hydrogen ion concentration in this pH region. Ionic strength effects support this interpretation.

The theoretical pH minimum for the degradation of I obtained by taking the derivative of Eq. 1 and equating it to zero was pH 5.13.

Temperature Dependency—The dependence of degradation of I on temperature was determined by measuring the rate of decomposition at 35, 45, and 55° at pH 2.23, 5.52, and 8.94 and ionic strength is 0.5. The values of k_{obs}, E_a, and log A are given in Table III, and the corresponding Arrhenius plots are shown in Fig. 7. The theoretical $k_{25°}$ calculated using the energy of E_a, activation was in good agreement with the experimentally determined $k_{25°}$ at each of the three pH values, indicating that the data can be used to predict the stability of I over a wide range of pH and temperature conditions.

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Synthesis and Biological Activity of an Amino Analogue of a Tripeptide Inhibitor of Angiotensin-Converting Enzyme

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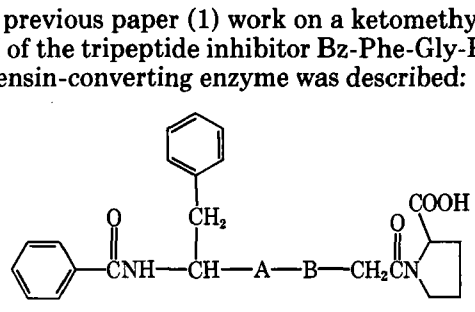
Received September 8, 1980, from the *Bio-Organic Chemistry Laboratory, SRI International, Menlo Park, CA 94025.* Accepted for publication February 25, 1982.

Abstract □ An amino analogue of *N*-benzoyl-phenylalanyl-glycyl-proline, a tripeptide inhibitor of angiotensin-converting enzyme, was synthesized. The analogue (III) has the phenylalanyl-glycine amide linkage of *N*-benzoyl-phenylalanyl-glycyl-proline reduced to a methylene amine. Compound III was tested as an inhibitor of porcine plasma angiotensin-converting enzyme and has an I_{50} of 620 μM compared with an I_{50} of 9.6 μM for its parent tripeptide. These results are explained in terms of a proposed model of the converting-enzyme active site.

Keyphrases □ Angiotensin-converting enzyme—synthesis and biological activity of an amino analogue of *N*-benzoyl-phenylalanyl-glycyl-proline, a tripeptide inhibitor □ *N*-Benzoyl-phenylalanyl-glycyl-proline—tripeptide inhibitor of angiotensin-converting enzyme, synthesis and biological activity of an amino analogue

In a previous paper (1) work on a ketomethylene analogue I of the tripeptide inhibitor Bz-Phe-Gly-Pro (II) of angiotensin-converting enzyme was described:



I: A = CO, B = CH_2
II: A = CO, B = NH
III: A = CH_2, B = NH

The replacement of the peptide amide linkage in II with a ketomethylene group was designed to stabilize this portion of the peptide molecule to peptidase cleavage. This substitution yielded a peptide analogue (I) with >100 times the inhibition activity against angiotensin-converting enzyme of the parent peptide.

A second approach to stabilizing this same peptide amide linkage in II to peptidase cleavage would be to reduce it to the corresponding methyleneamino compound (III). This compound has been synthesized and its inhibition activity against angiotensin-converting enzyme will be discussed.

BACKGROUND

A method for the synthesis of III was desired which would maintain the same stereochemistry around the optical centers as that found in the tripeptide (II). Initially, numerous attempts were made to selectively reduce the amide, but not the ester, group of *N*-benzyloxycarbonyl-phenylalanyl-glycine ethyl ester with borane, as reported previously (2), with *N*-benzyloxycarbonyl-glycyl-leucine methyl ester as the starting material. In contrast to those results the ester group of *N*-benzyloxycarbonyl-phenylalanyl-glycine ethyl ester was reduced more rapidly than the amide group by borane.

Because selective reduction of the desired amide functionality did not seem possible in this case, the dipeptide acid *N*-benzyloxycarbonyl-phenylalanyl-glycine (IV) was used as a starting material for the synthesis of III (Scheme I). The 9-fluorenyl-methoxycarbonyl amino blocking group (3) was used because of its stability to acidic conditions such as the Jones oxidation (4).

