base), 78010-24-9; **63**, 78010-21-6; **64**, 78010-22-7; **65**, 78010-20-5; **66**, 49755-48-8; **67**, 98051-89-9; **68**, 98051-90-2; **69**, 98064-12-1; **70**, 98064-13-2; **71**, 98051-91-3; **72**, 98051-92-4; **73**, 73845-37-1; **74**, 7035-94-1; **75**, 13947-20-1; **76**, 4299-09-6; **77**, 4299-13-2; $HNPh_2$, 122-39-4; $H_2NCH_2CH_2NHC_2CH_2OH$, 111-41-1; $H_2NCH_2CH_2OH$, 141-43-5; $CH_3(CH_2)_2COCl$, 141-75-3; $CH_3(CH_2)_4COCl$, 142-61-0; $CH_3(CH_2)_5COCl$, 2528-61-2; $CH_3(CH_2)_6COCl$, 111-64-8; $CH_3(CH_2)_8COCl$, 112-13-0; $CH_3(CH_2)_{18}COCl$, 40140-09-8; $H_2NCH(CH_3)CH_2CH_2Ph$, 22374-89-6; 4-$H_2NC_6H_4CH_2CO_2H$, 150-13-0; 4-$H_2NC_6H_4(CH_2)_3CH_3$, 104-13-2; 2,2′-dithiobis(benzoyl chloride), 19602-82-5; 1-amino-2-butanol, 13552-21-1; 2-amino-2-ethyl-1,3-propanediol, 115-70-8; 2,2′-dithiobis(5-chlorobenzoyl chloride), 64015-88-9; 2-ethylhexylamine, 104-75-6; 4-(aminomethyl)-piperidine, 7144-05-0; aminoacetaldehyde dimethyl acetal, 22483-09-6; glycine ethyl ester, 459-73-4; ethyl isocyanate, 109-90-0; lauroyl chloride, 112-16-3; palmitoyl chloride, 112-67-4; 3,5-diethylaniline, 1701-68-4; 4-bromo-1-naphthylamine, 2298-07-9; di-*n*-butylamine, 111-92-2; 1,2-benzisothiazolin-3-one, 2634-33-5; saccharin, 81-07-2; 2-propanolamine, 78-96-6; 4,4′-dithiobis(benzol chloride), 25717-23-1; 2,2′-dithiobis(3-hydroxypropylamino-carbonylbenzene), 36892-00-9; 2-(4-aminophenylsulfonyl-amino)benzothiazole, 6138-01-8; 2-(aminomethyl)furan, 617-89-0; 1-amino-4-chloronaphthalene, 4684-12-2; 1,4-diamino-7-hydroxynaphthalene, 98051-93-5; 3-amino-2-hydroxynaphthalene, 5417-63-0; 1-aminomethylnaphthalene, 118-31-0; 8-amino-quinoline, 578-66-5; 2-nitro-1-naphthylamine, 607-23-8; 3-amino-9-ethylcarbazole, 132-32-1; 1,2-dihydroxy-4-(2-amino-1-hydroxyethyl)benzene, 586-17-4; 2-amino-6-ethoxybenzothiazole, 94-45-1; 1,3-dioxoisoindole, 85-41-6; 1*H*-benzotriazole, 95-14-7.

Supplementary Material Available: NMR data for compounds [5], [8], [10], [24], [25], [28–30], [34], [47], [48], [50], [52], [53], [57], [58], [68], [69], and [73] (2 pages). Ordering information is given on any current masthead page.

Renin Inhibitors. Syntheses of Subnanomolar, Competitive, Transition-State Analogue Inhibitors Containing a Novel Analogue of Statine[1]

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Analogues of the renin octapeptide substrate were synthesized in which replacement of the scissile dipeptide with (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (statine, Sta) transformed the substrate sequence into potent, transition-state analogue, competitive inhibitors of renin. Synthesis and incorporation of the cyclohexylalanyl analogue of Sta, (3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoic acid (ACHPA), gave the most potent inhibitors of renin yet reported, including *N*-isovaleryl-L-histidyl-L-prolyl-L-phenylalanyl-L-histidyl-ACHPA-L-leucyl-L-phenylalanyl amide [Iva-His-Pro-Phe-His-ACHPA-Leu-Phe-NH_2, **3**], with renin inhibitions of $K_i = 1.6 \times 10^{-10}$ M (human kidney renin), $IC_{50} = 1.7 \times 10^{-10}$ M (human plasma renin), $IC_{50} = 1.9 \times 10^{-9}$ M (dog plasma renin), and $IC_{50} = 2.1 \times 10^{-8}$ M (rat plasma renin). This inhibitor **3**, containing ACHPA, was 55–76 times more potent vs. human renin than the comparable Sta-containing inhibitor **1** and 17 times more potent vs. dog renin than **1**. Inhibitor **3** lowered blood pressure in sodium-deficient dogs, with in vivo potency 19 times that shown by **1**, in close agreement with the relative in vitro potencies. Structure–activity results are presented that show the minimal N-terminus for these inhibitors. An ACHPA-containing pentapeptide, *N*-[(ethyloxy)carbonyl]-L-phenylalanyl-L-histidyl-ACHPA-L-leucyl-L-phenylalanyl amide [Etoc-Phe-His-ACHPA-Leu-Phe-NH_2, **8**], retained subnanomolar inhibitory potency. Molecular modelling studies are described that suggested the design of ACHPA.

The renin–angiotensin system (RAS) is a multiregulated proteolytic cascade that produces two potent pressor and aldosteronogenic peptides: an octapeptide, angiotensin II (AII), and a heptapeptide, angiotensin III (AIII).[2,3] Although the exact role that the RAS plays in the maintenance of normal blood pressure is unclear, it has been demonstrated that the pharmacological interruption of the RAS can lower blood pressure in a large majority of hypertensive patients.[4,5] Specifically, inhibitors have been developed of angiotensin converting enzyme,[6] the enzyme that cleaves the inactive decapeptide angiotensin I (AI) to yield AII. These angiotensin converting enzyme inhibitors may be a major advance in the treatment of hypertension and congestive heart failure.[5]

The first proteolytic step in the RAS is the renin enzyme reaction, in which the decapeptide AI is cleaved from a protein substrate, angiotensinogen. Competitive inhibitors of renin based upon the substrate peptide sequence have been reported from several laboratories.[7–12] We have described[13–15] the design of inhibitors of renin, with potencies around 10 nM ($K_i = 10^{-8}$ M), which are analogues of the minimum substrate octapeptide and which contain

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(1) Abbreviations follow IUPAC–IUB Joint Commission on Biochemical Nomenclature for amino acids and peptides; *Eur. J. Biochem.* 1984, *158*, 9–31. Additional abbreviations used are as follows: DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; Boc, *tert*-butyloxycarbonyl; Poc, (isopropyloxy)-carbonyl; Etoc, (ethyloxy)carbonyl; POA, phenoxyacetyl; TFA, trifluoroacetic acid; TEA, triethylamine; Iva, isovaleryl; Sta, (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.

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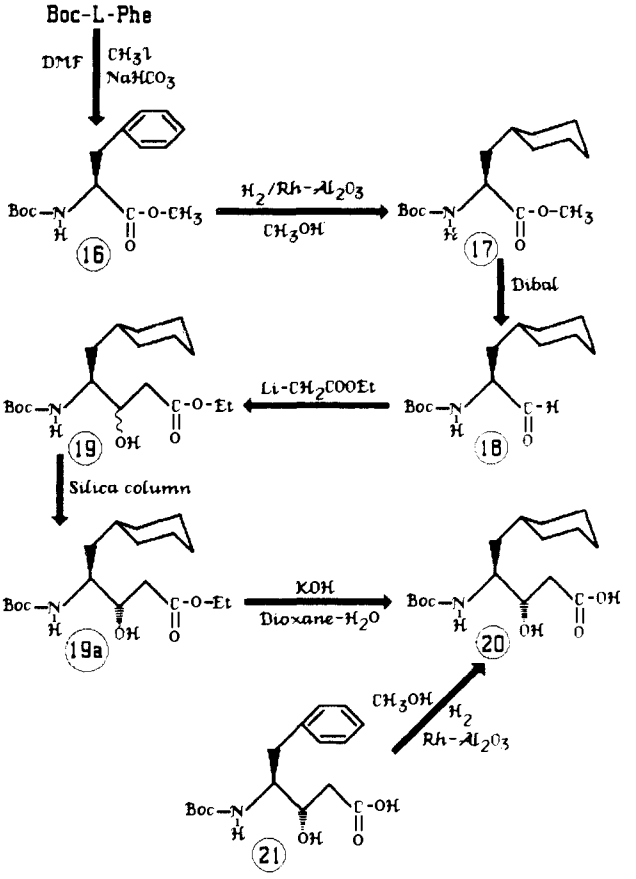
Scheme I. Synthesis of Boc-(3S,4S)-4-amino-5-cyclohexyl-3-hydroxypentanoic Acid (Boc-ACHPA)



the transition-state (or intermediate) dipeptide analogue statine, (3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid (Sta). This report describes the design and syntheses of inhibitors of renin containing a novel side-chain analogue, of Sta, called ACHPA, (3S,4S)-4-amino-5-cyclohexyl-3-hydroxypentanoic acid. Incorporation of ACHPA, as an analogue of the scissile dipeptide, into renin substrate sequences gives inhibitors with potencies approaching 100 pM (K_i = 10^{-10} M) for human renin, including the most potent inhibitor of human renin yet reported. These same compounds are nanomolar level inhibitors of a wide variety of primate and nonprimate renins and include the first reported potent inhibitor of rat renin.

Results

Synthesis: Boc-ACHPA. Synthesis of the Boc-protected Sta analogue *N*-(*tert*-butyloxycarbonyl)-(3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoic acid (Boc-ACHPA, **20**) is shown in Scheme I. Overall yield from Boc-L-Phe to the desired diastereomer **20** was 33%. Synthesis was begun from Boc-cyclohexylalanine methyl ester, **17**, produced in nearly quantitative yield from Boc-L-phenylalanine by esterification using methyl iodide and $NaHCO_3$ in dimethylformamide, producing **16**, followed by hydrogenation over Rh/Al_2O_3. For the hydrogenation of **16** to **17**, we noted some variability in the amount of time necessary for complete reduction, and the reduction time was extended for some batches. In all cases complete reduction could be attained. Conversion of **17** to the mixture of diastereomers, **19**, separation of diastereomers by column chromatography, and hydrolysis of the 3*S*,4*S* diastereomer **19A** to the free acid **20** followed the procedures described for Boc-Sta synthesis.[16] Diastereoselection in the aldol condensation step, **18** to **19**, was estimated from TLC to be approximately 55:45 in favor of the desired isomer, **19a**. After ester hydrolysis, the identity of the 3*S*,4*S* diastereomer **20** was confirmed by comparison of its properties with a sample produced by reduction of the authentic 3*S*,4*S* diastereomer of Boc-AHPPA (**21**, *N*-(*tert*-butyloxycarbonyl)-(3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid), prepared as described previously.[17] Assuming that no racemization takes place during reduction of **21** to **20**, then comparison of the optical rotations of the two samples of Boc-ACHPA, **20**, provides an estimate of the minimum enantiomeric purity produced by the route **16** to **20**. Thus, a diastereomerically-pure sample of **21** with $[\alpha]^{30}_D$ −36.2° (lit.[17] $[\alpha]^{30}_D$ −37.0), that was considered to have 99% ee on the basis of optical rotation, was converted by reduction to **20**, with $[\alpha]^{30}_D$ −32.2°. The route **16** to **20** (Scheme I) produced material with $[\alpha]^{30}_D$ −32.5°, indicating that the material produced by this route has >99% ee.

The optical purity of **19a** was established more rigorously by the preparation of diastereomeric derivatives, followed by analyses on HPLC, adapted (C. F. Homnick, unpublished) from the method of Gal and Sedman.[18] A sample of **19a** was deprotected, using cold trifluoroacetic acid followed by evaporation of the solvent under high vacuum, and reaction of the free amino acid with both the enantiomerically pure (*R*)-α-methylbenzyl isothiocyanate and the commercially available (*RS*)-α-methylbenzyl isothiocyanate. Analyses by HPLC indicated presence of 1.8% of a side product, assumed to be the diastereomer derived from the undesired 3*R*,4*R*-isomer impurity in **19a**, although identification of this diastereomeric product was based solely on its HPLC retention time. Since side reactions in the deprotection and derivatization chemistry have not been ruled out, unambiguous determination of the optical purity of **19a** must await independent preparation and analysis of the 3*R*,4*R* analogue of **19a**, beginning from Boc-D-phenylalanine.

