der Chemischen Industrie, and the Matthias Lackas-Stiftung for financial support.

Registry No. **1a**, 5883-83-0; **1b**, 62655-56-5; **1c**, 91444-16-5; **2a**, 115119-02-3; **2b**, 115119-03-4; **2c**, 115119-04-5; **3c**, 115119-05-6; **4a**, 115119-06-7; **4b**, 115119-07-8; **4c**, 115084-37-2; **5c**, 115119-08-9; **6a**, 115119-09-0; **6b**, 115119-10-3; **6c**, 115119-11-4; **7c**, 115119-12-5; **8a**, 115119-13-6; **8b**, 115119-14-7; **8c**, 115084-38-3; **9c**, 115119-15-8; **10a**, 115119-16-9; **10b**, 115119-17-0; **10c**, 115119-18-1; **11c**, 115119-19-2; **12a**, 115119-20-5; **12b**, 115119-21-6; **12c**, 115084-39-4; **13c**, 115119-22-7; **14a**, 115140-97-1; **14b**, 115140-98-2; **14c**, 115160-74-2; **15c**, 115140-99-3; **16a**, 115160-75-3; **16b**, 115141-00-9; **16c**, 115141-01-0; **17c**, 115141-02-1; $Br(CH_2)_4Br$, 110-52-1; $Br(CH_2)_5Br$, 111-24-0; $Br(CH_2)_6Br$, 629-03-8; $BrCH_2C_6H_4$-*p*-CH_2Br, 623-24-5; K_2PtCl_4, 10025-99-7; $NH_2(CH_2)_2NH_2$, 107-15-3; 17β-estradiol, 50-28-2.

Supplementary Material Available: ^1H NMR data of 1-(bromoalkyl)-2-phenylindoles **2–5**, 1-[[(2-aminoethyl)amino]alkyl]-2-phenylindoles **6–9**, dihydroxy-1-[[(2-aminoethyl)amino]-alkyl]-2-phenylindoles **10–13**, and *N*-[ω-(dihydroxy-2-phenylindol-1-yl)alkyl]-1,2-diaminoethane dichloroplatinum(II) complexes **14–17** (5 pages). Ordering information is given on any current masthead page.

Design of Rat Renin Inhibitory Peptides†

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Because several well-studied strains of rats manifest spontaneous hypertension, we set out to design a renin inhibitor suitable for use in this species. On the basis of the sequence of the renin substrate, a series of substrate analogue inhibitory peptides were synthesized by systematically modifying the P5, P3, P2, P1P1′, P2′, P3′, and P4′ positions. In assays against rat plasma renin, we found that modifications at the C-terminal segment have a marked influence on potency, and that a secondary butyl side chain at the P2′ position is important for obtaining optimal activity. The structure at the P3′ position, however, could vary considerably without significant effect. The steric effect of the P2 position was important; there an isopropyl side chain provided optimal binding between the inhibitor and the enzyme. At the P3 and P5 positions, potency appeared to depend on aromatic side chains. The effects at the P1P1′ position of the transition-state residue (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (statine) and its congeners (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA) and (3*S*,4*S*)-4-amino-3-hydroxy-5-cyclohexylpentanoic acid (ACHPA) were found to depend on the sequence of the C-terminal segment. For peptides with an unfavorable C-terminal segment (-Ile-Phe-NH_2), AHPPA and ACHPA resulted in a surprising retention of potency. For peptides with a favorable C-terminal segment (-Leu-Phe-NH_2), the effect of AHPPA was mild, even though ACHPA still significantly enhanced potency. The hypotensive and plasma renin inhibitory effects of three of the analogues were then studied in anesthetized sodium-depleted rats. One of the compounds, acetyl-His-Pro-Phe-Val-Statine-Leu-Phe-NH_2 (IC_{50} against rat plasma renin of 30 nM at pH 7.4), proved to be a potent hypotensive agent and a potentially useful probe for the study of the renin–angiotensin system in rats.

The renin–angiotensin system plays an important role in the maintenance of blood pressure and electrolyte balance.[1] Its precise contribution to the development of essential hypertension, however, is still not known. Because converting enzyme inhibitors (compounds in wide clinical use for the treatment of hypertension) also affect bradykinin[2] and prostaglandin,[3] they cannot be used as pharmacologic probes. For this reason potent and specific inhibitors of the renin–angiotensin system would help us understand a variety of clinical and experimental forms of the disease. In recent years transition-state substrate analogues have been shown to be effective inhibitors of primate renin, with IC_{50} values in the subnanomolar range.[4-9] Although some studies are being made of their hypotensive effect in vivo, little use has been made of these analogues in studies of the role of the renin–angiotensin system in the pathogenesis of hypertension.

Physiological studies in the primate are both difficult and expensive, and well-defined genetic models of hypertension have not been developed in this species. A potent inhibitor of rat renin, suitable for use in vivo, would be of great experimental value because of the considerable body of hypertensive studies in this species, the availability of a number of genetically determined models of spontaneous hypertension, and the relative abundance and low cost of these animals. However, because renins of various species differ considerably in both structure and substrate specificity, structural knowledge of primate renin inhibitors cannot be directly extrapolated to the design of rat renin inhibitors.

Recently a substrate analogue with a hydroxyethylene component at the scissile bond position was reported to have an IC_{50} value against rat plasma renin of 0.8 nM.[10]

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Table I. Synthetic Renin Substrate Analogues

