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# Modified Peptides which Display Potent and Specific Inhibition of Human Renin

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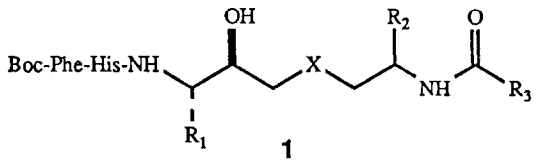
**Summary.** A new class of angiotensinogen analogues which contain heteroatom-methylene and retro-inverso amide bond replacements was synthesized and evaluated for renin inhibition. Selected compounds in the series were specific for renin over other aspartic proteinases, and the most potent inhibitor demonstrated hypotensive activity in a salt depleted monkey. 

Renin is an aspartic proteinase which is highly specific for its substrate, the plasma glycoprotein angiotensinogen. Cleavage by renin releases angiotensin I which is converted into the pressor hormone angiotensin II by angiotensin converting enzyme (ACE). The success of orally active ACE inhibitors in the control of renin-dependent hypertension and essential hypertension (1-5) has spawned an interest in the blockade of the preceding step in the renin-angiotensin system by utilizing renin inhibitors.

We wish to report the synthesis and biological evaluation of a novel class of renin inhibitors, 1 (6). Compounds of this class consist of an N-protected dipeptide (left-hand) portion coupled to a non-peptide (right-hand) portion. Perusal of the literature revealed that compounds of the class of statine-based inhibitors 2 represented the closest structural relatives (7). However, compounds from the former class differ from the latter in three important ways with respect to the right-hand portion of the molecule: a) a thiomethylene (8), sulfonylmethylene, or oxomethylene replaces the statine-leucine amide bond of 2, b) a retro-inverso amide (9) replaces the leucine-phenylalanine amide bond of 2, and c) the absence of the C-terminal carboxamide group of 2. These three differences serve to make the inhibitors lower in molecular weight, less peptidic in nature, and potentially more stable toward in vivo degradation.

0006-291X/87 $1.50

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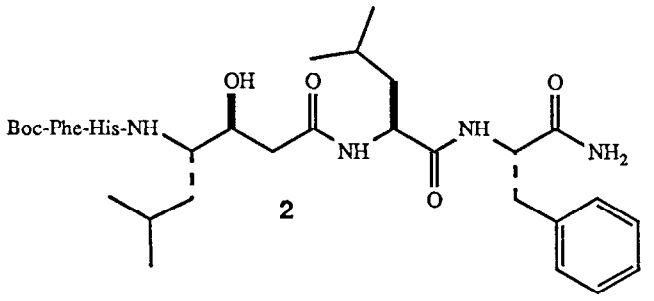



2

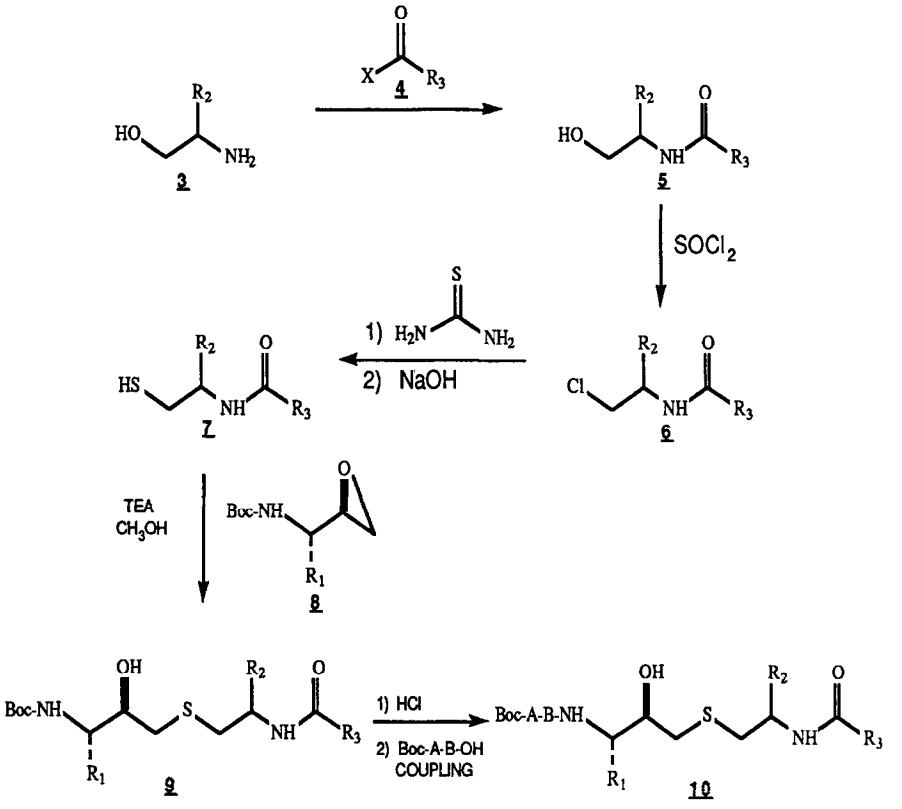
$IC_{50}$=190nM
(Ref. 7)