We found production of **20** by way of **21** (synthesized as described[17] also from Boc-L-Phe) gave overall inferior yields compared to **16** to **20** (results not shown) due to the comparatively poorer chemical yields in the synthesis of **21**, especially in the aldol condensation step, using Boc-phenylalaninal (vs. Boc-cyclohexylalaninal, **18**). In addition, the chirality of Boc-phenylalaninal is considerably

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Table I. Solid-Phase Synthesis: Schedule of Steps for 2-mmol Run

step	reagent/solvent	vol, mL	mix time, min
	Coupling: Program 1		
1	CH_2Cl_2	4 × 20	2
2	40% TFA in CH_2Cl_2	1 × 20	2
3	40% TFA in CH_2Cl_2	1 × 20	25
4	CH_2Cl_2	3 × 20	2
5	10% TEA in CH_2Cl_2	2 × 20	5
6	CH_2Cl_2	4 × 20	2
9	Boc-amino acid and HBT (2.5 equiv each) in 2:1 DMF/CH_2Cl_2, mix and hold (no drain)	1 × 15	5
10	1.0 M DCC in CH_2Cl_2	1 × 5	30
11	DMF	1 × 20	2
12	methanol	2 × 20	2
13	CH_2Cl_2	2 × 20	2
14	methanol	1 × 20	2
15	CH_2Cl_2	1 × 20	2
	Recoupling: Program 2		
1	CH_2Cl_2	3 × 20	2
2	10% TEA in CH_2Cl_2	2 × 20	5
3	CH_2Cl_2	3 × 20	2
4	Boc-amino acid and HBT (2.5 equiv each) in 2:1 DMF/CH_2Cl_2, mix and hold (no drain)	1 × 15	5
5	1.0 M DCC in CH_2Cl_2	1 × 5	30
6	DMF	1 × 20	2
7	methanol	2 × 20	2
8	CH_2Cl_2	2 × 20	2
9	methanol	1 × 20	2
	DNP Removal: Program 3		
1	CH_2Cl_2	1 × 20	2
2	DMF	2 × 20	2
3	10% thiophenol in DMF	1 × 20	25
4	DMF	1 × 20	2
5	10% TEA in CH_2Cl_2	1 × 20	5
6	DMF	1 × 20	2
7	10% thiophenol in DMF	1 × 20	25
8	DMF	3 × 20	2
9	methanol	2 × 20	2
10	CH_2Cl_2	3 × 20	2

more labile on silica (results not shown). Treatment of the crude Boc-amino acid aldehydes by a short pass down silica is necessary to liberate the free aldehyde, produced apparently as a mixture of aldehyde and methyl hemiacetal by the Dibal workup described. Even for the relatively chirally stable 18, racemization is promoted on silica by traces of acidic impurities found in commercial ethyl acetate, and it was crucial to use freshly distilled ethyl acetate for column chromatography.

Syntheses: Peptides. Syntheses of peptide renin inhibitors, listed in Table III, were most efficiently accomplished by standard solid-phase methodology[19] on a Merrifield resin, using protocols shown in Table I. Efficient and complete coupling of Boc-Sta and its analogues Boc-AHPPA and Boc-ACHPA was achieved with use of just 1.25 molar excess of the Boc-amino acid with prolonged coupling times and incorporation of a recoupling cycle using the retained coupling solution. No evidence was seen for byproducts that might arise from subsequent acylation on the unprotected 3-hydroxyl group, and thus use of these amino acid analogues in solid-phase syntheses was unremarkable. Peptides were cleaved efficiently and cleanly from the resin by acetic acid catalyzed aminolysis, and they were purified by silica gel chromatography.

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Several peptides were prepared by various solution methods, described in the Experimental Section. Characterization data are given in Table II and in the Experimental Section. Overall isolated yields calculated from the starting Boc-Phe resin were typically 40–60%.

In Vitro Enzyme Inhibition. The inhibitory potencies vs. human and dog renins of the renin substrate analogues containing Sta or the Sta analogues AHPPA and ACHPA are shown in Table III. The human and dog plasma inhibitions were measured by radioimmunoassay for AI production from the endogenous plasma substrate.[20] Inhibitions are expressed as IC_{50} values and were calculated for several inhibitor concentrations bracketing the IC_{50}. Independently measured values using slight variations in assay protocol generally gave resulting IC_{50} values in close agreement. A notable exception were four independent IC_{50} determinations for 1 in human plasma, measured at IC_{50} = 4.5 and 7.0 nM (from 10^{-3} M methanol solution) and as IC_{50} = 16 and 26 nM (from 10^{-3} M acetic acid solution). The value listed in Table III is the average (= 13 nM) of these four rather disparate determinations. No such variation was noted for several other inhibitors that were assayed from both initial solvents, and it is not clear why the variation was seen in this one case. Inhibition of highly purified human kidney renin was determined in a fluorometric assay using a synthetic tetradecapeptide substrate.[21] Analyses of the inhibition kinetics were consistent with competitive inhibition in all cases, and K_i values were calculated from fits of the experimental data to a standard competitive inhibition equation.[21] For the human plasma renin assay, the concentrations of human renin and renin substrate (angiotensinogen) were not measured. Therefore, the IC_{50} values determined for inhibition of human plasma renin (pH 7.4) cannot be converted to K_i values for direct comparison with the K_i values determined in the kidney renin assay (pH 7.2). However, since the renin concentration in human plasma is normally about 8×10^{-12} M (for a plasma renin activity of 3.5 ng of angiotensin 1 mL^{-1} h^{-1} assayed according to Slater and Strout[22] and using the enzyme kinetic constants cited therein) and since the concentration of human angiotensinogen (variously reported, for example, between 3.4 and 108 $\mu g/mL^{-1}$ in normotensive females[23]) is normally less than or equal to the K_m for angiotensinogen (K_m = 100 μg mL^{-1} for purified human renin), competitive inhibitors with $K_i \geq 1 \times 10^{-10}$ M would be expected ideally to have IC_{50} values in the human plasma renin assay between 1.05 and 2.1 times their K_i values in the human kidney assay, assuming the enzymes being measured in both assays are the same.[24] Only for the phenoxyacetylated ACHPA-containing compound 14 are the human kidney and plasma values widely disparate. The presence of the phenoxyacetyl group along with the cyclohexyl-containing ACHPA makes this analogue extremely hydrophobic. Although the water solubility is very low, no precipitate was noted in the plasma assay. In the presence of plasma, however, it is possible that the compound is adsorbed onto some plasma component and is thus unavailable for renin inhibition. This phenomenon, of a weak inhibition obtained in the human

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Table II. Characterization of Renin-Inhibitory Peptides Containing Statine, AHPPA, and ACHPA[a]

no.[b]	amino acid anal.[c]	HPLC, % purity	TLC, R_f[d]	formula[e]	$[\alpha]_D$,[f]
1	His, Pro, Phe, Sta, Leu	99.7	0.19C, 0.50D	$C_{54}H_{76}N_{12}O_9 \cdot 2H_2O \cdot 2C_2H_4O_2$	-43.7
2	His, Pro, Phe, Leu	99.2	0.21C, 0.43D	$C_{57}H_{74}N_{12}O_9 \cdot 3H_2O$	-43.9
3	His, Pro, Phe, Leu	98.0	0.21C, 0.50D	$C_{57}H_{80}N_{12}O_9 \cdot H_2O \cdot C_2H_4O_2$	-40.3
4	Phe, His, Leu	99.4	0.58C, 0.60D	$C_{43}H_{62}N_8O_8 \cdot 2H_2O$	-31.1[g]
5	Phe, His, Leu	98.0	0.49C, 0.59D	$C_{46}H_{60}N_8O_8 \cdot 0.5H_2O$	-35.0
6	Phe, His, Leu	96.3	0.48C, 0.63D	$C_{46}H_{66}N_8O_8 \cdot 1.5H_2O$	-32.1
7	Phe, His, Leu	97.3	0.45C, 0.52D	$C_{45}H_{64}N_8O_8 \cdot H_2O$	-35.4
8	Phe, His, Leu	98.5	0.34C, 0.38E	$C_{44}H_{62}N_8O_8 \cdot 2H_2O$	-34.3[g]
9	Phe, His, Sta, Leu	97.1	0.32C, 0.19E	$C_{40}H_{56}N_8O_7 \cdot 4H_2O$[h]	-27.8
10	Phe, His, Leu	94.4	0.31C, 0.46D	$C_{43}H_{54}N_8O_7 \cdot 2H_2O$	-28.6
11	Phe, His, Leu	90.8	0.39C, 0.55D	$C_{43}H_{60}N_8O_7 \cdot 2H_2O$	-26.6
12	His, Leu, Phe	98.3	0.36C, 0.21E	$C_{37}H_{51}N_7O_7 \cdot 2H_2O$	-30.9[i]
13	His, Leu, Phe	99.2	0.26C, 0.46D	$C_{40}H_{49}N_7O_7 \cdot 2H_2O$[j]	-24.4[k]
14	His, Leu, Phe	99.6	0.44C, 0.20E	$C_{40}H_{55}N_7O_7 \cdot 2H_2O$	-30.0[k]

[a] Sta, (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. [b] See Table III for sequence. [c] Values ± 4%. Sta, AHPPA, and ACHPA not usually determined quantitatively. NH_3 values found, 1.1–1.9, include varying background of 0.1–0.9. NMR found consistent with structure. [d] Solvent systems (A, B, etc.), see Experimental Section. [e] Analyses for C, H, N were ±0.4% of expected for formulae shown, unless noted. Acetic acid salts confirmed by NMR. Expected M + H ion seen in FAB-MS. [f] c 0.5, CH_3OH. [g] c 0.25, CH_3OH. [h] C, H; N: calcd, 13.45; found, 12.87. [i] c 0.25, CH_3OH–$CHCl_3$ (1:1). [j] C, N; H: calcd, 7.60; found, 6.74. [k] c 1.0, CH_3OH.

Table III. Renin Inhibition by Substrate Analogues Containing Statine, AHPPA, and ACHPA[a]

no.	compd	renin inhibition, 10^{-9} M: human kidney (K_i)	human plasma (IC_{50})	dog plasma (IC_{50})
1	Iva-His-Pro-Phe-His-Sta-Leu-Phe-NH_2	8.8[b]	13[c]	32
2	Iva-His-Pro-Phe-His-AHPPA-Leu-Phe-NH_2	18	2.2	100
3	Iva-His-Pro-Phe-His-ACHPA-Leu-Phe-NH_2	0.16[d]	0.17[e]	1.9[f]
4	Boc-Phe-His-Sta-Leu-Phe-NH_2	190	140	290
5	Boc-Phe-His-AHPPA-Leu-Phe-NH_2	49	45	68
6	Boc-Phe-His-ACHPA-Leu-Phe-NH_2	0.88	2.2	13
7	Poc-Phe-His-ACHPA-Leu-Phe-NH_2	1.1	5.1	20
8	Etoc-Phe-His-ACHPA-Leu-Phe-NH_2	0.52	0.76	9.5
9	Ac-Phe-His-Sta-Leu-Phe-NH_2	930	76	140
10	Ac-Phe-His-AHPPA-Leu-Phe-NH_2	210	100	64
11	Ac-Phe-His-ACHPA-Leu-Phe-NH_2	7.7	3.2	14
12	POA-His-Sta-Leu-Phe-NH_2	4100	nd	nd
13	POA-His-AHPPA-Leu-Phe-NH_2	580	>1000[g]	>1000[g]
14	POA-His-ACHPA-Leu-Phe-NH_2	14	1000	>1000[g]
15 (pepstatin)	Iva-Val-Val-Sta-Ala-Sta	13000	22000	1300

[a] Statine (Sta), (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; Poc, (isopropyloxy)carbonyl; Etoc, (ethyloxy)carbonyl; POA, phenoxyacetyl; Iva, isovaleryl; nd, not determined. [b] Combined data from two experiments. [c] Average of four determinations: IC_{50} = 7.0 and 4.5 nM (from methanol solution), and IC_{50} = 26 and 16 nM (from acetic acid solution). IC_{50} = 11, 12, and 18 nM, pH 6.0 (from acetic acid solutions). [d] Combined data from two independent determinations: K_i = 0.15 and 0.16 nM. [e] Average of six determinations: IC_{50} = 0.18, 0.22, 0.14, 0.11, 0.18, and 0.18 nM. IC_{50} = 0.078 nM, pH 6.0. [f] Average of four determinations: IC_{50} = 2.3, 2.3, 2.0, and 0.96 nM. [g] 20–40% inhibition at 1000 nM.

plasma assay (IC_{50}) for a compound determined to be a good inhibitor in the purified human renin assay (K_i), was seen for several other small, generally very hydrophobic compounds (data not shown), but we have been unable to provide a convincing explanation, since all small, hydrophobic inhibitors do not show the disparity. The phenomenon is under study.