no.[a]	purification[b] method	t[c]	amino acid anal.[d]
1	PHPLC (20)	6.57 (a)	Pro 2.10; His 2.11; Phe 1.07; Tyr 1.82; Sta 1.11
2	PHPLC (17)	6.27 (d)	Pro 1.70; His 2.00; Phe 1.07; Tyr 1.96; Lys 0.96; Sta 1.10
3	PHPLC (17)	6.22 (a)	Pro 2.00; His 2.30; Phe 1.18; Tyr 2.25; Lys 1.13; Ser 0.91; Sta 1.40
4[9]			
5	PHPLC (20)	5.99 (a)	Pro 2.28; His 2.00; Phe 1.04; Thr 2.00; Sta 1.20
6[9]			
7	PHPLC (25)	5.72 (b)	Pro 1.99; His 1.97; Phe 1.18; Leu 1.04; Sta 1.10
8	G-10	6.09 (b)	Pro 2.10; His 2.02; Phe 2.01; Leu 0.97; Sta 0.79
9	PHPLC (27)	6.38 (b)	Pro 1.02; His 1.98; Phe 2.00; Leu 1.03; Sta 1.01
10	PHPLC (35)	6.91 (b)	Pro 0.97; His 1.91; Phe 2.06; CHA 0.97; Sta 0.93
11	PHPLC (35)	6.14 (b)	Pro 0.96; His 1.92; Phe 2.08; tLeu 0.99; Sta 0.97
12	PHPLC (28)	6.06 (b)	Pro 0.95; His 1.88; Phe 2.15; Val 0.92; Sta 1.04
13	PHPLC (33)	6.76 (b)	Pro 0.97; His 1.88; Phe 1.13; Leu 0.90; CHA 0.97; Sta 0.99
14	PHPLC (23)	5.92 (b)	Pro 0.96; His 1.99; Phe 1.09; Leu 0.98; Val 0.94; Sta 0.99
15	G-10	7.13 (c) 2.45 (e)	Pro 1.07; His 1.00; Phe 2.23; Val 0.94; Leu 0.90; Sta 0.77
16	PHPLC (40)	7.71 (b)	Pro 0.98; His 1.02; Phe 2.16; Leu 1.77; Sta 1.02
17	G-10	7.40 (b)	Pro 1.00; His 1.03; Phe 2.14; tLeu 0.94; Leu 0.93; Sta 0.89
18	PHPLC (50)	8.38 (b)	Pro 0.93; His 0.98; Phe 2.06; Leu 0.88; CHA 1.00; Sta 1.00
19	G-10	7.67 (b)	Pro 0.93; His 0.96; Phe 2.22; Leu 1.88; Sta 1.08
20	PHPLC (33)	6.97 (b)	Pro 1.00; His 1.04; Phe 2.07; Ala 0.97; Leu 0.87; Sta 0.99
21	PHPLC (34)	6.25 (b) 4.81 (d)	Pro 0.95; His 1.86; Phe 1.13; Leu 0.99; Trp 0.52; Sta 1.04
22	PHPLC (33)	6.86 (b)	Pro 0.92; His 1.90; Phe 1.08; Leu 0.89; Napa 1.02; Sta 1.03
23	PHPLC (33)	6.74 (b)	Pro 0.92; His 2.02; Phe 1.08; Leu 0.88; CHA 1.02; Sta 0.96
24	PHPLC (33)	6.34 (b)	Pro 0.96; His 2.00; Phe 1.17; Leu 1.75; Sta 1.08
25	PHPLC (30)	5.68 (b)	Pro 1.00; His 2.20; Phe 1.04; Ala 0.95; Sta 0.92
26	PHPLC (29)	6.09 (b)	Pro 0.97; His 2.00; Phe 1.16; Leu 1.80; Sta 1.01
27	G-10	6.15 (e)	Pro 1.06; Tyr 0.82; Phe 2.10; Val 1.01; Leu 0.98; Sta 1.02
28	G-10	6.24 (e)	Pro 0.95; Phe 3.16; Val 0.93; Leu 0.95; Sta 1.08
29	G-10	7.34 (e)	Pro 0.99; Phe 2.00; Val 1.00; Leu 0.97; Napa 1.05; Sta 0.79
30	G-10	7.64 (e)	Pro 0.93; Phe 2.05; Leu 0.94; Val 0.93; CHA 1.50; Sta 1.02
31[9]			
32	PHPLC (33)	6.86 (b)	Pro 2.08; His 2.00; Phe 2.00; Ile 0.92; ACHPA 1.07
33[9]			
34	PHPLC (35)	6.18 (b)	Pro 1.00; His 2.05; Phe 1.80; Leu 1.10; AHPPA present
35	PHPLC (38)	7.71 (b)	Pro 1.10; His 1.01; Phe 2.01; Leu 0.96; Nle 0.96; AHPPA present
36	PHPLC (33)	6.77 (b)	Pro 0.93; His 1.85; Phe 1.84; Leu 0.97; ACHPA 0.74
37	PHPLC (40)	7.86 (b)	Pro 1.04; His 1.04; Phe 1.96; Val 0.97; Leu 0.99; ACHPA 1.12

[a] Peptides are designated by numbers and their sequences are listed in Table II. Peptides 4, 6, 31, and 33 are characterized in ref 9 (Table I, no. 4, 9, 18, and 20, respectively.). [b] PHPLC, preparative high-performance liquid chromatography on a Vydac C18 reverse-phase column (2.2 cm i.d. × 30 cm; 10-μm particle size); G-10, gel filtration chromatography on a Sephadex G-10 column (2.4 cm i.d. × 16 cm). Parentheses indicate isocratic condition in terms of the percentage of organic buffer B. The percentages of purity of the peptides after PHPLC and G-10 filtration were at least 99 and 95%, respectively, as determined from integration on a Hewlett Packard 3390A system of the analytical reverse-phase HPLC elution profile of a purified sample. [c] t is the HPLC retention time (min) obtained from a Vydac C18 analytical column eluted with the solvent gradient indicated in parentheses. [d] Sta (statine), (3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid; AHPPA, (3S,4S)-4-amino-3-hydroxy-5-phenylpentanoic acid; ACHPA, (3S,4S)-4-amino-3-hydroxy-5-cyclohexylpentanoic acid; CHA, cyclohexylalanine; tLeu, *tert*-leucine; Napa, 3-(1'-naphthyl)alanine.

This represents a major advance in the design of highly potent rat renin inhibitory peptides. Another compound, of a potency in the same range but with a longer duration of action, would be desirable. By studying the effect on inhibitory potency of a series of systematically modified peptides, we hoped to arrive at principles that could be used to design potent and long-acting rat renin inhibitors. After the IC_{50} value of each peptide had been measured against rat plasma renin, two potent inhibitors were selected for intravenous infusion into sodium-depleted rats. Their effects on blood pressure and associated changes in plasma renin activity were then determined.

