meter fitted with a combination electrode. The pH was maintained by the addition of NaOH. Experiments were carried out at 35 °C. The ionic strength was adjusted to 0.5 with potassium chloride. Initial β-lactam concentrations were 1×10^{-4} M.

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Registry No. 4a, 93712-30-2; 4b, 93712-27-7; 4c, 106139-91-7; 5a, 102652-83-5; 5b, 102652-84-6; 5c, 102652-85-7; 5d, 100239-03-0; 5e, 102652-81-3; 5f, 106139-92-8.

Supplementary Material Available: Table VI–X, unit cell parameters, fractional atomic coordinates, bond lengths, bond angles, and anisotropic temperature factors, respectively (5 pages). Ordering information is given on any current masthead page.

α-Methylproline-Containing Renin Inhibitory Peptides: In Vivo Evaluation in an Anesthetized, Ganglion-Blocked, Hog Renin Infused Rat Model

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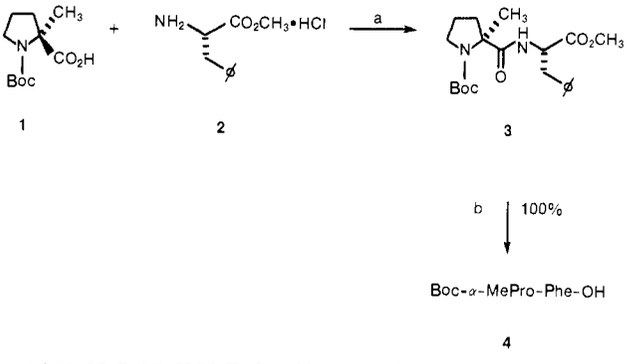
A structure–activity analysis of peptides containing backbone C_α-methyl modification at the P_4 site of the angiotensinogen sequence led to the discovery of potent renin inhibitors with apparent in vitro metabolic stability. Boc-α-MePro-Phe-His-Leuψ[$CHOHCH_2$]Val-Ile-Amp dicitrate (Va) is a potent inhibitor of human plasma renin with an IC_{50} value of 1.8 nM. This peptide was shown not to be degraded in vitro by chymotrypsin, elastase, pepsin, and a rat liver homogenate preparation. It is also a potent inhibitor of hog renin with an IC_{50} value of 1.6 nM and was shown to elicit in vivo activity and cause dose-dependent hypotensive responses when given intravenously to anesthetized ganglion-blocked, hog renin infused rats.

The renin–angiotensin system has been implicated in several forms of hypertension.[1] Renin is an aspartyl protease that is produced mainly in the juxtaglomerular apparatus of the kidney.[2] It is a highly specific proteolytic enzyme and cleaves the circulating α-globulin angiotensinogen, produced by the liver, to form the decapeptide angiotensin I.[3] The N-terminal sequence of human angiotensinogen is shown in Figure 1, the cleavage site being the peptidic bond between amino acids 10 and 11.[4] Angiotensin I has no known biological activity, but it is converted to the octapeptide angiotensin II by the angiotensin-converting enzyme present in lungs and other organs, as a result of the removal of the C-terminal dipeptide histidylleucine. Angiotensin II is a very potent vasoconstrictor and also stimulates the release of aldosterone from the adrenal gland. This mineralocorticoid induces sodium and water retention, contributing to an increase in blood pressure.[3]

The antihypertensive activity of inhibitors of converting enzyme is not clear mechanistically due to its involvement in the kinin system. Renin, however, is an enzyme of high substrate specificity and inhibitors of renin should affect only the renin–angiotensin system.[5] Interest in the blockade of renin has led to rapid development of potent inhibitors based on the angiotensinogen sequence. The most successful approach has been based upon the concept of a transition-state analogue[6] of the amide hydrolysis.

Scheme I. Synthesis of Dipeptide Subunit 4[a]



[a] (a) Et_3N, DCC, HOBT, CH_2Cl_2; (b) NaOH, H_2O, THF; aqueous HCl.

Modifications at the cleavage site to mimic the tetrahedral species have generated analogues of the minimum substrate with high inhibitory potency in vitro.[7]

Many renin inhibitors have been shown to lower blood pressure during intravenous infusion. However, blood pressure usually recovers within minutes after stopping an infusion.[8] Efforts to obtain renin inhibitors with longer duration of action have continued to make progress.[9,10]

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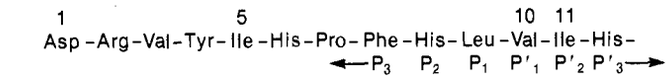
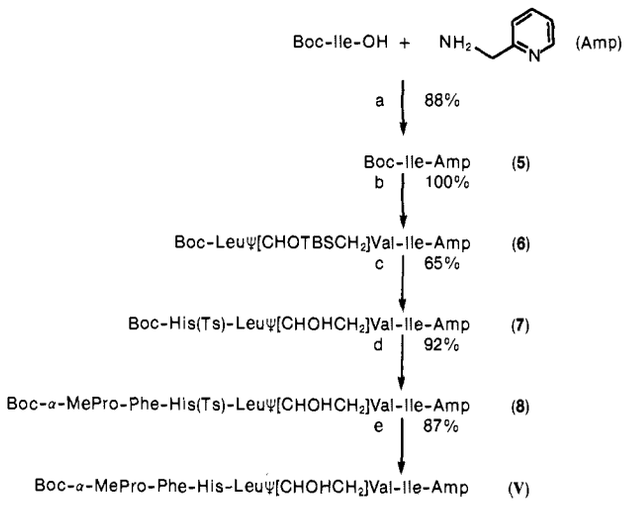


Figure 1. Human angiotensinogen.