A comparison of the enzyme inhibitions of the heptapeptide inhibitors 1, containing Sta, and 3, containing the cyclohexylalanine analogue ACHPA, is given in Table IV. Inhibition of human and dog plasma renins (IC_{50}) and human kidney renin (K_i) was calculated as in Table III. Plasma renin inhibition (IC_{50}) for rhesus monkey, rabbit, cat, and rat was determined similarly in high or low renin plasma, as noted. Inhibition of mouse submaxillary renin (K_i) was determined as for human kidney renin. Inhibition of hog kidney renin was determined as an IC_{50} value in an assay using a radioactively labeled synthetic decapeptide substrate, Ile-His-Pro-Phe-His-Leu-[^{14}C]Leu-Val-Tyr-Ser,[17] and IC_{50} values were converted to K_i values assuming competitive inhibition.[24] Inhibition of porcine pepsin and rhizopuspepsin was determined (IC_{50}) with use of a synthetic heptapeptide substrate, Phe-Gly-His-Phe-(4-NO_2)-Phe-Ala-Phe-OCH_3, as described previously,[25] and the IC_{50} values were converted to K_i values assuming competitive inhibition.[24] Inhibition of rabbit liver cathepsin D (K_i) was determined with use of [^{14}C-methyl]-glycinated hemoglobin substrate as described previously.[26] Inhibition of porcine plasma angiotensin converting enzyme (IC_{50}) was determined with use of (benzyloxycarbonyl)-Phe-His-Leu as substrate, as described.[27] The ratio Sta/ACHPA, given in Table IV, that

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Table IV. Comparison of Enzyme Inhibitions by Renin Substrate Analogues Containing Statine and ACHPA[a]

enzyme	enzyme inhibn, 10^{-9} M: Iva-His-Pro-Phe-His-*Xaa*-Leu-Phe-NH_2		
	Xaa = Sta (1)	Xaa = ACHPA (3)	STA/ ACHPA
human kidney renin	8.8	0.16[b]	55
human plasma renin	13[b]	0.17[b]	76
rhesus monkey plasma renin	6.2	0.19[c]	33
rabbit plasma renin	18	0.54[d]	33
dog plasma renin	32	1.9[b]	17
mouse submaxillary renin	14	2.1	6.7
hog kidney renin	10[e]	3.7[f]	2.7
rat plasma renin (pH 7.4)	630[g]	21[h]	30
rat plasma renin (pH 6.0)	115[i]	26[j]	4.4
porcine pepsin	27	28	0.96
rabbit liver cathepsin D	210	27	7.8
rhizopuspepsin	200	230	0.87
angiotensin converting enzyme	54000	12000	4.5

[a] For abbreviations, see Table III. Human kidney and mouse submaxillary gland renin: K_i; pH 7.2; 37 °C. Human, monkey, rabbit, dog, and rat plasma renins: IC_{50}; pH 7.4; 37 °C, except where noted. Hog kidney renin: K_i; pH 7.3, 30 °C. Porcine pepsin, rabbit liver cathepsin D and rhizopuspepsin: K_i; pH 4.0; 30 °C (cathepsin D). [b] Average of multiple determinations: see notes in Table III. [c] Averaged from two experiments: I_{50} = 0.12 nM, low renin plasma (PRA = 0.84 ng of Al mL^{-1} h^{-1}); and I_{50} = 0.27 nM, high renin plasma (PRA = 7.3 ng of Al mL^{-1} h^{-1}). [d] Average from two experiments: IC_{50} = 0.52 nM, low renin (PRA = 2.4 ng of Al mL^{-1} h^{-1}); and IC_{50} = 0.57 nM, high renin (PRA = 8.0 ng of Al mL^{-1} h^{-1}). [e] Averaged from four experiments: K_i = 10, 8.6, 10, and 12 nM. [f] Averaged from two experiments: K_i = 3.3 and 4.1 nM. [g] Averaged from two experiments: IC_{50} = 600 and 660 nM. [h] Averaged from three experiments: IC_{50} = 17, 16, and 29 nM. [i] Averaged from two experiments: IC_{50} = 130 and 110 nM. [j] Averaged from two experiments: IC_{50} = 17 and 35 nM.

is the ratio of K_i values for heptapeptide inhibitors 1 and 3 vs. renins and the other enzymes, indicates the potency effect of the ACHPA substitution. This potency-enhancing effect of ACHPA ranges from 2.3 to 76 times for renins, with the maximal effect seen for human renin. The effect on inhibition of other aspartic proteinases is generally less, with no additional inhibition seen for the ACHPA compound vs. the Sta compound for porcine pepsin and rhizopuspepsin.

In Vivo Renin Inhibition. The relative effects of 1 and 3 on blood pressure in four conscious, sodium-deficient dogs are shown in Figure 1. The compounds were administered intravenously to trained beagles at the indicated infusion rates. Each dose was preceded by a bolus injection of 4 times the per minute infusion rate to hasten the attainment of equilibrium, and each infusion was continued for 45 min. Two dogs received increasing doses of 1 first, followed by the doses of 3, and the order was reversed for the other two dogs. Full blood pressure recovery was attained prior to infusion of the other compound. Blood pressure was measured by tail cuff and recorded as an average of six readings 1 min apart after 45 min. For the ACHPA-containing 3, mean arterial blood pressure averaged 98 ± 4 mmHg before infusion and was reduced in a dose-dependent manner by 15 ± 4, 18 ± 4, and 29 ± 3 mmHg by 1.2, 2.4, and 4.8 μg kg^{-1} min^{-1}, respectively. Regression analysis indicated an ID_{15mmHg} for 3 of 1.4 μg kg^{-1} min^{-1} (95% confidence limit: 0.69–2.76 μg kg^{-1} min^{-1}) for blood pressure lowering by 3. For the Sta-containing 1, mean arterial blood pressure averaged

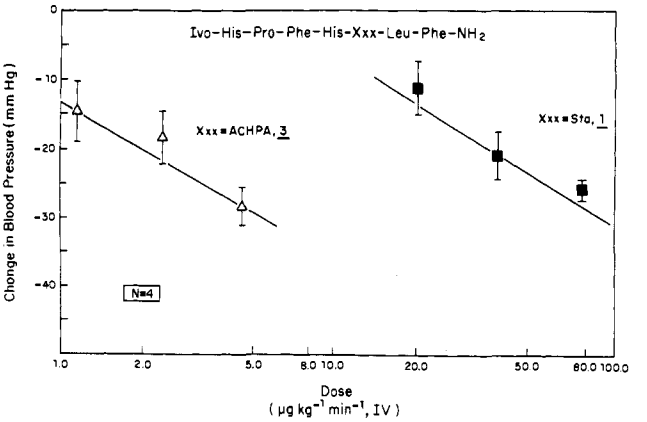

Figure 1. Effect of intravenous infusions of Iva-His-Pro-Phe-His-Sta-Leu-Phe-NH_2, 1 (■) and Iva-His-Pro-Phe-His-ACHPA-Leu-Phe-NH_2, 3 (△), on mean arterial blood pressure in sodium-deficient dogs.

96 ± 4 mmHg before infusion and was reduced in a dose-dependent manner by 11 ± 4, 21 ± 3, and 26 ± 1 mmHg by 20.0, 40.0, and 80.0 μg kg^{-1} min^{-1}. Regression analysis indicated an ID_{15mmHg} for 1 of 28 μg kg^{-1} min^{-1} (95% confidence limit: 18–42 μg kg^{-1} min^{-1}) for blood-pressure lowering by 1. For both compounds, there was no increase in heart rate at the lowest doses tested, and only a small increase in average heart rate (maximum +21 beats/min, not strictly dose related), even for the maximal blood-pressure-lowering doses (data not shown). In separate experiments (data not shown), it was found that plasma renin activity was inhibited almost completely at all infusion levels of either compound. The blood pressure dose–response lines for 1 and 3, shown in Figure 1, are parallel and indicate that 3 is 19 times more potent in this in vivo assay than 1 (95% confidence limits: 11–36 times more potent).

Discussion

Molecular modelling studies have been described that led to the hypothesis that statine (Sta) can be a dipeptide transition-state (or intermediate) analogue of the scissle dipeptide in renin substrate,[13-15] leading to heptapeptide inhibitors such as 1, isovaleryl-His-Pro-Phe-His-Sta-Leu-Phe-NH_2, a transition state analogue of the minimum octapeptide renin substrate His-Pro-Phe-His-Leu-Leu-Val-Tyr. These modelling studies used the X-ray structure of pepstatin (isovaleryl-Val-Val-Sta-Ala-Sta) bound in *Rhizopus chinensis* aspartic protease (rhizopuspepsin)[28] as a starting point to construct a model for the renin-bound conformation of a heptapeptide, statine-containing analogue of the renin octapeptide substrate. The natural-product pepstatin is a potent inhibitor of most aspartic proteinases, including rhizopuspepsin[15] (for example, for pepsin,[29] $K_i = 5 \times 10^{-11}$ M), but it is a relatively weak inhibitor of renin, a highly-specific aspartic proteinase (for human kidney renin,[13] $K_i = 1.3 \times 10^{-5}$ M). Nevertheless, pepstatin and pepstatin derivatives have been studied as renin inhibitors.[30-33] The molecular modelling used in our

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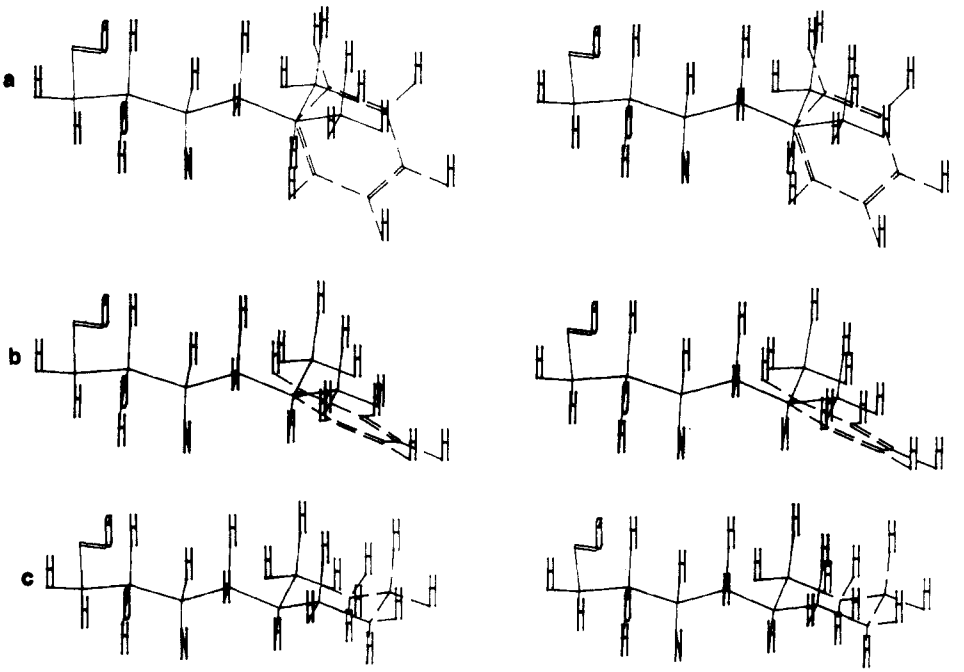