In the remarks that follow, we have adopted the current system of matching each amino acid position in the substrate analogue with its respective binding subsite on the enzyme. Thus S1 and S1' refer to the binding subsites on rat renin that accommodate the P1 amino acid residue (Leu-10) and the P1' amino acid residue (Leu-11) of rat angiotensinogen. Accordingly, subsites S2, S3, etc. correspond to amino acid residues P2, P3, etc. of the analogues.[11] The primed amino acid positions, P2', P3', etc., correspond to residues of the C-terminal sequence. In designing the rat renin inhibitors, we employed the approach we took in a previous study on human renin inhibitors.[9] There our three-dimensional model of human renin showed an extended binding site that could be favorably occupied by a substrate or inhibitor of at least eight amino acid residues. Here the substrate analogues are composed of seven to nine amino acid residues spanning the P6 through P4' positions. And in synthesizing the substrate analogues shown in Tables I and II, we accepted the hypothesis that replacement of the scissile-bond dipeptide by the unusual amino acid (3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid (statine)[12] and its congeners (3S,4S)-4-amino-3-hydroxy-5-phenylpentanoic acid

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Table II. Synthetic Renin Substrate Analogues

group	no.	sequence[a]									IC_{50},[b] nM
		P5	P4	P3	P2	P1	P1′	P2′	P3′	[c]	
	rat substrate[20]	His-	Pro-	Phe-	His-	Leu-	Leu-	Try-	Tyr-	Ser-Lys	
A	1	Pro-	His-	Pro-	Phe-	His-	Statine-	Tyr-	Tyr-	NH_2	1600
	2	---	---	---	---	---	----------	---	---	Lys-NH_2	35000
	3	---	----	---	---	---	----------	---	---	Ser-Lys-NH_2	7900
	4[9]	---	----	---	---	---	----------	Val	---	Lys	8900
	5	---	---	---	---	---	----------	Thr-	Thr-	NH_2	105000
	6[9]	---	---	---	---	---	----------	Ile-	Phe-	NH_2	10000
	7	---	---	---	---	---	----------	Leu-	Tyr-	Tyr-NH_2	775
	8	---	---	---	---	---	----------	---	Phe-	NH_2	240
B	9	Ac-	His-	Pro-	Phe-	His-	Statine-	Leu-	Phe-	NH_2	210
	10	--	---	---	---	---	----------	CHA	---	---	2700
	11	--	---	---	---	---	----------	*t*Leu	---	---	1900
	12	--	---	---	---	---	----------	Val	---	---	2800
C	9	Ac-	His-	Pro-	Phe-	His-	Statine-	Leu-	Phe-	NH_2	210
	13	--	---	---	---	---	----------	---	CHA	---	160
	14	--	---	---	---	---	----------	---	Val	---	270
D	9	Ac-	His-	Pro-	Phe-	His-	Statine-	Leu-	Phe-	NH_2	210
	15	--	---	---	---	Val	----------	---	---	---	30
	16	--	---	---	---	Leu	----------	---	---	---	310
	17	--	---	---	---	*t*Leu	----------	---	---	----	400
	18	--	---	---	---	CHA	----------	---	---	---	2500
	19	--	---	---	---	DLeu	----------	---	---	---	~1000000
	20	--	---	---	---	DAla	----------	---	---	---	~1000000
E	9	Ac-	His-	Pro-	Phe-	His-	Statine-	Leu-	Phe-	NH_2	210
	21	--	---	---	Trp	---	----------	---	---	---	240
	22	--	---	---	Napa	---	----------	---	---	---	220
	23	--	---	---	CHA	---	---------	---	---	---	650
	24	--	---	---	DLeu	---	----------	---	---	---	~1000000
	25	--	---	---	DAla	---	----------	---	---	---	~1000000
	26	--	---	---	Leu	---	----------	---	---	---	9100
F	15	Ac-	His-	Pro-	Phe-	Val-	Statine-	Leu-	Phe-	NH_2	30
	27	---	Tyr(OMe)	---	---	---	----------	---	---	---	34
	28	--	Phe	---	---	---	----------	---	---	---	42
	29	--	Napa	---	---	---	----------	---	---	---	50
	30	---	CHA	---	---	---	----------	---	---	---	280
G	6	Pro-	His-	Pro-	Phe-	His-	Statine-	Ile-	Phe-	NH_2	10000
	31[9]	---	---	---	---	---	AHPPA	---	---	---	370
	32	---	---	---	---	---	ACHPA	---	---	---	65
	4	Pro-	His-	Pro-	Phe-	His-	Statine-	Val-	Tyr-	Lys	8900
	33[9]	---	---	---	---	---	AHPPA	---	---	--- NH_2	1300
	9	Ac-	His-	Pro-	Phe-	His-	Statine-	Leu-	Phe-	NH_2	210
	34	--	---	---	---	---	AHPPA	---	---	---	150
	35	---	---	---	---	Nle	----------	---	---	---	1270
	36	--	---	---	---	His	ACHPA	---	---	---	50
	37	--	---	---	---	Val	----------	---	---	----	33

[a] Statine, (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid; AHPPA, (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid; ACHPA, (3*S*,4*S*)-4-amino-3-hydroxy-5-cyclohexylpentanoic acid; CHA, cyclohexylalanine, *t*Leu, *tert*-leucine; Napa, 3-(1′-naphthyl)alanine; DLeu, D-leucine; DAla, D-alanine; Ac, acetyl. [b] All IC_{50} values are the average of three to five experiments, with the exception of those for 9 and 15, which are the average of 10 experiments. [c] P1, P2, P3, P4, P5, P1′, P2′, and P3′ designate substrate positions that correspond to renin active site subsites S1, S2, S3, S4, S5, S1′, S2′, and S3′, respectively, after the convention of Schechter and Berger.[11] Dotted lines represent the same structure as above.

(AHPPA) and (3*S*,4*S*)-4-amino-3-hydroxy-5-cyclohexylpentanoic acid (ACHPA) improves inhibitory potency: when the scissile-bond dipeptide of renin substrate analogues is replaced by statine or its congeners, the inhibitors are more effective against human renin,[6] perhaps by acting as transition-state analogues[13,14] or "collected substrate" analogue inhibitors.[15]

Results and Discussion

Peptide Synthesis. The peptides were synthesized by the Merrifield solid-phase method[16] in a stepwise manner according to the general procedure of Stewart and Young.[17] No major difficulties were encountered in obtaining the desired sequences. The results of purification, reverse-phase HPLC characterization and amino acid analysis are summarized in Table I. The sequences of the synthetic

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peptides are listed in Table II. In general, the crude products extracted after HF cleavage showed 70–90% purity as determined by analytical reverse-phase HPLC. Peptides showing less than 95% purity after gel filtration chromatography on a Sephadex G-10 column were subjected to preparative reverse-phase HPLC under isocratic conditions, which yielded products of greater than 99% purity.