Scheme II. Synthesis of Peptide V[a]



[a] (a) DCC, HOBT, CH_2Cl_2; (b) TFA, CH_2Cl_2; Boc-Leuψ-[CHOTBSCH$_2$]Val-OH, DEPC, Et_3N, CH_2Cl_2; (c) HCl(g), ether; Boc-His(Ts)-OH, DEPC, Et_3N, CH_2Cl_2; (d) TFA, CH_2Cl_2; Boc-α-MePro-Phe-OH, DEPC, $(i\text{-Pr})_2NEt$, CH_2Cl_2; (e) HOBT, CH_3OH.

We have initiated a program with the intention of overcoming the problem of short biological half-life of these peptides, and in our previous reports,[10] we described our work on peptide backbone modification that led to the demonstration of an orally active renin inhibitory peptide. The present study reports a variant of this approach that has resulted in enzyme inhibitors with comparable biological half-life. It is our conviction that a focus on in vivo evaluation of compounds with high potency in vitro will be rewarded with better understanding in the design of therapeutically useful compounds in the renin inhibitor area.

Chemistry. The building blocks for the dipeptidic isosteres[11] of the scissile site, 4(*S*)-[(*tert*-butyloxycarbonyl)amino]-3(*S*)-hydroxy-6-methylheptanoic acid (Boc-Sta-OH),[12] *N*-[2(*S*)-[(*tert*-butyloxycarbonyl)-amino]-4-methylpentyl]-L-valine (Boc-Leuψ[CH_2NH]-Val-OH),[13] and 4(*S*)-[(*tert*-butyldimethylsilyl)oxy]-5(*S*)-[(*tert*-butyloxycarbonyl)amino]-2(*S*)-isopropyl-7-methyloctanoic acid (Boc-Leuψ[CHOTBSCH$_2$]Val-OH),[14] were prepared by known procedures. α-Methyl-L-proline was prepared according to Seebach et al.[15]

The N-terminal dipeptide, Boc-α-MePro-Phe-OH (**4**), was synthesized in a straightforward manner (Scheme I). Coupling of Boc-α-methyl-L-proline (**1**) to L-phenylalanine methyl ester (**2**) with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole gave Boc-α-MePro-Phe-OMe (**3**). Alkaline hydrolysis of the ester afforded the desired acid **4**. The peptides were synthesized by solution method, and intermediates from each coupling were purified by chromatography on silica gel. The synthetic sequence for a representative example, peptide V, is shown in Scheme II. The purity of the final peptides I–V was analyzed by reverse-phase analytical HPLC and their identity verified by high-resolution fast-atom-bombardment mass spectroscopy.

Table I. Inhibition of Human Plasma Renin

				IC_{50} (nM.)
Boc-Phe-Phe-	Sta	-Ile-Amp	(I)	24
Boc-α-MePro-Phe-Phe-	Sta	-Ile-Amp	(II)	36
Boc-α-MePro-Phe-His-	Sta	-Ile-Amp	(III)	3.5
Boc-α-MePro-Phe-His-Leuψ	[CH_2NH]	Val-Ile-Amp	(IV)	43
Boc-α-MePro-Phe-His-Leuψ[CHOHCH$_2$]Val-Ile-Amp			(V)	2.0

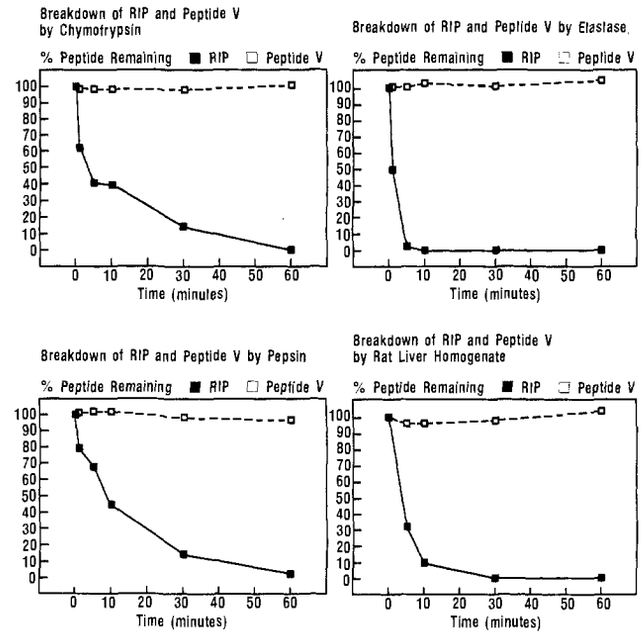


Figure 2. Degradation of RIP and compound V by selected proteases.