Figure 2. (a) Superposition of (3*S*,4*S*)-4-amino-5-phenyl-3-hydroxypentanoic acid (AHPPA, dashed lines) onto the X-ray structure of (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (Sta), as found in the central Sta of pepstatin (Iva-Val-Val-*Sta*-Ala-Sta) bound in the active site of rhizopuspepsin.[28] The dihedral angle (χ^{21}) of the phenyl ring of AHPPA was set at +90°. The attached hydrogens are at idealized positions. (b) Superposition of AHPPA (dashed lines) onto Sta as in (a), except χ^{21} = +137°. (c) Superposition of (3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoic acid (ACHPA, dashed lines) onto the X-ray structure of Sta, as in (a).

work assumed that the basic architectures of the active-site clefts were similar for renin and rhizopuspepsin, both of which are aspartic proteinases, and hypothesized that the differences in enzyme specificity shown by the two enzymes might be due to differences in active-site side chains within a broadly-similar aspartic proteinase active site. The model bound conformation of renin substrate, developed with use of these assumptions, was consistent with structure–activity relations of a number of linear and cyclic renin-inhibitor analogues.[14,15]

In the X-ray structure of pepstatin bound in rhizopuspepsin, the central Sta of pepstatin (Iva-Val-Val-*Sta*-Ala-Sta) is bound with its tetrahedral 3-hydroxyl group pointing at the two active-site, catalytically important, aspartic acid carboxyl groups. The leucine-type side chain of this Sta is bound underneath an enzyme hydrophobic flap, which covers one side of the active site cleft. This central Sta is thought to be bound in the same position as that of the amino acid on the N-terminal side of the scissile peptide bond of a rhizopuspepsin substrate.[28] Our model for the bound conformation of Sta-containing renin inhibitors has the Sta bound exactly as seen in the rhizopuspepsin X-ray structure. Confirmation of this hypothesized bound conformation awaits completion of the X-ray structure of the complex of Sta-containing renin inhibitors bound in mouse submaxillary renin.[34] Of great interest in the X-ray structure of rhizopuspepsin–pepsin is the large hydrophobic pocket that binds the Sta side chain. Modelling indicates that there is a large unfilled hydrophobic area extending past the Sta side-chain methyl groups. If a similar large extended hydrophobic pocket is present in renin, then inhibitors that can effectively present binding elements into that extended pocket could bind more tightly.

Not all large hydrophobic side chains would be expected to bind effectively in this hypothetical extended hydrophobic pocket. Since renin is one of the most specific proteinases known, recognizing a minimum octapeptide substrate, it is reasonable to expect considerable specificity for the side chains adjacent to the scissile bond. Modelling studies suggested two possibilities, shown in Figure 2. In Figure 2a is a view of the X-ray structure of the central Sta of pepstatin as bound in rhizopuspepsin active site. Matched onto it is a structure, in dashed lines, for the phenylalanine analogue of Sta, called AHPPA, (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid. The phenyl ring dihedral angle (χ^{21}, χ^{22} = ±90°) is that usually observed for Phe in proteins.[35] In Figure 2b, the phenyl ring has been rotated (X^{21} = +137°) so that the ring ortho carbons are equidistant from the Leu-type methyl groups. In either conformation, although the phenyl ring might provide an extended hydrophobic side chain for binding in an aspartic proteinase, it would do so necessarily at the expense of renin-specific recognition of the Leu-type side-chain methyl groups of Sta. In fact, previous work on close analogues of pepstatin had shown that replacement of Sta with AHPPA did not increase the inhibition of pepsin,[17] the prototypical aspartic proteinase. Formal reduction of this phenyl ring to a cyclohexane ring, as shown in Figure 2c, can restore two side-chain methylenes to the same location as found for the Leu δ-methyl groups, while adding three additional carbons for additional hydrophobic interactions. Thus, with the cyclohexylalanine analogue of Sta, called ACHPA, (3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoic acid, shown in Figure 2c, the Leu-type character of the side chain can be maintained in an extended hydrophobic side chain. Modelling of both of these Sta analogues into the rhizopuspepsin active site showed possible additional favorable interactions, but it was expected that renin, which requires the Leu side chain,

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would prefer the more Leu-like ACHPA side chain.

Replacement of Sta with either AHPPA or ACHPA in the heptapeptide renin inhibitor 1 gave potent inhibitors of human and dog renin (see Table III). The AHPPA-containing compound 2 inhibits the purified human kidney renin with a K_i close to that shown by 1. Human plasma renin is inhibited by 2 somewhat better than by 1 and dog plasma renin somewhat worse. However, replacement of Sta with the cyclohexylalanine analogue ACHPA, as in 3, gives the most potent inhibitors of human renin yet reported, with $K_i = 1.6 \times 10^{-10}$ M and $IC_{50} = 1.7 \times 10^{-10}$ M for the human kidney and plasma renin enzymes. This represents an improvement of potency of 55–76-fold, equivalent to a net gain in binding energy of at least 10.5 kJ mol^{-1} (= 2.5 kcal mol^{-1}). The human renin potency of 3 is approximately 100 000 times that shown by the general aspartic proteinase inhibitor pepstatin, 15. Dog plasma renin inhibition was increased for 3 vs. 1, from 3.2×10^{-8} to 1.9×10^{-9} M, a 17-fold increase in potency.

The effect of these Sta analogues, AHPPA and ACHPA, on renin-inhibitor analogues shortened at the N-terminus is shown by inhibitors 4–14 in Table III. As in 4, the N-terminal two amino acids of the minimum substrate sequence can be replaced by the *tert*-butyloxycarbonyl group (Boc), giving a pentapeptide inhibitor with potency reduced by about an order of magnitude. Substitution of Sta is 4 with AHPPA and ACHPA, as in 5 and 6, gives inhibitors of progessively increased potency, with 6 being about an order of magnitude less potent than the parent heptapeptide inhibitor 3. Replacement of the Boc group with *N*-acetyl, as in 9–11, gives inhibitors of roughly comparable potency in the human and dog plasma renin assays but with potency reduced by about 5 times in the human kidney renin K_i determinations. The reason for this discrepancy is unclear. Retaining the urethane-type N-terminus, as in Boc, but reducing the size from *tert*-butyl of Boc (as in 6) to isopropyl (as in 7) to ethyl (as in 8) shows the best N-terminus to be (ethyloxy)carbonyl. The acid-stable (ethyloxy)carbonyl group in 8, replacing the deleted His-Pro dipeptide, gives human and dog renin potencies only about 4 times less than in the parent heptapeptide 6.

Attempts to replace the N-terminal Phe of pentapeptide inhibitors 4–11 have been unsuccessful. Inhibitors 12–14 show the effect of replacing Phe with phenoxyacetyl, in effect deleting the acylated amino terminus while leaving a Phe-like side chain. Inhibition results shown in Table III for phenoxyacetylated tetrapeptides 12–14 indicate that in the human kidney assay, substitution of Sta with AHPPA and ACHPA gives dramatically more potent inhibitors, with the ACHPA-containing 14 having a $K_i = 1.4 \times 10^{-8}$ M, by far the most potent renin inhibitor of its size yet reported. Disappointingly, in the human plasma renin assay the IC_{50} value obtained was some 70-fold higher. This large discrepancy between these assays for 14 could represent a true difference in the renin found in plasma and that highly purified from kidney. However, the close correlation previously seen between these assays suggests that the explanation may lie instead with some property of the compound. Inhibitors 12–14 are extremely insoluble in water, and the ACHPA residue accentuates this property in 14. Although no precipitate was noted in the plasma assays, insolubility cannot be excluded as a cause for the poor IC_{50}. Another explanation is that some extremely tight specific or nonspecific absorption of 14 occurs to some plasma components, preventing the intrinsic renin inhibition from being manifested. A similar discrepancy has been noted in our laboratory for several other small, hydrophobic renin inhibitors (results not shown), but we have been unable to define the variable responsible, since not all such small, hydrophobic compounds show a discrepancy between plasma and purified-enzyme human renin assay results. The phenomenon is under study.

A more extensive comparison of the effect of ACHPA vs. Sta on inhibition of various renins and other related enzymes is shown in Table IV, which compares the inhibitions of the heptapeptide inhibitors 3 and 1. The ratio Sta/ACHPA indicates the relative potency gain of ACHPA vs. Sta. The ACHPA-containing inhibitor 3 is a potent inhibitor of human, monkey, rabbit, cat, dog, mouse, hog, and rat renins. The potency-enhancing effect of ACHPA (55–76-fold vs. Sta) is maximal for the human enzyme, intermediate (17–33-fold) for dog, rat, and monkey renins, and minimal (2.3-fold) for hog kidney renin.

Potent inhibitors of rat kidney renin have been unusually elusive. Considering the importance of rat models in antihypertensive research, it is fortunate that ACHPA exerts a potency-enhancing effect of 29-fold vs. Sta, bringing the inhibition for 3 to 2.1×10^{-8} M, making 3 the most potent reported inhibitor of rat renin. Inhibition values (IC_{50}) for rat renin measured at pH 7.4 were consistently higher than measurements at pH 6.0 for a wide variety of Sta-containing compounds (data not shown), but the ACHPA-containing 3 gives virtually identical IC_{50} values at the two pH values.

As described previously,[13] Sta-containing renin inhibitors based upon the renin substrate sequence are specific inhibitors of renin vs. other aspartic proteinases. This relative specificity is maintained or enhanced for ACHPA-containing compounds. Thus porcine pepsin inhibition (K_i) is unchanged when replacing Sta with ACHPA, as in 1 to 3 (see Table IV), although human renin inhibitory potency increases by more than 50-fold. Inhibition of rabbit liver cathepsin D increases modestly in the ACHPA-containing 3, but only by a factor of 8, generally less than the increase in potency vs. renins. Ironically, the substitution of ACHPA for Sta has no effect on inhibition of rhizopuspepsin, the X-ray structure of which was used to suggest the substitution originally. This irony does not negate the modelling work but only underscores the use of this type of molecular modelling as a suggestive rather than a predictive tool.

When tested against a wide variety of enzymes of other mechanistic classes, inhibitors such as 1 have little or no inhibitory activity.[13] Importantly, these compounds are exceedingly weak inhibitors of angiotensin converting enzyme (see Table IV), which is a metalloprotease and the enzyme immediately following renin in the renin angiotensin system. Thus 3 has a relative specificity for human renin vs. angiotensin converting enzyme of 75 000 times, a margin of specificity sufficient to insure that inhibition of the renin–angiotensin system caused by 3 can be ascribed unambiguously to renin inhibition.

The added in vitro potency of ACHPA-containing compounds, such as 3, translates into increased in vivo potency, as shown in Figure 1. Shown are the blood pressure dose–response curves of 1 and of 3 as continuous intravenous infusions in sodium-deficient dogs. The sodium-deficient dog is normotensive, but the renin system is activated, and the blood pressure is sensitive to changes in levels of activity of the renin–angiotensin system. A similar renin–angiotensin system activation takes place in hypertensive patients under treatment with diuretics.[2] Infusions of renin inhibitors, such as 1, in this model lead to large drops in blood pressure, and the reduced blood pressure levels can be maintained by adjusting the dose

of renin inhibitor.[36] As shown in Figure 1, blood pressure can be decreased more than 30 mmHg below the control value by either compound. However, the ACHPA-containing inhibitor **3** is 19 times more potent than **1**, a value in agreement with the 17-fold greater potency seen in vitro for dog renin inhibition (see Table IV).

In summary, incorporation of a cyclohexylalanyl analogue of Sta, ACHPA, (3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoic acid, into analogues of the renin octapeptide substrate gives specific inhibitors of renin with subnanomolar inhibitory potencies vs. human and monkey renins and with nanomolar potencies vs. a wide variety of other mammalian renins. Simplification of the N-terminus of our inhibitors has lead to pentapeptide inhibitors of nearly equivalent potency. It is likely as well that substitution of a cyclohexylalanyl side chain for the Leu side chain in non-Sta-containing renin inhibitors will lead to gains in potency, especially for transition-state or intermediate analogues, such as those containing reduced-bond and hydroxyethylene dipeptide analogues,[9,10] or the recently described aldehydic inhibitors.[11,12] Thus, these results may be of general applicability toward the development of orally effective renin inhibitors as antihypertensive agents.

