The First Mechanism-Based Inactivators for Angiotensin-Converting Enzyme

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The first example of mechanism-based inactivation of angiotensin-converting enzyme (ACE) is described for *N*-[*N*-(cyanoacetyl)-L-phenylalanyl]-L-phenylalanine (compound 1). It is proposed that an ACE-mediated deprotonation of 1 unmasks a ketenimine intermediate, which traps an active-site nucleophile, and hence irreversibly modifies the enzyme. In competition with the inactivation reaction, ACE also hydrolyzes 1 with a partition ratio of 8300 (i.e., k_{cat}/k_{inact}). Since the corresponding keto analogue, *N*-[(*R*)-2-benzyl-5-cyano-4-oxopentanoyl]-L-phenylalanine (compound 4), does not inactivate the enzyme, it is suggested that the NH in compound 1 is critical for the proper active-site anchoring of the inhibitor for the inactivation process to take place.

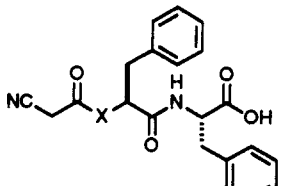
Introduction

A wealth of studies have established the crucial role of angiotensin-converting enzyme (ACE, peptidyldipeptide hydrolase, EC 3.4.15.1) in human hypertension of renovascular origin. Furthermore, the ACE activity in vitro indicates its involvement in metabolism of other biologically active peptides.[1] Inhibitors of this zinc metalloprotease have been successfully used as antihypertensive agents, which exert their effect by inhibiting the formation of the potent vasoconstrictor angiotensin II and by curtailing the degradation of the vasodilator, bradykinin.[2] The efficacy of these compounds is derived from their potent competitive inhibition of ACE.

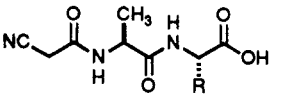
The active sites of ACE and carboxypeptidase A (CPA), a prototypic zinc protease, are understood to be very similar. This similarity was exploited in the design of the first generation of ACE inhibitors as antihypertensive agents, as stated above. The similarity of the active sites and the mechanistic processes of the two enzymes were further documented by the demonstration that both ACE[3] and CPA[4] catalyze the stereospecific enolization of ketonic substrate analogues. We have recently demonstrated that this enzymic characteristic of CPA can be exploited in the design of specific mechanism-based inactivators for the enzyme, wherein a deprotonation step leads to the formation of transient intermediates as the putative inactivating species.[5,6] We have been interested in extending this design principle for the development of irreversible inactivators for therapeutically important zinc metalloproteases, and in this vein, we report here the first example of mechanism-based inactivators targeted specifically for inactivation of ACE.

Results

Compound 1 was obtained in two steps by the following synthetic scheme. *N*-Cyanoacetyl-L-phenylalanine, prepared as reported earlier,[6] was coupled to phenylalanine *tert*-butyl ester using a water-soluble diimide procedure in 69% yield. Acidolysis of the resulting dipeptide *tert*-butyl ester using trifluoroacetic acid (TFA) in methylene chloride provided 1 in 51% yield. *N*-Cyanoacetyl-L-alanine was prepared by the two-step diimide/acidolysis procedure described above in 75% overall yield. The same procedure was used to prepare the leucine and phenylalanine adducts of *N*-cyanoacetyl-L-alanine, compounds 2 and 3. *N*-[(*R*)-2-Benzyl-5-cyanopentanoyl]-L-phenylalanine (4), the ketomethylene analogue of 1, was synthesized in 49% yield by diimide-mediated coupling of (*R*)-2-benzyl-5-cyano-4-oxopentanoic acid with L-phenylalanine methyl ester. The resulting product was subjected to alkaline hydrolysis to provide 4 in 66% yield.



