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New renin inhibitors homologous with pepstatin

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Four homologues of pepstatin, the potent but poorly soluble inhibitor of aspartic proteinases, were synthesized by coupling to the *C*-terminus of the natural pentapeptide the following amino acid residues: L-arginine methyl ester, L-aspartic acid, L-glutamic acid and the dipeptide L-aspartyl-L-arginine. The peptide-coupling reagent we used, benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate, allowed us to obtain readily pure pepstatin homologues with high yields (60–83%). Pepstatylarginine methyl ester and pepstatylglutamic acid were about one order of magnitude more water-soluble than pepstatin. The four homologues and pepstatin were tested *in vitro* as inhibitors for highly purified pig and human renins acting on the *N*-acetyltetradecapeptide substrate. The 50% inhibitory concentrations (IC_{50}) of the homologues were ranged from 0.01 to 1 μM against porcine renin at pH 6.0 (pepstatin $IC_{50} \sim 0.32\,\mu$M) and from 5.8 to 41 μM against human renin at pH 6.5 (pepstatin $IC_{50} \sim 17\,\mu$M). By three different graphical methods we showed that pepstatin and the four homologues behaved as competitive inhibitors for porcine renin. The most potent inhibitors were pepstatylaspartic acid and pepstatylglutamic acid, with inhibitory constants respectively 2- and 10-fold smaller than that of pepstatin. By coupling glutamic acid to pepstatin, the ratio solubility/K_i was increased by two orders of magnitude.

Renin (EC 3.4.99.19), an aspartic proteinase, plays an important role in the control of blood pressure in mammals (Cowley *et al.*, 1971; Barrett, 1980). It is involved in reno-vascular hypertensive diseases and may contribute to the maintenance of high blood pressure in essential hypertension (Haber, 1976). The inhibition of the renin–angiotensin system at the level of the renin–angiotensinogen reaction is therefore of considerable interest for the study of this system in experimental situations, and may have important therapeutic implications.

Pepstatin (Umezawa *et al.*, 1970), an *N*-acylpentapeptide of microbial origin, is the most potent renin inhibitor (Aoyagi *et al.*, 1971), but is not widely used for experiments *in vivo* because of its low solubility in physiological media. Its structure:

Isovaleryl-L-valyl-L-valylstatyl-L-alanylstatine

where statine is (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid, can explain its hydrophobic character.

By coupling charged hydrophilic amino acid residues to the *C*-terminus of pepstatin, we hoped to obtain soluble homologues. In the present paper we report the synthesis of four pepstatin homologues and the study of their properties as renin inhibitors.

Abbreviations used: OMe, methyl ester; -(2)HCl, (di)hydrochloride; BOP-PF_6, benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate ('Le BOP'); -OBu$^t_2$, di-t-butyl ester; (β-Bzl), β-benzyl; OBzl, benzyl ester or benzyloxy-; (NO_2), nitro; Boc-, butoxycarbonyl; Ac-, acetyl.

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Materials and methods

Synthesis of pepstatin homologues

Materials. Pepstatin was generously given by Dr. H. Umezawa and Dr. T. Aoyagi, Institute of Microbial Chemistry, Tokyo, Japan. Amino acid derivatives were purchased from Bachem, Bubendorf, Switzerland, and trifluoroacetic acid from Fluka, Buchs, Switzerland. Dimethylformamide was redistilled under reduced pressure and stored over a molecular sieve [0.4 nm (4Å)]. Triethylamine was distilled over KOH pellets. BOP-PF_6 was prepared in the laboratory as previously described (Castro *et al.*,

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1976). T.l.c. was performed on silica-gel plates (Merck Kieselgel G) with two solvent systems [butanol/acetic acid/water (4:1:1, by vol.) (BAW), and butanol/pyridine/acetic acid/water (15:10:3:12) (BΠAW). The peptides were revealed with ninhydrin or by the Cl_2/KI/starch method (Rydon & Smith, 1952). Melting points were measured on a Köfler plate and were not corrected. Optical rotations were determined at 20°C on a Perkin–Elmer 451 automatic polarimeter. I.r. spectroscopy was performed by using a Perkin–Elmer 457 spectrophotometer. Amino acid composition was determined with a Technicon analyser after hydrolysis in 6M-HCl under vacuum for 24h at 110°C.