Experimental Section

Peptides were prepared most efficiently using an automatic peptide synthesizer (Beckman, Model 990B) according to protocols shown in Table I for a 2-mmol solid-phase synthesis. Synthesis of **1** at a 6-mmol scale used 3 times the volumes indicated for 2 mmol. Solid-phase syntheses on a scale 0.5–1.0 mmol used similar protocols with the same volume (20 mL) of total solvent in each step and using 0.25–0.5 M DCC in CH_2Cl_2 solution for coupling, as appropriate. The larger scale (20 mmol) synthesis of **3** used a larger preparative mixing chamber for the synthesizer and was run in a total volume of 130 mL of solvent in each step. Peptide characterization data are given below and in Table II. Boc was used for α-amino protection. The side chain of His was protected as the 2,4-DNP derivative, removed as a final resin program with two treatments with 10% thiophenol in DMF. Protected amino acids, except for Sta, AHPPA, and ACHPA, were purchased from Bachem (Torrance, CA). *N*-Boc-(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (Sta) and *N*-Boc-(3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA, **21**) were prepared as described previously.[16,17] Synthesis of *N*-Boc-(3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoic acid (ACHPA, **20**) is described below. Solvents and other reagents for peptide synthesis were reagent grade and used without further purification. Tetrahydrofuran was distilled from sodium/benzophenone ketal. Ethyl acetate for column chromatography was distilled from K_2CO_3. Dioxane was freed of peroxides by passage through alumina. Other reagents and solvents were reagent grade. Pepstatin (**15**) was purchased from Sigma Chemical Co. (St. Louis, MO). Ac-Phe was purchased from Aldrich Chemical Co. (Milwaukee, WI) and HCl·Phe-NH_2 from Bachem.

HPLC analyses were performed on either a Hewlett-Packard 1084-B or a Spectro-Physics SP8000 instrument, a Waters C-18 column, and a pH 3.2 trimethylamine–phosphate buffer–acetonitrile gradient elution system, with UV detection at 210 nM. NMR spectra (^1H) were recorded on a Nicolet NT 560 or a Varian 300 spectrometer and are expressed in ppm from sodium 2,2-dimethyl-2-silapentane-5-sulfonate or tetramethylsilane as internal standard. Optical rotations were recorded on a Perkin-Elmer 141 polarimeter and are expressed as specific rotations. Amino acid analyses were done on either a Beckman 121MB or Beckman 6300 analyzer after 70-h hydrolysis in 6 N HCl. Quantitative determination of Sta, AHPPA, and ACHPA was complicated by extensive degradation during hydrolysis and was not performed routinely. Fast atom bombardment mass spectrometry (FAB-MS) was performed on an adapted Varian 731 spectrometer. Thin-layer chromatography (TLC) was done on silica gel plates (E. Merck or, when noted, on Whatman K1) and components were visualized by *tert*-butyl hypochlorite–KI reagents[37] (for NH) and by Sanger spray[38] (for His). Systems used in TLC were as follows: $CHCl_3$–CH_3OH–H_2O, 80:20:2 (A), 80:10:1 (B); $CHCl_3$–CH_3OH–H_2O–acetic acid, 80:20:2:1 (C); $CHCl_3$–CH_3OH–concentrated aqueous NH_3, 80:20:2 (D), 80:10:1 (E); ethyl acetate–pyridine–acetic acid–H_2O, 10:5:1:3 (F); 1-butanol–acetic acid–H_2O, 10:2:3 (G); hexanes–ethyl acetate, 1:1 (H); and $CHCl_3$–CH_3OH–H_2O–acetic acid, 80:10:1:1 (I). Column chromatography was performed on silica gel 60, 0.04–0.063 mm (E. Merck), eluting by gravity flow with system A, C, or D, adjusted with suitable additional $CHCl_3$ to obtain an R_f value of 0.2–0.3.

Isovaleric Acid (Iva, 3-Methylbutanoic Acid). A solution of 3,3-dimethylacrylic acid (40 g, 0.4 mol) in 100 mL of ethanol and 25 mL of H_2O was hydrogenated over 0.1 g of PtO_2 (60 lbs of H_2, 18 h). The solution was filtered and concentrated, and the oily residue was distilled, giving 32 g (78%) of isovaleric acid, bp 177–178 °C. NMR showed no traces of starting material or of isomeric products: ($CDCl_3$) δ 0.99 (6 H, d, J = 7 Hz), 2.12 (1 H, m, J = 7 Hz), 2.24 (2 H, d, J = 7 Hz), 12.0 (1 H, s). The 2-methylbutanoic acid present in commercial samples is detected readily by the 2-methyl doublet at δ 1.17.

***N*-Boc-L-phenylalanine Methyl Ester (16).** To a solution of Boc-L-phenylalanine (265.3 g, 1 mol) in 800 mL of dry, degassed DMF were added $NaHCO_3$ (252 g, 3 mol) and CH_3I (156.1 g, 68.5 mL, 1.1 mol). After stirring under N_2 for 5 days, the reaction mixture was filtered, and the precipitate was washed with CH_2Cl_2. The combined solutions were stripped, covered with 400 mL of H_2O, and extracted with 4 × 100 mL of ethyl acetate. The combined organic layers were washed with 5% sodium thiosulfate, 5% $NaHCO_3$, saturated NaCl, 10% citric acid, and H_2O and dried over Na_2SO_4. The colorless solution was filtered, evaporated to an oil, and dried to constant weight, giving 277.9 g (99.5% yield): HPLC 99.9% pure; TLC R_f 0.60 (H); $[\alpha]_D$ −2.2° (*c* 10, CH_3OH). Anal. ($C_{15}H_{21}NO_4$) C, H, N.

***N*-Boc-L-cyclohexylalanine Methyl Ester (17).** A solution of Boc-L-phenylalanine methyl ester (**16**; 108.4 g, 0.388 mol) in 50 mL of CH_3OH was hydrogenated for 18 h in a Parr apparatus using 5% Rh/Al_2O_3 (5 g). The H_2 pressure was periodically adjusted to maintain 30–55 psi. The solution was filtered through Celite, evaporated, dissolved in hexanes (600 mL), and washed with 3 × 25 mL of H_2O. The hexane solution was dried over Na_2SO_4, filtered, and stripped to a clear oil: 108.7 g (98.2% yield); HPLC 99.7% (<0.1% remaining starting material); TLC R_f 0.68 (H); ^1H NMR (CD_3OD) δ 4.15 (1 H, dd), 3.69 (3 H, s), 1.44 (9 H, s), 1.85–0.85 (13 H, m); $[\alpha]_D$ −20.1° (*c* 10, CH_3OH). Anal. ($C_{15}H_{27}NO_4$) C, H, N.

***N*-Boc-L-cyclohexylalaninal (18).** To a 3-L, three-neck flask, under a positive flow dry N_2 atmosphere and equipped with overhead stirring, was added *N*-Boc-L-cyclohexylalanine methyl ester (**17**; 108.6 g, 0.381 mol) in 600 mL of dry toluene. This and subsequent transfers were done with 16-gauge double-tipped needles, through rubber septa, by positive N_2 pressure. The solution was stirred and cooled to −78 °C, monitored by an internal thermometer, in a large acetone/dry ice bath. To a 1-L, open-jacketed, pressure-equalizing funnel was added diisobutylaluminum hydride (Dibal) in hexanes (892 mL of 1 M solution, 2.3 equiv). To the outside jacket was added cold (−78 °C) acetone, followed by dry ice. The Dibal was allowed to cool to −78 °C for 20 min, and then it was added to the stirring reaction over 5 min. After an additional 5 min, cold (−78 °C), dry CH_3OH (50 mL) was added in a thin stream. The considerable gas evolved was allowed to escape though a 1 cm bore glass outlet tube. Immediately after CH_3OH addition was complete, the reaction was quenched by addition of 1 L of saturated Rochelle salt (≈660 g L^{-1}). Stirring was continued with monitoring of temperature. The dry ice/acetone bath was maintained, but the reaction temperature rose to −10 °C and ice formation was evident, in addition to a copious gel precipitate. The bath was then replaced with an ice–salt bath, and stirring was continued for 2 h, until two layers

(36) Blaine, E. H.; Schorn, T. W.; Boger, J. *Hypertension* 1984, *6* (Suppl. 1), I-111.

(37) Mazur, R. H.; Ellis, B. E.; Cammarata, P. S. *J. Biol. Chem.* 1962, *237*, 1619.

(38) Sanger, F.; Tuppy, H. *Biochem. J.* 1951, *49*, 463.

were evident. The reaction mixture was combined with ether (1 L) in a 4-L flask, and the mixture was stirred for 1 h, at which time both layers were clear and without precipitate. The organic layer was removed, and the aqueuous layer was washed twice with ether (500 mL). The combined organic layers were dried over Na_2SO_4, filtered, stripped to an oil, cooled to −78 °C, and stored overnight at −78 °C prior to columning. The crude aldehyde was chromatographed on a 100-mm Still-type silica column,[39] eluted with hexanes–ethyl acetate (5:1). Residence time on the column was minimized, and product fractions were combined and stripped (at room temperature or below) to give a clear oil: 78 g (80% yield); TLC R_f 0.3, hexanes–ethyl acetate (4:1); ^{1}H NMR ($CDCl_3$) δ 9.50 (1 H, s), 4.8 (1 H), 4.2 (1 H), 1.4 (9 H, s), 1.8–0.8 (13 H, m). Traces of starting ester and minor byproducts (totalling <1%) were found to affect the optical rotation, such that characterization by optical rotation was unreliable at this stage. This material was used without further purification.

***N*-Boc-(3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoic Acid Ethyl Ester (Boc-ACHPA-OCH$_2$CH$_3$, 19A).** A dry three-neck, round-bottom flask with a magnetic stirring bar was fitted with a rubber septum, a 0.5-L open-jacketed pressure-equalizing funnel, and a nitrogen inlet and outflow. All transfers to the system were by 16-gauge double-tipped needles, using positive N_2 pressure. Transferred to the flask were dioxane (125 mL) and diisopropylamine (39.6 g, 54.9 mL, 0.391 mol). The solution was cooled to −25 °C in a CCl_4–dry ice bath. To the addition funnel was added *n*-butyllithium (150 mL, 2.6 M in hexane, 0.390 mol), and the jacket was half-filled with cold (−78 °C) acetone and dry ice. The chilled-*n*-butyllithium was added to the reaction, in a slow stream, with stirring, and the addition funnel was rinsed with hexane. After 1 h, the CCl_4–dry ice bath was replaced with an acetone–dry ice bath. In a dry graduated cylinder fitted with a septum, freshly distilled ethyl acetate (34.5 g, 38.2 mL, 0.391 mol) was cooled to −78 °C, transferred to the reaction by needle, and stirred for 15 min. Meanwhile, Boc-L-cyclohexylalaninal (18; 63.5 g, 0.261 mol) was dissolved in 300 mL of tetrahydrofuran and the solution transferred to the jacketed addition funnel and cooled to −78 °C. This was added rapidly to the reaction. After stirring for 5 min following completion of the addition, the reaction mixture was quenched by the addition of prechilled (−5 °C) 1 N HCl (783 mL, 0.783 mol), in one portion, added by pouring through an opened neck of the flask. The cooling bath was removed, and the "pH", measured on wide-range pH paper, was adjusted to near pH 3. This solution was extracted with ethyl acetate (3 × 300 mL), and the ethyl acetate layers were combined, washed with saturated NaCl, dried over Na_2SO_4, filtered, and stripped to clear oil, 80.0 g. Analysis by TLC, on Whatman K1 plates in 4:1 hexanes–ethyl acetate, showed predominantly the two spots for the diastereomeric products, R_f 0.28 (isomer A) and 0.21 (isomer B). A minor impurity at R_f 0.45 was the aldehyde.