1: X = NH
4: X = CH_2



2: R = $CH_2CH(CH_3)_2$
3: R = $CH_2C_6H_5$

The peptidic compounds 1–3 inactivated ACE in a time-dependent manner. When 18 mM concentrations of the

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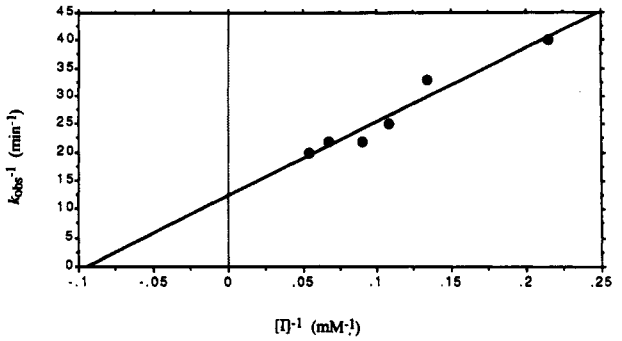

Figure 1. Double-reciprocal plot of the first-order inactivation rates for ACE as a function of concentration of compound 1.

inactivators were used, the first-order rate constant (k_{obs}) for loss of enzymic activity was measured to be 0.05, 0.03, and 0.015 min^{-1} for **1**, **2**, and **3**, respectively. The evidence for the creation of a reactive intermediate at the active site was provided by a complete protection from inactivation afforded by captopril, a known competitive inhibitor for ACE. The captopril protection study, however, does not provide conclusive proof for a mechanism entailing an active-site quenching of the reactive intermediate. It is conceivable that the thiol group of captopril potentially traps the electrophilic species that is generated from compounds **1–3** in the active site in competition with the binding of captopril to the enzyme. In any event, protection from inactivation by added nucleophiles (e.g., thiols) to the mixture of enzyme and inactivators does corroborate the process of mechanism-based inactivation. A detailed kinetic analysis of the inactivation reaction, according to the method of Ghosh et al.[6] was then undertaken for the best peptidic inactivator, compound **1**. Enzyme inactivation rates were pseudo-first-order and obeyed saturation kinetics for concentrations of the inactivator ranging between 4.6 and 18.6 mM. The inactivation was irreversible and extensive dialysis failed to regenerate the enzyme activity. A double-reciprocal plot of the first-order inactivation rate constant (k_{obs}) as a function of inactivator concentration was linear (Figure 1), and the kinetic parameters k_{inact} and K_m were evaluated to be 0.08 min^{-1} and 10.5 mM, respectively.

Compound **1** was treated as an inhibitor for hydrolysis of *N*-[3-(2-furyl)acryloyl]-Phe-Gly-Gly[7] by ACE at concentrations well below the K_m for the inactivation reaction. Analysis by the method of Dixon[8] revealed a competitive mode of inhibition, and the reversible inhibition constant (K_i) was determined to be 65 μM (Figure 2).

A titration method[9] was utilized for evaluating the partition ratio (k_{cat}/k_{inact}) for the inactivation process. A series of inactivation experiments were performed where the $[I]_0/[E]_0$ ratio was varied from 50 to 8200; however, linearity was seen only within the range of 400–4100. The deviation from linearity at $[I]_0/[E]_0 > 4100$ is attributed to product inhibition. Figure 3 shows the plot of $[I]_0/[E]_0$ versus percent enzymatic activity remaining at the end of a 20-h incubation period. A partition ratio of 8300 was

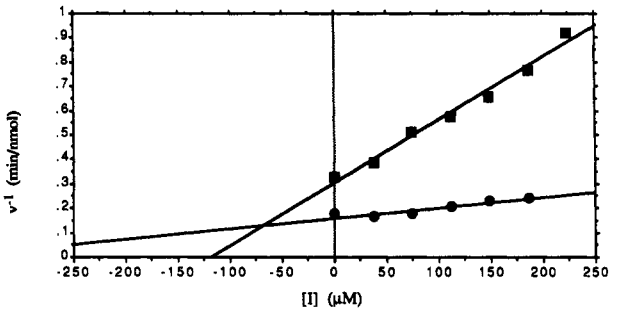

Figure 2. Plot of the reciprocal of velocity as a function of concentration of **1** with 120 μM (■) and 150 μM (●) substrate.