Amino acid analysis was performed on a PICO-TAG system. For all the unusual amino acids [statine, AHPPA, ACHPA, *tert*-leucine, cyclohexylalanine, norleucine and 3-(1′-naphthyl)alanine] standard chromatograms were obtained from the acid hydrolysates of their Boc-protected derivatives. We previously reported that statine and AHPPA are partially degraded by acid hydrolysis.[9] In the work described here, ACHPA was also found to undergo degradation during acid hydrolysis. Because of the degradation, in the case of statine, AHPPA, and ACHPA the major peak of the amino acid analysis was termed the reference peak. Under the chromatographic conditions of the PICO-TAG system, the major-peak retention times of statine, AHPPA, and ACHPA were 10.5, 11.2, and 13.3 min, respectively. The retention times of the norleucine, *tert*-leucine, cyclohexylalanine, and 3-(1′-naphthyl)alanine peaks were 8.0, 9.0, 12.6, and 12.8 min, respectively.

Because the extent of the partial degradation of statine, AHPPA, and ACHPA depends on the conditions of hydrolysis, the value of the reference peak (expressed as area/picomole of compound) cannot be used to accurately determine the amino acid content of the peptides. Thus for calculations of the amino acid ratio of these three compounds, the average value (in area/picomole of compound) obtained from peptides hydrolyzed under identical conditions was used.

Inhibition of Rat Plasma Renin Activity. The peptides were tested against samples of pooled rat plasma in a renin activity assay, the final step of which is an immunoassay for angiotensin I. None of the peptides interfered with this step, and so none possessed immunologic cross-reactivity with angiotensin I. Assay mixture containing 1.25% DMSO, which was required to dissolve some of the peptides, also had no effect. Because the enzymatic reaction was quenched by raising the pH of the mixture to 8.5 and the immunodetection of angiotensin I was also performed at pH 8.5,[9] a control study was conducted with high-renin rat plasma incubated at 37 °C for 1 h at pH 8.5, which verified that no angiotensin I was generated (as compared with a sample incubated at 0 °C). The IC_{50} of each peptide listed in Table II is defined as the molar concentration of peptide that results in a 50% decrease in renin activity as compared with a sample that does not contain inhibitory peptide (but does contain 1.25% DMSO).

For the structure–activity analysis, we classified the peptides into seven groups (A–G, Table II) according to their sequence relationships. Peptides in group A were modified from the P2′ to P4′ positions along the C-terminal segment. In groups B, C, D, E, and F, peptides were modified systematically at the P2′, P3′, P2, P3, and P5 positions, respectively, to define the structural requirement for optimal inhibitory activity against rat renin at each position of the analogue. In group G, the effects on inhibitory activity of statine, AHPPA, and ACHPA at the P1P1′ position were compared. In groups A and G, proline was generally incorporated at the N-terminus to increase the aqueous solubility of the peptide.[18]

Role of the C-Terminal Segment. In our previous report we showed that the species specificity of renin substrate analogue inhibitory peptides is determined by the P2′P3′ amino acid residues. This suggests that the differences in substrate specificity among renins from various species are the result of differences in the S2′S3′ subsites.[9] In the present study on rat renin, changes were made in the P2′, P3′, and P4′ positions only. These modifications (group A) had a remarkable influence on the potency of the analogues. In studies on human renin inhibitors, incorporation of an Ile-His segment at the P2′P3′ position (as in the natural human substrate) produced potent inhibitors, with IC_{50} values in the nanomolar range.[4,9,19] To our surprise, the same strategy of incorporating the natural rat sequence at the P2′P3′ position (Tyr-Tyr) does not produce potent rat renin inhibitors. Compound 1, which contains Tyr-Tyr at the P2′P3′ position, is only a relatively weak inhibitor of rat renin. In primate renin inhibitors, extending the peptide with Lys in the P4′ position had been shown to enhance potency.[18] But in rat renin inhibitors, incorporating a Lys residue in the P4′ position (**2**) dramatically lowers potency [however when Ser-Lys is incorporated at the P4′P5′ position (**3**), some of the loss in potency is recovered]. This discrepancy suggests that there may be a specific binding site for the P4′ position that cannot be favorably occupied by Lys. A comparison of 1 and **5** shows that replacement of the Tyr residues by Thr causes a 65-fold decrease in potency. One possible hypothesis is that hydrophobicity makes an important contribution to analogue binding to rat renin, even though hydrogen binding between the enzyme and the OH groups of the position 12 and 13 Tyr residues in rat angiotensinogen may exist. The insertion of a Leu residue between the statine and Tyr in 1 caused a 2-fold increase in potency (**7**). A further increase in potency (3.7-fold) was achieved by replacing the P3′P4′ Tyr-Tyr in **7** by a single Phe (**8**).

Replacing the Ile at the P2′ position by Leu (**8** vs **6**) has a striking effect on potency. Compound **8** is approximately 50 times more active than **6**. One possible explanation is that the Leu in **8** (as well as in **7**) may occupy the S1′ subsite of the rate enzyme, the natural counterpart of which is, according to the system of Schechter and Berger,[11] a Leu residue. Thus statine would take the place of a single amino acid residue, the P1 leucine, in binding to the S1 subsite of rat renin and the Leu at the position C-terminal to statine would take the P1′ position (which would occupy the S1′ subsite).

In group B, changes have been restricted to the P2′ residue and all the amino acids in that position have aliphatic side chains of differing size and shape. The most potent inhibitor in this series has Leu in the P2′ position (**9**). A larger cyclohexylalanine (CHA) side chain (**10**) or a smaller Val side chain (**12**) decreases binding significantly. Likewise, a change in the shape of the side chain from an isobutyl to a *tert*-butyl group (**9** vs **11**) decreases binding significantly. The effect of these changes implies that, for optimal binding, the steric requirements of the S2′ position are stringent in rat renin. In contrast, the change from a Phe at the P3′ position to either a CHA or a Val (group C) has only a minimal influence on potency. Thus aromaticity at the P3′ position does not appear to be necessary for optimal activity (**9** vs **13**) and steric factors have only a minimal influence on potency. These results show that the S3′ subsite may be capable of accommodating hydrophobic forms of various structures.