The low yield obtained for the transformation of VI to VIII was

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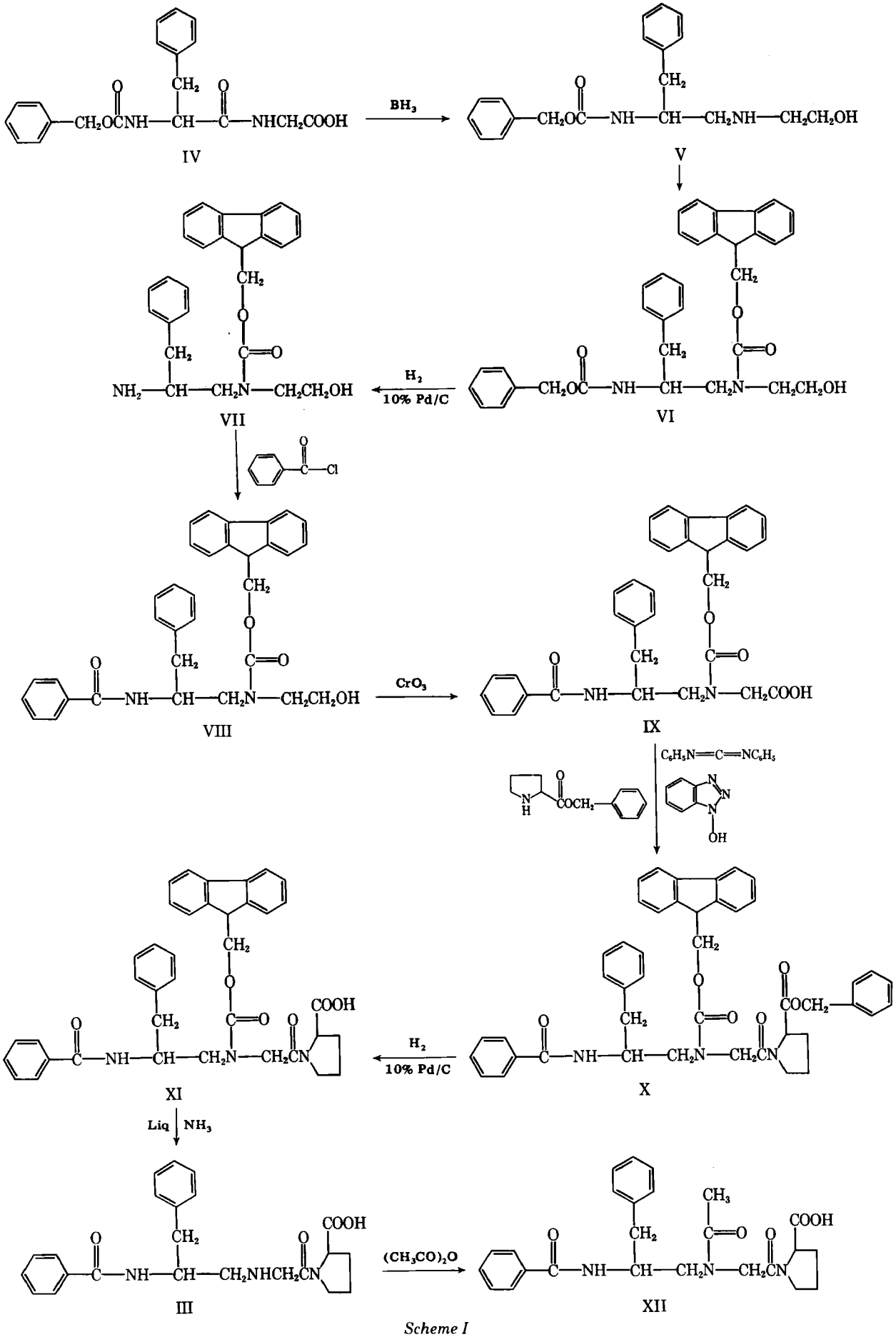


Scheme I

Table I—Inhibition of Porcine Plasma Angiotensin-Converting Enzyme

Compound	I_{50} (μM)[a]
Captopril	0.30
I	0.07
II	9.40
III	620.00
XII	2500.00

[a] Concentration required for 50% enzyme inhibition. All values are the average of results obtained in two or more experiments.

probably due to partial removal of the 9-fluorenylmethoxycarbonyl group during the hydrogenolysis step (5).

