H, pyridine protons), 8.78 (s, 1 H, NH), 10.40 (s, 1 H, NH); mass spectrum, m/e 322 (M^+).

Pharmacological Methods. 1. Anesthetized Rat Model. Male SPF Sprague–Dawley rats (350–450 g, Charles River WIGA, 8741 Sulzfeld, FRG) were anesthetized with thiabutabarbital sodium (80–100 mg/kg ip). After tracheotomy and insertion of a cannula to maintain airway patency, a Millar Microtip manometer PR-249 was introduced into the left ventricle via the right carotid artery for recording left ventricular pressure (LVP). Arterial blood pressure (BP) was registered via a polypropylene catheter (PP 50) which had been inserted into the femoral artery and connected to a Statham Transducer P 23 Db.

Change in left ventricular pressure was determined by electronic differentiation of pressure signal with a physiodifferentiator and taken as an index of inotropic state. Heart rate (HR) was deduced from the LVP signal by a pulse counter. All parameters were continuously recorded by a universal amplifier 47/Varioscript V 8008. All test doses of compounds 1–**25** were injected in a volume of 0.5 mL/kg into the jugular vein as single bolus injection in increasing doses at an interval of 10 min. The hemodynamic parameters were always determined 10 min after application and represent "steady state effects" and not the maximum change in hemodynamics which occurred about 1–3 min after the bolus injections.

2. Anesthetized Cat Model. Cats of both sexes were anesthetized with pentobarbitone sodium (45 mg/kg ip) and after tracheotomy respirated by a Starling pump with open air (10 mL/kg and 30 strokes/min). Anesthesia was continued by a maintenance infusion of pentobarbitone sodium at a dose of 0.1 mg/kg × min. After thoracotomy, LVP and HR were recorded by means of a Millar Tip manometer, PC-350, which had been introduced into the left ventricle via the auricle. BP and dP/dt_{50} were recorded as described in rats. The animals received dextran (MW 75000) at a dose of 10 mL/kg via a jugular vein to stabilize the hemodynamic situation. This was followed by an injection of desacetylmetipranolol (0.3 mg/kg iv) to induce cardiovascular failure by β-blockade.

The test compounds were intravenously injected at an interval of 10 min in increasing doses. Again the hemodynamic parameters obtained 10 min after the bolus injection were taken as results.

3. Intravenous Administration to Conscious Dogs. In mongrel dogs of both sexes, weighing 25–30 kg, a left-side thoracotomy was performed under aseptic conditions and general anesthesia (haloethane, N_2O, O_2). The dogs were implanted with polyethylene catheters to measure BP in the femoral artery, to guide a Millar Tip PC-350, and to inject the test compounds in the femoral vein. dP/dt_{60} was obtained as described before, but at a LVP of 60 mmHg. All parameters were continuously recorded on a universal amplifier 47/Varioscript 8008. The dogs were recruited for the experiments after a recovery period of 10 days at least. The animals received the test compounds at an interval of 10 min in increasing doses intravenous hemodynamic parameters were determined every 10 min after each drug administration.

4. Oral Administration to Conscious Dogs. The dogs were instrumented as described before and a Konigsberg manometer guided into the left ventricle. Each animal received 1 mg/kg 1, milrinone, and pimobendan by gavage in a suspension of 1% of methylcellulose or an equivalent volume of methylcellulose as placebo for control experiments. LV dP/dt_{60} and blood pressure were recorded for 6 h after drug administration. During the experiments, the dogs were kept in small cages permitting only limited movement. In addition, the dogs were largely shielded from environmental effects (noise, moving people, etc.).

Analysis of Structure–Activity Relationships in Renin Substrate Analogue Inhibitory Peptides†

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On the basis of the minimal octapeptide sequence of the renin substrate, a series of peptides was synthesized containing (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (statine) or (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA) at the P1P1′ position. Some of these peptides also contained N^{in}-formyltryptophan at the P5, P3, or P3′ position. Renin-inhibitory potency varied over a wide range (from inactive to IC_{50} = 3 nM). Potency was reduced by at least 10-fold when the peptide was shortened by two residues at either the amino or carboxy terminus. The AHPPA-containing inhibitors were several-fold less potent than the statine-containing inhibitors. Analysis of models for the three-dimensional structure of inhibitors at the active site of human renin suggests that the diminished potency of the AHPPA peptides in comparison with the statine-containing peptides was caused by a shift in the peptide backbone due to a steric conflict between the phenyl ring of the AHPPA residue and the S1 subsite. The importance of the side chain and the 3(*S*)-hydroxyl group of the statine residue was demonstrated by substituting 5-aminovaleric acid for a dipeptide unit at the P1P1′ position, which resulted in a peptide devoid of renin-inhibitory activity. Substitutions of other basic amino acids for histidine at the P2 position caused a great loss in potency, possibly due to disruption of a hydrogen bond as suggested by molecular modeling. Studies on the plasma renins of four nonhuman species suggest that the isoleucine-histidine segment at the P2′P3′ position is important to defining the human specificity of the substrate. This work suggests a number of properties important to the design of potent renin inhibitors, and demonstrates the usefulness of three-dimensional models in the interpretation of structure–activity data.

RIP (Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys), a competitive renin inhibitory peptide based on the sequence of equine angiotensinogen, was developed in this laboratory several years ago.[1,2] This substrate analogue has been studied both in primates[1,2] and in humans.[3,4] Although it appears to be a specific renin inhibitor, it is of relatively low potency (K_i = 5 μM) and may exhibit a lack of selectivity for renin when studied in vivo at high doses.[3,4] Others subsequently reported the synthesis of more potent renin inhibitors containing the amino acid residue (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (sta-

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Table I. Synthetic Renin Substrate Analogues