In Vitro Renin Inhibition. Competitive inhibitors based upon the N-terminal sequence (Figure 1) of angiotensinogen are the focus of current renin inhibitor design. On the basis of active-site chemistry, transition-state analogues have generated numerous compounds with high inhibitory potency in vitro. We chose to focus our attention on the backbone modification on the N-terminus of the cleavage site in an effort to discover renin inhibitors with greater proteolytic resistance and yet retain high inhibitory potency. As an extension of our previous investigative effort,[10] we replaced the proline residue at P_4 site by α-methyl-L-proline to increase steric hindrance so as to render the resulting peptide more resistant to enzymatic degradation. It was hoped that such structural change would not preclude the bioactive conformer as a consequence of the added conformational constraint.

As shown in Table I, the peptide I[10a] with an IC_{50} value of 2.4×10^{-8} M is a good renin inhibitor and was our

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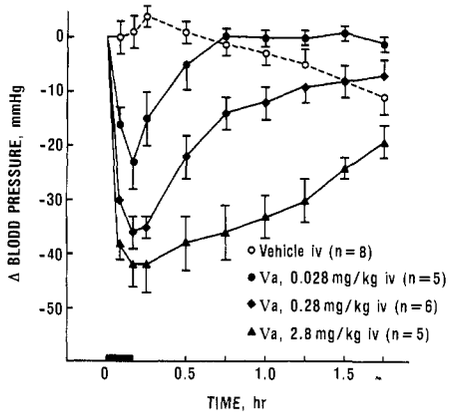

Figure 3. Intravenous administration of compound Va in hog renin infused rats.

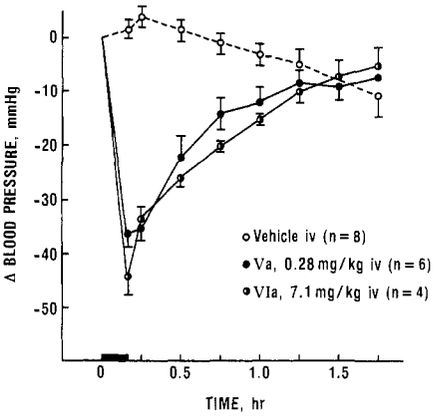

Figure 4. Comparison between compounds Va and VIa at equally potent dose by intravenous administration in hog renin infused rats.

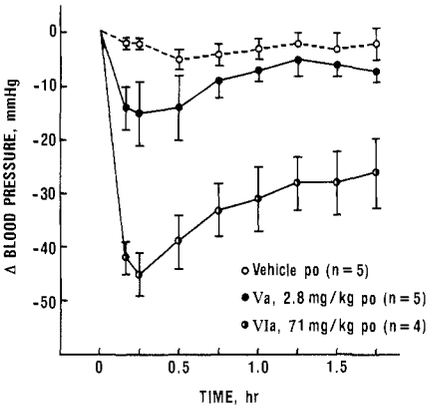

Figure 5. Comparison between compounds Va and VIa at equally potent dose by oral administration in hog renin infused rats.

starting point. Addition of an α-methyl-L-proline residue resulted in peptide II with comparable renin inhibitory activity as that of peptide I. On the basis of this encouraging result, we examined a series of peptides with three different known dipeptide isosteres of the scissile site. Among the peptides III–V, the hydroxyethylene isostere-containing peptide V is the most potent compound with an IC_{50} value of 2 nM.

In Vitro Metabolic Stability. In order to assess some indication of increased metabolic stability, the peptide V was subjected to possible reactions with selected proteases. As shown in Figure 2, it was found to remain essentially unchanged after incubation with chymotrypsin, elastase, and pepsin over 60 min. In contrast, the breakdown of RIP[16] (Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys) by chymotrypsin, elastase, or pepsin was essentially complete. In addition, the peptide V was also found to be completely resistant to a rat liver homogenate preparation over 60 min. With this finding, we proceeded to evaluate the hypotensive efficacy of this compound in vivo.

Hypotensive Efficacy in a Rat Model. The peptide Va which is the dicitrate salt of the parent peptide V of better solubility characteristics happened to be a potent inhibitor of hog renin with an IC_{50} value of 1.6 nM. This presented us with an opportunity to evaluate the in vivo consequence of the apparent in vitro metabolic stability via experiments in a rat model.[10b]

Male Sprague–Dawley rats were anesthetized with dial-urethane and then ganglion blocked with mecamylamine after surgical preparation. An infusion of hog renin was used to raise the blood pressure to approximately 120 mmHg, the level usually present prior to ganglionic blockade. The magnitude of the hog renin dependent blood pressure component was approximately 60 mmHg. Validation of the model for oral absorption studies was effected by the oral administration of Captopril at 5 mg/kg and resulted in a complete neutralization of the pressor effect of the hog renin infusion within 60 min.

Figure 3 illustrates the results of experiments with the peptide Va. The intravenous administration of 0.028, 0.28, and 2.8 mg/kg in the 0.1 M citric acid vehicle elicited dose-dependent hypotensive responses. Both the magnitude of the hypotension and the duration of the responses appeared to be a function of dose.