RESULTS AND DISCUSSION

The results of testing some of the compounds for inhibition of angiotensin-converting enzyme are shown in Table I. The amino analog (III) has 1/66th the inhibition activity of its parent tripeptide (II) but is about four times more active than the *N*-acetyl derivative (XII). Kinetic studies on III (Figs. 1 and 2) show it to be a competitive inhibitor of converting enzyme with either hippuryl-histidyl-leucine or angiotensin I as substrates.

The low activity of III is interesting in light of its structural similarities to the potent inhibitors (Table II) reported previously (6). Table II shows structure–activity data for some of these compounds that exhibit inhibition of the angiotensin-converting enzyme. One noteworthy conclusion from this table is that a phenethyl group in the R_1 position (*i.e.*, compound XV) yielded a more potent inhibitor than that obtained with other alkyl R_1 substituents. In previous studies on peptides (7) and tripeptide inhibitors (1), optimum activity was also obtained when the inhibitor contained an aromatic group (*i.e.*, compound II) that was the same distance from the carboxylic acid group of proline as was the phenyl group in XV. It was also found that an aromatic acyl group at the amino terminus yielded a more active inhibitor than tripeptides with either a nonaromatic or no acyl group in this position (1). Therefore, there appear to be binding points within the converting enzyme active site for groups located at the amino terminal portion of III.

Examination of the hypothetical active site of the angiotensin-converting enzyme (Fig. 3) proposed previously (8) might explain the low activity of III. One important binding functionality that all the known potent inhibitors and substrates of the angiotensin-converting enzyme have in common is a functionality that is positioned properly to interact with the enzyme-bound zinc ion. Such functionalities vary from a carbonyl (as in II) or a carboxylic acid (as in XIII–XV) to a thiol (as in captopril). The amino group in III must not be positioned properly to fulfill the role of a zinc-binding functionality. The low enzyme-inhibition activity of III exemplifies the major importance of the zinc interaction for good enzyme inhibition.

EXPERIMENTAL[1]

***N*-(2*S*-2-Benzyloxycarbonylamino-3-phenylpropyl)aminoethanol (V)**—To an ice-cold solution of *N*-benzyloxycarbonyl-L-phenylalanyl-glycine (5) (IV, 700 mg, 1.96 mmoles) in 8 ml of dry (lithium aluminum hydride-distilled) tetrahydrofuran was added 8.50 ml (8.50 mmoles) of 1 *M* borane under a nitrogen atmosphere. The solution was refluxed for 1 hr, then cooled in ice and treated dropwise with 0.5 *N* ethanolic HCl to pH 4. The mixture was stirred at room temperature for 2 hr, then cooled in ice, and filtered to give 408 mg (56% yield) of the hydrochloride as a white solid, mp 183–184°.

[1] Melting points were determined on a Thomas-Hoover Uni-melt and are uncorrected. Optical rotations were measured using a Perkin-Elmer 141 automatic polarimeter. Mass spectra were taken on an LKB 9000 GC–MS spectrometer. The ^{1}H-NMR spectra were taken on a Varian EM390 spectrometer. TLC was carried out on Uniplates from Analtech coated with 250 μm of silica gel GF. Evaporations were performed at 40° under house vacuum on a Buchi Rotavapor unless otherwise stated. Elemental analyses were conducted by Mr. Eric Meier, Stanford University, Stanford, California. Preparative high-pressure liquid chromatography (HPLC) was performed using the Waters Prep LC/System 500 and silica gel cartridges. The existence of solvents of crystallization was confirmed by ^{1}H-NMR whenever possible. Many of the compounds in this series had a strong tendency to trap solvents within their solid structure. Heating these compounds at 60–100° under 0.1-mm Hg vacuum for 24 hr would not completely remove the last traces of solvent. Continued heating under vacuum beyond this point began to decompose the compounds. All of these compounds were homogeneous by TLC and had spectra (mass spectra and/or ^{1}H-NMR) characteristics of the desired compounds. Elemental analyses have, therefore, been reported with solvents of crystallization.