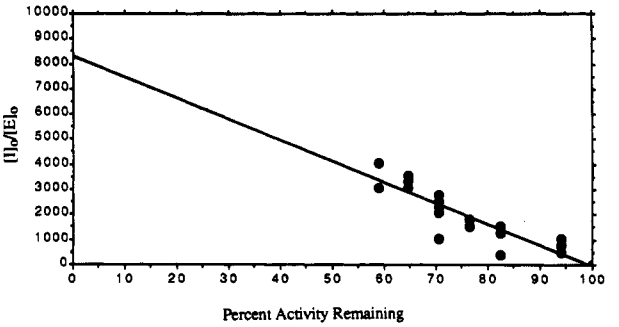

Figure 3. Plot of $[I]_0/[E]_0$ versus the percent of ACE activity remaining after 20 h exposure to compound **1**.

determined, from which the catalytic constant (k_{cat}) was calculated as 11.1 s^{-1}.

No time-dependent inactivation of ACE was observed when the enzyme was incubated with the ketomethylene analogue, *N*-[(*R*)-2-benzyl-5-cyano-4-oxopentanoyl]-L-phenylalanine (**4**). For these experiments, concentrations higher than 10 mM could not be attained, due to low solubility of the compound in the inactivation mixture. Further, less than 10% reversible inhibition of the ACE-catalyzed peptide hydrolysis was observed in the presence of 10 mM **4**, thereby suggesting that **4** does not even bind effectively to the enzyme active site.

Discussion

A major goal of our research has been the development of strategies for the mechanism-based inactivation of zinc proteases. Our approach for the design of inactivators for ACE is based on (i) the use of the hypothetical active site model of ACE, originally proposed by Ondetti and coworkers,[10] as a template for choosing the structural motifs in the molecules deemed necessary for providing the binding interactions in the enzyme active site, and (ii) incorporating latent functional groups, determined from our mechanism-based inactivation studies of the model zinc protease, CPA, in substrate analogues for ACE. It was anticipated that unmasking of these latent functionalities by ACE would generate electrophilic reactive intermediates, which would inactivate the enzyme.

The similarity of ACE and CPA in their hydrolytic mechanism of action was instrumental in the proposal of the hypothetical active site model of ACE, which allowed for the rational design of the potent antihypertensive drugs, captopril[10] and enalapril.[11] In addition, ACE shares a

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common feature with CPA in its ability to carry out enolization of ketonic substrate analogues, which was demonstrated in the α,β-elimination of *p*-nitrothiophenolate from *N*-[3-benzoyl-2-(*p*-nitrothiophenoxy)propanoyl]-L-phenylalanine.[3] This observation suggested to us that the appropriate placement of a latent functionality in a substrate analogue may lead to selective deprotonation, and the subsequent unmasking of the reactive electrophilic intermediate in the substrate analogue. Our recent report on a ketonic molecule [(*R*)-2-benzyl-5-cyano-4-oxopentanoic acid],[5] and peptidic compounds,[6] *N*-(cyanoacetyl)-L-phenylalanine and *N*-(3-chloropropionyl)-L-phenylalanine, as irreversible mechanism-based inactivators for CPA suggested the possibility that dipeptide derivatives of these compounds may function as such inactivators for ACE. Compounds 1 and 4 were designed to be analogous to the ACE ketomethylene substrate, *N*-[3-benzoyl-2-(*p*-nitrothiophenoxy)propanoyl]-L-phenylalanine,[3] in retaining the benzyl groups for interaction with the S_1' and S_2' subsites. The dipeptide derivatives of *N*-(cyanoacetyl)-L-alanine (compounds 2 and 3) were prepared to provide a preferred smaller hydrophobic P_1' residue, based on the ACE active site model.[10] The terminal carboxyl group in these molecules was expected to be involved in ion-pairing interaction with a positively charged active site residue. Finally, the α-cyanomethyl moiety was incorporated for the requisite ACE-catalyzed isomerization of the nitrile to the ketenimine intermediates, resulting from an initial enzymic deprotonation step at the α position to carbonyl function. Based on the precedent reported for the ketomethylene and peptidic inactivators for CPA,[5,6] it was envisioned that these electrophilic ketenimine intermediates would irreversibly trap an active site residue, hence leading to irreversible inactivation of the enzyme. Such rearrangements of nitriles have been previously utilized for inactivation of other enzymes.[12]