To place the in vivo results of Boc-α-MePro-Phe-His-Leuψ[$CHOHCH_2$]Val-Ile-Amp dicitrate (Va) in proper perspective, we compared the pharmacological response to that elicited by Boc-Pro-Phe-N-MeHis-Leuψ-[$CHOHCH_2$]Val-Ile-Amp dicitrate (VIa). Compound VI has been previously reported[10b] as an orally active renin inhibitor. Figure 4 compares the hypotensive responses in this rat model after intravenous administration of 0.216 μmol/kg (0.28 mg/kg) dose of compound Va and 5.4 μmol/kg (7.1 mg/kg) dose of compound VIa in 0.1 M citric acid vehicle. These are presumed equipotent doses since the IC_{50} values against hog renin for compounds Va and VIa were found to be 1.6 and 40 nM, respectively. The hypotensive responses were nearly identical in magnitude and duration of action.

We have further compared the hypotensive efficacy of these two compounds after oral administration. It has been demonstrated previously[10b] that compound VI at 54 μmol/kg dose given orally evoked approximately the same hypotensive response as an intravenous administration of 5.4 μmol/kg dose in 0.1 M citric acid vehicle. Again making provision for the differential in vitro inhibitory potency, compound Va was given at 2.16 μmol/kg dose orally as an equipotent dose to compound VIa at 54 μmol/kg. Figure 5 illustrates the comparative responses. The results indicate that compound Va proved to be much less efficacious than the previously reported compound VIa after oral administration of equipotent doses.

Discussion

As emphasized in our previous report,[10a] we cannot overlook the *tert*-butyloxycarbonyl group, the 2-pyridylmethylamine functionality, and the dipeptide isostere as contributors to the metabolic stability of peptide V. We should also bear in mind that apparent in vitro stability is not necessarily sufficient for a compound to elicit the expected pharmacological response. In this study, the

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modification at P_4 site with an α-methyl-L-proline moiety resulted in peptide Va, which was demonstrated to effect the desired pharmacological response.

It is gratifying to observe that compound Va given intravenously elicited a hypotensive response in a manner comparable to that of a previously reported compound VIa, which was given at 25 times higher dose. The apparently simple extrapolation of the in vitro inhibitory potency to the in vivo pharmacological responses between the two compounds might not have been totally anticipated. It suggests that these two renin inhibitors are removed from circulation at a similar rate, resulting in a comparable duration of action. It remains to be seen whether this will be generally observed for class of compounds in this series. Related work on proteolytically stable renin inhibitors[17] suggested that biliary clearance was the major factor effecting the observed duration of action.

In sharp contrast, results from the oral study pointed to a significant difference in their hypotensive responses after oral administration to rats. Compound Va proved to be much less efficacious than an equally potent dose of compound VIa (given at 25 times the dosage of compound Va). We do not as yet have data to suggest factors contributing to this observed disparity. Since these two compounds have the same molecular weight and are structurally closely related, it would be of interest to pursue other structural characteristics that perhaps contribute to the absorption process and the apparent metabolic stability after oral administration. We plan to continue evaluating a number of renin inhibitors in vivo to gather more information in the hope of addressing questions relevant to this problem.

Summary. We have demonstrated that substituting an α-methyl-L-proline at the P_4 site of the angiotensinogen sequence gave potent renin inhibitors with apparent in vitro metabolic stability. Boc-α-MePro-Phe-His-Leuψ-[$CHOHCH_2$]Val-Ile-Amp (V) is a potent inhibitor of renin and showed enzymatic resistance in vitro. The high inhibitory potency of the corresponding dicitrate Va (1.6 nM) against hog renin made it possible for it to be evaluated as a hypotensive agent in an anesthetized, ganglion-blocked, hog renin infused rat model. Dose-dependent hypotensive responses were elicted after bolus intravenous administration. The magnitude and duration of response were comparable to those of Boc-Pro-Phe-*N*-MeHis-Leuψ[$CHOHCH_2$]Val-Ile-Amp dicitrate (VIa), a previously reported orally active congener. The in vivo responses after intravenous administration could be directly correlated to the in vitro renin inhibitory potencies and suggested similar rates of removal from circulation. Compound Va was much less efficacious, however, when administered orally, and the reasons for this disparity remained to be elucidated.

Experimental Section

Chemistry. Mass spectra, infrared spectra, optical rotations, melting points, and combustion analyses were obtained by the Physical and Analytical Chemistry Department of The Upjohn Co. ^1H NMR spectra were recorded at 80 MHz with a Varian Model CFT-20 Fourier transform spectrometer. Chemical shifts were reported as δ units relative to tetramethylsilane as internal standard.

Thin-layer chromatography was conducted with Analtech 0.25-mm glass plates precoated with silica gel GF. For column chromatography, E. Merck silica gel 60, 230–400 mesh, was used. All solvents for chromatography were Burdick and Jackson reagent grade distilled in glass.

Dichloromethane was distilled from phosphorus pentoxide. Triethylamine and diisopropylethylamine were distilled from calcium hydride. Diethylphosphoryl cyanide was freshly distilled before use.

Peptides I–V were analyzed on a Perkin-Elmer Series 4 liquid chromatograph with a Kratos Spectroflow 773 detector (254 nm) and a Perkin-Elmer LCI-100 integrator using a Brownlee RP-18, 10 μm, 25 cm × 4.6 mm analytical column at a flow rate of 1.5 mL/min. The mobile phase for peptides I and II was an isocratic mixture of 75% methanol and 25% aqueous phosphate pH 3 buffer and for peptides III–V was an isocratic mixture of 90% methanol and 10% aqueous phosphate pH 3 buffer.