no.[a]	purification method[b]	% purity[c]	t[d]	amino acid anal.[e]
1	IE	99.5	5.50 (a) W	Pro 0.93; His 0.98; Phe 3.10; Val 1.01; Tyr 0.91; Lys 1.00
2	IE	99	6.60 (e) W	Pro 0.91; His 0.99; Phe 1.03; Val 1.01; Tyr 0.92; Lys 1.04; Orn 1.27
3	IE	97.8	5.33 (c) W	Pro 1.08; His 1.90; Phe 1.06; Val 0.99; Tyr 0.88; Lys 1.09; Sta 1.26
4	IE	98.3	5.79 (f) W	Pro 2.15; His 1.91; Phe 1.04; Val 1.05; Tyr 0.76; Lys 1.08; Sta 1.23
			7.26 (f) V	
5	IE	>94	5.18 (c) V	Pro 2.09; His 3.03; Phe 1.02; Ile 0.81; Lys 1.06; Sta 1.20
	PHPLC (15% B)	100	6.38 (b) V	
6	IE	98.7	7.25 (f) V	Pro 2.17; His 3.05; Phe 0.96; Ile 0.86; Lys 0.96; Sta 0.76
7	IE	>95	6.00 (g) W	Pro 1.01; His 2.07; Phe 0.99; Ile 0.90; Lys 1.04; Sta 0.81
8	IE	95	8.75 (c) W	Pro 1.10; His 1.99; Phe 1.07; Ile 0.90; Lys 1.05; Sta 1.01
9	IE	>99	5.00 (a) V	Pro 2.02; His 2.02; Phe 2.07; Ile 0.96; Sta 1.06
	PHPLC (25% B)	100	6.44 (f) W	
10	IE	98.6	5.85 (h) W	Pro 1.11; His 1.07; Phe 1.91; Ile 0.91; Sta 0.85
11	IE	100	6.27 (k) V	Pro 1.90; His 1.00; Phe 3.05; Ile 0.94; Sta 0.80
12	IE	>95	7.33 (k) V	Pro 0.91; Phe 3.10; Ile 0.9; Sta 1.07
13	IE	98.5	6.09 (d) V	Phe 2.10; Ile 0.88; Sta 0.99
14	IE	98.5	6.05 (c) W	Pro 2.20; His 1.97; Phe 1.97; Ile 0.86; AVA 1.24
15	IE	100	6.47 (i) V	Pro 2.14; His 1.00; Phe 3.07; Val 0.88; Tyr 0.82; Arg 1.07; Lys 1.02
16	IE	99.6	7.26 (d) W	Pro 1.05; His 0.99; Phe 0.98; Sta 1.07
17	IE	96	6.71 (d) W	Pro 1.90; His 1.98; Phe 0.90; Sta 1.10
18	IE	>99	5.12 (a) V	Pro 2.22; His 1.91; Phe 1.87; Ile 0.86; AHPPA present
19	PHPLC (25% B)	100	6.64 (a) V	Pro 2.01; His 1.06; Phe 1.97; Ile 0.91; Lys 1.05; AHPPA present
20	IE	92.6	5.06 (l) V	Pro 2.09; His 2.04; Phe 1.05; Val 1.03; Tyr 0.80; Lys 0.91; AHPPA present
21	IE	96.5	5.50 (j) V	Pro 2.27; His 3.17; Phe 1.00; Ile 0.81; Lys 1.05; AHPPA present
22	PHPLC (20% B)	100	3.70 (a) V	Pro 1.15; His 1.96; Phe 1.09; Ile 0.80; Lys 1.11; AHPPA present
23	PHPLC (35% B)	100	6.58 (a) V	Pro 2.03; His 1.04; Phe 0.93; Ile 0.89; Trp(CHO) 1.55; AHPPA present
24	PHPLC (30% B)	100	5.69 (a) V	Pro 1.04; His 1.01; Phe 0.94; Ile 0.89; Trp(CHO) 0.74; AHPPA present
25	PHPLC (30% B)	100	5.45 (a) V	Pro 1.04; His 0.96; Ile 0.78; Trp(CHO) 2.23; AHPPA present
26	PHPLC (30% B)	100	6.62 (a) V	Pro 2.04; His 1.04; Ile 0.93; Trp(CHO) 2.23; AHPPA present
27	PHPLC (30% B)	100	6.38 (a) V	Pro 2.00; His 1.10; Phe 0.91; Ile 0.88; Trp(CHO) 1.60; AHPPA present
28	PHPLC (30% B)	100	6.50 (a) V	Pro 2.00; His 1.10; Phe 0.96; Ile 0.95; Trp(CHO) 1.75; Sta 0.89

[a] Peptides are designated by number and their sequences are listed in Table II. [b] IE, ion-exchange chromatography using (carboxymethyl)cellulose as the stationary matrix; PHPLC, preparative high-performance liquid chromatography on a Vydac, C18 reverse-phase column (2.2 cm i.d. × 30 cm; 10-μm particle size). Parentheses indicate isocratic condition in terms of the percentage of organic buffer B. [c] Percent purity is reported as determined from integration, by a Hewlett Packard 3390A integrator, of the reverse-phase HPLC elution profile of an analytical sample. [d] t is the HPLC retention time (min) using the solvent gradient indicated in parentheses. W., Waters μBondapak C18 analytical column. V, Vydac C18 analytical column. [e] Sta, 4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic acid; AVA, 5-aminovaleric acid; AHPPA, 4(*S*)-amino-3(*S*)-hydroxy-5-phenylpentanoic acid; Orn, ornithine.

tine) in place of the P1P1′ residues in substrate or pepstatin sequence analogues.[5,6] These compounds may act as a transition-state analogue[7,8] or "collected substrate" analogue inhibitor.[9] Studies of aspartyl protease inhibition have showed that the statine residue is important to the inhibitory mechanism (ref 9 and references therein). Using both the structure–activity relationships of published compounds[5] and molecular modeling to direct our work, we designed and synthesized a number of substrate analogues that contain statine and its congener, (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA), and then compared their renin-inhibitory activities.

Although no X-ray diffraction data on human renin are presently available, X-ray diffraction studies on a number of related aspartyl proteases reveal the existence of hydrophobic regions within the S1 and S1′ subsites of the active site.[10–12] From the three-dimensional structures of the aspartyl proteases, models for the three-dimensional structure of human renin have been constructed. These models suggest that similar hydrophobic pockets are present in renin, and that they are responsible for the binding of substrate and specific inhibitors.[13–15] To directly compare the importance of the hydrophobic side chain as well as the 3(*S*)-hydroxy group of statine at the P1P1′ position, 5-aminovaleric acid, which can act as a replacement for the scissile dipeptide unit in the backbone but lacks a hydrophobic side chain and the 3(*S*)-hydroxyl group, was substituted for the statine residue. The importance of other residues was also studied by either shortening the sequence from both the amino and carboxyl termini or substituting various amino acid residues in the substrate sequence. The relative potencies of the inhibitors were determined by measuring their IC_{50} values for human renin. A computer model of the three-dimensional structure of human renin[13] and its inhibitors at the active site was then used to analyze the structure–activity relationships. The model predicts certain interactions between the inhibitors and the subsites in the catalytic center of human renin and explains some of the differences in inhibitory activity. Sequence changes at the P2′P3′ position

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Table II. Substrate Analogue Sequences and Their Inhibitory Potencies on Human Plasma Renin