It should be noted that when X=S in compound **1**, the thiomethylene ($S-CH_2$) replacement for the amide (CO-NH) group is retro-inverted with respect to the isomeric thiomethylene ($CH_2-S$) usually incorporated into the peptide backbone. This uncommon replacement was chosen due to its ready accessibility from the synthetic route discussed below. Additionally, one has the flexibility of being able to oxidize the sulfur to the sulfoxide or sulfone oxidation state and probe the statine carbonyl-like hydrogen bond accepting capability of the resulting inhibitor with renin. The synthetic strategy also lends itself to the preparation of the corresponding oxomethylene analogues (X=O) which are likely to be more stable to in vivo oxidation/reduction conditions. Finally, since most of the non-peptide portion of the molecule is constructed from building blocks ultimately derived from natural and unnatural amino acids, a wide variety of side chains $R_1$ and $R_2$ are readily available with well-defined absolute stereoconfigurations.

## Materials and Methods

Synthesis: The preparation of the sulfur-containing inhibitors is outlined in Scheme 1. The appropriate amino alcohol **3** was acylated with acid chloride **4** to give amide **5** in good yield. Conversion to chloride **6** with thionyl chloride, followed by treatment with thiourea and hydrolysis of the resulting isothiuronium salt with sodium hydroxide provided mercaptan **7**. Opening of epoxide **8** gave adduct **9** which was deprotected with HCl and then coupled to dipeptide Boc-A-B-OH using either standard mixed anhydride or DCC/HOBT (B=His) coupling conditions. Oxidation with either one or two equivalents of 3-chloroperoxybenzoic acid (MCPBA) gave the corresponding sulfoxide or sulfone. Over oxidation with excess MCPBA provided His(N-OH) sulfone **18**.

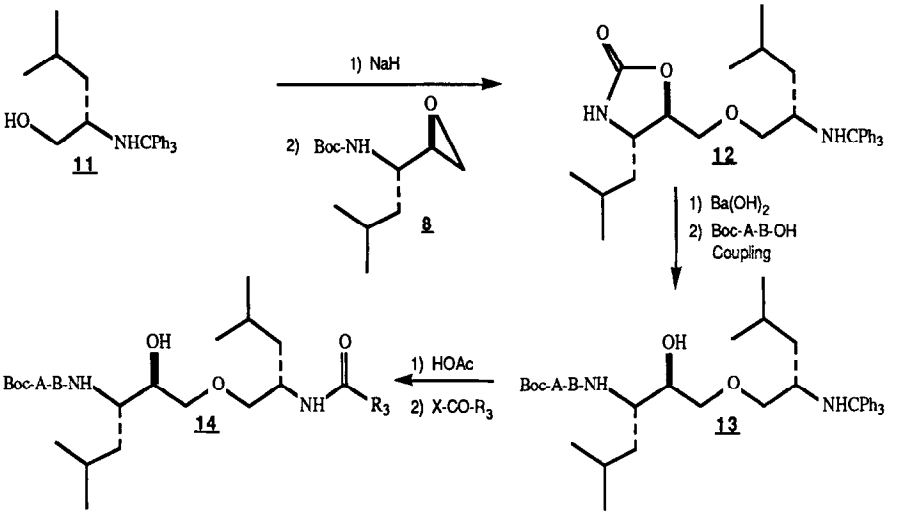
The synthesis of the oxy-analogues is summarized in Scheme 2. N-Trityl-L-leucinol was treated sequentially with sodium hydride and epoxide **8** to give oxazolidin-2-one **12**. Hydrolysis to the amino alcohol and dipeptide coupling to Boc-A-B-OH using mixed anhydride conditions provided **13**. The N-trityl




**SCHEME 1**

group was removed with acetic acid, and the resulting product was acylated to give oxy-analogues **14**.