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Table III. Effects of P2 Val on the Potency of Substrate Analogues Used as Rat and Human Plasma Renin Inhibitors[a]

compd	sequence	IC_{50}, nM rat	human
9	Ac-His-Pro-Phe-His-Statine-Leu-Phe-NH_2	210	14
15	-- --- --- --- Val -------- --- --- ---	30	3.2
36	Ac-His-Pro-Phe-His-ACHPA-Leu-Phe-NH_2	50	0.5
37	-- --- --- --- Val -------- --- --- ---	33	15

[a] See footnote to Table II.

Role of the P2 Position. We recently published a study on the role of the P2 His in substrate analogues. Substitution of His at this position by Phe, Lys, or Arg resulted in a profound loss of inhibitory activity against human plasma renin. Our computer modeling explained the loss of activity as having been due to the disruption of the hydrogen bond between the histidyl imidazole of the inhibitor and the Tyr-230 of the human enzyme.[9] As evidenced by the relative potencies of **9**, **15**, **16**, and **17** (group D), rat renin inhibitors do not appear to require a hydrogen bond at the P2 position. Instead this position appears to have stringent steric requirements in the case of the rat. A comparison of **15** and **16** shows that a shorter side chain improves potency by 15 times. The bulkiness of the side chain also affects potency (**18** is 8 times less potent than **16**). Similarly, the Nle side chain (**35**, group G), which is longer than the His side chain (**34**), caused an 8.5-fold decrease in potency. The important steric effect of the P2 residue is also demonstrated by a comparison of **15** and **17**. The favorable interaction between the isopropyl side chain (**15**) and S2 subsite is disrupted when the side chain is replaced by a *tert*-butyl group (**17**).

Although Val at the P2 position increases the potency of statine-containing analogues (group D), it has no clearly beneficial effect in the ACHPA-containing analogues (**37** vs **36**, group G). We presume that the bulky cyclohexylmethyl side chain of the transition-state residue adjacent to the P2 Val created too much steric hindrance for the valine isopropyl side chain to accommodate the stringent requirements of the S2 subsite. Another possibility is that a slight difference in alignment between the P1 residue backbones of the two transition-state analogues may also have shifted the valine side chain away from its optimal binding position. It is therefore not surprising that in **37** the beneficial effect of the P2 Val is partially neutralized.

Although the species specificity of both human and rat substrate analogues is determined mainly by the sequence of the P2′ and P3′ residues,[9] the effect on potency of a change from a P2 His to a P2 Val differs in each. In Table III a comparison between **9** and **15** (both contain statine) shows that the Val residue enhances inhibitory potency by 7 times against rat plasma renin but by only 4.4 times against human plasma renin. Nisato et al.[21] recently combined the Free–Wilson and correlation analyses to study the contribution of P2 side chains to the inhibitory potency against human plasma renin in a series of pepstatin analogues. Their results revealed that valine, cyclopentylglycine, and norvaline are favorable substitutions at position 2. The high potency of **15** against human plasma renin (Table III) agrees with their prediction. However, in the Nisato group's analysis the observed high activity of analogues with His at the P2 position cannot be explained. Other properties of the imidazole ring, such as the propensity to form hydrogen bonds, were put forward as explanations.

In ACHPA-containing peptides, a P2 Val has yet another effect. A comparison of **36** and **37** shows that even though a P2 Val enhances the inhibitory potency of the peptide against rat plasma renin by about 1.5 times, it actually lowers the potency of the peptide against human plasma renin by about 30 times. These results suggest, with our evidence reported previously,[9] that the S2 subsite is one area of difference between rat and human renins, and that it is a site that must be given careful consideration in the design of rat renin inhibitors.

Examination of the results for **19** also demonstrates that the proper spatial orientation of the P2 side chain is essential to activity. Its influence is remarkable, although not unexpected. A comparison between **16** and **19** shows that a change from an L to a D configuration converts a less potent inhibitor (**16**) to a compound almost devoid of inhibitory activity (**19**), in spite of the presence of the same core transition-state residue, statine.

Role of the P3 Position. The peptides in group E demonstrate the effect of modifications to the P3 side chain. Here three observations can be made. The first is that even though the size of the side chains in **9**, **21**, and **22** differs, the inhibitory potency of the three peptides is virtually the same. This is in contrast to the findings of Kokubu et al.,[22] who reported that the introduction of a large aromatic residue [3-(1′-naphthyl)alanine] in place of the Phe in the P3 position of a tripeptide aldehyde derivative (Z-Phe-His-leucinal) significantly increased inhibitory potency against human renin. Our data show that applying the same strategy to a rat renin inhibitor [by replacing the P3 Phe with a 3-(1′-naphthyl)alanine in a peptide spanning from positions P3′ to P5] is not rewarding. The second observation is that changing from an aromatic residue to an aliphatic residue (both hydrophobic) in the P3 position causes a substantial decrease in affinity. Peptide **23** (containing CHA at the P3 position) was approximately 3 times less potent than **9**, **21**, or **22** (which contain an aromatic side chain at the P3 position). This observation about rat renin inhibitors agrees with the report of Fehrentz et al.[23] on human renin inhibitors, which states that an aromatic residue at the P3 position is required for activity. It also suggests that in conformation and electron distribution, the S3 subsites of rat and human renins are similar. The third observation underscores the importance of hydrophobicity to binding affinity, which is demonstrated when CHA is replaced by Leu (which has a less hydrophobic side chain). Molecular modeling studies on human renin show the S3 subsite as a large hydrophobic pocket[24] and point to hydrophobic forces as probably the most important factor in tight binding between the P3 residue and the S3 subsite.[25] As in the case of the P2 residue, a specifically oriented P3 side chain appears mandatory for binding. Peptide **24** (with D-Leu at P3) and peptide **25** (with D-Ala at P3) are virtually inactive.

Role of the P5 Position. In group F peptides, we studied the influence on potency against rat renin of changes in aromaticity, hydrophobicity, and steric configuration at the P5 side chain. Compounds **15** and **27**,

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which both contain an aromatic side chain with a hydrophilic component, exhibit very similar binding affinities for rat renin. When the OMe group of **27** is deleted (as in **28**), the peptide becomes less hydrophilic and the potency of the compound decreases. The effect of steric hindrance is observed when the P5 Phe is replaced by a residue carrying a larger hydrophobic side chain (a naphthyl group, in compound **29**). The potency of **29** is also less. A comparison of **28** and **30** indicates that an aromatic side chain is required to maintain potency: the presence of a cyclohexyl group in **30** causes 1 order of magnitude decrease in binding affinity. We also noted that the change in potency of the group F peptides parallels their change in hydrophilicity. HPLC retention times (see Table I) show that the more hydrophilic a compound is, the more potent it is. The IC_{50} values of **30**, **29**, **28**, **27**, and **15** decrease in proportion to their retention times. Thus in the design of rat renin inhibitors, the choice of a hydrophilic aromatic side chain at the P5 position is important for maintaining potency.