group	no.	sequence	IC_{50},[a] nM
		P5 P4 P3 P2 P1 P1' P2' P3'[b]	
A	equine substrate[20]	His-Pro-Phe-His-Leu-Leu-Val-Tyr	
	RIP	Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys	5200
	1	Pro-Phe-His-Phe-Phe-Val-Tyr-Lys-NH_2	~100 000
	15	Pro-His-Pro-Phe-Arg-Phe-Phe-Val-Tyr-Lys	200 000
	29[h]	D-His-Pro-Phe-His-Phe-$\overset{R}{}$Phe-Val-Tyr[c]	580
	3	His-Pro-Phe-His-Statine--Val-Tyr-Lys[d]	400
	4	Pro-His-Pro-Phe-His-Statine--Val-Tyr-Lys	10
	2	Pro-Phe-His--δ-Orn---Val-Tyr-Lys-NH_2[e]	~100 000
B	human substrate[21,22]	His-Pro-Phe-His-Leu-Val-Ile-His	
	7	Pro-Phe-His-Statine--Ile-His-Lys	900
	8	acetyl-Pro-Phe-His-Statine--Ile-His-Lys	40
	5	Pro-His-Pro-Phe-His-Statine--Ile-His-Lys	8
	6	acetyl-Pro-His-Pro-Phe-His-Statine--Ile-His-Lys	20
C	10	Pro-Phe-His-Statine--Ile-PheNH_2	220
	9	Pro-His-Pro-Phe-His-Statine--Ile-PheNH_2	3
	14	Pro-His-Pro-Phe-His--AVA---Ile-PheNH_2[f]	>1 000 000
D	13	Phe-Statine--Ile-PheNH_2	>100 000
	12	Pro-Phe-Phe-Statine--Ile-PheNH_2	430
	11	Pro-His-Pro-Phe-Phe-Statine--Ile-PheNH_2	30
E	16	Pro-Phe-His-StatineNH_2	68000
	17	Pro-His-Pro-Phe-His-StatineNH_2	900
F	18	Pro-His-Pro-Phe-His-AHPPA-Ile-PheNH_2[g]	30
	19	Pro-His-Pro-Phe-Lys-AHPPA-Ile-PheNH_2	3900
	20	Pro-His-Pro-Phe-His-AHPPA-Val-Tyr-LysNH_2	27
	21	Pro-His-Pro-Phe-His-AHPPA-Ile-His-LysNH_2	30
	22	acetyl-Pro-Phe-His-AHPPA-Ile-His-LysNH_2	46
G	28	Pro-Trp(CHO)-Pro-Phe-His-Statine--Ile-Trp(CHO)NH_2	16
	27	Pro-Trp(CHO)-Pro-Phe-His-AHPPA-Ile-Trp(CHO)NH_2	27
	26	Pro-Trp(CHO)-Pro-Trp(CHO)-His-AHPPA-Ile-Trp(CHO)NH_2	49
	23	Pro-Trp(CHO)-Pro-Trp(CHO)-His-AHPPA-Ile-PheNH_2	30
	24	Pro-Trp(CHO)-His-AHPPA-Ile-PheNH_2	67
	25	Pro-Trp(CHO)-His-AHPPA-Ile-Trp(CHO)NH_2	240

[a] All IC_{50} values are the average of three experiments, with the exception of **9**, the IC_{50} of which is the average of 20 experiments. [b] P1, P2, P3, P4, P5, P1', P2', and P3' designate substrate subsites with respect to renin active site subsites S1, S2, S3, S4, S5, S1', S2', and S3', respectively, after the convention of Schechter and Berger.[45] [c] R, reduced amide bond $-CH_2NH-$. [d] Statine, 4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic acid. [e] δ-Orn, δ-ornithine. [f] AVA, 5-amino-valeric acid. [g] AHPPA, 4(*S*)-amino-3(*S*)-hydroxy-5-phenylpentanoic acid. [h] This peptide is a gift from the UpJohn Co.

were also correlated with species specificity.

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 described by 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 peptides are listed in Table II.

In general, the crude products extracted after HF cleavage showed 40–70% purity as determined by analytical reverse-phase HPLC. Low-purity crude products, such as **5**, as well as the hydrophobic peptides of relatively low aqueous solubility, **22–28**, were purified by preparative reverse-phase HPLC under isocratic conditions. In such cases, 2–10 mg of crude peptide dissolved in 50–200 μL of 20% acetonitrile in water was usually used for each injection. Compound **9** was normally purified from 100 to 250 mg of crude material by ion-exchange chromatography, achieving products of greater than 99% purity.

Statine and AHPPA were difficult to quantify by conventional amino acid analysis. Statine is partially degraded by acid hydrolysis and therefore the amino acid could not be used as a standard. The area of the peak appearing in the statine position on the amino acid analyses of hydrolysates from 10 highly purified peptides containing one residue of statine varied by ±19% and was used to calculate the ratio of the statine residue reported for each peptide.

On an amino acid analyzer, the hydrochloride salt of 4(*S*)-amino-3(*S*)-hydroxy-5-phenylpentanoic acid (HCl·AHPPA) gave a single peak with a retention time of 77 min 13 s. The retention time for ammonia produced during acid hydrolysis of the peptides was 78 min 30 s. In acid hydrolysates of HCl·AHPPA, or Boc-AHPPA-OEt, the AHPPA peak overlapped the ammonia peak. A second peak corresponding to the degradation product, which was

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approximately 28% of the total area of both peaks, had a retention time of 74 min 30 s. Since the major AHPPA peak overlapped the ammonia peak after both 6 N HCl or 4 N methanesulfonic acid hydrolysis, the presence of the AHPPA residue was determined by the peak of the degradation product.

Umezawa et al.[18] reported that the pepsin-inhibitory potency of pepstatin decreased when the peptide contained O-acetylated statine. To exclude O-acetylation of either statine or AHPPA in the peptides reported here, each peptide was subjected to treatment with 250 equiv of 0.25 M Na_2CO_3 at 47 °C for 30–60 min and then subjected to analytical HPLC. There was no change in the elution position of the purified peptides as used in the inhibition studies. However, the AHPPA-containing peptides obtained after HF cleavage showed the presence of a significant amount of saponifiable (O-acylated) material. In contrast, crude statine-containing peptides showed no significant O-acylation.

The conditions for O-acylation of the AHPPA residue were examined. *N*-(*tert*-Butyloxycarbonyl)-(3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid ethyl ester (Boc-AHPPA-OEt)[19] was subjected to various acylation conditions at room temperature in dichloromethane. Results were monitored by reverse-phase analytical HPLC. No reaction occurred in the presence of 12.5% acetic anhydride over a period of 5 h. When diisopropylethylamine (DIEA) (5.6%) was added to the reaction mixture, a new reaction product (presumably the O-acetylated Boc-AHPPA-OEt) was formed with approximately 25% conversion after 2.5 h. However, in the presence of 4-(dimethylamino)pyridine (DMAP) and in the absence of DIEA, the same reaction product was detectable after 1 min. After 45 min of reaction, over 50% of the starting ester was acetylated. (DMAP was not used in the synthesis of the reported peptides, except in the esterification step during the incorporation of the first Boc-amino acid to the hydroxymethyl resin.) Another study was carried out with 2.5 equiv of Boc-glycine as the acylating compound and 2.5 equiv of DCC as the condensing reagent. After 4 h of mixing, no change was observed in the Boc-AHPPA-OEt HPLC peak.