Our analysis of the kinetics of interactions of 1 with ACE indicated that compound 1 behaves very much like a typical substrate with a k_{cat}/K_m value (1.1×10^3 M^{-1} s^{-1}) comparable to those reported for some relatively poor ACE substrates, such as *N*-[3-(2-furyl)acryloyl]-Gly-Gly-Gly, and *N*-[3-(2-furyl)acryloyl]-Gly-Leu-Gly.[13] However, in a diversion from the turnover processes, compound 1 undergoes the chemistry envisioned to lead to covalent modification of the enzyme (vide supra). It is noteworthy that the kinetic parameters for inactivation and turnover of ACE by 1 (e.g., k_{inact} = 0.08 min^{-1}, K_m = 10.5 mM, k_{cat} = 11.1 s^{-1}) were quite similar to those reported for the mechanism-based inactivation of CPA by *N*-(cyanoacetyl)-L-phenylalanine (k_{inact} = 0.035 min^{-1}, K_m = 4.19 mM, k_{cat} = 0.7 s^{-1}). Treatment of compound 1 as a competitive inhibitor of ACE hydrolysis of the peptide substrate, *N*-[3-(2-furyl)acryloyl]-Phe-Gly-Gly gave a K_i of 65 μM, indicative of a reasonably high affinity for the enzyme.

Since compound 1 is a peptidic molecule, deprotonation at the α-carbon and the peptide bond hydrolysis were expected to be competing reactions. The measured partition ratio of 8300 indicates that the hydrolysis reaction predominates over the inactivation process. Whereas in general it is desirable to have low partition ratios for mechanism-based inactivators, one finds exceptions among such bioactive molecules. For example, sulbactam, a clinically useful mechanism-based inactivator for β-lactamases, shows partition ratios in the range of 1000–7000.[14] It should be desirable, especially for metabolically essential enzymes such as ACE, to have inactivators that have the ability to lower the activity of the target enzyme, but do not completely abolish the enzymic activity. In our view, compound 1 and others like it meet this expectation.

The ketomethylene analogue 4 is not a mechanism-based inactivator for ACE and also appears to bind very poorly to the enzyme as a reversible inhibitor. This observation differentiates ACE from CPA since *N*-(cyanoacetyl)-L-phenylalanine and its ketomethylene analogue, (*R*)-2-benzyl-5-cyano-4-oxopentanoic acid, exhibit comparable rates for inactivation of CPA and are similar in their reversible binding constants for the enzyme.[5,6] The marked difference observed upon replacement of the NH in compound 1 by a CH_2 group (compound 4) emphasizes the critical importance of the NH group for active-site binding and inactivation and is reminescent of the requirement of an NH at the corresponding position in *N*-carboxymethyl-dipeptide inhibitors[11] and peptide substrates[15] of ACE. The crystal structure of the complex of CPA and a protein inhibitor from potato indicated two hydrogen bonds between the inhibitor and the side chain hydroxyl of Tyr-248 of the enzyme.[16] One of these hydrogen bonds is with the NH of the incipient amine of the hydrolytic reaction, whose contribution to the stability of the complex was estimated at 2.5 kcal/mol.[17] Similarily, chemical modification experiments have identified the existence of a tyrosine residue in the active site of ACE.[18] It is conceivable that a hydrogen bond from this tyrosine, or possibly other active site residues, to the NH of our peptidic compound is indispensible for proper alignment of the inactivator(s) in the active site, and hence for the chemistry of inactivation. This interaction would not be possible with the ketonic compounds.