Pepstatyl-Arg-OMe-HCl. Pepstatin (69mg, 0.1mmol) and L-Arg-OMe-2HCl (70mg, 0.2mol) were dissolved in dimethylformamide (5ml). BOP-PF_6 (88mg, 0.2mmol) was added, followed by an excess of triethylamine (50mg, 0.5mmol). The reaction mixture was stirred at room temperature for 72h. Distilled water (20ml) was then added and the reaction solution chilled. The resulting precipitate was filtered off, washed three times with 10ml of ether and dried under reduced pressure overnight. The crude material was then dissolved in 50ml of ethanol. The solution was filtered and evaporated to dryness. Yield: 69mg (76%); m.p. 262–263°C; $[\alpha]_D^{20}-40.1$ (*c* 0.18 in methanol); i.r. ester band 1740cm^{-1}; R_F 0.55 (BAW); 0.95 (BΠAW); $Arg_{0.95}$, Ala_1, $Val_{2.1}$.

Pepstatyl-Glu. Pepstatin (130mg, 0.2mmol) and L-Glu-OBu^t_2-HCl (120mg, 0.4mmol) were dissolved in dimethylformamide (8ml) in the presence of triethylamine (57mg, 0.6mmol). BOP-PF_6 (180mg, 0.4mmol) was then added. After 72h of stirring, 15ml of distilled water was added and the reaction mixture was chilled. The product, which formed as a precipitate, was collected by filtration, washed with 35ml of diethyl ether and dissolved in 30ml of methanol. The alcoholic solution was filtered and evaporated to dryness under reduced pressure. Treatment with 10ml of 50% trifluoroacetic acid in methylene chloride gave pepstatyl-Glu, which was precipitated by ether, filtered off, washed with ether and dried under vacuum. Yield: 130mg (83%); m.p. 130°C; $[\alpha]_D^{20}-79.0$ (*c* 0.55 in methanol); Ala_1, $Val_{1.97}$, $Glu_{0.99}$.

Pepstatyl-Asp. Pepstatin (137mg, 0.2mmol) and L-Asp-OBu^t_2-HCl (98mg, 0.4mmol) were coupled as described above. After treatment with 50% trifluoroacetic acid in methylene chloride, pepstatyl-Asp was obtained. Yield: 109mg (60%), m.p. 238–240°C; $[\alpha]_D^{20}-67.6$ (*c* 0.51 in methanol); $Asp_{0.92}$, $Ala_{1.12}$, $Val_{2.0}$.

Boc-Asp(β-Bzl)-Arg(NO_2)-OBzl. Arg(NO_2)-OBzl trifluoroacetate (1.06g, 2.5mmol) and Boc-L-Asp-Oβ Bzl (0.807g, 2.5mmol) were dissolved in acetonitrile (10ml) in the presence of triethylamine (0.505g, 5mmol) and BOP-PF_6 (1.1g, 2.5mmol) was added. After 4h stirring at room temperature, 50ml of water was added and the dipeptide extracted with ethyl acetate. The organic solution was washed three times with 2M-HCl and a saturated $NaHCO_3$ solution and, finally, once with water. After being dried over $MgSO_4$, the solution was evaporated under reduced pressure to give a solid product, shown by t.l.c. to be homogenous (in ethyl acetate). Yield: 1.32g (98%).

