site of ACE, which may stabilize the captopril derivatives having an L-Phe residue. However, it appears that the interaction of the carboxylic group of Se-captoril (**2**) with the enzyme active site is more important for the inhibition than the interaction of the Phe residue. Recently, Akif *et al.*[20] have reported the crystal structure of AnCE (a homologue of ACE) in complex with RXP380, a C-terminal specific inhibitor of ACE containing a L-Trp residue. It has been shown that the aromatic group of L-Trp interacts

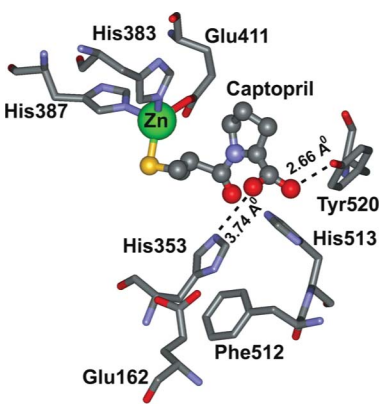


Fig. 3 Crystal structure of captopril binding to the active site of ACE. Captopril binds to ACE through thiolate coordination to Zn(II) and there are additional hydrogen bonding interactions to carbonyl and carboxylate groups of captopril through various amino acid residues present at the active site.[9,19]

with the subsite having aromatic residues such as Phe363. In testicular ACE, these positions are occupied by Val379 and Val380, which may be involved in interactions with the L-Phe residue in compound **9** (Fig. 4). However, these interactions may not be sufficient to stabilize the ACE-**9** complex as the size of L-Phe is significantly smaller than that of the L-Trp residue.

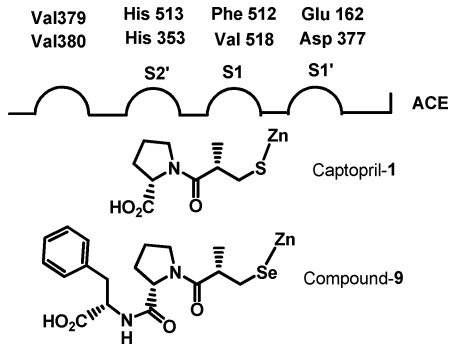
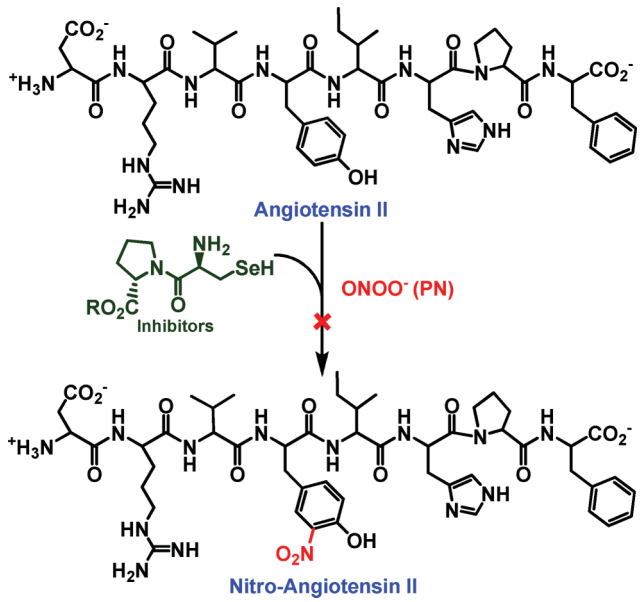


Fig. 4 Possible binding of captopril (**1**) and compound **9** to the ACE active site. Val379 and Val380 are in close proximity to the proline moiety of the captopril–ACE complex,[19] which may play an important role in forming a Phe binding pocket.

PN-mediated nitration assays

To understand the effect of additional amino acid in captopril analogues on the antioxidant activity,[21–24] we carried out the inhibition of peroxynitrite (PN)-mediated nitration of the tyrosine residues in Ang II and bovine serum albumin (BSA). Briefly, the nitration of Ang II was studied by an HPLC method (Scheme 5) and BSA was studied by an immunoblotting method. 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 only a trace amount of the dinitro compound was produced. The decrease in the concentration of nitro-Ang II with an increase



Scheme 5 Peroxynitrite-mediated nitration of Ang II to mono-nitro-Ang II.