4(*S*)-[(*tert*-Butyloxycarbonyl)amino]-3(*S*)-hydroxy-6-methylheptanoic acid (Boc-Sta-OH) was prepared according to Rich et al.[12] *N*-[2(*S*)-[(*tert*-Butyloxycarbonyl)amino]-4-methylpentyl]-L-valine (Boc-Leuψ[CH_2NH]Val-OH) was prepared in a similar manner to that of Szelke et al.[13] 4(*S*)-[(*tert*-Butyldimethylsilyl)oxy]-5(*S*)-[(*tert*-butyloxycarbonyl)amino]-2(*S*)-isopropyl-7-methyloctanoic acid (Boc-Leuψ[CHOTBS-CH_2]Val-OH) was prepared according to Hester and Emmert.[14] α-Methyl-L-proline was prepared according to Seebach et al.[15]

General Procedure A. Removal of the *tert*-Butyloxycarbonyl Group with Dry HCl. Dry HCl gas was passed into a solution of the substrate in ether. The resulting mixture was allowed to stir at room temperature for an additional 20 min, and then the volatile components were removed with a stream of nitrogen. The resulting solid residue was dried in vacuo.

General Procedure B. Removal of the *tert*-Butyloxycarbonyl Group with Trifluoroacetic Acid. A solution of the substrate in equal volume of dichloromethane and trifluoroacetic acid was allowed to stir at room temperature for 0.5–2 h. The reaction mixture was then concentrated and the residue treated with excess aqueous $NaHCO_3$. The aqueous phase was extracted with several portions of dichloromethane. The combined organic phase was dried ($MgSO_4$) and then concentrated to give the free amine.

General Procedure C. Coupling Reaction with Dicyclohexylcarbodiimide/1-Hydroxybenzotriazole. To a stirred solution of the acid component and the amine component in dichloromethane was added a slight excess of 1-hydroxybenzotriazole, followed by a slight excess of dicyclohexylcarbodiimide. After stirring at room temperature for 10–24 h, the resulting mixture was filtered and the filtrate washed with saturated aqueous $NaHCO_3$. The organic phase was dried ($MgSO_4$) and then concentrated. The residue was then purified by chromatography on silica gel.

General Procedure D. Coupling Reaction with Diethylphosphoryl Cyanide. To a stirred solution of the acid component and the amine component in dichloromethane was added a slight excess of triethylamine, followed by slow addition of a slight excess of diethylphosphoryl cyanide. After stirring at room temperature for 2–12 h, the reaction mixture was diluted with dichloromethane and then washed with saturated aqueous $NaHCO_3$. The organic phase was dried ($MgSO_4$) and then concentrated. The residue was then purified by chromatography on silica gel.

General Procedure E. Removal of the *p*-Toluenesulfonyl Group from Histidine. A solution of the peptide substrate and 3–5 equiv of 1-hydroxybenzotriazole in methanol was allowed to stir at room temperature for 12–24 h. The reaction mixture was then concentrated and the residue purified by chromatography on silica gel.

***N*-(*tert*-Butyloxycarbonyl)-α-methyl-L-proline (1).** To a stirred solution of 796 mg (3.8 mmol) of α-methyl-L-proline hydrobromide in 4.0 mL of 4:1 (v/v) DMF–water was added 306 mg (7.65 mmol) of NaOH in 1.0 mL of water. To this was added 1.173 g (4.76 mmol) of 2-[[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile, and the mixture was heated with stirring overnight. The mixture was then partitioned between ether and water and the aqueous phase withdrawn, acidified with dilute HCl, and extracted repeatedly with dichloromethane. The organic extracts were dried ($MgSO_4$), and the solvent was removed under reduced pressure to give 0.775 g (3.38 mmol, 89%) of a white solid (1): ^1H NMR ($CDCl_3$) δ 1.46 (s, 9 H), 1.58 (s, 3 H), 1.65–2.8 (m, 4 H), 3.24–3.64 (m, 2 H), IR (mull) 1735, 1625 cm^{-1}; $[\alpha]_D$ −45° (*c*

(17) Boger, J.; Bennett, C. D.; Payne, L. S.; Ulm, E. H.; Blaine, E. H.; Homnick, C. F.; Schorn, T. W.; Lamont, B. I.; Veber, D. F. *Regul. Pept.* **1985**, Suppl 4, 8.

Table II. Characterization of Renin Inhibitory Peptides

peptides[a]	HPLC:[b] k' (partition ratio)	amino acid anal.			FAB MS	
		Phe	His	Ile	calcd	found
II	8.39	1.91		1.00	884.5285	884.5249
III	7.72	1.00	0.95	0.99	874.5190	874.5172
IV	11.7	1.07	1.01	1.03	915.5820	915.5788
V	11.3	0.97	0.97	0.97	930.5816	930.5841

[a] ^{1}H NMR spectra were consistent with the assigned structures. [b] See conditions at the beginning of the Experimental Section.