Pepstatyl-Asp(β-Bzl)-Arg(NO_2)-OBzl. Boc-Asp-(βBzl)-Arg(NO_2)-OBzl (106mg) was treated with 50% trifluoroacetic acid in 10ml of dichloromethane for 10min. Addition of 50ml of diethyl ether gave a precipitate, which was isolated by filtration, washed with ether and dissolved in 5ml of dimethylformamide in the presence of triethylamine (0.5mmol, 50mg). Pepstatin (69mg, 0.1mmol) was then dissolved in the solution and BOP-PF_6 (88mg, 0.2mmol) added. After the mixture had been stirred for 3 days at room temperature, 20ml of chilled water was added to the reaction mixture. The precipitate obtained was isolated by filtration and washed with 20ml of cold water, 10ml of 2M-HCl and 10ml of saturated $NaHCO_3$ solution. It was then again washed in 5ml of water, 5ml of methanol and 10ml of ether. The resulting white powder was dried under reduced pressure to yield 101mg (91%).

Removal of the protecting groups was achieved by catalytic-transfer hydrogenolysis (Kahn & Sivanandaiah, 1978). Part of the above material (40mg) was suspended in 2ml of ethanol with Pd/charcoal (10%, w/w) (30mg). Cyclohexene was added (1ml) and the mixture was heated under reflux for 24h. The solution was filtered through filter paper and the catalyst washed three times with 10ml of methanol. After evaporation, the solid material was dissolved in 50ml of twice-distilled water in the presence of arginine (20mg, 0.2mmol). The solution was then filtered and freeze-dried. Yield: 31mg; $Ala_{1.1}$, $Val_{2.04}$, $Asp_{0.92}$, $Arg_{4.95}$.

Determination of the solubility of pepstatin homologues in water. A saturated solution of each of the above products was prepared in a precise volume of twice-distilled water, pH5.8. The solution was warmed to 40°C to speed up solubilization, cooled to room temperature and spun for 30min at 6000rev./min ($r_{av.}$ 10cm.). The clear supernatant was frozen and freeze-dried to yield a fluffy material, which was weighed.

Enzymic assays

Materials. Pig renal renin was highly purified and had a specific activity of 135 Goldblatt units (G.U.)/mg of protein (Corvol *et al.*, 1977). Pure human renin (800G.U./mg of protein) was prepared as previously described (Galen *et al.*, 1979). When

tested with [^{14}C]dimethylcasein as substrate, the preparation was devoid of proteinase activity (Galen *et al.*, 1979).

N-Acetyltetradecapeptide substrate (Ac-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) and the tetrapeptide Leu-Val-Tyr-Ser were purchased from Bachem, Bubendorf, Switzerland, and the fluorescamine (Fluram) from Roche, Basel, Switzerland.

Fluorescent measurements were done at 405–478nm by using a Jobin–Yvon JY-3 automatic spectrofluorimeter.

Renin assay. We used the fluorimetric assay previously described (Galen *et al.*, 1978). This assay is based on the cleavage of the leucine–leucine bond of the *N*-acetyl synthetic substrate to produce *N*-acetylangiotensin I and Leu-Val-Tyr-Ser. The latter NH_2-free tetrapeptide is made to react with fluorescamine and can be quantified by spectrofluorimetry.

In each assay, 20mG.U. of pig renin and 7nmol of substrate were incubated in the presence of various inhibitor concentrations (0.2–5μM) in a 1ml final volume of 0.02M-citrate/phosphate buffer, pH6.0, for 1h at 37°C. Control incubations were run in the absence of inhibitor. The reaction was terminated by immersion in a boiling-water bath for 5min. The pH was then raised to 7.3 by adding 1ml of 0.1M-citrate/phosphate buffer, pH7.3. The fluorimetric reaction was developed at room temperature by adding 0.2ml of Fluram solution (0.3mg/ml of dioxan).

Conditions for kinetic studies. For K_m determinations, renin (20mG.U./ml) was incubated with four different substrate concentrations (9.3, 13.8, 18.6 and 27.6μM) at several inhibitor concentrations (around IC_{50}). The assay was then continued as described above.

Calculation of kinetic constants. Data were analysed by using three different graphical methods: a double-reciprocal plot ($1/v$ versus $1/[S]$), a Dixon (1953) ($1/v$ versus [I]) and a Cornish-Bowden (1974) plot {$[S]/v$ versus [I]}.