Table 2 IC_{50} values for the inhibition of ACE by compounds **5–14**, and their corresponding diselenides and disulfides

Compound	IC_{50}/nM	Compound	IC_{50}/nM
5	4.5 ± 0.2	**19**	3.4 ± 1.5
6	15.2 ± 0.6	**20**	100[a]
7	14.1 ± 1.0	**23**	45.4 ± 1.5
8	21.9 ± 0.6	**22**	40.8 ± 1.4
9	6.0 ± 0.5	**31**	3.6 ± 0.1
10	6.2 ± 0.4	**28**	2.9 ± 0.1
11	5.6 ± 0.5	**32**	5.8 ± 0.4
12	7.2 ± 0.6	**29**	4.0 ± 0.1
13	6.4 ± 0.3	**33**	3.4 ± 0.2
14	4.5 ± 0.1	**30**	2.0 ± 0.1

[a] Only 30% of the activity was inhibited at this concentration. 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, 300 μM PN and different concentrations of the inhibitors.

in the concentrations of inhibitor was followed by measuring the peak area at 215 nm. The concentrations of test compounds required for the inhibition of 50% of nitration are represented as IC_{50} values. The IC_{50} values obtained for the inhibition by different sulfur and selenium compounds are summarized in Table 2.

In contrast to the ACE inhibition, the PN-scavenging activity does not depend significantly on the nature of the amino acid side chain. The dipeptides **5** and **6** and the corresponding diselenides and disulfides having additional amino acid residues exhibit similar effects on the nitration reactions. As expected, the PN-scavenging activity of all the selenium compounds was found to be much higher than that of the sulfur analogues. Most of the diselenides exhibited better activity than the corresponding selenols. This is in contrast to the captopril analogues in which the selenols were found to be more active than the corresponding diselenides.[17] It should be noted that the thiols **7** and **8** were found to be much better scavengers of PN than the corresponding disulfides **23** and **22**, respectively. This is in agreement with our previous report that the antioxidant activity of captopril is significantly higher than that of the corresponding disulfide.

It is known that PN inactivates several proteins by tyrosine nitrations. PN can effectively nitrate one or more tyrosyl residues in bovine serum albumin (BSA) depending on the PN concentration. Therefore, we have studied the effect of diselenides and disulfides on PN-mediated nitration of BSA by immunoblotting experiments. In a typical experiment, 100 μM BSA was incubated with 1.2 mM PN and 60 μM of inhibitor and the reaction was incubated at 20 °C for 30 min. At this concentration of the inhibitors, ~20–50% inhibition of tyrosine nitration was observed (Fig. 5). Similar to the inhibition of Ang II, the selenium compounds (**19**, **28–33**) exhibited better inhibition of PN-mediated nitration of BSA as compared to the sulfur-containing compounds **22** and **23**.

To understand the mechanism of inhibition of PN-mediated nitration by the diselenides and disulfides, compounds **22** and **28** were treated with PN in 50 mM phosphate buffer of pH 7.5 and the reaction products were analyzed by a mass spectrometer. It was observed that the reaction of the disulfide **22** with PN essentially produced only one oxidized product (compound **34**, Fig. 6). Although several peaks were observed in the mass spectrum (Fig. S3, ESI†), all these peaks can be assigned either to the starting disulfide or to the oxidized product **34**, which are complexed with

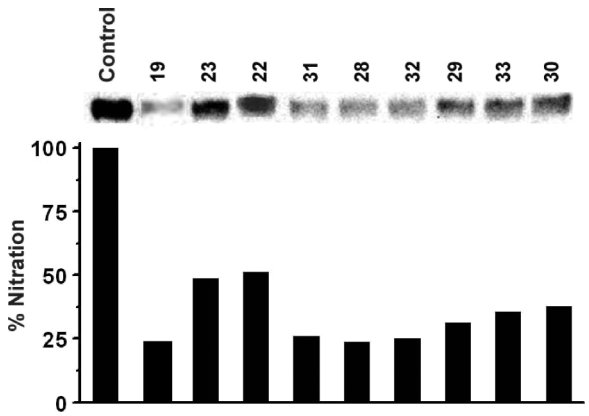


Fig. 5 Immunoblots for the inhibition of PN-mediated nitration of BSA. 100 μM of BSA was incubated with 60 μM of inhibitor and 1.2 mM of PN for 30 min at 20 °C and then subjected to gel electrophoresis.