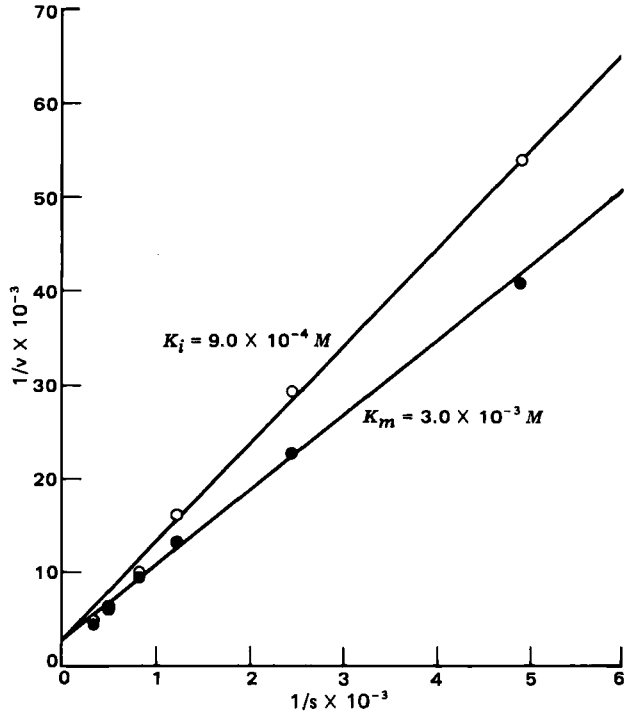


Figure 1—*Double-reciprocal plots of the effect of compound III as an inhibitor of porcine plasma angiotensin-converting emzyme with hippuryl-histidyl-leucine as substrate. Key: (●) no inhibitor; (○) with compound III at a concentration of 0.33 m*M. *Lines were drawn by the method of least squares.*

The hydrochloride was mixed with water (20 ml) and chloroform (20 ml). The aqueous layer was adjusted to pH 11 with 1 *N* NaOH while stirring vigorously. The chloroform layer was separated and the aqueous phase was extracted twice with chloroform (2 × 20 ml). The combined chloroform extracts were dried with calcium sulfate and evaporated to dryness to give 298 mg (46%) of the free base as a white solid, mp 93–95°; IR (mineral oil): 3300 and 3250 (amide NH), 1695 (CO), 1550 (amide CNH).

Anal.—Calc. for $C_{19}H_{24}N_2O_3$: C, 69.49; H, 7.37; N, 8.53. Found: C, 69.16; H, 7.26; N, 8.70.

***N*-(2*S* - 2-Benzyloxycarbonylamino-3-phenylpropyl) - *N* - (9-fluorenylmethyloxy-carbonyl)aminoethanol (VI)**—To an ice-cold solution of V (1.68 g, 5.12 mmoles) in 49 ml of 4Å sieve-dried acetone was added sodium bicarbonate (2.24 g, 26.7 mmoles) followed dropwise by a solution of 9-fluorenylmethyl chloroformate (1.98 g, 7.63 mmoles) in 5 ml of dry acetone. The mixture was stirred at 0° for 5 hr, then at room temperature for 1.5 hr, and finally stored at 4° for 64 hr.

The solid was removed by filtration. The filtrate was treated with methanol (20 ml) and evaporated to dryness. The residue was redissolved in methanol (20 ml) and again evaporated to dryness. This oil was dissolved in 20 ml of 50% ether in chloroform and added dropwise with rapid stirring to 400 ml of petroleum ether (bp 30–60°). The mixture was cooled at 4° for 20 hr. The solvent was decanted and the residual oil was dissolved in chloroform (50 ml), washed twice with water (2 × 50 ml), dried with calcium sulfate, and evaporated to dryness to give 2.54 g of a foam. This was chromatographed on silica gel preparative plates with 2% methanol in chloroform. The UV absorbing band at R_f 0.40 was extracted with 20% methanol in chloroform to give 2.07 g (73% yield) of VI as a foam; ^{1}H-NMR ($CDCl_3$): δ 4.90 (2 H, s, benzyloxy CH_2), 7.13 (18 H, m, aromatic).