P1 Position–S1 Subsite Interaction. In assays of inhibitory potency against human renin, the incorporation of the transition-state residue statine at the P1P1′ position (scissile bond) has been found to increase potency.[5,7,9,26] The statine congeners AHPPA[27] and ACHPA[6] have also been used to replace the scissile-bond dipeptide unit. Although the effect of AHPPA on the inhibitory potency of human renin inhibitors is moderate,[6,9] the remarkable potentiative effect of ACHPA[6] at the same position has led to the almost universal employment of a cyclohexylmethyl P1 side chain in the design of renin inhibitors.

Our results for group G show considerable variation in the contribution made by each statine congener to the inhibitory potency of the rat renin substrate analogues. Such variation depends on the C-terminal sequence to the right side of the transition-state residue. On the basis of this study, a C-terminal sequence can be termed either favorable or unfavorable. The -Leu-Phe-NH_2 sequence is favorable because when all other factors remain unchanged, analogue **9** exhibits the highest potency. The -Ile-Phe-NH_2 sequence is unfavorable because when comparing compounds **6** and **8**, the former is approximately 2 orders of magnitude less potent than the latter. In peptides with the unfavorable C-terminal sequence, the presence of AHPPA and ACHPA (in comparison with statine) dramatically increases potency: **31** and **32** are 27 and 29 times more potent than **6**. In peptides with the favorable C-terminal sequence, AHPPA and ACHPA (again in comparison with statine) confer only a mild advantage: **34** and **36** are 1.4 and 4.2 times as potent as **9**.

Another unfavorable C-terminal sequence is -Val-Tyr-Lys. In this case the AHPPA-containing inhibitor (**33**) is 6.8 times more potent than the statine-containing analogue (**4**). Apparently the tighter binding produced by the favorable, -Leu-Phe-NH_2 C-terminal sequence restricts the ability of the inhibitor to accommodate a wider range of side chains in the P1 position. Recently a renin inhibitor with the sequence Iva-His-Pro-Phe-His-Statine-Leu-Phe-NH_2, which, with the exception of the P6 residue, is the same as compound **9**, was reported to have an IC_{50} value against rat plasma renin of 630 nM[6]—a value considerably lower than that of compound **8** or **9**. However, replacing the statine residue by the ACHPA residue compensates for the disadvantage of an Iva at the P6 position. The IC_{50} value of the ACHPA-containing sequence was reported to be 21 nM, which is comparable to that of compound **36**.

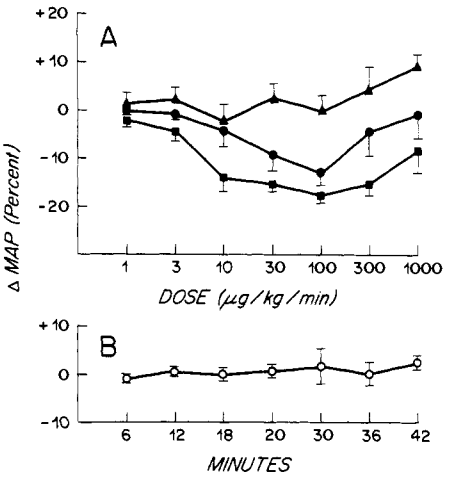

Figure 1. Dose–response curves for change in mean arterial pressure (ΔMAP, %) after 6-min intravenous infusion of renin substrate analogues and vehicle into sodium-depleted rats. A, closed square, compound **15** (n = 19); closed circle, compound **9** (n = 13); triangle, compound **20** (n = 7); B, open circle, 5% dextrose in water (D5W). Each value is the mean ± SEM.

Thus in the design of rat renin inhibitors, the potentiation of activity resulting from the modification of a P1 side chain in one transition-state analogue cannot necessarily be extrapolated directly to another sequence, i.e., the overall potency of an inhibitor is not necessarily the summation of each favorable factor. This principle also holds true in the design of cathepsin D inhibitors.[28]

In Vivo Rat Studies. The in vitro inhibition assays showed that compound **15** is the most potent rat renin inhibitor, with an IC_{50} value of 30 nM when assayed at neutral pH. Compound **9** is the parent structure from which **15** was derived (**9** is 7 times less potent than **15** in the inhibition assay). To evaluate the hypotensive potency of these two compounds in association with their renin inhibitory activity, they were infused over 6 min into sodium-depleted, anesthetized rats. To confirm the renin dependency of changes in blood pressure, compound **20**, which has no significant rat renin inhibitory activity, was also included in the study. Compound **15** was studied in sodium-replete rats as a control. To reveal its possible contribution to changes in blood pressure, the vehicle (D5W, 5% dextrose in water) was infused over 42 min at the same rate as the peptide solution, in both sodium states.

Figure 1 shows the dose–response relationships of each of these compounds with respect to blood pressure. Compound **15** requires a minimal dose of 3 μg/kg per min to attain an average decrease in mean arterial pressure of 4.9%. At 10 μg/kg per min, the decrease in mean arterial pressure averaged 14.2%. The maximal hypotensive efficacy (a 17.7% average decrease in mean arterial pressure) occurred at a dose of 100 μg/kg per min. Also at this dose an average 90% inhibition of plasma renin activity was obtained. In the case of **9**, a maximal decrease in mean arterial pressure occurred at a dose of 100 μg/kg per min, although the maximal hypotensive efficacy was less than that of **15**. The 13.2% decrease in mean arterial pressure attained by **9** at 100 μg/kg per min could be attained by **15** at 10 μg/kg per min. D5W had no effect on the change in blood pressure (see Figure 1B), and up to a dose of 100

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μg/kg per min, control compound **20** also had no effect. Beyond the 100 μg/kg per min dose, the lowering of blood pressure attended by the administration of both **9** and **15** ceased. Two explanations come to mind for the reversal of this tendency at doses higher than 100 μg/kg per min. One is that the measurement of the plasma renin activity of **9** at 1000 μg/kg per min shows a residual renin activity of 30%. This activity suggests that a feed-back mechanism activated by inhibition of the renin-angiotensin system may stimulate the secretion of renin.[29] The other is that, as evidenced by the mildly hypertensive effect of compound **20** when infused at high doses, renin substrate analogues may possess a blood pressure elevating component whose effect emerges at supramaximal doses. Data are not shown for the study on sodium-replete rats, in which, as expected, infusion of **15** at 300 μg/kg per min over 6 min caused no significant change in mean arterial pressure (2.2% decrease on average, $n = 5$).