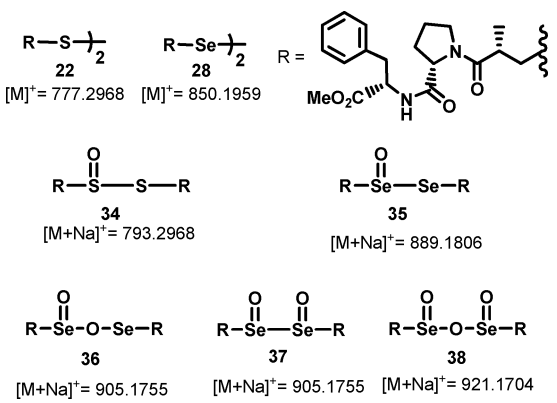


Fig. 6 Proposed oxidation products from the reaction of **22** and **28** with PN.

one or two sodium ions under mass conditions. In contrast, the treatment of diselenide **28** with PN led to a rapid oxidation of the selenium center to higher oxidation states. Because of the presence of two selenium centers, the compound can form multiple oxidized products. Mass spectral analysis of the reaction mixture indicates the formation of three distinct products (Fig. S4, ESI†). These data indicate the formation of compounds **35–38** upon treatment of the diselenide **28** with PN. As the molecular masses of compounds **36** and **37** are identical, it is difficult to assign the peak at *m/z* 905.1755 to one of these species. However, these observations suggest that one molecule of diselenide can scavenge up to three molecules of PN, which may account for the higher activity of the diselenides as compared to the disulfides.

Conclusions

This study suggests that the presence of a free carboxylate at the proline moiety of captopril analogues is important for an efficient inhibition of angiotensin converting enzyme. While the introduction of a phenylalanine residue to captopril drastically reduces the ACE inhibition activity of the parent compound, such replacement only marginally affects the activity of Se-captopril. The introduction of tyrosine or valine amino acid residues to captopril or Se-captopril remarkably reduces the inhibitory activity. These observations suggest that the proline carboxylate plays an important role in the ACE inhibition and there may be a phenylalanine binding pocket at the active site of ACE. The antioxidant activity of captopril analogues was studied using PN-mediated nitration of Ang II and BSA. It was observed that the introduction of an additional amino acid to proline does not affect the PN-scavenging activity of the selenium compounds. The better antioxidant activity of the diselenides as compared to the disulfides is mainly due to the facile oxidation of the selenium center in the diselenides

Experimental section

General procedure

Angiotensin converting enzyme (ACE) and captopril (compound **1**) were purchased from Sigma-Aldrich Chemical Co. Compounds **2–4**, **15–18** were synthesized by known methods.[17] Compounds **5–14** were synthesized *in situ* by reducing the corresponding disulfides and diselenides with two equivalents of $NaBH_4$ in methanol. 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) by using pre-loaded silica cartridges. ^1H (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 (^1H 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.