Anal.—Calc. for $C_{34}H_{34}N_2O_5 \cdot 0.40\ CHCl_3$: C, 69.05; H, 5.79; N, 4.68. Found: C, 69.13; H, 6.04; N, 4.41. Subsequent larger runs were purified by HPLC with 20% ethyl acetate in chloroform.

***N*-(2*S*-2-Benzamido-3-phenylpropyl)-*N*-(9-fluorenylmethyloxy-carbonyl)aminoethanol (VIII)**—A solution of VI (3.09 g, 5.62 mmoles) in 300 ml of glacial acetic acid was hydrogenated at atmospheric pressure with 2.90 g of 10% palladium-on-carbon for 2 hr. The mixture was filtered through diatomaceous earth. The filtrate was evaporated to dryness to give the crude amine (VII) as an oil (2.65 g).

Sodium bicarbonate (1.13 g, 13.5 mmoles) was added to an ice-cold solution of the amine in 360 ml of 4Å sieve-dried dichloromethane fol-

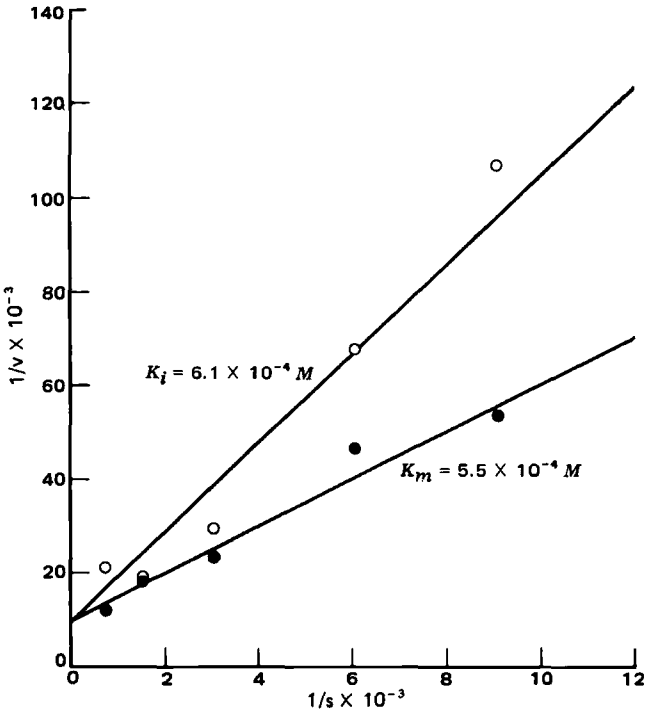


Figure 2—*Double-reciprocal plots of the effect of III as an inhibitor of porcine plasma angiotensin-converting enzyme with angiotensin I as substrate. Key: (●) no inhibitor; (○) with compound III at a concentration of 0.66 mM. Lines were drawn by the method of least squares.*

lowed dropwise by benzoyl chloride (0.785 ml, 6.74 mmoles). The mixture was stirred at room temperature for 18 hr. Another 0.523 ml of benzoyl chloride was added and stirring was continued for an additional 3 hr.

The solid was removed by filtration. The filtrate was treated with methanol (50 ml) and evaporated to dryness. The residue was redissolved in methanol (50 ml) and again evaporated to dryness. This oil was dissolved in 10 ml of chloroform and added dropwise with rapid stirring to 500 ml of petroleum ether (bp 30–60°). The mixture was cooled at 4° for 64 hr. The solvent was decanted and the residual oil was dissolved in chloroform (50 ml), washed with 0.2 *N* HCl (2 × 50 ml), saturated sodium bicarbonate solution (50 ml), and water (50 ml). The chloroform extract was dried with calcium sulfate and evaporated to dryness to give 2.06 g of a foam. This was purified by preparative HPLC using 1:1 chloroform–ethyl acetate, which afforded 1.05 g of VIII (36% yield from VI) as a white solid foam; R_f 0.5 (1:1 chloroform–ethyl acetate): mass spectrum 593 (M^+ + trimethylsilyl + H).