Summary

The species specificity of renin substrate analogue inhibitory peptides has been reported to depend on the P2′P3′ amino acid residues.[9,19] In the case of the rat, modifications at the C-terminal segment of substrate analogues appear to have a marked influence on potency. Contrary to our expectations, the strategy of incorporating Tyr-Tyr residues at the P2′P3′ position (as in the natural rat substrate) did not yield a potent inhibitor. Instead we found that a secondary butyl side chain at the P2′ position is important for optimal activity. At the P3′ position, substitutions of the hydrophobic residues phenylalanine, cyclohexylalanine, and valine did not cause significant variance in potency even though the aromaticity and size of these residues differ. This suggests that the S3′ subsite of rat renin is capable of accommodating hydrophobic forms of various structures.

The P2 position appears to be critical to the design of rat renin inhibitors. As expected, the L configuration at the P2 side chain is required for activity. Although in the case of human renin inhibitors a hydrogen bond between the P2 His of the inhibitor and the 230 Tyr of the enzyme is important for high binding affinity, steric considerations at the same position appear to be more important in the case of rat renin inhibitors. Substitution of Val for His at the P2 position can increase the potency of an inhibitor by 1 order of magnitude. A P2 Val was also observed to have differing effects on the inhibition of rat and human renins in a comparison of statine-containing and ACHPA-containing peptides. A P2 Val always potentiates activity in tests against rat plasma renin; however, this is not necessarily so in tests against human plasma renin. This suggests that the S2 subsites in rat and human plasma renins differ.

Studies of structure–activity relationships at the P3 position show that, in addition to an L configuration, an aromatic side chain is required for optimal activity. This observation about aromatic side chains in rat renin inhibitors is comparable to one made of human renin inhibitors,[9] and suggests that the conformation and electron distribution at the S3 subsite in rat and human renins are analogous. Similar to the case of the P3 position, an aromatic side chain is also necessary at the P5 position for retention of potency. The hydrophilicity of a P5 aromatic side chain also contributes to optimal potency.

The effects of scissile-bond modifications appear to be sequence dependent. For inhibitors with an unfavorable C-terminal segment (-Ile-Phe-NH_2), ACHPA and AHPPA cause a surprising retention of potency. For inhibitors with a favorable C-terminal segment (-Leu-Phe-NH_2), AHPPA has only a mild advantage over statine, even though ACHPA still enhances potency by 1 order of magnitude. If another beneficial structural incorporation is made at some other position, ACHPA's prominent contribution to activity against rat plasma renin may be partially neutralized.

Of the two renin inhibitors (compounds **9** and **15**) studied in sodium-depleted, anesthetized rats, acetyl-His-Pro-Phe-Val-Statine-Leu-Phe-NH_2 (**15**) was the most potent, with an IC_{50} value of 30 nM against rat renin. The in vivo activities of **9** and **15** parallel their in vitro activities, and their hypotensive effects proved to be due to the inhibition of rat plasma renin. Compound **15** should be a useful pharmacologic probe in studies of the renin-angiotensin system in rat models.

Experimental Section

Synthesis of Renin Inhibitory Peptides. All commercial protected amino acids were obtained from Peninsula Laboratories (San Carlos, CA) with the exception of Boc-cyclohexylalanine, which came from Bachem (Torrance, CA) and Boc-*tert*-leucine, which was from Advanced ChemTech, Inc. (Louisville, KY). The side-chain protecting groups were tosyl for histidine and benzyl for serine, threonine, and tyrosine. Other reagents were dichloromethane (Dow Chemical, Midland, MI), *N,N′*-dicyclohexylcarbodiimide (Fluka, New York), trifluoroacetic acid and *N,N*-diisopropylethylamine (Aldrich, Milwaukee, WI), both distilled before use, acetic anhydride (Fisher Chemical, Fairlawn, NJ), HF (Matheson, Secaucus, NJ), HPLC-grade acetonitrile (Baker, Phillipsburg, NJ), and *p*-methylbenzhydrylamine resin hydrochloride (United States Biochemical, Cleveland, OH). *N*-Boc-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic acid (Boc-statine) was either synthesized according to Rich et al.[30] or purchased from Advanced ChemTech. *N*-Boc-4(*S*)-amino-3(*S*)-hydroxy-5-phenylpentanoic acid (Boc-AHPPA) was synthesized according to Rich et al.[27] *N*-Boc-4(*S*)-amino-3(*S*)-hydroxy-5-cyclohexylpentanoic acid (Boc-ACHPA)[6] was synthesized by a procedure similar to that for Boc-statine,[30] starting from Boc-cyclohexylalanine.

The renin inhibitory peptides were synthesized in a stepwise manner according to the Merrifield solid-phase method.[16] Each synthesis was begun with 0.4 g of resin and 2.5 equiv of Boc-amino acid, with the exception of Boc-statine, Boc-AHPPA, and Boc-ACHPA syntheses, in which 1.25 equiv were used. In each coupling reaction, 2.5 equiv of *N,N′*-dicyclohexylcarbodiimide (DCC) was used as a condensing reagent, except for the coupling of Boc-statine, Boc-AHPPA, and Boc-ACHPA, in which 1.25 equiv of DCC was used. The reaction time for each coupling step was 1 h except for the coupling of Boc-statine, Boc-AHPPA, and Boc-ACHPA, the reaction time of which was 10 h.