Separation of the diastereomers 19, the 3*R*,4*S* and 3*S*,4*S* isomers, was by gravity flow silica gel chromatography, with a column (3 kg of silica, 0.04–0.063-μm particle size, 15 × 52 cm) packed in 8:1 hexanes–ethyl acetate. The crude oil was applied in 150 mL and eluted with a stepped gradient from 6:1 to 1:1 hexanes–ethyl acetate. Fractions containing the pure isomers were combined and stripped to dryness, giving 38.2 g (43% yield) of pure isomer **19A**, 18.2 g (21% yield) of pure isomer **19B**, and 11.1 g of mixed fractions (mostly isomer B). Isomer A was identified as the desired 3*S*,4*S* diastereomer by comparison of the free acid (see below) with a sample produced by reduction (using Rh/Al_2O_3 as described below) from Boc-(3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid[17] (see Results). The product material (isomer A) was a single component on HPLC (99+%) and TLC (99+%): ^{1}H NMR (CD_3OD) δ 4.14 (2 H, OCH_2, q, J = 8.4 Hz), 3.98 (1 H, C*H*(OH), m), 3.66 (1 H, NHC*H*(R), m), 2.42 (2 H, CH_2CO, abx), 1.95–0.8 (13 H, br m), 1.45 (9 H, s), 1.25 (3 H, t, CH_3, J = 8.4 Hz); FAB-MS, M + H at 344; $[\alpha]^{30}_D$ −31.2° (c 1.1, CH_3OH). Anal. ($C_{18}H_{31}NO_4$) C, H, N.

***N*-Boc-(3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoic Acid (Boc-ACHPA, 20).** Pure Boc-(3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoic acid ethyl ester (**19A**; 38.0 g, 0.111 mol) was dissolved in 150 mL of fresh dioxane, and 50 mL of H_2O was added. A 2 N NaOH solution was added dropwise, with stirring, over several hours. The drop rate was adjusted to keep the "pH" near 12, measured on a pH meter adjusted to read "pH" 10 in a 1:1 mixture of dioxane and pH 10 borate/carbonate buffer. After addition of the theoretical amount of base (55.5 mL), the reaction mixture was allowed to stir for an additional 1 h. Analysis by TLC (Whatman K1 plates, I) showed only a faint spot remaining at R_f 0.77, with the product acid at R_f 0.37. To the basic solution was added 5 N HCl (2 mL), and the dioxane was removed by rotary evaporation. The aqueous solution was extracted with CH_2Cl_2 (4 × 50 mL). The aqueous layer was then stripped of residual CH_2Cl_2, cooled to −5 °C, and acidified to pH 2.5 with 1 N HCl. A heavy, white precipitate was extracted into freshly distilled ethyl acetate, dried over Na_2SO_4, filtered, and stripped to an oil. The oil was triturated with hexanes and stripped to a foam, which was dried 48 h in vacuo at room temperature, giving 33.8 g (97% yield) of a white powder: TLC 99+% (I); HPLC (99+%); ^{1}H NMR (CD_3OD) δ 6.11 (1 H, NH, d, J = 9.6 Hz, exchangeable overnight), 3.97 (1 H, C*H*(OH), m), 3.65 (1 H, NHC*H*(R), m), 2.45 (1 H, C*H*HCO, abx, J = 4.2 and −15.8 Hz), 2.34 (1 H, C*H*HCO, abx, J = 8.8 and −15.8 Hz), 1.95–0.8 (13 H, br m), 1.45 (9 H, s); $[\alpha]^{30}_D$ −32.5°; $[\alpha]^{30}_{578}$ −32.4°; $[\alpha]^{30}_{546}$ −36.8°; $[\alpha]^{30}_{436}$ −62.1°; $[\alpha]^{30}_{365}$ −95.8° (c 1.4, CH_3OH). Anal. ($C_{16}H_{29}NO_5$) C, H, N.

***N*-Boc-(3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoic Acid (Boc-ACHPA, 20). Preparation from *N*-Boc-(3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic Acid (Boc-AHPPA, 21).** A sample of isomerically pure Boc-(3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid (**21**; 2.72 g, 8.9 mmol, $[\alpha]^{30}_D$ −36.2° (c 1.0, CH_3OH); lit.[17] $[\alpha]^{24}_D$ −37.0° (c 1.0, CH_3OH)) was hydrogenated in ethanol (10 mL) with Rh/Al_2O_3 (0.5 g) for 18 h at 40–50 psi of H_2. The reaction was filtered through Celite, stripped to dryness, precipitated as an oil from CH_2Cl_2–hexanes, and evaporated to a foam to give, after 48 h of drying in vacuo at room temperature, a quantitative yield (2.81 g): TLC, one spot, R_f 0.34 (I), vs. the starting material R_f 0.38; HPLC 99+% pure, with no trace (<0.1%) of the starting material; $[\alpha]^{30}_D$ −32.2° (c 1.2, CH_3OH). Anal. ($C_{16}H_{29}NO_5$) C, H, N.

General Procedure for N-Terminal Acylation. N-terminal acylation with isovaleric acid (Iva, in **1**, **2**, and **3**) or phenoxyacetic acid (POA, in **13**, and **14**) was performed on solid phase using two treatments with 5 equiv of the acid in CH_2Cl_2, coupling with 2.5 equiv of DCC. Commercial samples of IVA examined were found to contain 7–40% contamination with 2-methylbutanoic acid isomer, and the synthesis of pure Iva is given above. Acetylated peptides **9**–**11** were prepared with Ac-Phe (Aldrich, Milwaukee, WI). (Isopropyloxy)carbonyl peptide **8** was acylated on the resin with isopropyl *p*-nitrophenyl carbonate (5.0 equiv, 18 h, itself prepared from *p*-nitrophenyl chloroformate and 1.3 equiv of 2-propanol in toluene containing 1.1 equiv of pyridine, 18 h, room temperature). N-terminal acylation with ethyl chloroformate (for **7**) was performed in solution as described below.

General Procedure for Removal of Peptide from Resin: Aminolysis and Purification. Removal of the crude peptide, as its C-terminal amide, from the resin was achieved by aminolysis in methanol containing a small amount of acetic acid. Briefly, 1 mmol of completed resin was suspended in a pressure bottle in 20 mL of dry methanol, under N_2, and 0.1 mL of glacial acetic acid was added. The mixture was cooled in dry ice/acetone, and ca. 20 mL of NH_3 was condensed into the bottle. The bottle was sealed, warmed slowly to room temperature, and stirred magnetically for 3 days. After release of the pressure with continued stirring, the suspension was filtered, and the resin was washed repeatedly with alternating methanol and CH_2Cl_2. The combined filtrates were evaporated, dissolved in 4:1 ethyl acetate–methanol, and washed with H_2O. Compounds containing two His residues, as in **1**–**3**, were extracted additionally into 10% citric acid, neutralized with solid K_2CO_3, extracted back into 4:1 ethyl acetate–methanol, and washed with H_2O. This procedure removes much of the non-peptide yellow color associated with various dinitrophenyl byproducts, which are otherwise troublesome to remove in these compounds. The organic layer was dried over Na_2SO_4, filtered, and stripped to dryness. This crude peptide was applied to a silica gel column (0.04–0.063-mm silica, E. Merck) and eluted with System A, C, or E, adjusted with suitable ad-

(39) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

ditional $CHCl_3$ to obtain an R_f value of 0.2–0.3 as determined previously by TLC. Product fractions judged pure by TLC were combined, evaporated, and precipitated from CH_2Cl_2/ether or CH_2Cl_2/hexanes. The product peptide was dried in vacuo at room temperature. Typical overall isolated yields, calculated from the starting C-terminal amino acid ester resin, were 40–60%.

Isovaleryl-His-Pro-Phe-His-Sta-Leu-Phe-NH_2 (1). Iva-His-Pro-Phe-His-Sta-Leu-Phe-NH_2 (4.45 g, 64%) was prepared from Boc-Phe resin (6 mmol) according to the general solid-phase procedures, cleaved from the resin by aminolysis, purified on silica gel (1500 g) in chloroform–methanol–H_2O–acetic acid (80:20:2.5:1), and freeze-dried from 5% acetic acid: $[\alpha]_D$ −66.8° (c 0.5, 1 N acetic acid). Amino acid analysis: $His_{2.01}$, $Pro_{1.00}$, $Phe_{2.00}$, $Sta_{1.02}$, $Leu_{0.98}$.

Isovaleryl-His-Pro-Phe-His-AHPPA-Leu-Phe-NH_2 (2). Iva-His-Pro-Phe-His-AHPPA-Leu-Phe-NH_2 (0.49, 43%) was prepared from Boc-Phe resin (1.0 mmol) according to the general solid-phase procedures, cleaved by aminolysis, purified on silica gel (900 g) in chloroform–methanol–H_2O–acetic acid (100:20:2:1), and precipitated from CH_2Cl_2/ethyl acetate/hexanes: $[\alpha]_D$ −68.0° (c 0.5, 1 N acetic acid). Amino acid analysis: $His_{2.00}$, $Pro_{0.99}$, $Phe_{1.98}$, $AHPPA_{present}$, $Leu_{1.00}$.

Isovaleryl-His-Pro-Phe-His-ACHPA-Leu-Phe-NH_2 (3). Iva-His-Pro-Phe-His-ACHPA-Leu-Phe-NH_2 (10.4 g, 44%) was prepared from Boc-Phe resin (20 mmol) according to the general solid-phase procedures, except 25% trifluoroacetic acid was used for Boc deprotection. The peptide was cleaved from the resin by aminolysis, purified on silica gel (2500 g) in chloroform–methanol–H_2O–acetic acid (110:20:2:1), and freeze-dried from 1% acetic acid: $[\alpha]_D$ −63.4° (c 0.5, 1 N acetic acid). Amino acid analysis: $His_{1.99}$, $Pro_{1.03}$, $Phe_{1.96}$, $ACHPA_{1.02}$, $Leu_{1.02}$. A single major impurity in the crude peptide was isolated separately (1.6 g, 8%) and was identified by amino acid analysis and NMR as Boc-His-Pro-Phe-His-ACHPA-Leu-Phe-NH_2. The only detectable impurity in **3** was this Boc-terminal analogue.