Inhibition of Human Plasma Renin Activity. Plasma renin activity assays were performed on a pool of human plasma characterized by high renin activity. The final step of this procedure is an immunoassay for angiotensin I. None of the peptides studied showed immunologic cross-reaction with angiotensin I and consequently did not interfere with this step. Buffers containing 3% Me_2SO, which was required to dissolve some of the peptides, also had no effect on the assay. Control studies of high-renin human plasma incubated at 37 °C for 1 h at pH 8.5 did not generate angiotensin I, as compared with the 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 compared with a sample that does not contain inhibitory peptide. Compound **9** was the most potent analogue studied, with an IC_{50} of 3 ± 1.5 nM. Its inhibitory potency is very similar to that of Iva-His-Pro-Phe-His-Sta-Ile-Phe-NH_2 in human plasma at pH 7.4 (IC_{50} = 1.9 nM).[5]

To compare structure–activity relationships, we classified the peptides into seven groups (A–G, Table II) according to their sequence relationships. Peptides in group A include the dipeptide Val-Tyr at the P2′ and P3′ positions, as in the equine sequence of angiotensinogen.[20] Group B peptides include the Ile-His dipeptide, as in the human sequence of angiotensinogen.[21,22] Groups C and D comprise analogues that have Ile-Phe-NH_2 at the C-terminus. Group E contains peptides with statine-NH_2 at the C-terminus. The peptides in group E, assuming that statine occupies the S1S1′ subsites, have no residues to occupy the S2′ and S3′ subsites of the active site of renin. Group F shows the influence of the AHPPA residue in comparison with that of the statyl residue (groups A, B, and C). Group G contains N^{in}-formyltryptophan in various positions of the inhibitor sequence. In each group, proline was generally incorporated at the N-terminus, and in some cases, lysine was incorporated at the C-terminus so as to increase the aqueous solubility of the peptide.[2]

P5, P4, and P3 Positions. Table II show several sets of homologous statine-containing peptides that vary in the P5, P4, and P3 positions (**5** and **7**; **11**, **12**, and **13**; **9** and **10**; **17** and **16**). Inhibitory potency is diminished as the peptide is shortened progressively by removing residues from the amino terminus. This observation is also consistent with other reports.[5,23] An examination of the interactions of these peptides with the renin model shows multiple, important interactions between residues at the P5 to P3 positions and the active site groove, readily explaining the loss of binding efficiency as these residues are deleted.

We were intrigued by the observation that the inhibitory potency of **7** is 22-fold less than that of the acylated analogue **8**. In comparing **5** with **7**, the deletion of the P5 His and P6 Pro residues results in a reduction in potency of more than 100-fold. This is partially compensated for by simply acylating the amino terminus of **7**, as shown in **8**. A comparison between **21** and **22** also shows the effectiveness of acetylation at the amino terminus in restoring potency lost by deletion. An examination of the model shows that when the amino terminus is at the P4 position, it is surrounded by hydrophobic residues in the active site of the protein, with little space to accommodate solvent. No counter charge is present on the protein in this region, and the positively charged amino group can only be partially neutralized by the effects of solvation. This sets up an unfavorable electrostatic interaction. When the amino terminus is acetylated, the positive charge is eliminated from the amino terminus and replaced by an aliphatic group that enhances the interaction of the N-acetylated group with the environment of the S4 subsite of renin.

Role of the P2′P3′ Position. The importance of the P2′P3′ position in determining potency can be readily appreciated by comparing the following two pairs: **16** with **10**, and **17** with **9**. In each pair the sequences are identical, with the exception that in the first member the residues at P2′P3′ were deleted. In both cases, this deletion resulted in a 300-fold loss of potency. The loss of potency incurred by inhibitors that lack amino acids in the P2′ and P3′ positions is consistent with the early observations[20] on the length of the peptide substrate necessary for the maximal activity, which suggested the presence of binding sites on the enzyme for amino acids from the P5 to the P3′ posi-

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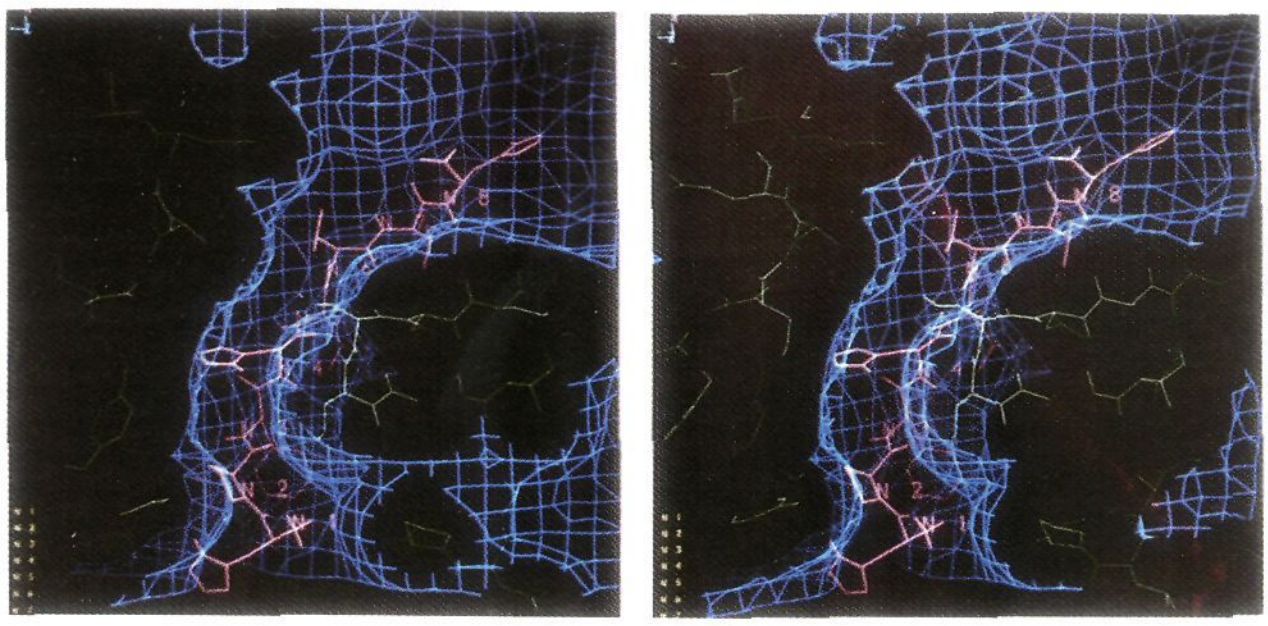

Figure 1. Stereoscopic computer images of the three-dimensional models for the octapeptide fragment of the natural substrate at the active site of human renin. The substrate fragment is shown in red and the protein in green. The blue wire mesh represents an envelope around the protein at a distance of 1.4 Å from the van der Waals surface. The N-terminus of the substrate fragment is at the bottom and the C-terminus at the top. The nitrogen atoms of the peptide backbone are labeled N1 through N8, starting from the N-terminus (ref 45):

N1	N2	N3	N4	N5	N6	N7	N8
His	Pro	Phe	His	Leu	Val	Ile	His
P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'