Synthesis of 19

0.15 g (0.27 mmol) of compound **17** was dissolved in 10 mL ethanol and lithium hydroxide (17 mg, 0.4 mmol) was added to it. 10 mL of water was added to the reaction mixture and allowed to stir for 3 h. Ethanol was evaporated under reduced pressure and the aqueous layer was lyophilized to remove water. The dipeptide **19** was purified by reverse phase flash chromatography (water : methanol; 1 : 3). Yield 0.10 g (71%); ^1H NMR (D_2O) δ (ppm): 1.76–1.89 (m, 3H), 2.14–2.21 (m, 1H), 2.91–2.97 (m, 1H), 3.05–3.11 (m, 1H), 3.24–3.29 (dd, 1H), 3.6–3.48 (m 1H), 3.59–3.62 (m, 1H), 4.50–4.52 (dd, 2H); ^{13}C NMR (D_2O) δ (ppm): 25.6, 27.7, 28.8, 44.0, 52.8, 55.7, 158.8, 171.7; ^{77}Se NMR (D_2O) δ (ppm) 305; ESI-MS: *m/z* calcd. for $C_{16}H_{26}N_4O_6Se_2$ $[M + H]^+$ 531.0261, found 530.8870.

Synthesis of 20

This compound was synthesized following a similar method to that given for compound **19** using compound **18** as starting material. Yield 79%; ^1H NMR (D_2O) δ (ppm):1.84–1.95 (m, 3H), 2.22–2.26 (m, 1H), 3.14–3.22 (m, 2H), 3.38–3.50 (m, 2H), 4.18–4.21 (m, 2H), 4.42–4.44 (m, 1H); ^{13}C NMR (D_2O) δ (ppm): 27.5, 31.6, 39.0, 50.5, 53.7, 62.6, 169.3, 178.1; ESI-MS: *m/z* calcd. for $C_{16}H_{26}N_4O_6S_2$ $[M + H]^+$ 435.1372, found 434.9422.

Synthesis of 22

To a solution of **21** (0.14 g, 0.3 mmol) in chloroform, DCC (0.21 g, 1.0 mmol) and HOBt (0.15 g, 1.0 mmol) were added successively. The mixture was stirred at 0 °C for 20 min. L-Phe-OMe (0.27 g, 1.0 mmol) was added to the above reaction mixture and allowed to attain the room temperature slowly. The solution was further stirred for 10 h. The precipitate was filtered and the filtrate was washed three times each with $KHSO_4$ solution, sodium carbonate solution and brine. The organic layer was dried over Na_2SO_4 and the compound was purified with the help of flash chromatography (hexane : ethyl acetate, 1 : 3). Yield 0.15 g (62%); ^{1}H NMR ($CDCl_3$) δ (ppm):1.08–1.09 (d, 6H), 1.80–1.85 (m, 2H), 1.92–1.97 (m, 4H), 2.28–2.32 (m, 2H), 2.57–2.59 (m, 2H), 2.98–3.03 (m, 6H), 3.13–3.18 (m, 2H), 3.45–3.46 (m, 2H), 3.53–3.55 (m, 2H), 3.69 (s, 6H), 4.57–4.59 (d, 2H), 4.75–4.80, (q, 2H), 7.09–7.11 (d, 4H), 7.20–7.29 (m, 6H); ^{13}C NMR ($CDCl_3$) δ (ppm): 17.8, 25.3, 38.2, 38.3, 41.4, 47.8, 52.8, 53.8, 60.2, 127.4, 128.9, 129.7, 136.6, 171.5, 172.4, 175.4; HRMS (ESI mode) calcd. for $C_{38}H_{50}N_4O_8S_2$ $[M + Na]^+$ 777.2968, found 777.2960.

Synthesis of 23

0.10 g (0.13 mmol) of **22** was dissolved in 10 mL ethanol. 14 mg (0.33 mmol) lithium hydroxide was added to it followed by 10 mL of water. The mixture was stirred for 3 h. Ethanol was evaporated under reduced pressure. The aqueous mixture was acidified to a pH of 2 by adding $KHSO_4$. The compound was then extracted with ethyl acetate and the organic layer was dried over Na_2SO_4. The compound was purified by flash chromatography (hexane : ethyl acetate, 1 : 5). Yield 0.08 g (83%); ^{1}H NMR ($CDCl_3$) δ (ppm):1.05–1.06 (d, 3H), 1.80–1.82 (m, 3H), 2.10–2.12 (m, 1H), 2.47–2.51 (m, 1H), 2.84–2.95 (m, 3H), 3.09–3.14 (m, 1H), 3.34–3.47 (m, 2H), 4.46–4.47 (m, 1H), 4.67–4.69 (dd, 1H), 7.05–7.16 (m, 5H); ^{13}C NMR ($CDCl_3$) δ (ppm):14.7, 17.6, 21.5, 25.2, 37.8, 41.3, 48.0, 53.8, 60.9, 127.3, 128.8, 129.9, 136.8, 171.8, 174.0, 175.7; ESI-MS: *m/z* calcd. for $C_{36}H_{46}N_4O_8S_2$ $[M - H]^-$ 725.2679, found 725.3537.