1.01, $CHCl_3$); FAB MS, $[M + H]^+$ at m/z 230.1392 (calcd 230.1392).

***N*-(*tert*-Butyloxycarbonyl)-α-methyl-L-prolyl-L-phenylalanine Methyl Ester (3).** According to general procedure B, to 118 mg (0.513 mmol) of Boc-α-MePro-OH (1), 123 mg (0.568 mmol) of phenylalanine methyl ester hydrochloride, and 83.8 mg (0.62 mmol) of 1-hydroxybenzotriazole with 79 μL (0.57 mmol) of triethylamine in 5.0 mL of dichloromethane was added 117 mg (0.567 mmol) of dicyclohexylcarbodiimide. Chromatography of the residue on silica with 2% methanol–dichloromethane provided 211 mg (quantitative) of the dipeptide **3**: ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 9 H), 1.56 (s, 3 H), 1.25–2.5 (m, 4 H), 3.09 (t, J = Hz, 2 H), 3.20–3.53 (m, 2 H), 3.74 (s, 3 H), 4.60–4.95 (m, 1 H), 7.37 (br s, 5 H); IR (mull) 3330, 1740, 1690, 1650 cm^{-1}; $[\alpha]_D$ −58° (*c* 0.744, $CHCl_3$); FAB MS, $[M + H]^+$ at m/z 391.2254 (calcd 391.2233).

***N*-(*tert*-Butyloxycarbonyl)-α-methyl-L-prolyl-L-phenylalanine (4).** To 200 mg (0.512 mmol) of the dipeptide ester **3** in 3 mL of THF was added 3 mL of 1 N aqueous NaOH. The heterogeneous mixture was stirred vigorously for 2.5 h and then most of the THF was removed under a stream of N_2. The residue was partitioned between ether and water, and the aqueous phase was separated, acidified with dilute HCl, and extracted with CH_2Cl_2. Drying ($MgSO_4$) and solvent removal afforded 197 mg (quantitative) of a clear, colorless oil (**4**): ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 9 H), 1.53 (s, 3 H), 1.25–2.4 (m, 4 H), 3.17 (t, J = 7 Hz, 2 H), 3.1–3.52 (m, 2 H), 4.65–5.00 (m, 1 H), 7.4 (br s, 5 H); IR (mull) 1880, 1740, 1700, 1680, 1640 cm^{-1}; $[\alpha]_D$ −67° (*c* 0.44, $CHCl_3$); FAB MS, $[M + H]^+$ at m/z 377.2073 (calcd 377.2076).

Preparation of Peptide V as a Representative Example. *N*-[*N*-(*tert*-Butyloxycarbonyl)-L-isoleucyl]-2-pyridylmethylamine (5). According to general procedure C, 9.25 g (40 mmol) of Boc-Ile-OH, 4.2 mL (40.7 mmol) of 2-(aminomethyl)-pyridine, 5.95 g (44 mmol) of 1-hydroxybenzotriazole, and 8.66 g (42 mmol) of dicyclohexylcarbodiimide in 40 mL of dichloromethane afforded 11.34 g (35.3 mmol, 88%) of Boc-Ile-Amp after chromatography on silica gel with ethyl acetate: ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3 H, J = 7 Hz), 0.93 (d, 3 H, J = 7 Hz), 1.42 (s, 9 H), 4.05 (dd, 1 H, J = 6, 9 Hz), 4.57 (d, 2 H, J = 5 Hz), 5.12 (br d, 1 H, J = 9 Hz), 7.21 (m, 2 H), 7.65 (m, 1 H), 8.54 (br d, 1 H, J = 5 Hz).

***N*-[*N*-[4(*S*)-[(*tert*-Butyldimethylsilyl)oxy]-5(*S*)-[(*tert*-butyloxycarbonyl)amino]-2(*S*)-isopropyl-7-methyloctanoyl]-L-isoleucyl]-2-pyridylmethylamine (6).** According to general procedure B, 11.3 g (35.2 mmol) of Boc-Ile-Amp (**5**) in 25 mL of dichloromethane and 25 mL of trifluoroacetic acid afforded 7.76 g (35.1 mmol, 100%) of H-Ile-Amp after the aqueous phase was continuously extracted with dichloromethane for 1 day and the resulting residue chromatographed on silica gel with 3% methanol and 3% methanol (saturated with ammonia) in dichloromethane.

According to general procedure D, 4.457 g (10.0 mmol) of Boc-Leuψ[CHOTBS-CH_2]Val-OH, 2.434 g (11.0 mmol) of H-Ile-Amp, 1.53 mL (11.0 mmol) of triethylamine, and 1.69 mL (11.0 mmol) of diethylphosphoryl cyanide in 60 mL of dichloromethane afforded 6.50 g (10.0 mmol, 100%) of compound **6** after chromatography on silica gel with 20% hexane in ethyl acetate: ^{1}H NMR ($CDCl_3$) δ 0.11 (s, 6 H), 0.91 (m, 18 H), 1.43 (s, 9 H), 7.20 (m, 2 H), 7.64 (m, 1 H), 8.54 (br d, 1 H, J = 5 Hz).