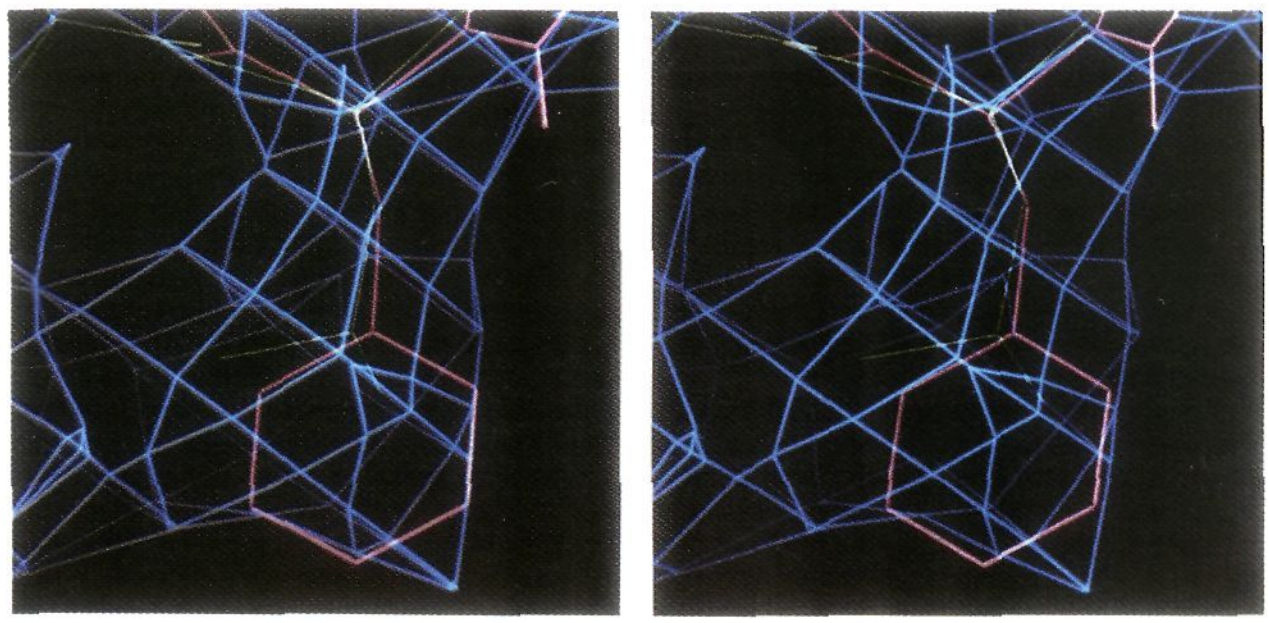

Figure 2. Stereoscopic computer images of the three-dimensional models for the P_1 side chains in the S_1 subsite of human renin. The statine side chain is shown in green and the AHPPA side chain in red. The blue wire mesh represents an envelope around the protein at a distance of 1.4 Å from the van der Waals surface. Any inhibitor atom that penetrates this envelope violates van der Waals contact and is thus in steric conflict with the protein. The statine side chain does not penetrate the envelope and is therefore not in steric conflict in the S_1 subsite. The phenylalanine in the S_1 subsite, however, clearly penetrates the envelope and is therefore in steric conflict with the protein. Because of these steric conflicts, shifts in the backbone would be necessary for binding of inhibitors that contain phenylalanine side chains in the S_1 subsite.

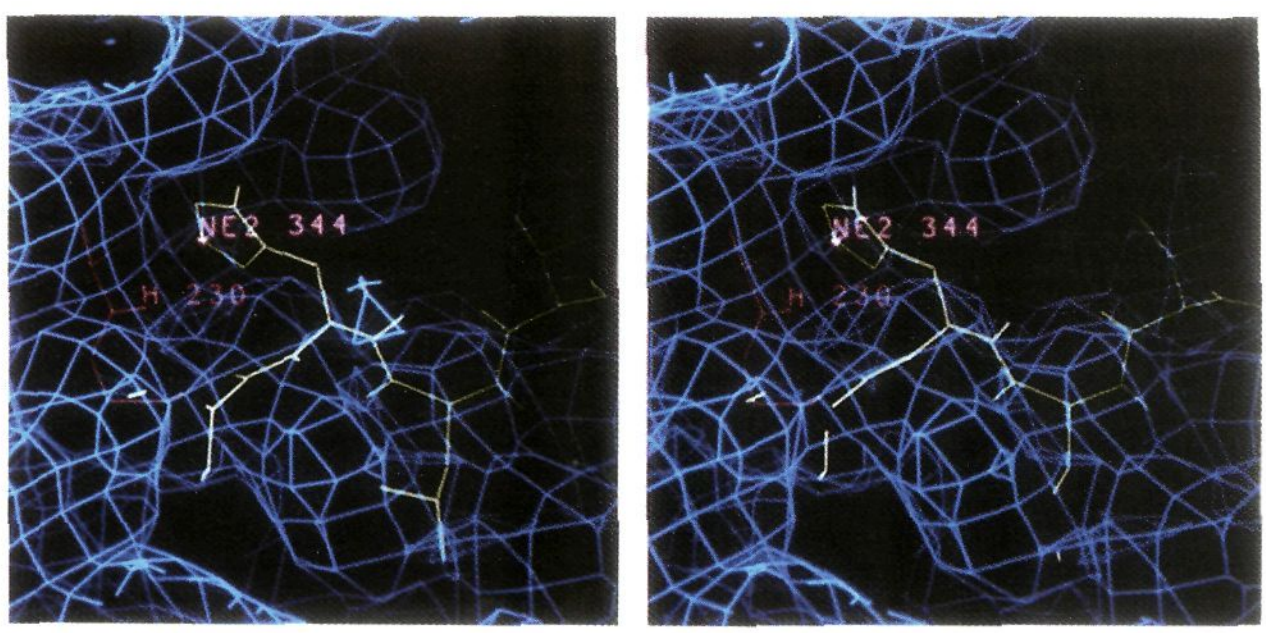

Figure 3. Stereoscopic computer images of the three-dimensional models for the octapeptide fragment of the natural substrate of human renin showing the hydrogen bond between the histidine in the P_2 position and the backbone nitrogen of Tyr-230. The octapeptide fragment is shown in green and the backbone of Tyr-230 is shown in red. The nitrogen of the histidine is labeled NE 2 344 and the hydrogen of the backbone nitrogen of Tyr-230 is labeled H 230. The blue wire mesh represents the van der Waals surface of the protein.

tions of the substrate. Our model (Figure 1) also shows an extended binding site that can be occupied favorably by at least eight amino acid residues.

This enhancement of binding by amino acid residues at the P2′ and the P3′ positions has been observed[5] for inhibitors in which the scissile peptide bond has been replaced by putative transition-state analogues. Potent aldehydic inhibitors that do not have amino acids in the P1′, P2′, etc., positions have been synthesized.[24,25] These inhibitors contain an aldehydic group at the C-terminus that does not span the scissile bond position. It is likely that the enhanced potency of the aldehydic inhibitors involves a binding mechanism different from that of the statine-containing inhibitors, and the contribution of residues in the P1′, P2′, or P3′ positions in this case does not apply.