Boc-Phe-His-Sta-Leu-Phe-NH_2 (4). Boc-Phe-His-Sta-Leu-Phe-NH_2 (0.153 g, 69%) was prepared from 2HCl·His-Sta-Leu-Phe-NH_2 (0.200 g, 0.271 mmol) and Boc-Phe (0.080 g, 0.30 mmol) with diphenylphosphoryl azide (0.165 g, 0.130 mL, 0.60 mmol), LiN_3 (0.03 g, 0.60 mmol), and diisopropylethylamine (0.129 g, 0.175 mL, 1.00 mmol) at −20 °C in DMF (1 mL) for 48 h. The reaction mixture was stripped to dryness, taken up in ethyl acetate, washed with 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4, stripped to dryness, and purified on a silica column (40 g) in chloroform–methanol–H_2O (80:7:0.7). Pure fractions were combined, stripped, precipitated from CH_2Cl_2–hexanes, and dried in vacuo, giving **4**. Amino acid analysis: $Phe_{2.02}$, $His_{0.99}$, $Sta_{present}$, $Leu_{1.02}$. Overall yield from Boc-Sta-OEt (see below) was 28%. The starting 2HCl·His-Sta-Leu-Phe-NH_2, used also for the preparation of **9** and **12**, was prepared by solution methods. Boc-His-Sta. Boc-Sta ethyl ester (Boc-Sta-OEt, 3.2 g, 10.5 mmol), prepared according to Rich et al.,[16] was deprotected in HCl-saturated ethyl acetate (6 mL) at −20 °C for 1 h, after which most of the HCl was removed by a vigorous bubbling of dry N_2 for 1 h. The product, HCl·Sta-OEt, was precipitated as a clear oil by the addition of petroleum ether, the remaining solution was decanted, and the oil was dried in vacuo over KOH to give a single spot product on TLC (2.7 g, D, R_f 0.29). This material was dissolved in DMF (50 mL) cooled to −20 °C under N_2, to which was added Boc-(DNP)-His (5.3 g, 12.6 mmol), NaN_3 (0.8 g, 16 mmol), diisopropylethylamine (3.0 g, 40 mL, 23 mmol), and diphenylphosphoryl azide (7.0 g, 5.5 mL, 27.5 mmol). After 72 h at −20 °C, the reaction mixture was stripped, dissolved in ethyl acetate, washed with 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4, and stripped to a red oil, giving crude Boc-His(DNP)-Sta-OEt (6.6 g, TLC: E, R_f 0.71). A portion of this red oil (4.5 g) in DMF (50 mL) was stirred for 18 h with 1-pentanethiol (25 mL), after which only one Sanger-positive spot was present by TLC (E, R_f 0.27). The reaction was stripped repeatedly to dryness, with additions on ethanol, precipitated from ethyl acetate–hexanes, and purified on silica (500 g, E), giving pure (by TLC) Boc-His-Sta-OEt (2.4 g, 5.4 mmol, 76% yield from Boc-Sta-OEt). A portion (2.0 g, 4.5 mmol) was hydrolyzed in 50% dioxane (24 mL) by addition of 1 N NaOH (4.6 mL), added over 20 min. After an additional 20 min, 1 N HCl (4.6 mL) was added, the dioxane evaporated in vacuo, and the cloudy solution extracted with 1-butanol (4 × 50 mL), and the 1-butanol was evaporated to 30 mL. The product was precipitated by the addition of hexanes (200 mL), filtered, and dried, giving Boc-His-Sta (1.70 g, 3.4 mol; 75% yield, overall yield from Boc-Sta-OEt: 56%): HPLC 99.5%. Amino acid analysis: 82% peptide based on His. HCl·Leu-Phe-NH_2. To Boc-Leu·H_2O (12.0 g, 48 mmol) in DMF–CH_2Cl_2 (50 mL, 1:1) at 0 °C was added 1-hydroxybenzotriazole hydrate (7.66 g, 50 mmol), followed by 2 N DCC solution in CH_2Cl_2 (24 mL, 48 mmol). After the mixture was stirred for 30 min, Phe-NH_2 (8.21 g, 50 mmol, Bachem) was added, and the solution was stirred at 0 °C for 18 h. The reaction was filtered, stripped to dryness, redissolved in $CHCl_3$ (200 mL), cooled to −50 °C, and filtered again. The solution was evaporated, dissolved in 1-butanol–ethyl acetate (1:1), and washed with 5% $NaHCO_3$, 10% citric acid, 5% $NaHCO_3$, and H_2O. Ethanol (50 mL) was added, and the organic layer was evaporated, redissolved in 1-butanol, and precipitated with petroleum ether, giving Boc-Leu-Phe-NH_2 (17.6 g, 47 mmol): TLC, single spot, chloroform–methanol (95:5), R_f 0.40. A portion of this material (5.0 g) was deprotected in 20 mL of HCl-saturated ethyl acetate (0 °C, 1 h) and worked up by removal of HCl in an N_2 stream and precipitation with petroleum ether, giving, after drying in vacuo over KOH, HCl·Leu-Phe-NH_2 (4.2 g, 12.7 mmol, 93% yield from Boc-Leu): TLC, E, R_f 0.31. Amino acid analysis: $Leu_{0.99}$, $Phe_{1.01}$; 95% peptide based on formula weight 313.8, HCl·Leu-Phe-NH_2. To a portion of Boc-His-Sta (0.73 g, 1.45 mmol) in CH_2Cl_2 was added HCl·Leu-Phe-NH_2 (0.48 g, 1.45 mmol, prepared as described above, diisopropylethylamine (0.74 g, 1.0 mL, 5.8 mmol), and (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate ("Le Bop",[40] 1.29 g, 2.90 mmol), which were stirred for 1 h at room temperature for 72 h at 0 °C. The reaction solution was evaporated, extracted extensively in ethyl acetate (20 mL) with H_2O and 5% $NaHCO_3$, dried over Na_2SO_4, stripped to a yellow powder, and purified by silica chromatography (200 g, E), giving Boc-His-Sta-Leu-Phe-NH_2 (0.82 g). The Boc group was removed in HCl-saturated ethyl acetate (20 mL, −20 °C, 1 h), and the product was precipitated by removal of most of the HCl by vigorous N_2 bubbling, followed by addition of hexanes (100 mL), giving 2HCl·His-Sta-Leu-Phe-NH_2 (0.74 g, 1.03 mmol, 71% yield, overall yield 40% from Boc-Sta-OEt): TLC, single spot, D, R_f, 0.38; A, R_f 0.08; HPLC 92.3%. Amino acid analysis: $His_{0.96}$, $Sta_{1.02}$, $Leu_{1.01}$, $Phe_{1.01}$; 90% peptide based on formula weight 644.6.

Boc-Phe-His-AHPPA-Leu-Phe-NH_2 (5). Boc-Phe-His-AHPPA-Leu-Phe-NH_2 (0.52 g, 51%) was prepared from Boc-Phe resin (1.0 mmol) according to the general solid-phase procedures, cleaved by aminolysis, purified on silica gel (300 g) in chloroform–methanol–concentrated ammonium hydroxide (100:10:1), and precipitated from $CHCl_3$–ether–hexanes. Amino acid analysis: $Phe_{1.96}$, $His_{1.02}$, $AHPPA_{present}$, $Leu_{1.01}$.

Boc-Phe-His-ACHPA-Leu-Phe-NH_2 (6). Boc-Phe-His-ACHPA-Leu-Phe-OCH_3 (0.48 g, 53%) was prepared from Boc-Phe resin (1.0 mmol) according to the general solid-phase procedures, cleaved by methanolysis, purified on silica gel (90 g) in chloroform–methanol–H_2O–acetic acid (100:10:1:1), and precipitated from $CHCl_3$–ether–hexanes. A portion (0.19 g) of this methyl ester analogue was converted to **6** by the general aminolysis procedure and freeze-dried from dioxane–H_2O, giving pure **6** (0.130 g, 70%). Amino acid analysis: $Phe_{1.96}$, $His_{1.02}$, $AHPPA_{present}$, $Leu_{1.01}$. Overall yield of **6** from Boc-Phe resin was 37%.

[(Isopropyloxy)carbonyl]-Phe-His-ACHPA-Leu-Phe-NH_2 (7). [(Isopropyloxy)carbonyl]-Phe-His-ACHPA-Leu-OCH_3 (0.611 g, 46%) was prepared from Boc-Leu resin (2.0 mmol) according to the general solid-phase procedures, cleaved by methanolysis, purified on silica gel (200 g) in chloroform–methanol–H_2O–acetic acid (90:10:1:1), and precipitated from $CHCl_3$–ether–hexanes. A portion (0.580 g, 0.67 mmol) of this methyl ester analogue was converted to the hydrazide [(isopropyloxy)carbonyl]-Phe-His-ACHPA-Leu-$NHNH_2$ (0.500 g, 0.65 mmol, 98%), using 20% anhydrous hydrazine in methanol (30 min), stripping to dryness, dissolving in ethyl acetate, and washing extensively with H_2O. A portion (0.150 g, 0.21 mmol) of the hydrazide was converted to the azide by using 4 N HCl in tetrahydrofuran (0.5 mL) and isoamyl nitrite (27 mg, 0.031 mL, 0.23 mmol) in DMF (3 mL) for

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3 h at −20 °C. To the azide was added HCl·Phe-NH_2 (0.063 g, 0.315 mmol, Bachem) and the solution was brought to "pH 10" (on wet paper) with diisopropylethylamine (0.29 g, 0.39 mL). After the mixture was stirred at −20 °C for 1 h, the "pH" had fallen below 7, and additional diisopropylethylamine was added to bring the "pH" to 10. Workup after 24 h and purification by silica gel chromatography (150 g), in chloroform–methanol–concentrated ammonium hydroxide (100:10:1), gave pure **7** (0.097 g, 54%). Amino acid analysis: $Phe_{1.93}$, $His_{1.04}$, $ACHPA_{present}$, $Leu_{1.03}$. Overall yield of **7** from Boc-Leu resin was 24%.

[(Ethoxy)carbonyl]-Phe-His-ACHPA-Leu-Phe-NH_2 (8). [(Ethyloxy)carbonyl]-Phe-His-ACHPA-Leu-Phe-NH_2 was prepared from **6** by solution methods. Boc-Phe-His-ACHPA-Leu-Phe-NH_2 **6** (0.050 g, 0.057 mmol) was deprotected in 10 mL of HCl-saturated ethyl acetate (−20 °C, 1 h) and precipitated by addition of hexanes, after removal of most of the HCl by vigorous bubbling of dry N_2. The crude product, 2HCl·Phe-His-ACHPA-Leu-Phe-NH_2, was dried in vacuo over KOH and dissolved in CH_2Cl_2 (10 mL), to which was added diisopropylethylamine (0.038 g, 0.050 mL, 0.29 mmol) and ethyl chloroformate (0.038 g, 0.033 mL, 0.34 mmol). After 30 min, the reaction was stripped, and the product was purified on silica gel chromatography (30 g) in chloroform–methanol–H_2O–acetic acid (100:10:1:1), giving **8** (0.035 g, 68%). Amino acid analysis: $Phe_{1.96}$, $His_{1.01}$, $ACHPA_{present}$, $Leu_{1.02}$. Overall yield of **8** from Boc-Phe resin was 25%.

Ac-Phe-His-Sta-Leu-Phe-NH_2 (9). Ac-Phe-His-Sta-Leu-Phe-NH_2 was prepared from 2HCl·His-Sta-Leu-Phe-NH_2 (see synthesis of **4**) and Ac-Phe by solution methods. To a solution in DMF (3 mL, −20 °C) of 2HCl·His-Sta-Leu-Phe-NH_2 (0.194 g, 0.271 mmol), diisopropylethylamine (0.140 g, 0.189 mL, 1.08 mmol), and diphenylphosphoryl azide (0.186 g, 0.146 mL, 0.678 mmol) was added Ac-Phe (0.062 g, 0.300 mmol), and the reaction mixture was kept at −20 °C for 24 h. The reaction mixture was stripped, and the product was purified on silica gel chromatography (50 g) in solvent I, giving **9** (0.138 g, 56%). Amino acid analysis: $Phe_{2.01}$, $His_{1.00}$, $Sta_{1.05}$, $Leu_{1.00}$. Anal. ($C_{40}H_{56}N_8O_7 \cdot 4H_2O$) C, H; N: calcd, 13.45; found, 12.87. Overall yield of **9** from Boc-Sta-OEt was 22%.

Ac-Phe-His-AHPPA-Leu-Phe-NH_2 (10). Ac-Phe-His-AHPPA-Leu-Phe-NH_2 (0.38 g, 47%) was prepared from Boc-Phe resin (1.0 mmol) according to the general solid-phase procedures, cleaved by aminolysis, purified on silica gel (175 g) in solvent E, and precipitated from $CHCl_3$–methanol–hexanes. Amino acid analysis: $Phe_{1.95}$, $His_{1.02}$, $AHPPA_{present}$, $Leu_{1.03}$.

Ac-Phe-His-ACHPA-Leu-Phe-NH_2 (11). Ac-Phe-His-ACHPA-Leu-Phe-OCH_3 (0.47 g, 45%) was prepared from Boc-Phe resin (1.0 mmol) according to the general solid-phase procedures, cleaved by methanolysis with 20% diisopropylethylamine in methanol (18 h), purified on silica gel (200 g) in chloroform–methanol–H_2O–acetic acid (100:10:1:1), and precipitated from $CHCl_3$–hexanes. Amino acid analysis showed 79% peptide content based on a molecular weight of 816.0. A portion of this methyl ester (0.135 g, 0.130 mmol) was quantitatively converted to the amide **11**, using the general aminolysis procedure, which gave, after workup, pure **11** (0.119 g, 0.130 mmol, 45% overall from Boc-Phe resin), freeze-dried from dioxane–H_2O. Amino acid analysis: $Phe_{1.98}$, $His_{1.02}$, $ACHPA_{present}$, $Leu_{1.01}$.

(Phenoxyacetyl)-His-Sta-Leu-Phe-NH_2 (12). (Phenoxyacetyl)-His-Sta-Leu-Phe-NH_2 was prepared from 2HCl·His-Sta-Leu-Phe-NH_2 (see synthesis of **4**) and phenoxyacetic acid by solution methods. To a solution in DMF (3 mL, −20 °C) of 2HCl·His-Sta-Leu-Phe-NH_2 (0.194 g, 0.271 mmol), diisopropylethylamine (0.140 g, 0.189 mL, 1.08 mmol), and diphenylphosphoryl azide (0.186 g, 0.146 mL, 0.678 mmol) was added phenoxyacetic acid (0.046 g, 0.300 mmol), and the reaction mixture was kept at −20 °C for 24 h. The reaction was stripped, and the product was purified on silica gel chromatography (50 g) in solvent I, giving **12** (0.154 g, 77%). Amino acid analysis: $His_{0.98}$, $Sta_{present}$, $Leu_{1.02}$, $Phe_{1.02}$.