***N*-[*N*-[*N*-(*tert*-Butoxycarbonyl)-N^{im}-tosyl-L-histidyl]-5(*S*)-amino-4(*S*)-hydroxy-2(*S*)-isopropyl-7-methyloctanoyl]-L-isoleucyl]-2-pyridylmethylamine (7).** According to general procedure A, 6.36 g (9.8 mmol) of compound **6** in 150 mL of ether saturated with gaseous HCl afforded the amine hydrochloride.

According to general procedure D, 5.2 g (12.7 mmol) of *N*-(*tert*-butyloxycarbonyl)[-N^{im}-tosyl-L-histidine, the above amine, 4.2 mL (30 mmol) of triethylamine, and 1.9 mL (12.7 mmol) of diethylphosphoryl cyanide in 50 mL of dichloromethane afforded 5.3 g (6.4 mmol, 65%) of compound **7** after chromatography on silica gel with 85% ethyl acetate in hexane: ^{1}H NMR ($CDCl_3$) δ 0.8 (m, 27 H), 1.30 (s, 9 H), 2.30 (s, 3 H), 5.83 (d, 1 H, J = 7.5 Hz), 6.22 (d, 1 H, J = 9 Hz), 6.56 (d, 1 H, J = 9 Hz), 8.38 (d, 1 H, J = 5 Hz).

***N*-[*N*-[*N*-[*N*-[(*tert*-Butyloxycarbonyl)-α-methyl-L-prolyl-L-phenylalanyl]-N^{im}-tosyl-L-histidyl]-5(*S*)-amino-4(*S*)-hydroxy-2(*S*)-isopropyl-7-methyloctanoyl]-L-isoleucyl]-2-pyridylmethylamine (8).** According to general procedure B, 3.30 g (4.0 mmol) of compound **7** in 10 mL of dichloromethane and 10 mL of trifluoroacetic acid afforded 3.42 g of the free amine after neutralization with aqueous $NaHCO_3$.

According to general procedure D, 1.57 g (4.2 mmol) of the acid **4**, the above amine, 0.77 mL (4.4 mmol) of diisopropylethylamine, and 0.67 mL (4.4 mmol) of diethylphosphoryl cyanide in 40 mL of dichloromethane afforded 3.99 g (3.68 mmol, 92%) of compound **8** after chromatography on silica gel with 5% methanol in ethyl acetate: ^{1}H NMR ($CDCl_3$) δ 0.90 (m, 18 H), 1.31 (s, 9 H), 1.48 (s, 3 H), 2.39 (s, 3 H), 8.47 (d, 1 H, J = 5 Hz).

***N*-[*N*-[*N*-[*N*-[(*tert*-Butyloxycarbonyl)-α-methyl-L-prolyl-L-phenylalanyl]-L-histidyl]-5(*S*)-amino-4(*S*)-hydroxy-2(*S*)-isopropyl-7-methyloctanoyl]-L-isoleucyl]-2-pyridylmethylamine (V).** According to general procedure E, 3.99 g (3.68 mmol) of compound **8** and 2.0 g (14.8 mmol) of 1-hydroxybenzotriazole in 30 mL of methanol afforded 2.99 g (3.21 mmol, 87%) of peptide V after chromatography on silica gel with 7% methanol (saturated with gaseous ammonia) in ethyl acetate: ^{1}H NMR ($CDCl_3$) δ 0.87 (m, 18 H), 1.39 (s, 9 H), 1.50 (s, 3 H), 8.47 (d, 1 H, J = 5 Hz).

The dicitrate Va was prepared by mixing 2.99 g (3.21 mmol) of compound V and 1.35 g (6.42 mmol) of citric acid monohydrate in 20 mL of methanol. The resulting solution was concentrated and the residue was dissolved in 60 mL of water with sonication. The aqueous solution was then lyophilized to give 4.2 g of a white solid, HPLC and FAB MS of which were identical with that of compound V.

Peptides II–IV were prepared in a similar manner to the synthesis of peptide V. The physical characteristics for the final products II–V are listed in Table II.

Biology. Inhibition of Human Plasma Renin. Compounds I–V were assayed for plasma renin inhibitory activity as follows: Lyophilized human plasma with 0.1% EDTA was obtained commercially (New England Nuclear). The angiotensin I generation step utilized 250 μL of plasma, 2.5 μL of phenylmethanesulfonyl fluoride, 25 μL of maleate buffer (pH 6.0), and 10 μL of an appropriate concentration of inhibitor in a 1% Tween 80 in water vehicle. Incubation was for 90 min at 37 °C. Radioimmunoassay for angiotensin I was carried out with a commercial kit (Clinical Assays). Plasma renin activity values for inhibitor tubes were compared to control tubes to estimate percent inhibition. The inhibition results were expressed as IC_{50} values, which were obtained by plotting three to four inhibitor concentrations on semilog graph paper and estimating the concentration producing 50% inhibition.

Inhibition of Hog Renin. IC_{50} values of the renin inhibitors against hog renin were determined in the following manner. One unit of lyophilized hog renin (Sigma, catalog no. R-6127) was reconstituted with 1.0 mL of Tris-acetate pH 7.4 buffer. This solution was further diluted 8100× with additional Tris-acetate buffer. Tetradecapeptide (TDP; Peninsula, code no. 7004) was used as the substrate. TDP was prepared as a 0.155 mg/mL solution in sodium phosphate buffer. The sodium phosphate buffer consisted of 0.15 M Na_2HPO_4, 0.16 M NaCl, 3 mM EDTA, and 0.4% NaN_3. The buffer was adjusted to pH 6.0 with HCl.