Synthesis of 25

To a solution of **24** (0.50 g, 1.3 mmol) in chloroform, DCC (0.41 g, 2.0 mmol) and HOBt (0.31 g, 2.0 mmol) were added successively. The mixture was stirred at 0 °C for 20 min. L-Phe-OMe (0.55 g, 2.0 mmol) was added to the above reaction mixture and allowed to attain the room temperature. The solution was further stirred for 10 h. Precipitate was filtered and the filtrate was washed three times each with $KHSO_4$ solution, sodium carbonate solution and brine. The organic layer was dried over Na_2SO_4 and the compound was purified with the help of flash chromatography (hexane : ethyl acetate, 1 : 3). Yield 0.48 g (68%); ^{1}H NMR ($CDCl_3$) δ (ppm): 1.01–1.03 (d, 3H), 2.35–2.38 (m, 1H), 2.46–2.49 (m, 1H), 2.09–2.12 (m, 1H), 2.81–2.85 (m, 1H), 2.98–3.01 (m, 1H), 3.11–3.19 (m, 3H), 3.29–3.32 (m, 2H), 3.69 (s, 3H), 3.73 (s, 3H), 3.78 (s, 2H), 4.61–4.62 (d, 1H), 4.75–4.79 (m, 1H), 4.86–4.89 (m, 1H), 6.81–6.82 (dd, 2H), 7.07–7.11 (m, 2H), 7.18–7.29 (m, 5H); ^{13}C NMR ($CDCl_3$) δ (ppm): 17.2, 25.1, 27.3, 28.1, 28.6, 36.9, 40.1, 47.5, 52.9, 54.3, 56.3, 60.1, 114.9, 116.2, 127.3, 128.9, 130.1, 130.9, 132.9, 155.9, 172.3, 172.8, 176.5; ^{77}Se NMR ($CDCl_3$) δ (ppm): 246; ESI-MS: *m/z* calcd. for $C_{27}H_{34}N_2O_5Se$ $[M - H]^-$ 545.1555, found 545.0529.

Synthesis of 26

This compound was synthesized following a similar method to that given for compound **25** by using L-Tyr-OMe instead of L-Phe-OMe. Yield 63%; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.04–1.16 (d, 3H), 1.24–1.28 (m, 2H), 2.29–2.33 (m, 1H), 2.46–2.49 (m, 1H), 2.61–2.65 (m, 1H), 2.82–2.85 (m, 1H), 2.91–2.96 (m, 2H), 3.05–3.09 (dd, 1H), 3.32–3.34 (m, 2H), 3.70 (s, 3H), 3.75 (s, 2H), 3.81 (s, 3H), 4.57–4.59 (m, 1H), 4.70–4.72 (m, 1H), 6.68–6.70 (d, 2H), 6.82–6.84 (d, 2H), 6.95–6.97 (d, 2H), 7.20–7.22 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 18.9, 25.2, 27.3, 28.0, 28.4, 37.5, 39.9, 47.9, 52.8, 54.1, 55.8, 60.3, 114.4, 116.0, 127.2, 130.4, 130.8, 131.9, 156.3, 158.9, 171.9, 172.5, 176.3; ^{77}Se NMR ($CDCl_3$) δ (ppm): 247; ESI-MS: *m/z* calcd. for $C_{27}H_{34}N_2O_6Se$ $[M + Na]^+$ 585.1480, found 585.2116.