Role of the P1–S1 Subsite Interaction. Table II shows that **4**, **5**, **8**, and **9**, which contain statine at the scissile bond, are approximately 2–10-fold-better inhibitors than their corresponding analogues (**20**, **21**, **22**, and **18**), which contain (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA). The AHPPA residue differs from the statine residue by replacing the isobutyl side chain of statine with a benzyl group. From an examination of our three-dimensional model, it is apparent that the benzyl side chain does not fit well into the S1 subsite when the side chain has the preferred orientation with χ_1 at −60° and χ_2 at 90° (see Figure 2). If the benzyl side chain maintains these preferred angles, then it would have to either shift the AHPPA backbone away from the aspartic acids or alter the conformation of the enzyme in order to alleviate the steric conflicts in the S1 subsite. The result would be a destabilization of the hydrogen bond that would otherwise occur between the 3-hydroxyl of statine and the aspartyl residues 37 and 225 in the binding pocket. A similar backbone shift was reported earlier when we noted that RIP, a weak competitive inhibitor of renin that contains two Phe residues at the P1 and P1′ positions, causes the backbone to shift and prevents the aspartic acid interaction present with the natural substrate.[26] Alternatively, the benzyl side chain could rotate about the χ_1 and χ_2 angles and alleviate the steric conflicts in this fashion. In either case the conformation would be energetically unfavorable and therefore the binding would not be as tight as in the case of the statyl counterpart, as would be expected from the addition of a large hydrophobic side chain that did not cause any structural distortions. Boger et al.,[5] on the basis of modeling from the X-ray structure of pepstatin bound to rhizopuspepsin, have pointed out that the phenyl ring of the AHPPA residue cannot be superimposed with leucine-type methyl groups found in statine. Such reasoning was used to account for the extremely potent and enhanced inhibition of renin by peptides containing the cyclohexylalanine analogue of Sta (ACHPA) when compared to the corresponding Sta or AHPPA peptides.[23] These data are all consistent with an important and highly specific interaction of the P1 residue side chain with the S1 subsite.

To probe the importance to binding at the P1 site of either the 3(*S*)-hydroxyl residue and the hydrophobic isobutyl side chain of statine, we synthesized two compounds that lacked a peptide bond at P1P1′; one had no side chain (**14**), the other an amino side chain (**2**). Neither showed inhibitory activity. This indicates that an inhibitor cannot be created by simply deleting the scissile peptide bond, and that the 3(*S*)-hydroxyl or a hydrophobic side chain, or both, are essential to tight binding of the enzyme.

Role of the P2 Histidine. Table II indicates that substitutions of the P2 histidine with basic residues such as lysine or arginine, or with an uncharged hydrophobic side chain such as phenylalanine, result in a profound loss of inhibitory activity (RIP vs. **15**, **9** vs. **11**, **18** vs. **19**). This is true for the competitive inhibitors containing conventional amino acids and for the compounds thought to be transition-state analogues that contain statine or AHPPA. In the model, there is a hydrogen bond between the ring nitrogen of the P2 histidine and residue 230 of the protein (Figure 3). Such a hydrogen bond cannot be made with the substituted residues in this position without causing a disruption of other intramolecular hydrogen bonds.

***N*^{in}-Formyltryptophan at P3, P5, or P3.** Sawyer et al.[27] report that for inhibitors containing either Phe-Phe or Pheψ(CH$_2$NH)Phe in the P1P1′ position within the P5-P3′ octapeptide template, *N*^{in}-formyltryptophan substituted at P5 is superior to histidine. They also found that RIP with *N*^{in}-formyltryptophan at position P3′ was significantly more potent than the corresponding inhibitor with tryptophan or tyrosine (>5- and 3-fold, respectively). An N- and C-terminal *N*^{in}-formyltryptophan-modified statine-containing substrate analogue, acetyl-Trp(For)-Pro-Phe-His-Statine-Val-Trp(For)-NH2, was reported to have highly potent inhibitory activity (IC_{50} = 0.38 nM).[27] To further examine the effect of this residue on the potency of renin inhibitors, we synthesized a series of *N*^{in}-formyltryptophan-containing analogues with proline as the N-terminal residue and isoleucine at the P2′ position (group G). Among the longer peptides that contain AHPPA at the position of the scissile bond, multiple substitutions of *N*^{in}-formyltryptophan appear to have little effect on potency. For example, a comparison between **27** and **18** shows that *N*^{in}-formyltryptophan substituted at the P5 and P3′ positions has no significant effect on inhibitory potency. Substitution of *N*^{in}-formyltryptophan for residues at the P5, P3, and P3′ positions (**18** vs. **26**) also has little effect. Also, **23** has the same potency as **18**. On the other hand, when **28** is compared with the statine-containing **9**, in which *N*^{in}-formyltryptophan is substituted at the P5 and P3′ positions, a 5-fold reduction in potency is seen.

As noted earlier, deletion of the P6 and P5 residues generally results in a marked loss of potency. Yet the combined deletion of Pro-Phe from the N-terminus of **18** and substitution of *N*^{in}-formyltryptophan at the P3 position (**24**) results in only a 2-fold loss in potency. This suggests that the *N*^{in}-formyltryptophan residue at the P3 position in shorter peptides may have a more significant effect on potency than it does in longer peptides. For a short peptide (**24**), substitution of *N*^{in}-formyltryptophan at the P3 position compensates for the loss of potency anticipated from the deletion of the P5 and P6 residues. This is consistent with the observation reported by Kokubu et al.[24] that the introduction of a more hydrophobic

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Table III. Species Specificity of Renin Inhibitory Peptides[a]

	IC_{50}[b] M				
no.	human	dog	cat	rabbit	rat
4	1×10^{-8}	3.7×10^{-8} (3.7)	9×10^{-8} (9)	1×10^{-7} (10)	$>10^{-6}$ (>100)
5	8×10^{-9}	5×10^{-7} (63)	1.25×10^{-6} (156)	2×10^{-6} (250)	$>10^{-5}$ (>1250)
9	3×10^{-9}	5×10^{-8} (16.7)	8.1×10^{-8} (27)	2×10^{-7} (67)	$>10^{-6}$ (>333)

[a] Studies were done on plasma obtained from hypertensive humans, mongrel dogs, New Zealand white rabbits, and Sprague–Dawley rats, with the exception that a mixture of purified renin and anephric dog plasma was used in the cat study. [b] All IC_{50} values are the average of three experiments, with the exception of **9**, the IC_{50} of which is the average of 20 experiments. Values in parentheses are the relative potency ratios calculated by (species IC_{50}) divided by (human IC_{50}).

residue, 3-(1-naphthyl)alanine, in place of phenylalanine at the P3 position significantly increases potency.

Species Specificity in Relation to P2′P3′. Three of the potent, statine-containing peptides (**4**, **5**, and **9**) were assayed for species specificity with dog, rabbit, and rat plasma as well as purified cat renin. These results are summarized in Table III. The peptides vary only in the P2′P3′ position, which defines the difference between the P5–P3′ segments of equine and human angiotensinogens. All three peptides show a decreasing order of inhibitory potency with respect to human, dog, cat, rabbit, and rat renins. Compound **5** shows the greatest range of inhibitory constants. Inhibitory potency with respect to canine, feline, rabbit, and rat renins is respectively 63, 156, 250, and more than 1250 times less than that of human renin. Compound **4** shows the least species specificity and **9** is intermediate. These data suggest that major differences among species may exist in the S2′S3′ subsites. An inference that can be drawn from this data is that canine renin is most homologous to human renin in this region of the molecule, while rat renin is least similar.