In summary, we propose the following mechanism for the inactivation of ACE. Subsequent to enzymic deprotonation, initiated either by promoted water attack or by an active-site basic residue, compound 1 undergoes a

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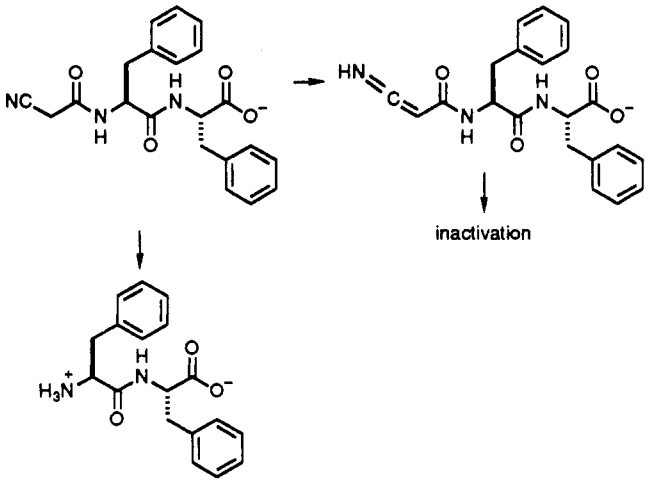
Scheme I



rearrangement to a ketenimine. This reactive intermediate, in turn, traps an active site nucleophile to irreversibly inactivate the enzyme (Scheme I). This mechanistic scheme has ample precedent in our mechanism-based inactivation studies with CPA,[5,6] and more recently, with renal dipeptidase.[19]

Experimental Section

Infrared spectra were obtained on a Mattson Cygnus 25 FT-IR instrument and ^{1}H NMR spectra were recorded on a Nicolet 360 MHz instrument using tetramethylsilane as an internal standard. Mass spectra were obtained on a Kratos MS 80RFA instrument. Kinetic studies were performed on a Perkin-Elmer Lambda 3B UV/vis spectrometer. Purified bovine lung ACE was provided by Dr. Irwin Wilson of the University of Colorado. *N*-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly was purchased from Sigma. The active enzyme concentration was determined from first-order rate constants with *N*-[3-(2-furyl)acryloyl]-Phe-Gly-Gly using the method of Bull et al.,[20] where [active enzyme] = $k/(k_{cat}/K_m)$ assuming K_m = 300 μm and k_{cat} = 317 s^{-1} for *N*-[3-(2-furyl)-acryloyl]-Phe-Gly-Gly.[7] The hydrochloride salts of amino acid *tert*-butyl esters were purchased from Bachem and all other chemicals were obtained from Aldrich. Melting points were determined in open capillaries in a Thomas Hoover melting point apparatus and are uncorrected. Analytical HPLC analysis of the compounds were performed on a C-18 reverse-phase column (Altex, 0.46 × 25 cm), using a 1 mL/min flow rate and a 20–75% linear gradient of acetonitrile in 0.1% aqueous TFA over 25 min. Compounds were detected at 214 or 254 nm.

***N*-(Cyanoacetyl)alanine.** To a solution of 500 mg (2.75 mmol) of alanine *tert*-butyl ester hydrochloride in 11 mL of *N*,*N*-dimethylformamide (DMF) under argon was added 383 μL of triethylamine followed by 234 mg (2.75 mmol) of cyanoacetic acid and 442 mg (2.89 mmol) of 1-hydroxybenzotriazole hydrate (HOBt). The resulting mixture was cooled to 0 °C and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC, 527 mg, 2.75 mmol) was added. The mixture was stirred at 0 °C for 1 h followed by 12 h at 23 °C. The solvent was removed in vacuo, and the resulting residue was suspended in 20 mL of ethyl acetate. The organic layer was extracted three times each with 3 mL of 1 N $NaHSO_4$, 5% $NaHCO_3$, and brine, dried over anhydrous $MgSO_4$, and concentrated to a white oil weighing 520 mg (90%). The product, *N*-(cyanoacetyl)alanine *tert*-butyl ester, was found to be pure by TLC (ethyl acetate, R_f 0.62), and was submitted for deprotection without further purification.