Synthesis of 27

This compound was synthesized following a similar method to that given for compound **25** by using L-Val-OMe instead of L-Phe-OMe. Yield 71%; ^{1}H NMR ($CDCl_3$) δ (ppm): 0.86–0.91 (m, 6H), 1.14–1.16 (d, 3H), 1.80–1.83 (m, 1H), 1.96–1.97 (m, 1H), 2.03–2.15 (m, 2H), 2.48–2.50 (m, 1H), 2.51–2.53 (m, 1H), 2.77–2.80 (m, 1H), 2.87–2.92 (m, 1H), 3.38–3.44 (m, 2H), 3.71 (s, 3H), 3.76 (s, 2H), 3.80 (s, 3H), 4.39–4.43 (m, 1H), 4.69–4.71 (m, 1H), 6.81–6.84 (d, 2H), 7.20–7.22 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 18.1, 19.2, 19.5, 25.5, 27.3, 28.0, 31.3, 39.9, 47.8, 52.5, 55.7, 57.8, 59.8, 114.3, 130.3, 131.9, 158.9, 171.6, 172.6, 176.2; ^{77}Se NMR ($CDCl_3$) δ (ppm): 247; ESI-MS: *m/z* calcd. for $C_{23}H_{34}N_2O_5Se$ $[M + Na]^+$ 521.1531, found 521.1171.

Synthesis of 28

To a 10 mL methanolic solution of **25** (0.41 g, 0.75 mmol), iodine (0.23 g, 0.9 mmol) was added. Water (10 mL) was added to the reaction mixture and stirred for 15 min. To this, 0.5 mL of hydrazine hydrate was added to destroy 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 0.27 g (85%); ^{1}H NMR ($CDCl_3$) δ (ppm): 1.11–1.13 (d, 3H), 1.85–1.94 (m, 3H), 2.29–3.01 (m, 1H), 2.81–2.85 (m, 1H), 2.99–3.04 (m, 2H), 3.14–3.23 (m, 2H), 3.46–3.56 (m, 2H), 3.69 (s, 3H), 4.57–4.59 (m, 1H), 4.78–4.79 (d, 1H), 7.10–7.12 (m, 2H), 7.23–7.29 (m, 3H); ^{13}C NMR ($CDCl_3$) δ (ppm):18.4, 25.2, 27.9, 32.8, 38.2, 39.7, 47.8, 52.7, 53.8, 60.1, 127.3, 128.8, 129.6, 136.4, 171.5, 172.3, 175.5; ^{77}Se NMR ($CDCl_3$) δ (ppm): 301; HRMS (ESI-mode) *m/z* calcd. for $C_{38}H_{50}N_4O_8Se_2$ $[M + Na]^+$ 873.1857, found 873.1855.

Synthesis of 29

This compound was synthesized following a similar method to that given for compound **28** by using compound **26**. Yield 78%; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.13–1.14 (d, 3H), 1.89–1.94 (m, 3H), 2.13–2.16 (m, 1H), 2.81–2.84 (m, 1H), 2.95–3.01 (m, 2H), 3.20–3.23 (m, 1H), 3.52–3.57 (m, 3H), 3.67 (s, 3H), 4.54–4.56 (m, 1H), 4.75–4.77 (m, 1H), 6.69–6.71 (d, 2H), 6.90–6.92 (d, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm): 18.4, 25.3, 28.6, 30.2, 33.0, 39.9, 48.0, 52.9, 53.9, 60.4, 115.9, 127.1, 130.8, 156.4, 172.0, 172.5, 175.6; ^{77}Se NMR

($CDCl_3$) δ (ppm): 306; ESI-MS: m/z calcd. for $C_{38}H_{50}N_4O_{10}Se_2$ $[M + Na]^+$ 905.1755, found 905.3151.