Summary

The interactions between a model of human renin and a number of peptides have been examined and compared to the apparent renin inhibitory effectiveness of these compounds in vitro. Studies on the inhibitory potency of homologous sets of peptides lacking the P5, P4, and P3 residues show progressively lower binding affinities. A study of the model shows that important contributions to binding are made by these residues. It seems that the acetyl group can replace Pro-His at the P5P6 position. A study of the model shows that a protonated amine at the amino terminus of P5 or P4 causes unfavorable electrostatic interactions in the binding cleft. At P6, the protonated amino-terminal nitrogen is far enough away from the protein to be solvated.

The substitution of a carbon–carbon bond for an amide bond at the P1P1′ position in the absence of any side chain or in the presence of an amino side chain results in compounds that do not inhibit renin. This points to the importance to renin binding of either the 3(*S*)-OH group or a hydrophobic side chain, or both. Further insight into hydrophobic interactions at this position may be gained by comparing statine and AHPPA at the P1P1′ position. AHPPA-containing peptides are generally less potent than homologous statine-containing peptides. A study of the model shows that the benzyl side chain of AHPPA cannot fit into the S1 subsite pocket, although the isobutyl side chain of statine does fit. The shift of the backbone disrupts a hydrogen bond between the OH of statine and aspartic acids 37 and 225 at the catalytic site.

The substitution of the histidine at the P2 position by other basic or uncharged residues results in decreased inhibitory potency. A study of the model shows that histidine forms a hydrogen bond with residue 230 of the enzyme that cannot be formed by the other basic residues at that position.

N^{in}-Formyltryptophan at P5, P3, or P3′ has little effect on inhibitory activity in the longer AHPPA-containing peptides. In statine-containing peptides, N^{in}-formyltryptophan seems to reduce activity. In shorter AHPPA-containing peptides, the presence of N^{in}-formyltryptophan at the P3 position seems to diminish the unfavorable effect of shortening a peptide from the amino terminus.

Not surprisingly, in view of species differences among angiotensinogens, inhibitors are markedly affected in their species specificities by changes in the P2′P3′ position.

Experimental Section

Synthesis of Renin-Inhibitory Peptides. All commercial protected amino acids were obtained from Peninsula Laboratories (San Carlos, CA) with the exception of *N*-δ-Boc-*N*-α-Cbz-ornithine and *N*-α-Boc-ε-(2-Cl-Cbz) lysine, which were obtained from Bachem (Torrance, CA). The side-chain protecting groups were tosyl for histidine and arginine and benzyl for tyrosine. The purity of the protected amino acids was checked by TLC prior to use. Other reagents were dichloromethane (Dow Chemical, Midland, MI) distilled from Na_2CO_3, *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 HCl (United States Biochemical, Cleveland, OH). *N*-Boc-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic acid (Boc-statine) was synthesized according to Rich et al.;[28] *N*-Boc-4(*S*)-amino-3(*S*)-hydroxy-5-phenylpentanoic acid (Boc-AHPPA) was synthesized according to Rich and Sun.[19] *N*-Boc-5-aminovaleric acid was synthesized according to the general procedure of Moroder et al.[29]

The renin-inhibitory peptides were synthesized in a stepwise manner according to the standard Merrifield solid-phase method.[16] Each synthesis was begun with 1 g of resin and 2.5 equiv of Boc-amino acid, with the exception of the Boc-statine and Boc-AHPPA syntheses, in which 1.5 equiv were used. In the case of the synthesis with hydroxymethyl-resin, the first Boc-amino acid was incorporated by the esterification procedure described by Wang[30] and Chang et al.[31] 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 and Boc-AHPPA, in which 1.5 equiv of DCC were used. The reaction time for each coupling step was 2 h. The coupling and deprotecting cycles were monitored with the Kaiser ninhydrin test.[32] To remove the Boc group, the resin was treated with 50% trifluoroacetic acid in DCM 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 (20 mL) treatment at 0 °C for 45 min in the presence of 10% anisole. After the HF was removed in vacuo,

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the residue was washed three times with anhydrous ether and air-dried. The peptide was extracted with water and then glacial acetic acid. The extracts were pooled and lyophilized. The crude peptide was gel-filtered in a Sephadex G-10 (Sigma) column (2.4 cm i.d. × 16 cm), which was equilibrated and eluted with 1 M acetic acid. The fractions containing the peptide were located by the Pauly[17] or ninhydrin tests. The peptide was subsequently purified by ion-exchange chromatography or reverse-phase preparative HPLC. For ion-exchange chromatography, the peptide was eluted from a (carboxymethyl)cellulose column (Whatman CM52, 2.4 cm i.d. × 30 cm) with a linear gradient of 0.01–0.3 M ammonium acetate buffer, pH 5.0. Preparative HPLC was carried out on a Vydac C18 column (2.2 cm i.d. × 25 cm, particle size 10 μm). Each peptide was eluted with a water–acetonitrile mixture containing 0.05% trifluoroacetic acid at a flow rate of 5 mL/min (under isocratic conditions with acetonitrile concentrations ranging from 15% to 30%). The absorbance of the effluent was measured at 214 nm. Before lyophilization, the purity of the fractions containing the desired peptide was assessed by analytical reverse-phase HPLC performed on a Vydac C18 or a Water 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) 25 to 60 to 80 to 25%, (b) 5 to 50 to 100 to 5%, (c) 20 to 60 to 100 to 20%, (d) 25 to 65 to 100 to 25%, (e) 0 to 40 to 80 to 0%, (f) 0 to 50 to 100 to 0%, (g) 10 to 50 to 100 to 10%, (h) 20 to 60 to 80 to 20%, (i) 30 to 70 to 100 to 30%, (j) 40 to 70 to 100 to 40%, (k) 15 to 60 to 80 to 15%, and (l) 15 to 50 to 80 to 15%.

For amino acid analysis, each peptide was hydrolyzed with 6 N HCl in evacuated, sealed tubes for 24 h at 110 °C. Peptides containing formyltryptophan were hydrolyzed with 4 N methanesulfonic acid.[33] The amino acid content of the hydrolysates was determined with a Dionex Model 500C analyzer. The formyltryptophan residue was identified by referring to a standard sample in which a known quantity of Boc-formyltryptophan had been hydrolyzed with 4 N methanesulfonic acid.

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 0–40% Me_2SO. These stock solutions were then subjected to a series of 1:10 dilutions with Tris buffer containing 0–10% Me_2SO. The plasma pool used in this study was collected over a year and a half from approximately 500 hypertensive patients. Blood samples obtained by renal vein catheterization were collected into Terumo Venoject tubes (Terumo Medical, Elkton, MD) containing EDTA chilled to 0 °C. The blood samples were centrifuged at 4 °C to separate the plasma, which was then pooled and activated with trypsin.[34] After activation, 5 mM phenylmethylsulfonyl 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 AI mL^{-1} h^{-1}. Blood samples from mongrel dogs, New Zealand white rabbits, and Sprague–Dawley rats were collected in EDTA (5 mM) at 0 °C, centrifuged at 4 °C to separate the plasma, and then mixed in PMSF (5 mM) and 8-hydroxyquinoline sulfate (3 mM).