To a solution of 260 mg (1.23 mmol) of *N*-(cyanoacetyl)alanine *tert*-butyl ester in 9 mL of methylene chloride was added dropwise 9 mL of trifluoroacetic acid (TFA). The reaction mixture was stirred for 30 min at 0 °C followed by 2 h at 23 °C. The solvent was then removed under reduced pressure, and the crude product was purified by flash chromatography using sequential elutions with ethyl acetate and a mixture containing 85:10:5 chloroform/methanol/acetic acid. *N*-(Cyanoacetyl)alanine was obtained as a clear oil (160 mg, 83%). ^{1}H NMR ($CDCl_3$): δ 6.55 (bs, 1 H), 4.70 (m, 1 H), 3.45 (s, 1 H), 1.51 (d, 3 H). IR (neat): 3308, 2959, 2264, 1714, 1651 cm^{-1}.

Synthesis of Peptidic Mechanism-Based Inactivators, 1–3. Compounds 1–3 were prepared by a two-step water-soluble carbodiimide/acidolysis procedure as exemplified by the synthesis of 1 described below.

***N*-[*N*-(Cyanoacetyl)-L-phenylalanyl]-L-phenylalanine *tert*-Butyl Ester.** To a solution of 59.2 mg (0.23 mmol) of phenylalanine *tert*-butyl ester in 1 mL of DMF under argon was added 32 μL (0.23 mmol) of triethylamine, followed by 54 mg (0.23 mmol) of *N*-(cyanoacetyl)-L-phenylalanine and 33 mg (0.24 mmol) of HOBt. The resulting mixture was cooled to 0 °C, and EDC (44 mg, 0.23 mmol) was added. The mixture was stirred for 1 h at 0 °C and then for 12 h at 23 °C. The DMF was removed in vacuo, and the resulting residue was suspended in 10 mL of ethyl acetate. The organic layer was extracted three times each with 3 mL of 1 N $NaHSO_4$, 5% $NaHCO_3$, and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated. The crude product was taken up in chloroform and was precipitated by the addition of hexane. After filtration, the product was washed thoroughly with hexane to furnish 70 mg of pure *N*-[*N*-(cyanoacetyl)-L-phenylalanyl]-L-phenylalanine *tert*-butyl ester in 69% yield; R_f 0.70 (chloroform/methanol/acetic acid, 85:10:5). ^{1}H NMR ($CDCl_3$): δ 7.40 (d, 1 H), 7.34–7.10 (m, 10 H), 6.70 (d, 1 H), 4.85 (m, 1 H), 4.75 (m, 1 H), 3.30 (s, 2 H), 3.16–2.90 (m, 4 H), 1.40 (s, 9 H). IR (neat): 3300, 2960, 2260, 1732, 1651, 1538, 1155, 700 cm^{-1}.

***N*-[*N*-(Cyanoacetyl)-L-phenylalanyl]-L-phenylalanine (1).** A solution of 70 mg of *N*-[*N*-(cyanoacetyl)-L-phenylalanyl]-L-phenylalanine *tert*-butyl ester (0.16 mmol) in 1.4 mL of methylene chloride was cooled to 0 °C and 1.4 mL of TFA was added dropwise under an argon atmosphere. The reaction mixture was stirred for 30 min at 0 °C and then for 2.5 h at 23 °C. The solvent was removed under vacuum, and the crude product was purified by flash chromatography using 0.5% acetic acid in ethyl acetate as solvent to yield 31 mg (51%) of *N*-[*N*-(cyanoacetyl)-L-phenylalanyl]-L-phenylalanine (1), as an oil. HPLC: single peak, t_R = 10.7 min. ^{1}H NMR (CD_3OD): δ 7.35–7.20 (m, 12 H), 4.75–4.60 (m, 2 H), 3.45 (s, 2 H), 3.25–3.15 (dd, 1 H), 3.15–3.05 (dd, 1 H), 3.01–2.94 (dd, 1 H), 2.85–2.75 (dd, 1 H). IR (neat): 3288, 2263, 1714, 1651, 1557, 700 cm^{-1}; FAB$^-$ MS *m*/*z* 378.4 (100%, M − 1).