Synthesis of 30

This compound was synthesized following a similar method given for compound **28** by using compound **27**. Yield 86%; ^{1}H NMR ($CDCl_3$) δ (ppm): 0.86–0.91 (m, 6H), 1.22–1.24 (d, 3H), 1.83–1.86 (m, 1H), 2.00–2.16 (m, 3H), 2.36–2.39 (m, 1H), 2.83–2.87 (dd, 1H), 3.07–3.9 (m, 1H), 3.23–3.29 (m, 1H), 3.57–3.59 (m, 2H), 3.70 (s, 3H), 4.40–4.43 (m, 1H), 4.66–4.68 (d, 1H); ^{13}C NMR ($CDCl_3$) δ (ppm): 18.1, 18.8, 19.5, 25.5, 27.4, 31.4, 32.7, 39.9, 48.0, 52.6, 57.8, 60.0, 171.6, 172.7, 175.8; ^{77}Se NMR ($CDCl_3$) δ (ppm): 305; HRMS (ESI mode) m/z calcd. for $C_{30}H_{50}N_4O_8Se_2$ $[M + Na]^+$ 777.1857, found 777.2000.

Synthesis of 31

Compound **31** was synthesized following a similar method to that given for compound **23** by using **28** as starting material. Yield 69%; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.02–1.04 (d, 3H), 1.81–1.83 (m, 3H), 2.10–2.12 (m, 1H), 2.73–2.75 (d, 1H), 2.89–2.97 (m, 2H), 3.10–3.15 (m, 2H), 3.36–3.38 (m, 1H), 3.46–3.48 (m, 1H), 4.47–4.49 (d, 1H), 4.67–4.72 (m, 1H), 7.06–7.20 (m, 5H); ^{13}C NMR ($CDCl_3$) δ (ppm): 18.4, 25.2, 28.3, 30.2, 32.7, 37.9, 48.0, 53.8, 60.5, 127.4, 128.9, 129.9, 136.8, 171.9, 174.1, 175.9; ^{77}Se NMR ($CDCl_3$) δ (ppm): 306; HRMS (ESI mode) m/z calcd. for $C_{36}H_{46}N_4O_8Se_2$ $[M + Na]^+$ 845.1544, found 845.1724.

Synthesis of 32

This compound was synthesized from compound **29** by following a similar method to that given compound **23**. Yield 71%; ^{1}H NMR (MeOH-d_4) δ (ppm): 1.17–1.19 (d, 3H), 1.92–1.95 (m, 3H), 1.99–2.01 (m, 1H), 2.89–2.96 (m, 2H), 3.02–3.07 (m, 3H), 3.62–3.65 (m, 2H), 4.424.45 (m, 1H), 4.53–4.56 (m, 1H), 6.66–6.72 (m, 2H), 7.03–7.07 (m, 3H); ^{13}C NMR (MeOH-d_4) δ (ppm): 17.1, 24.7, 29.4, 29.8, 32.6, 36.5, 39.6, 54.4, 60.3, 115.2, 128.1, 130.4, 156.4, 173.2, 173.6, 175.5; ^{77}Se NMR (MeOH-d_4) δ (ppm): 301; HRMS (ESI mode) m/z calcd. for $C_{36}H_{46}N_4O_{10}Se_2$ $[M + Na]^+$ 877.1442, found 877.2111.

Synthesis of 33

This compound was synthesized from compound **30** by following a similar method to that given for compound **23**. Yield 61%; ^{1}H NMR ($CDCl_3$) δ (ppm): 0.85–0.90 (m, 6H), 1.14–1.16 (d, 3H), 1.86–1.96 (m, 2H), 2.06–2.20 (m, 3H), 2.76–2.81 (m, 1H), 2.98–3.00 (m, 1H), 3.14–3.17 (m, 1H), 3.55 (s, 2H), 4.39–4.42 (dd, 1H), 4.60–4.61 (d, 1H); ^{13}C NMR ($CDCl_3$) δ (ppm): 17.9, 18.6, 19.6, 25.5, 28.2, 30.2, 31.5, 40.0, 48.2, 57.6, 60.4, 172.2, 174.8, 175.8; ^{77}Se NMR ($CDCl_3$) δ (ppm): 304; HRMS (ESI mode) m/z calcd. for $C_{28}H_{46}N_4O_8Se_2$ $[M + Na]^+$ 749.1544, found 749.1534.

Synthesis of peroxynitrite (PN)

Peroxynitrite was synthesized following a literature procedure.[17]

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 a 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, 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. 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

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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