Enzyme Assays: Purified human renal renin (10) was assayed utilizing pure human angiotensinogen (11) at pH 6.0 in maleate buffer. Test compounds were dissolved in DMSO and diluted so that prior to addition to the assay




**SCHEME 2**

system the solutions were 10% in DMSO and 0.5% in BSA. The final incubation mixture (100 µl) contained maleate buffer, pH 6.0, 0.135 M; EDTA, 3 mM: PMSF, 1.4 mM; angiotensinogen, 0.21 µM; renin, 0.24 mGU (12); BSA, 0.44%; DMSO, 1%. At least 3 different concentrations of inhibitor which bracketed the $IC_{50}$ were preincubated with renin for 5.0 min at 37°C, substrate was added and the incubation was allowed to proceed for 10.0 min. The reaction was stopped by freezing the solution in a methanol-dry ice bath, and after thawing at 4°C an aliquot was analyzed for angiotensin I generation by radioimmunoassay utilizing a commercial kit (NEN Research). The percent inhibition of the reaction was determined and the $IC_{50}$ was calculated by regression analysis. The reaction time of 10 min was on the linear portion of the incubation time - angiotensin I generation curve, and at the highest concentrations tested none of the compounds cross-reacted with the antibody to angiotensin I. The presence of 1% DMSO in the final incubation mixture caused no statistically significant effect on the renin activity. Mouse submaxillary gland renin was assayed with the same concentrations of maleate, EDTA, PMSF, BSA and DMSO plus 3.45 mM 8-hydroxyquinoline; the enzyme was 20 ng of purified protein (13). Dog plasma, diluted 1.5:10, served as the substrate.

Bovine cathepsin D (Sigma) and porcine pepsin (Sigma) activities were assessed by the hydrolysis of hemoglobin at pH 3.1 and 1.9, respectively, at 37°C, and measurements of the absorbance at 280 nm of the supernatant after precipitation with trichloroacetic acid (14).

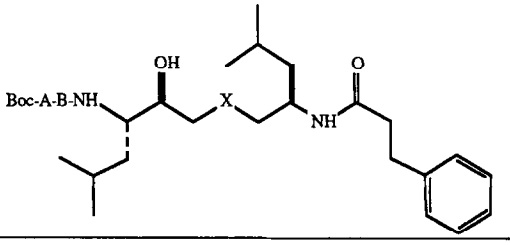
Cardiovascular Effects: Intravenous activity was determined in anesthetized cynomolgus monkeys (M. fascicularis), weighing 4-6 kg. The monkeys were maintained on a low salt chow and fruit diet and treated with furosemide (5 mg/kg, p.o.) on days 8 and 1 prior to the experiment. This regimen enhanced renin secretion and elevated basal plasma renin activity. On the morning of the experiment, each monkey was sedated (ketamine, 10 mg/kg, i.v.), anesthetized (Nembutal® 15 mg/kg bolus + 0.1 mg/kg/min maintenance i.v. infusion), and instrumented with an intravenous catheter as an access route for drug delivery. Blood pressure and heart rate were monitored non-invasively at 2 minute intervals using an arm cuff with a microphone and a sphygmomanometer (Narco Biosystems, Houston, Texas). Venous blood samples were collected before and after **30** was given, as indicated in Figure 1, for the determination of plasma renin activity (15). Blood samples were not obtained during captopril treatment.

Stability Studies. The degradation of 0.05 mg/mL **30** with 0.1 mg/mL bovine chymotrypsin (Sigma #C-3142) or bovine pancreatic proteases (Sigma #P4630) in phosphate buffer was monitored by high pressure liquid chromatographic analysis of the incubations (Waters µBondapak C18 analytical column; $CH_3CN/H_2O$/TFA, 35/65/0.1-80/20/0.1, 20 min gradient, 214 nM detection).

## Results and Discussion

As can be seen in Table 1, compound **15**, which differs from compound **2** in that it contains thiomethylene and retro-inverso amide replacements and lacks the C-terminal carboxamide group, still maintains the *in vitro* potency. Oxidation of **15** to a mixture of diastereomeric sulfoxides **16** resulted in a fall in biological activity, as did oxidation to sulfone **17**. Treatment of **15** with 3 equivalents of MCPBA provided **18**, the result of oxidation of the sulfide to the sulfone and the imidazole to the imidazole hydroxylamine. The dramatic fall in biological activity of **18** compared to **17** demonstrates the sensitive nature of the histidine side chain interactions, either electronic or hydrogen bonding, with the $S_2$ binding site of renin. Substitution of Phe with 3-(2'-