Anal.—Calc. for $C_{33}H_{32}N_2O_4 \cdot 0.10\ CHCl_3$: C, 74.65; H, 6.08; N, 5.26. Found: C, 74.50; H, 6.44; N, 5.16.

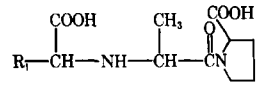
***N*-(2*S*-2-Benzamido-3-phenylpropyl)-*N*-(9-fluorenylmethyloxycarbonyl)aminoacetic Acid (IX)**—To an ice-cold solution of compound VIII (1.01 g, 1.94 mmoles) in 38 ml of acetone was added dropwise a solution of chromium trioxide (457 mg, 4.57 mmoles) in 9.7 ml of 35% H_2SO_4. The reaction was stirred at room temperature for 1 hr and then added to 200 ml of water and extracted with chloroform (3 × 100 ml). The chloroform extracts were washed with water (100 ml), dried with sodium sulfate, and evaporated to dryness to give compound IX as a foam (1.07 g), which was used directly in the next step.

An analytical sample was obtained in an earlier, small-scale run by crystallization from chloroform–ether following chromatography on a preparative silica gel plate with 10% methanol in chloroform. The tan solid softened at 139–142° and melted at 167–171°; R_f 0.4 (10% methanol chloroform): mass spectrum 606 (M^+ + trimethylsilyl).

Anal.—Calc. for $C_{33}H_{30}N_2O_5 \cdot 0.25\ CHCl_3$: C, 70.22; H, 5.40; N, 4.96. Found: C, 70.06; H, 5.37; N, 4.98.

***N*-[*N*-(2*S*-2-Benzamido-3-phenylpropyl)-*N*-(9-fluorenylmethyloxycarbonyl)aminoacetyl]-L-proline Benzyl Ester (X)**—To a stirred ice-cold solution of compound IX (1.07 g, 2.01 mmoles), L-proline benzyl ester hydrochloride (0.485 g, 2.01 mmoles), 1-hydroxybenzotriazole (0.300 g, 2.01 mmoles), and triethylamine (0.280 ml, 2.01 mmoles, distilled from phthalic anhydride) in 34 ml of 4Å sieve-dried dichloromethane was added a solution of dicyclohexylcarbodiimide (0.415 g, 2.01 mmoles) in 10 ml of dry dichloromethane. The mixture was stirred at 5° for 0.5 hr, then at room temperature for 45 hr.

Table II—Inhibition of Porcine Plasma Angiotensin Converting Enzyme



R_1	Compound	I_{50} (μM)[a]
H	XIII	2.4 (36)
CH_3	XIV	0.09 (1.3)
$-(CH_2)_2C_6H_5$	XV	0.0038 (0.057)
	Captopril	0.02 (0.30)

[a] Concentration required for 50% enzyme inhibition (1, 6). The numbers in parentheses are relative to a captopril I_{50} of 0.30 μM as reported in Table I.

The reaction mixture was cooled and filtered. The filtrate was washed with ice-cold 2 *N* HCl (25 ml), 0.3 *N* NaOH (25 ml), and water (25 ml), then dried with calcium sulfate and evaporated to dryness. The light yellow solid foam was purified by preparative HPLC with chloroform as solvent. The yield of white solid foam X was 1.06 g (73%); ^{1}H-NMR ($CDCl_3$): 7.00–7.85 (23 H, m, aromatic), 1.85 (4 H, m, proline CH_2, s); R_f 0.30 (chloroform).

Anal.—Calc. for $C_{45}H_{43}N_3O_6 \cdot 0.20\ CHCl_3$: C, 72.48; H, 5.84; N, 5.63. Found: C, 72.81; H, 5.82; N, 5.60.