(Phenoxyacetyl)-His-AHPPA-Leu-Phe-NH_2 (13). (Phenoxyacetyl)-His-AHPPA-Leu-Phe-NH_2 (0.10 g, 27%) was prepared from Boc-Phe resin (0.5 mmol) according to the general solid-phase procedures, cleaved by aminolysis, purified on silica gel (500 g) in chloroform–methanol–H_2O–acetic acid (100:20:2:1), and precipitated from CH_2Cl_2–ethyl acetate–hexanes. Amino acid analysis: $His_{1.05}$ (interference from AHPPA degradation products in hydrolysis), $Phe_{0.98}$, $AHPPA_{present}$, $Leu_{0.97}$. Anal. ($C_{40}H_{49}N_7O_7 \cdot 2H_2O$) C, N; H: calcd, 7.60; found, 6.74.

(Phenoxyacetyl)-His-ACHPA-Leu-Phe-NH_2 (14). (Phenoxyacetyl)-His-ACHPA-Leu-Phe-NH_2 (0.39 g, 48%) was prepared from Boc-Phe resin (1.0 mmol) according to the general solid-phase procedures, cleaved by aminolysis, purified on silica gel (250 g) in chloroform–methanol–H_2O–acetic acid (100:16:2:1), and precipitated from CH_2Cl_2–ethyl acetate–hexanes. Amino acid analysis: $His_{1.00}$, $Phe_{1.00}$, $ACHPA_{present}$, $Leu_{1.01}$.

Enzyme Assays. Human kidney and mouse submaxillary gland renin assays were performed with synthetic tetradecapeptide renin substrate by a fluorometric method,[41] pH 7.20 (citrate/phosphate), 37 °C. Human kidney renin was International Standard WHO human kidney renin,[42] which was purified additionally by affinity chromatography on pepstatin–aminohexyl-Sepharose[21] or was fully purified human kidney renin according to Slater and Strout.[22] Both renins gave identical results in the inhibition assays. Inhibition data, analyzed to give K_i values,[21] were consistent with competitive inhibition in all cases. When noted in Tables II and III, values of K_i were computed with data combined from two independent experiments. Values for K_i were found generally to be reproducible within ±20%. Plasma renin inhibition in human, dog (mongrel), monkey (rhesus), rat, and rabbit plasma were determined as an ID_{50} value by a radioimunoassay for angiotensin I, as described by Haber et al.,[20] using a commercial kit (Clinical Assays, Cambridge, MA) at pH 7.4 (phosphate), 37 °C, or, when noted, at pH 6.0 (maleate), 37 °C. Lyophilized human plasma was obtained from Clinical Assays (for high renin) and Ortho Diagnostics (Raritan, NJ, for low renin) and reconstituted with cold distilled H_2O on the day of assay. Plasma from dogs and rats was collected from anesthetized animals that had been treated with furosemide. Blood was drawn from conscious rabbits and monkeys tranquilized with ketamine. For the latter two species, blood was obtained both from normal and from furosemide-treated animals, and data from these low and high renin sources are noted in Tables II and III. All blood was collected in chilled containers treated with NaEDTA. Pooled plasma was separated by centrifugation at 4 °C, and aliquots were stored frozen. Plasma inhibition values (IC_{50}) were obtained as described[20] and generally were reproducible within ±30%. The 95% confidence limits obtained by analysis of data of a single experiment were routinely ±10%. Inhibitors were dissolved initially at 1.00×10^{-3} M in methanol (or acetic acid, where noted) before dilution to the assay concentrations. For the plasma assays at pH 7.4, 8-hydroxyquinoline (8-HQ) or phenylmethanesulfonyl fluoride (PMSF) were used as angiotensinase inhibitors and both were found to give similar results in human and dog plasma at pH 7.4. In rat plasma at pH 7.4, 8-HQ was used.[13] At pH 6.0, PMSF was used. Inhibition of hog kidney renin was measured as described previously,[17] using a ^{14}C-labeled decapeptide substrate, except that the pH was raised to pH 7.3 by 0.05 M citrate/phosphate buffer, 30 °C. Inhibition values as IC_{50} were calculated as described[13] and converted to K_i values according to the method of Cha,[24] assuming competitive inhibition: $K_i = (IC_{50} - E_t/2)(1 + S_0/K_m)^{-1}$, where E_t (enzyme concentration) = 9×10^{-9} or 9×10^{-10} M, S_0 (substrate concentration) = 8.0×10^{-5} M, and $K_m = 5.0 \times 10^{-5}$ M. Assays of hog kidney renin at $E_t = 9 \times 10^{-10}$ M, incubated for 5 h instead of 30 min as for the higher concentration, were used to preserve the supply of hog kidney renin and were necessary for meaningful IC_{50} data below 10^{-8} M. Calculated hog kidney renin K_i values were shown to be independent of assay time within this range. The validity of the Cha expression was confirmed for IC_{50} values $> E_t$ for 1 by assays using varying enzyme concentrations and extrapolation to E_t = zero. Porcine pepsin (Sigma Chemical Co.) and rhizopuspepsin (gift of D. Davies, National Institutes of Health) were assayed at pH 4.0 (formate), 30 °C, with use of a heptapeptide substrate, Phe-Gly-His-Phe-(4-NO_2)-Phe-Ala-Phe-OCH_3, followed at 310 nm.[25] With use of several inhibitor concentrations, IC_{50} values were calculated as for the hog kidney renin assay. With use of the Cha

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equation as described above, K_i values were calculated, using for pepsin: $E_t = 1.2 \times 10^{-8}$ M, $S_0 = 1.0 \times 10^{-4}$ M, and $K_m = 1.25 \times 10^{-5}$ M. Values for rhizopuspepsin were $E_t = 6.0 \times 10^{-8}$ M, $S_0 = 1.0 \times 10^{-4}$ M, and $K_m = 5.0 \times 10^{-5}$ M. Values of K_m were determined under the conditions of the assay. Rabbit liver cathepsin D was purified, and compounds were assayed for inhibition as described previously,[26] using [^{14}C-methyl]-glycinated hemoglobin substrate, pH 4.0 (citrate), 37 °C, giving K_i values from Dixon plots.[43] Porcine plasma angiotensin converting enzyme was assayed at pH 7.65 (Tris-HCl), 30 °C, as described previously, with (benzyloxycarbonyl)-Phe-His-Leu as substrate.[27]

In Vivo Renin Inhibition. The relative effects of 1 and 3 on blood pressure was determined in four conscious, sodium-deficient dogs. The dogs were prepared essentially as described previously.[36] Sodium deficiency was established with use of a low-sodium diet and furosemide as described. The compounds were administered intravenously through an indwelling venous catheter by peristaltic pump to trained beagles in a Pavlov sling. Compounds were administered at various infusion rates as solutions in 5% dextrose, containing up to 0.1% acetic acid. No effect of vehicle was noted (results not shown). Each dose was preceded by a bolus injection of 4 times the per minute infusion rate to hasten the attainment of equilibrium, and each infusion was continued for 45 min. Two dogs received increasing doses of 1 first, followed by the doses of 3, and the order was reversed for the other two dogs. Full blood pressure recovery was attained prior to infusion of the other compounds. Blood pressure and heart rate were measured by tail cuff and recorded as an average of six readings, 1 min apart at post 45 min.

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Registry No. 1, 86153-45-9; 1·2$C_2H_4O_2$, 98168-92-4; **2**, 98105-33-0; **3**, 98105-34-1; **3**·$C_2H_4O_2$, 98168-93-5; **4**, 87063-27-2; **5**, 98105-35-2; **6**, 98105-36-3; **7**, 98105-37-4; 8, 98126-19-3; **9**, 87063-45-4; **10**, 98105-38-5; 11, 98105-39-6; **12**, 93962-09-5; **13**, 98105-40-9; **14**, 93961-79-6; **16**, 51987-73-6; **17**, 98105-41-0; **18**, 98105-42-1; **19** (isomer 1), 98105-43-2; **19** (isomer 2), 98105-44-3; **20**, 98105-45-4; **21**, 72155-48-7; CH_3I, 74-88-4; Boc-Phe-OH, 13734-34-4; His-Sta-Leu-Phe-NH_2·2HCl, 98168-94-6; Boc-Sta-OEt, 67010-43-9; Sta-OEt·HCl, 84851-46-7; Boc-His(DNP)-OH, 25024-53-7; Boc-His-Sta-OEt, 98105-46-5; Boc-His-Sta, 92608-47-4; Boc-Leu-OH, 13139-15-6; Phe-NH_2, 5241-58-7; Boc-Leu-Phe-NH_2, 33900-15-1; Leu-Phe-NH_2·HCl, 74214-38-3; Boc-His-Sta-Leu-Phe-NH_2, 93962-08-4; Boc-Phe-His-ACHPA-Leu-Phe-OCH_3, 98105-47-6; [(isopropyloxy)carbonyl]-Phe-His-ACHPA-Leu-OCH_3, 98126-20-6; [(isopropyloxy)carbonyl]-Phe-His-ACHPA-Leu-$NHNH_2$, 98105-48-7; Phe-NH_2·HCl, 65864-22-4; Phe-His-ACHPA-Leu-Phe-NH_2·2HCl, 98105-49-8; Ac-Phe, 2018-61-3; Ac-Phe-His-ACHPA-Leu-Phe-OCH_3, 98105-50-1; Boc-Sta-OH, 58521-49-6; Boc-Pro-OH, 15761-39-4; Boc-His(DNP)-Sta-OEt, 98105-51-2; 3,3-dimethylacrylic acid, 541-47-9; ethyl acetate, 141-78-6; phenoxyacetic acid, 122-59-8; isovaleric acid, 503-74-2; isopropyl *p*-nitrophenyl carbonate, 90923-15-2; renin, 9015-94-5; ethyl chloroformate, 541-41-3.

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1,2-Dihydro-2-oxo-6-(2,2-dimethylpropyl)-3-pyridinecarboxylic Acid, Analogues, and Derivatives. A New Class of Oral Hypoglycemic Agents

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1,2-Dihydro-2-oxo-6-(2-methylpropyl)-3-pyridinecarboxylic acid was found to be a hypoglycemic agent but not to have the undesirable mechanism of action possessed by nicotinic acid. A series of 1,2-dihydro-2-oxo-3-pyridinecarboxylic acids with a substituent primarily at the 6-position was prepared by hydrolysis of the corresponding nitriles. The nitriles were prepared by reaction of the sodium enolate of the appropriate 3-substituted 3-oxopropionaldehyde with cyanoacetamide. The sodium enolates were synthesized from ethyl formate and the appropriate ketone and sodium or sodium hydride. The active 1,2-dihydro-2-oxo-3-pyridinecarboxylic acids, listed in order of decreasing hypoglycemic potency, had the following substituents: 6-(2,2-dimethylpropyl), 6-(2,2-dimethylbutyl), 6-(1,1-dimethylethyl), 6-(2-methylpropyl), 6-(1,1-dimethylpropyl), 1-methyl-6-(2-methylpropyl), 6-hydrogen. The inactive compounds were those with 6-methyl, 6-(1-methylethyl), 6-pentyl, 4-(2,2-dimethylpropyl), 6-(3-methylbutyl), 6-(1,1-dimethylheptyl), 6-(2,2-dimethyloctyl), 6-(1-cyclobutylmethyl), and 1-methyl-6-(2,2-dimethylpropyl) substituents. The corresponding alcohol, aldehyde, tetrazole, sodium salt, and ethyl ester of the most potent acid were also active compounds. The corresponding amide, decarboxyl compound, and 2-deoxo compound were inactive.

During the course of screening for oral hypoglycemic agents in the 18-h fasted normal rat,[1] 1,2-dihydro-2-oxo-6-(2-methylpropyl)-3-pyridinecarboxylic acid[2] (**13**) was found to be active. A search of the literature disclosed only two 1,2-dihydro-2-oxo-3-pyridinecarboxylic acids which are reported to be associated with hypoglycemic activity. Fang[3] reported that 1,2-dihydro-2-oxo-6-methyl-3-pyridinecarboxylic acid[4] (**10**) was inactive and that 1,2-dihydro-2-oxo-3-pyridinecarboxylic acid[5] (**9**) was active in the diabetic rat. Fang[6] concluded that the hypoglycemic action of **9** was achieved through the suppression of the release of free fatty acids from the adipose tissues. Since nicotinic acid[7] also displays this activity and nicotinic acid has been reported to be ineffective as a hypoglycemic agent in man,[8,9] this mechanism of action for hypoglycemic ac-

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