**TABLE 1**



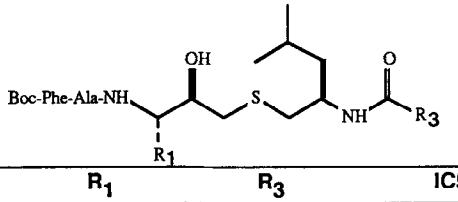

| COMPOUND # | A-B | X | $IC_{50}$,nM |
|---|---|---|---|
| 15 | PHE-HIS | S | 200 |
| 16 | PHE-HIS | SO | 3500 |
| 17 | PHE-HIS | $SO_2$ | 850 |
| 18 | PHE-HIS (N-OH) | $SO_2$ | >10000 |
| 19 | (2)NAL-HIS | $SO_2$ | 700 |
| 20 | PHE-ALA | S | 2000 |

naphthyl)-alanine ((2)Nal), a change which has provided up to a 10-fold boost in activity in other renin inhibitors (16), led to a slight loss in activity. The Phe-Ala analogue, **20**, was associated with a 10-fold loss in activity. However, the ease of synthesis and purification of molecules containing Ala rather than His suggested that further right-hand structural studies be done initially on Phe-Ala compounds and then optimized with Phe-His.

As shown in Table 2, changing the $R_3$=phenethyl group of **20** to the cyclohexylethyl group of **21** maintained potency while the benzylamino group of urea **22** increased potency slightly. Building on this finding, the $R_1$=benzyl analogue, **23**, was prepared. Although this change has been associated with enhanced activity in statine-based inhibitors of aspartic proteinases (17,7), a slight loss in activity was observed in our series.

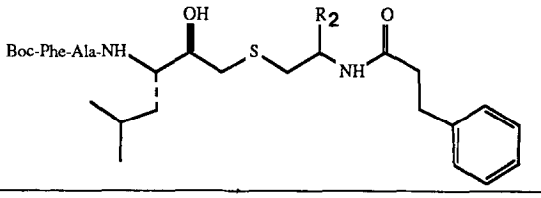
We next explored the structural requirements of the $R_2$ substituent. Changing the isobutyl side chain of **20** to either the benzyl or cyclohexylmethyl side chain of inhibitors **24** and **25** respectively, resulted in activity

**TABLE 2**




| COMPOUND # | $R_1$ | $R_3$ | IC50,nM |
|---|---|---|---|
| 20 | ISOBUTYL | $(CH_2)_2Ph$ | 2000 |
| 21 | ISOBUTYL | $(CH_2)_2$-$C_6H_{11}$ | 2500 |
| 22 | ISOBUTYL | $NHCH_2Ph$ | 1000 |
| 23 | BENZYL | $NHCH_2Ph$ | 2000 |

**TABLE 3**




| COMPOUND # | $R_2$ (*) | $IC_{50}$, nM |
|---|---|---|
| 20 | ISOBUTYL (R) | 2000 |
| 24 | BENZYL (R) | 10000 |
| 25 | CYCLOHEXYLMETHYL (R) | 5000 |
| 26 | ISOBUTYL (S) | 650 |

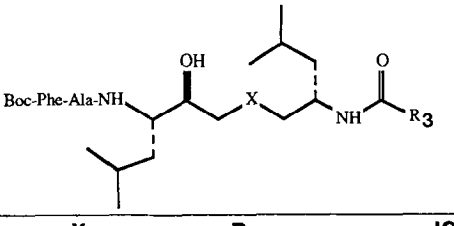
* = Absolute configuration of carbon bearing $R_2$

loss whereas simply changing the "R" configuration of the carbon bearing the $R_2$=isobutyl side chain of **20** to the "S" configuration (18) resulted in a 3-fold gain in activity (Table 3). The change of the backbone sulfide to the corresponding ether (X=O, **27**, Table 4) was not well tolerated; however, shortening the $R_3$ substituent from phenethyl to methyl (inhibitor **28**) led to no further activity loss.

To more fully optimize the activity, we replaced the $R_1$=isobutyl group with an $R_1$=cyclohexylmethyl group. This replacement, which is known to enhance the potency of statine-based renin inhibitors (7), provided a 10-fold boost in the series at hand (Table **5**, **29**). Finally, we reinstated the His residue to give the expected 10-fold increase in activity.