Renin activity was determined by a radioimmunoassay for angiotensin I based on the method of Haber et al.[35] Plasma samples (700 μL, pH 7.4) were mixed with 17.5 μL of serial concentrations of peptide inhibitors. In the case of the cat renin inhibition assay, 20-μL aliquots of purified cat renin[36] (which generates approximately 4000 ng AI mL^{-1} h^{-1} at a cat renin substrate concentration of 14000 ng/mL) were mixed with 100-μL samples of anephric dog plasma containing serial concentrations of peptide inhibitors. The mixture was divided into two portions for incubation at 37 °C 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 mixture). Plasma renin activity, measured as the rate of angiotensin I generation, was obtained by a radioimmunoassay in which aliquots of reaction mixture in 1 mL of [^{125}I]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 (Travenol-Genentech Diagnostics, Cambridge, MA). The renin-activity value was obtained from a standard curve generated from a parallel experiment with known quantities of angiotensin I.

Molecular Modeling. X-ray diffraction studies of three aspartyl proteases (rhizopuspepsin, penicillopepsin and endothiapepsin) have been completed to a resolution of 3.0[12] and 2.8 Å[10] and recently extended to 2.1 and 1.8 Å.[37-39] Models have been built from the electron-density maps of these proteins and from maps of inhibitors at their active sites.[40-42] From the three-dimensional structures of these three aspartyl proteases, models for the three-dimensional structure of human renin have been constructed for use in the design of potent renin inhibitors.[13,15] The coordinates of the three-dimensional models for the aspartyl proteases and pepstatin were obtained from the individual investigators (D. Davies and T. Blundell) and from the Brookhaven Protein Databank. The coordinates of the three-dimensional model for human renin constructed in this laboratory and of the aspartyl proteases were displayed on an Evans and Sutherland PS340 high-performance graphics monitor using FRODO[43] software, and were superimposed so that pepstatin could be displayed in the active site of human renin.

The three-dimensional model of pepstatin was used as a starting structure for the construction of models for the octapeptide fragment of the natural substrate of human renin. Pepstatin is a naturally occurring peptide-like compound that contains two unique statine residues. With pepstatin at the active site of human renin as a template, a model of an octapeptide with the amino acid sequence of the naturally occurring substrate for human renin was constructed. The peptide backbone of pepstatin was used directly for those residues from the amino terminus of the peptide up to the scissile bond. The amino acid side chains were then constructed by replacing the amino acid side chains of pepstatin with those of the natural substrate. This was done in a manner that preserved the χ_1 and χ_2 angles of the corresponding side chains in pepstatin. For the amino acid residues in the substrate that lie between the scissile bond and the carboxy terminus of the substrate, the backbone had to be constructed in a slightly different manner because the statine residue of pepstatin that spans the scissile bond contains two more carbon atoms than does a natural amino acid. The presence of these two carbon atoms in the backbone of pepstatin prevents hydrolysis by the aspartyl proteases, but they also distort the conformation of the carboxy terminal residues so that it differs from that of the natural substrate. Because of the difference between pepstatin and the natural substrate in the backbone, the residues of the substrate in this part of the molecule had to be graphically modeled in the active site of human renin. The modeling was done in a manner that maximizes the hydrogen bonds between the peptide backbone of the substrate and the enzyme, and maximizes the hydrophobic interactions of the amino acid side chains, while minimizing any

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unfavorable steric interactions.

With the octapeptide fragment of the natural substrate at the active site of human renin as a template, models for several inhibitors discussed here were constructed at the active site of human renin. Inhibitors representative of different structural classes were chosen for construction. These inhibitors are RIP, **9**, 18, and **28**. The peptide backbone and the amino acid side chains of the natural substrate or of pepstatin were used directly where they were identical with the inhibitor. For the phenylalanines in the P1 and P1′ positions of RIP, the χ_1 and χ_2 angles for the corresponding residues of the model for the natural substrate were used to orient the phenylalanines in the preliminary model. The phenylalanine side chains were then adjusted by rotating the side chain around the Cα–Cβ bond (χ_1) and the Cβ–Cγ bond (χ_2) to minimize unfavorable steric contacts.

After preliminary models were constructed of pepstatin, the natural substrate and the inhibitors, the structures were refined by using the energy refinement program CHARMM.[44] The refinement was done in a manner similar to that used for the structure of human renin.[13] Constraints were placed on the movement of atoms from the initial positions and reduced every 25 cycles for the first 100 cycles. Another 100 cycles were completed without constraints on either the protein or the substrate.

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Limited Nerve Impulse Blockade by "Leashed" Local Anesthetics

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To measure the depth of the local anesthetic binding site within the neuronal membrane, biotin-containing polyethylene glycols having zero, three, and six ethylene glycol subunits were added to the *p*-amino termini of tetracaine and procaine, thereby interposing a pharmacologically inert "spacer" molecule between the local anesthetic and the biotin moiety. These biotinyl–local anesthetic derivatives produced "tonic" inhibition of the compound action potential of split, desheathed frog sciatic nerves in a concentration-dependent, reversible manner. However, no inhibition of the action potential occurred when sufficient avidin, a 66 000-MW protein that binds four biotins, was present to bind and anchor the biotin-containing end of each derivative outside the plasma membrane. Increasing the "leashed" anesthetic derivative's concentration to 4 times that which reduced impulse height by 50% in the absence of avidin still produced no detectable block when equimolar avidin was present. Apparently, the "spacer" in the derivative compound was too short to permit the avidin-complexed anesthetic to reach its site of action on the sodium channel. In a similar fashion, the local anesthetic derivatives produced "use-dependent" block when drug-treated nerves were stimulated at 40 Hz in the absence of equimolar avidin, but failed to produce "use-dependent" block when equimolar avidin was present. In common with others, we assume that tertiary amine local anesthetics may reach their binding site via hydrophobic (transmembrane) pathways without necessarily entering the cytoplasm. Thus, since our longest local anesthetic derivative, that containing six ethylene glycol subunits, placed the local anesthetic group a maximum of 15–18 Å from the surface of the avidin moiety, we conclude that the local anesthetic binding site for block of sodium channels of amphibian nerve must be $\gtrsim$15 Å from the outer surface of the plasma membrane.

Since local anesthetics were first identified as having an effect on nerves, several notable advances have been made toward defining their locus of action. The first advance was the demonstration by Taylor in 1959 that, under voltage clamp conditions, the primary effect of procaine on the squid axon was to decrease the sodium current activated by depolarizations.[1]

Experiments with permanently charged, quaternary amine derivatives of local anesthetics and with tertiary amine local anesthetics at different axoplasmic and extracellular pH values demonstrated a general lack of potency of charged local anesthetics applied to the external surface of neuronal membranes.[2,3] This contrasted markedly with both the potency of permanently uncharged local anesthetics (e.g., benzocaine) and of tertiary amine local anesthetics (at physiological pH) when applied on either side of the plasma membrane, and the potency of charged local anesthetics when directly applied inside the

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