***N*-[*N*-(2*S*-2-Benzamido-3-phenylpropyl)-*N*-(9-fluorenylmethyloxycarbonyl)aminoacetyl]-L-proline (XI)**—A solution of X (1.01 g, 1.40 mmoles) in 100 ml of glacial acetic acid was hydrogenated at atmospheric pressure with 1 g of 10% palladium on carbon for 2 hr. The reaction was filtered through diatomaceous earth and evaporated to dryness to give 0.900 g of crude compound XI as a gum. A white solid (mp 151–152°) analytical sample of compound XI was obtained by chromatography on a silica gel preparative plate with 7% methanol in chloroform followed by crystallization from chloroform–ether; ^{1}H-NMR ($CDCl_3$): 7.00–7.85 (18 H, m, aromatic), 1.95 (4 H, m, proline CH_2, s); R_f 0.2 (7% methanol in chloroform).

Anal.—Calc. for $C_{38}H_{37}N_3O_6 \cdot 0.60\ CHCl_3$: C, 65.92; H, 5.39; N, 5.97. Found: C, 65.52; H, 5.41; N, 5.90.

***N*-[*N*-(2*S*-2-Benzamido-3-phenylpropyl)aminoacetyl]-L-proline (III)**—Crude compound XI (836 mg, 1.32 mmoles) was stirred in 40 ml of liquid ammonia under nitrogen atmosphere for 3 hr. The ammonia was evaporated and the residue was stirred vigorously for 10 min in a mixture of 20 ml of water and 20 ml of ethyl acetate. The water layer was separated and evaporated to dryness to give a white solid. This was triturated with methanol (10 ml) to give 280 mg (52% yield) of III, mp 200–201°; mass spectrum 553 (M^+ + 2 trimethylsilyl); $[\alpha]_D^{31}$ −92.4° (*c* = 0.953, 1 *N* NH_4OH).

Anal.—Calc. for $C_{23}H_{27}N_3O_4 \cdot 0.25\ CH_3OH$: C, 66.82; H, 6.76; N, 10.06. Found: C, 66.94; H, 6.64; N, 10.00.

***N*-[*N*-Acetyl-*N*-(2*S*-2-benzamido-3-phenylpropyl)aminoacetyl]-L-proline (XII)**—The amino acid (III) (100 mg, 0.244 mmoles) was stirred in 10 ml of water and 10 ml of acetic anhydride for 2-⅓ hr at room temperature. The reaction was evaporated to dryness at 35°. Another 10 ml of water was added to the residue and the mixture was again evaporated to dryness. The residue was dissolved in chloroform (20 ml) and washed with cold 2 *N* HCl (20 ml) and water (20 ml), dried with calcium sulfate, and evaporated to dryness. The gummy residue was crystallized from chloroform–ether to give 58 mg (53% yield) of compound XII as a white solid, mp 156–158°; ^{1}H-NMR ($CDCl_3$–methanol-d_4 2:1): δ 2.01 (3 H, d, acetamide CH_3); mass spectrum 523 (M^+ + trimethylsilyl).

Anal.—Calc. for $C_{25}H_{29}N_3O_5 \cdot 0.25\ H_2O$: C, 65.85; H, 6.52; N, 9.21. Found: C, 65.88; H, 6.39; N, 9.20.

Fluorometric Assay of Angiotensin-Converting Enzyme—Materials used were porcine plasma-converting enzyme[2], hippuryl-histidyl-leucine[3], histidyl-leucine[3], *o*-phthaldehyde[3], and angiotensin I[4]. All chemicals used were reagent grade.

The enzyme assay (9) activity was conducted with fluorometric determination of histidyl-leucine, a product of the enzyme reaction. The substrate used was hippuryl-histidyl-leucine or angiotensin I. The concentration ranges were 2.6×10^{-4}–2.04×10^{-3} *M* and 1.1×10^{-4}–1.4×10^{-3} *M* for hippuryl-histidyl-leucine and angiotensin I, respectively. The

[2] Miles Laboratories.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Bachem Fine Chemicals, Marina Del Rey, Calif.