***N*-[*N*-(Cyanoacetyl)-L-alanyl]-L-leucine (2).** *N*-[*N*-(Cyanoacetyl)-L-alanyl]-L-leucine *tert*-butyl ester was obtained in purified form in 34% yield following the EDC/HOBt-mediated coupling of *N*-(cyanoacetyl)-L-alanine with leucine *tert*-butyl ester. TFA hydrolysis of *N*-[*N*-(cyanoacetyl)-L-alanyl]-L-leucine *tert*-butyl ester furnished 2 in 43% yield as a white solid: mp 168–170 °C. HPLC: single peak, t_R = 4.9 min. ^{1}H NMR ($CDCl_3$): δ 4.41 (m, 1 H), 4.31 (m, 1 H), 3.30 (s, 2 H), 1.70–1.59 (m, 2 H), 1.37 (d, 3 H, *J* = 7.1 Hz), 0.93 (dd, 6 H). IR (neat): 3301, 2264, 1731, 1652 cm^{-1}; FAB$^-$ MS *m*/*z* 268.3 (20%, M − 1).

***N*-[*N*-(Cyanoacetyl)-L-alanyl]-L-phenylalanine (3).** *N*-[*N*-(Cyanoacetyl)-L-alanyl]-L-phenylalanine *tert*-butyl ester was synthesized by condensation of *N*-(cyanoacetyl)-L-alanine with phenylalanine *tert*-butyl ester in 90% yield using the EDC/HOBt coupling procedure. Compound 3 was obtained following TFA hydrolysis of *N*-[*N*-(cyanoacetyl)-L-alanyl]-L-phenylalanine *tert*-butyl ester in 39% yield. HPLC: single peak, t_R = 5.7 min. ^{1}H NMR ($CDCl_3$): δ 7.3–7.15 (m, 7 H), 4.62 (m, 1 H), 4.35 (m, 1 H), 3.43 (s, 1 H), 3.20 (dd, 1 H, *J* = 8.7, 5.1 Hz), 3.03 (dd, 1 H, *J* = 5.5, 8.2 Hz), 1.29 (d, 3 H, *J* = 7.1 Hz). IR (neat): 3281, 3061, 2934, 2261, 1665, 1543, 701 cm^{-1}; FAB$^-$ MS *m*/*z* 302.3 (100%, M − 1).

***(R)*-2-Benzyl-5-cyano-4-oxopentanoic Acid.** The synthesis of this compound was reported earlier by us.[5] We report below a much shorter and simpler synthesis for the title compound.

n-Butyllithium (1.25 mL of a 2.31 M solution in hexane, 2.88 mmol) was rapidly added to a solution of acetonitrile (150 μL, 2.88 mmol) in 5 mL of dry THF at −78 °C under an atmosphere

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of argon. A white precipitate formed after the addition was completed, and the mixture was allowed to stir for 1 h at −78 °C. A solution of methyl (*R*)-2-benzyl-3-carbomethoxypropionic acid[5] (320.5 mg, 1.44 mmol) in 4 mL of THF was added dropwise, and the reaction mixture was stirred for 1 h at −78 °C, followed by 0.5 h at 0 °C. The reaction was quenched by the addition of 1.3 mL of acetic acid, and subsequently, the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate and washed three times with brine. The organic layer was then dried over $MgSO_4$ and filtered, and the solvent was evaporated in vaccuo to yield a yellow oil. The crude product was purified by preparative TLC using 1% acetic acid in ethyl acetate as eluent, and the title compound was obtained as a clear oil weighing 150 mg (45%). The spectroscopic properties of the product were identical to those reported previously.[5]