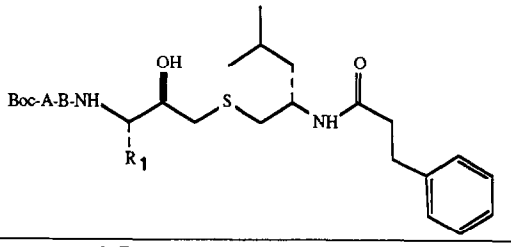
The resulting inhibitors **29** and **30** were of sufficient potency to warrant further in vitro and in vivo evaluation. Table 6 shows the high degree of specificity of the inhibitors toward human renin over other selected aspartic proteinases. We chose the more potent inhibitor, **30**, for evaluation in monkeys by intravenous administration. It led to a rapid drop in both mean blood pressure and plasma renin activity (Figure I); the magnitude of the blood

**TABLE 4**




| COMPOUND # | X | $R_3$ | $IC_{50}$, nM |
|---|---|---|---|
| 26 | S | $(CH_2)_2Ph$ | 650 |
| 27 | O | $(CH_2)_2Ph$ | 3000 |
| 28 | O | $CH_3$ | 3000 |

TABLE 5




| COMPOUND # | A-B | $R_1$ | $IC_{50}$, nM |
|---|---|---|---|
| 26 | PHE-ALA | ISOBUTYL | 650 |
| 29 | PHE-ALA | CYCLOHEXYLMETHYL | 70 |
| 30 | PHE-HIS | CYCLOHEXYLMETHYL | 7 |

TABLE 6 ENZYME SPECIFICITY

| | $IC_{50}$,nM | | | |
|---|---|---|---|---|
| COMPOUND # | HUMAN RENIN | PORCINE PEPSIN | BOVINE CATHEPSIN D | MOUSE SUBMAX. RENIN |
| 29 | 70 | >100,000 | >100,000 | 2400 |
| 30 | 7 | >100,000 | >100,000 | 1100 |

pressure drop, but not the duration of action, was comparable to that elicited by captopril. Note that the mean arterial pressure returned to baseline values within 30 minutes following a bolus dose of **30**, but the plasma renin activity was still depressed.

In part, the short half-life could be due to cleavage of the only "normal" peptide bond in the molecule, Phe-His. Although **30** was found to

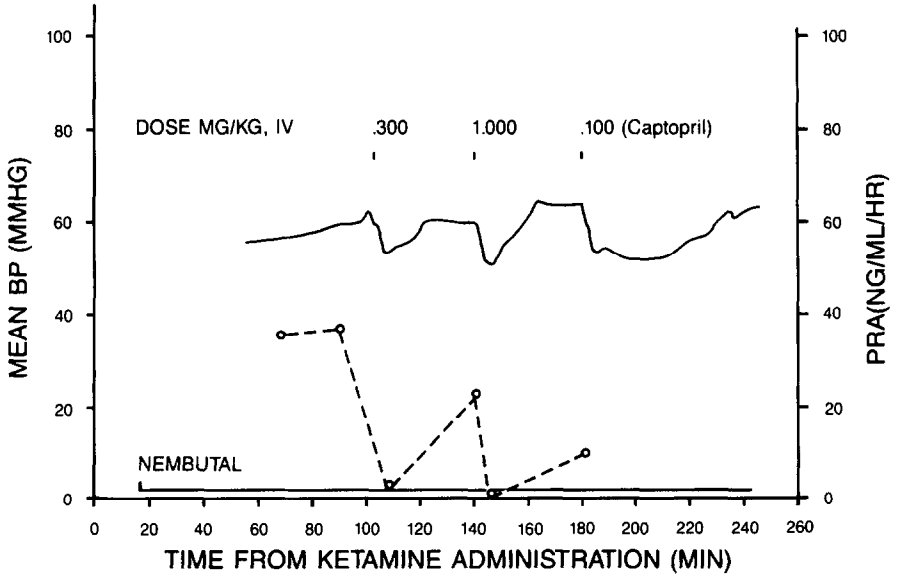



**Figure 1.** Effect of intravenous, bolus administration of **30** at 0.3 and 1.0 mg/kg or 0.1 mg/kg captopril on mean blood pressure (—) and plasma renin activity (o---o) in a salt-depleted monkey. Circles in the plasma renin activity graph indicate points of blood sampling. This experiment was repeated in a second monkey and produced similar effects to those shown here.

undergo no reaction in the presence of liver and intestine homogenates, disappearance of the parent compound with appearance of Boc-Phe was observed to be catalyzed by a commercial preparation of pancreatic proteases or by chymotrypsin, indicating cleavage of the Phe-His amide bond. Further studies directed toward the optimization of biological activity and stability of the renin inhibitors are in progress.

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