The Michaelis constant (K_m) and maximal velocity ($V_{max.}$) were then calculated from a weighted Lineweaver–Burk plot (Wilkinson, 1961). K_i was calculated from the formula:

$$K_p = K_m\left(1+\frac{[I]}{K_i}\right)$$

where K_p is the apparent Michaelis constant in the presence of inhibitor and [I] the inhibitor concentration. K_i was also determined directly from the Dixon plot as the intersection point on the abscissa.

To determine the molecular order of participation (n) of the inhibitors in the enzymic inhibition, the method of Lotfield & Eigner (1969) was used according to the formula:

$$\log\left(\frac{1}{v}-\frac{1}{v_0}\right) = n\log[I] + \log\left(\frac{K_m}{V[S]K_i}\right)$$

where v is the rate in the presence of inhibitor, v_0 the rate in the absence of inhibitor, n the interaction coefficient, [S] the substrate concentration and V the inhibited rate at maximum substrate concentration. The slope of the Lotfield plot:

$$\log\left(\frac{1}{v}-\frac{1}{v_o}\right) \text{ versus } \log[I]$$

gave the interaction coefficient.

Results

Peptide synthesis and solubility of pepstatin derivatives

All peptide couplings were performed with two equivalents of carboxy-group-protected amino acid or dipeptide, and two equivalents of condensation reagent in order to obtain quantitative reactions. BOP-PF_6 (Castro *et al.*, 1975) does not generate any insoluble material, so the pepstatin homologues were easily isolated from the reaction mixture by precipitation with small amounts of water. Table 1 summarizes the yields in each final product, cal-

Table 1. *Yield and solubility of the pepstatin homologues*

Pepstatin homologues	Yield (%)	Solubility in water	
		mg·ml^{-1}	mM
Pepstatin		0.27	0.40
Pepstatin arginine salt		5	5.8
Pepstatyl-Arg-OMe	76	4.0	4.5
Pepstatyl-Asp	60	2.8	3.5
Pepstatyl-Asp diarginine salt		15.0	12.5
Pepstatyl-Glu	83	3.8	4.7
Pepstatyl-Asp(β-Bzl)-Arg(NO_2)-OBzl	91		
Pepstatyl-Asp-Arg tetra-arginine salt		6.5	3.9

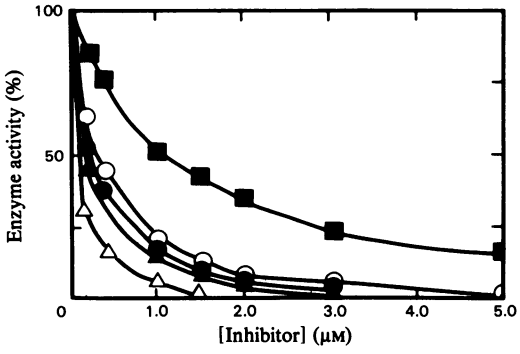

Fig. 1. *Inhibition of pig renin by pepstatin and the four new homologues*
Incubation mixture contained 20mG.U. of highly purified pig renin and 7nmol of *N*-acetyltetradecapeptide in 1ml of phosphate buffer, pH6.0, with various inhibitor concentrations. Incubations performed without inhibitors produced 3.5nmol of Leu-Val-Tyr-Ser/h per ml. ■, Pepstatyl-Arg-OMe; ○, pepstatin; ●, pepstatyl-Asp-Arg; ▲, pepstatyl-Asp; △, pepstatyl-Glu.

Table 2. *IC_{50} values for the pepstatin homologues towards porcine and human renins*

Inhibitor \ Renin ...	IC_{50} (μM) Porcine (pH6.0)	Human (pH6.5)
Pepstatin	0.32	15
Pepstatyl-Arg-OMe	1.1	41
Pepstatyl-Asp	0.19	—
Pepstatyl-Glu	0.10	5.8
Pepstatyl-Asp-Arg	0.23	—