Compounds were dissolved in 100 μL of dimethyl sulfoxide and then diluted with Na_2HPO_4 buffer to achieve the desired concentrations. Several inhibitor concentrations were run in an attempt to bracket the estimated IC_{50} values.

Each incubation mixture of 340 μL contained the following components: 200 μL of sodium phosphate buffer, 10 μL of pmsf, 20 μL of hog renin, 50 μL of TDP, and 60 μL of the inhibitor. Incubation mixtures without inhibitor were run for comparison and contained 60 μL of Na_2HPO_4 buffer in place of the inhibitor. The reaction mixtures were incubated at 37 °C for 30 min in a shaking water bath. An identical set of incubation mixtures remained on ice during the generation step.

Following the incubation period, each mixture was radioimmunoassayed in duplicate as described above. The amount of angiotensin I formed in tubes that contained inhibitor was compared to the reaction tubes without inhibitor to yield a percent inhibition. IC_{50} values were obtained by plotting several inhibitor concentrations and estimating the concentration producing 50% inhibition.

Susceptibility of RIP and Peptide V to Proteolytic Actions. Preparation of Rat Liver Homogenate. A male rat (Upjohn, Sprague-Dawley) weighing 300 g was sacrificed by decapitation, and the liver was quickly removed and chilled on ice in an aluminum dish. The liver was homogenized in 10 volumes (w/v) of 50 mM Tris·HCl, pH 7.5, using a Brinkman Polytron at setting 8 for 60 s. The homogenate was centrifuged at 30000*g* for 60 min and the supernatant collected and stored at −90 °C in 1-mL aliquots.

Preparation of Peptide Solutions. Peptides to be tested against chymotrypsin, elastase, or liver homogenate were first dissolved in Me_2SO, then 50 mM Tris·HCl, pH 7.5 buffer was added to make a 0.125 mM solution of peptide containing 10% Me_2SO. Peptides to be tested against pepsin were dissolved in 50 mM HCl to give a concentration of 0.25 mM.

Digestion of Peptides with Chymotrypsin and Elastase. To 0.25 mL of peptide solution was added either 5 μL of 25 units/mL α-chymotrypsin (E.C. 3.4.21.1, Sigma), dissolved in 50 mM Tris·HCl, pH 7.5, or 5 μL of 50 units/mL elastase (E.C. 3.4.21.11, Sigma), dissolved in 50 mM Tris·HCl, pH 7.5. Incubation was performed at 37 °C and terminated by the addition of 0.25 mL of 80% acetonitrile–20% water containing 0.2% TFA.

Digestion of Peptides with Pepsin. To 0.25 mL of peptide solution was added 0.25 mL of 0.01 mg/mL pepsin (E.C. 3.4.23.1, Difco) dissolved in 50 mM HCl. Samples were incubated at 37 °C, and the digestion reaction was terminated by the addition of 0.5 mL of 80% acetonitrile–20% water containing 0.2% TFA.

Digestion of Peptides with Liver Homogenate. To 0.2 mL of peptide solution was added 0.05 mL of liver homogenate (as described above) and the mixture was incubated at 37 °C. Proteolytic enzymes in the homogenate were inactivated by the addition of 0.25 mL of 80% acetonitrile–20% water containing 0.2% TFA.

HPLC Conditions for Analysis of Reaction Mixtures. The proteolytic digestion of RIP and peptide V was monitored by using HPLC separation of the parent compound and any peptide fragments generated during incubation under the four assay conditions described above. Separation was performed on a 25 cm × 4.1 mm SynChropak RP-P column using a flow rate of 1.5 mL/min and a gradient starting at 10% solvent B and increasing linearly to 75% solvent B over 35 min. HPLC solvent A was 50 mM NaH_2PO_4, 1% H_3PO_4, in water. HPLC solvent B was 12.5 mM NaH_2PO_4, 0.25% H_3PO_4, in 25% water–75% acetonitrile. Column effluent was monitored by UV absorption at 204 nM.

Hypotensive Evaluation in the Rat Model. The ganglion-blocked hog renin infused rat model has been described in detail elsewhere.[10b] Briefly, Sprague–Dawley rats were anesthetized with dial-urethane, 100 mg/kg ip. Each animal was tracheostomized and bilaterally vagotomized. One carotid artery and both jugular veins were catheterized. In some animals that had fasted for 24 h before being anesthetized, an infant feeding tube was passed into the stomach through the mouth for the oral administration of compounds. Mecamylamine at 1.25 mg/kg iv was utilized to elicit ganglionic blockade. An intravenous infusion of partially purified hog renin at 0.15 goldblatt unit kg^{-1} min^{-1} was used to maintain blood pressure at the level present before ganglionic blockade. The partially purified hog kidney renin had been prepared by a slightly modified literature procedure.[18] The compounds were dissolved in 0.1 M citric acid and infused intravenously at 0.05 mL/min for 10 min or administered into the stomach as a 5 mL/kg bolus.

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