The coupling and deprotecting cycles were monitored with the Kaiser ninhydrin test.[31] To remove the Boc group, the resin was treated with 50% trifluoroacetic acid in dichloromethane for 2 and then 15 min. Before the coupling of the next Boc-amino acid, the resin was neutralized with 5% *N,N*-diisopropylethylamine (DIEA) in DCM for 1 and then 5 min. After Boc-removal of the last amino acid residue, N-acetylation was performed by treating the resin with 25 mL of acetic anhydride for 5 min. The completed peptide was simultaneously deprotected and cleaved from the resin by HF (10 mL) treatment at 0 °C for 45 min in the presence of 5% anisole. After the HF was removed in vacuo, the residue was washed 3 times with anhydrous ether and air-dried. The peptide was extracted with water and then glacial acetic acid, and the extracts were pooled and lyophilized. The crude peptide was

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dissolved in 50% ethanol at a concentration of 25 mg/mL and then treated with dilute NaOH (0.16 N) for approximately 15 min before neutralization with glacial acetic acid. The solution was then gel filtered in a Sephadex G-10 (Sigma) column (2.4 cm i.d. × 16 cm), which was equilibrated and eluted with 5 M acetic acid. The fractions containing the peptide (1.5 mL each) were located by the Pauly[17] or ninhydrin tests. The purity of the first fraction containing the desired peptide was assessed by analytical reverse-phase HPLC performed on a Vydac C18 or a Waters μBondapak C18 column (4.6 mm i.d. × 25 cm) connected to a 214-nm detector in a Beckman instrument (Model 110A). The chromatograms were recorded over a 10-min period on a Hewlett-Packard 3390A integrator. Solutions A and B were water and acetonitrile, respectively, each containing 0.05% trifluoroacetic acid. The flow rate was 2 mL/min. The cycle of each HPLC program was 7 min. The first gradient lasted for 5 min, the second and third for 1 min each. The following cycles show changes in the percentage of solution B: (a) 5 to 50 to 80 to 5%, (b) 15 to 60 to 80 to 15%, (c) 15 to 60 to 90 to 15%, (d) 25 to 70 to 90 to 25%, and (e) 40 to 80 to 95 to 40%. Fractions showing at least 95% purity were lyophilized for biological assays. Peptides of less than 95% purity were further purified by preparative reverse-phase HPLC as described.[9]

For amino acid analysis on a PICO-TAG work station (Millipore), each peptide was hydrolyzed with 6 N HCl in evacuated, sealed reaction vials for 24 h at 110 °C. Peptides containing tryptophan were hydrolyzed with 4 N methanesulfonic acid.[32] The tryptophan content of the hydrolysates was determined with a Beckman Model 6300 analyzer.

In Vitro Assay. Peptide stock solutions were prepared by dissolving the peptide in Tris buffer (1.0 M, pH 7.4, 0.02% azide) containing 50% DMSO. These stock solutions were then subjected to a series of 1:10 dilutions with Tris buffer containing 25% DMSO. Blood from ether-anesthetized rats was collected via a carotid cannula into tubes containing EDTA chilled to 0 °C. The blood samples were centrifuged at 4 °C to separate the plasma. Before the assay, 5 mM phenylmethanesulfonyl fluoride (PMSF; 0.3 M in ethanol), 3 mM 8-hydroxyquinoline sulfate, and 5 mM more EDTA were added. Plasma renin activity assays (at zero concentration of peptide inhibitor) showed an average activity of 10 ng of angiotensin I mL^{-1} h^{-1}. Plasma was prepared as described before, with the exception that it was not necessary to perform trypsin activation.[9]

Renin activity was determined by a radioimmunoassay for angiotensin I, based on the method of Haber et al.[33] For the in vitro evaluation of the inhibitory potency of the peptides, rat plasma samples (200 μL, pH 7.4) were mixed with 10 μL of serial concentrations of peptide inhibitors. The mixtures were divided into two portions for incubation at 37 and 0 °C for 1 h. The renin enzymatic reaction was quenched at pH 8.5 by adding saturated Tris solution (approximately 2 μL/100 μL of mixture) and the solution was then diluted with an equal volume of 0.1 M Tris (pH 8.5, containing 0.02% sodium azide). Aliquots of reaction mixture in 1 mL ^{125}I-labeled angiotensin I tracer (1% BSA in 0.1 M Tris, pH 8.5, 0.02% azide) were incubated in tubes coated with rabbit anti-angiotensin I antibody (Travenol-Genentech Diagnostics, Cambridge, MA). The renin activity value was obtained from a standard curve generated by a parallel experiment with known quantities of angiotensin I. To determine the renin activity of rat plasma obtained from the infusion studies (see below), 100–150 μL of plasma was directly assayed without additional peptide. Inhibition assays on human plasma renin were performed as described before.[9]

In Vivo Rat Studies. The experiments were carried out in sodium-depleted and sodium-replete Wistar-Kyoto rats (Charles River Laboratories, Wilmington, IN) weighing from 180 to 250 g. In the sodium-replete state, the animals were fed rat chow containing 1.6% sodium (Purina, St. Louis, MO). In the sodium-depleted state, the animals were fed low-sodium rat chow (0.07%, Purina, St. Louis, MO) for 4–7 days before the infusion experiments. Tap water was given ad libitum until the day of the experiment.

The animals were anesthetized with Inactin-Byk (Byk Gulden, Konstanz, Federal Republic of Germany) at a dose of 100 mg/kg (intraperitoneally) and then placed on a surgical table under a heat lamp to maintain a rectal temperature of 37–38 °C. The animal was then tracheotomized. A carotid artery was cannulated with a PE-50 tube to allow continuous direct measurement of blood pressure with a transducer connected to a Grass 7 polygraph recorder (Quincy, MA). A jugular vein was cannulated with a PE-10 tube for the administration of drugs.

To the sodium-depleted animals furosemide was administered intravenously at a dose of 2 mg/kg. Thirty minutes after the administration of furosemide, 0.4 mL of blood was withdrawn from the arterial cannula and measured for plasma renin activity. Forty-five minutes after the administration of furosemide, when the blood pressure was stabilized, the peptide (dissolved in 5% dextrose in water) was delivered into the jugular vein with a constant-rate infusion pump (Harvard Bioscience, South Natick, MA). Doses of from 1 to 1000 μg/kg per min were administered intravenously by constant infusion (at an average flow rate of 0.044 mL/min) for 6 min each. Arterial blood (0.4 mL) was withdrawn at the end of the 100 and 1000 μg/kg per min infusions for assay of plasma renin activity. The blood was collected into a tube containing EDTA chilled in an ice bath. The plasma was isolated by centrifugation at 4 °C and then stored at −20 °C until assay. To evaluate the vehicle's blood pressure lowering effect, 5% dextrose in water was infused for 42 min at the same rate as the peptide solution.

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