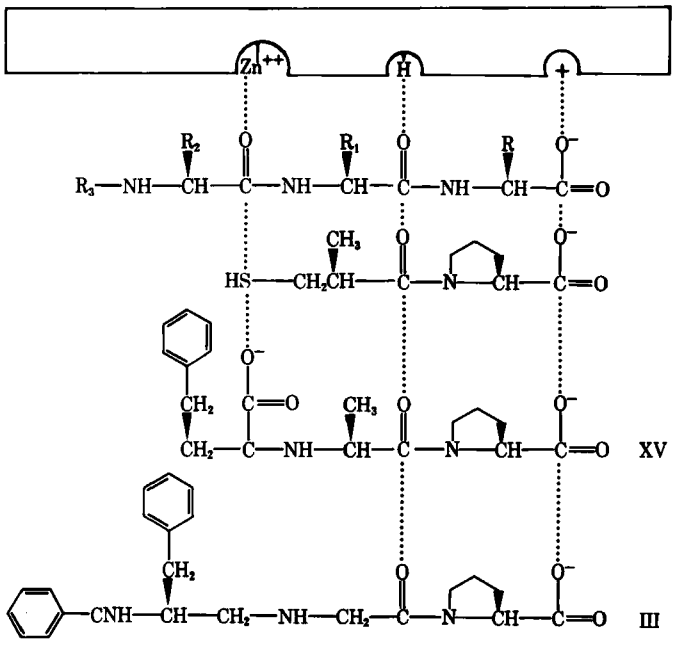


Figure 3—*Schematic representation of the binding of inhibitors and substrates at the hypothetical active site of angiotensin-converting enzyme as proposed previously (8).*

assay was carried out by mixing 10 μl of phosphate buffer (0.1 *M*, pH 7.6, containing 0.3 *M* NaCl) containing the testing inhibitor with substrate dissolved in 10 μl of phosphate buffer. Then 1 mg of porcine plasma-converting enzyme in 50 μl of buffer was added. The mixture was incubated at 37° for 90 min with constant shaking, and the reaction was stopped by adding 50 μl of 10% trichloroacetic acid. The samples were then diluted with 0.7 ml of water followed by 0.4 ml of 2 *N* NaOH. To the alkalized mixture, 0.1 ml of 1% (w/v) *o*-phthaldehyde in methanol was added. After exactly 4 min, 0.2 ml of 6 *N* HCl was added. The contents of all tubes were thoroughly mixed after each addition. The samples were then centrifuged at 10,000×*g* for 10 min, and the fluorescence of the supernatant was measured[5] with excitation at 365 nm and emission at 495 nm. The fluorescent product of histidyl-leucine with *o*-phthaldehyde is not stable in alkaline solution, but is stabilized upon acidification. The fluorescence of the acidified solution is stable up to 1 hr, so all readings should be made within that period (10).

A standard curve of histidyl-leucine was prepared for each assay by mixing various amounts of histidyl-leucine with 1 mg of enzyme in 70 μl of phosphate buffer. The tubes containing the standards were treated identically to those containing the samples. A reagent blank containing all reagents but no substrate was also run for each assay.

Test for the Effect of Inhibitors—For testing the effect of inhibitors on angiotensin-converting enzyme, two assays were run in parallel. One contained the substrate (2 m*M*), enzyme (1 mg), and various concentrations (1 n*M* to 10 m*M*) of an inhibitor; the other contained only the substrate and enzyme. The assay conditions were the same as described above. The product formed with an inhibitor relative to that without an inhibitor was calculated to give the percent of inhibition. By plotting the percent inhibition *versus* various concentrations of an inhibitor, the I_{50} was obtained.

For determination of the kinetics and the K_i of an inhibitor, the enzyme assay was carried out as described above using various concentrations of substrate with and without inhibitor. When hippuryl-histidyl-leucine was used as substrate, the concentrations were 2×10^{-4}–3×10^{-3} *M*. When angiotensin I was used as substrate, the concentrations were 1.1×10^{-4}–1.3×10^{-3} *M*. By using the Michaelis–Menten equation (11) and double-reciprocal plot (12), the K_m and K_i can be calculated.

[5] Aminco Bowman fluorometer.

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