***N*-[(*R*)-2-Benzyl-5-cyano-4-oxopentanoyl]-L-phenylalanine (4).** The methyl ester of the title compound was synthesized by coupling (*R*)-2-benzyl-5-cyano-4-oxopentanoic acid to L-phenylalanine methyl ester using the carbodiimide coupling procedure and was purified by preparative thin-layer chromatography on silica gel using 1:1 ethyl acetate/hexane to give the product in 49% yield. ^{1}H NMR ($CDCl_3$): δ 7.34–7.07 (m, 10 H), 5.96 (d, 1 H, J = 7.8 Hz), 4.77 (m, 1 H), 3.67 (s, 3 H), 3.40 (s, 2 H), 3.13–2.87 (m, 5 H), 2.68 (dd, 1 H), 2.45 (dd, 1 H). IR (neat): 3352, 3029, 2953, 2261, 1738, 1661 cm^{-1}.

Saponification of the methyl ester in aqueous methanolic sodium hydroxide provided the desired product 4, in 66% yield after preparative TLC (0.1% acetic acid in ethyl acetate; R_f 0.52). HPLC: single peak, t_R = 14.5 min. ^{1}H NMR ($CDCl_3$): δ 7.4–7.1 (m, 10 H), 6.25 (d, 1 H), 4.8 (m, 1 H), 3.38 (s, 2 H), 3.15 (dd, 1 H, J = 5.3, 8.8 Hz), 3.05–2.88 (m, 4 H), 2.66 (dd, 1 H, J = 6.9, 6.4 Hz), 2.46 (m, 1 H). IR (neat): 3314, 2925, 2265, 1733, 1653, 1559, 702 cm^{-1}. EI MS: m/z 360.4 (10%, M − 18), EI MS m/z 360.4 (95%, M − 18).

Enzyme Assay. Spectrophotometric assay according to the method of Holmquist et al. was used.[7] A typical assay mixture contained 1.4 pM enzyme and 60 μM *N*-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly in 150 mM MOPS, 0.3 M KCl, pH 7.5. The decrease of the chromophore at 328 nm ($\Delta\epsilon_{328}$ = 2300) was monitored as a function of time.

Kinetic Experiments. Inactivation experiments were carried out according to the method of Ghosh et al.[6] The ketonic and peptidic inactivators were dissolved in *p*-dioxane and DMSO, respectively, to give the final concentrations of inactivator of 4.6–18.6 mM. The inactivation mixture contained 140 pM ACE in 150 mM MOPS, 0.3 M KCl, pH 7.5 and no more than 10% organic cosolvent. Aliquots (15 μL) were withdrawn at specific intervals and diluted 100-fold serially into the final assay mixture. The enzyme activity was monitored immediately.

Competition experiments were performed in the presence of captopril [(2*S*)-1-(3-mercapto-2-methylpropionyl)-L-proline]. A 10 nM solution of ACE in 150 mM MOPS, 0.3 M KCl, pH 7.5 was incubated with 55 nM captopril (K_i = 2 nM) for 15 min at room temperature. Subsequently, compound 1 was mixed into the mixture (1.5 mM final), and the enzymic activity was monitored periodically. Since captopril is a potent slow-binding inhibitor of ACE, we carried out a similar experiment with ACE in the absence of compound 1. The rate of remaining activity at each time point was identical whether the experiment was carried out in the presence or the absence of peptide 1.

The partition ratio of 1 was determined by the titration method.[9] A series of solutions containing $[I]_0/[E]_0$ ranging from 50 to 8200 were incubated at 4 °C for 20 h. Subsequently, the enzymic activity was measured for each given mixture. To determine the extent of nonspecific protein inactivation, control experiments were carried out in the absence of the inactivator.

Reversible inhibition of ACE by the inactivators was studied by the Dixon method.[8] The assays were conducted with two different substrate concentrations (120 and 150 μM). Inactivator concentrations were in the range of 35–225 μM. An enzyme concentration of 0.9 pM was used in these experiments, and each assay was monitored for 30–45 min.

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