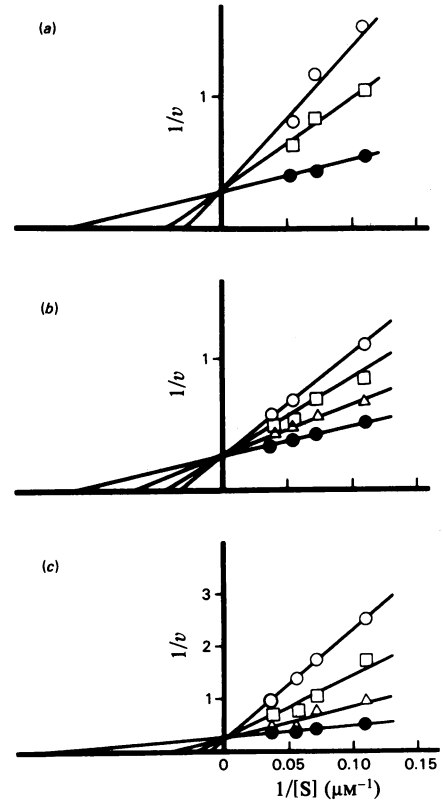

Fig. 2. *Lineweaver–Burk plot for inhibition of pig renin by pepstatin and its homologues*
(*a*) Pepstatin (□, 0.3μM; ○, 0.4μM); (*b*) pepstatyl-Asp (△, 0.05μM; □, 0.10μM; ○, 0.15μM); (*c*) pepstatyl-Glu (△, 0.025μM; □, 0.05μM; ○, 0.1μM). ●, Control.

culated from the starting amount of pepstatin and gives homologue solubility in twice-distilled water at pH5.8. To increase the solubility, certain homologues were prepared as arginine salts.

The extension of the *C*-terminus of pepstatin by hydrophilic amino acid residues increased its solubility by one order of magnitude.

Enzymatic assays

All the pepstatin homologues we synthesized inhibited pig renin and, as shown in Fig. 1, the homologue inhibition curves have the same profiles as that for pepstatin. Table 2 gives the (IC_{50}) values towards pig renin at the enzyme's optimum pH (6.0). They were in the same order of magnitude as pepstatin (0.01–1μM). Aspartate and glutamate derivatives were the most efficient (with K_i values below that of pepstatin). A 100% inhibition was obtained with pepstatyl-Glu at a concentration of 0.2μM.

Inhibition characteristics were studied on pig renin at different concentrations of substrate and inhibitors. Data were analysed by three graphical methods. The double-reciprocal plot showed a competitive pattern for the four homologues as well as for pepstatin. Three examples are shown in Fig. 2. We checked that pepstatin and its homologues caused a marked increase in the K_m values without altering those of V. The Dixon plot was also in accord with competitive inhibition (Fig. 3). The intersection point of the lines on the abscissa gave the K_i values that were identical with those obtained by the calculation from the Lineweaver–Burk plot (Table 3). The Cornish-Bowden plot showed parallel lines, confirming that the inhibition was of the competitive type (Fig. 4).

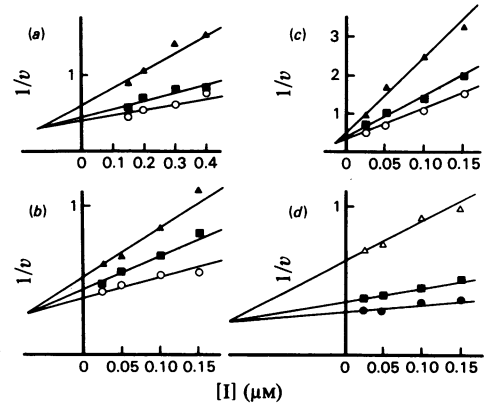

Fig. 3. *Dixon plot for inhibition of pig renin by pepstatin and its homologues*

(*a*) Pepstatin; (*b*) pepstatyl-Asp; (*c*) pepstatyl-Glu; (*d*) pepstatyl-Asp-Arg. Inhibition at various substrate concentrations is shown (△, 6.9 μM; ▲, 9.2 μM; ■, 13.8 μM; ○, 18.4 μM; ●, 27.6 μM).

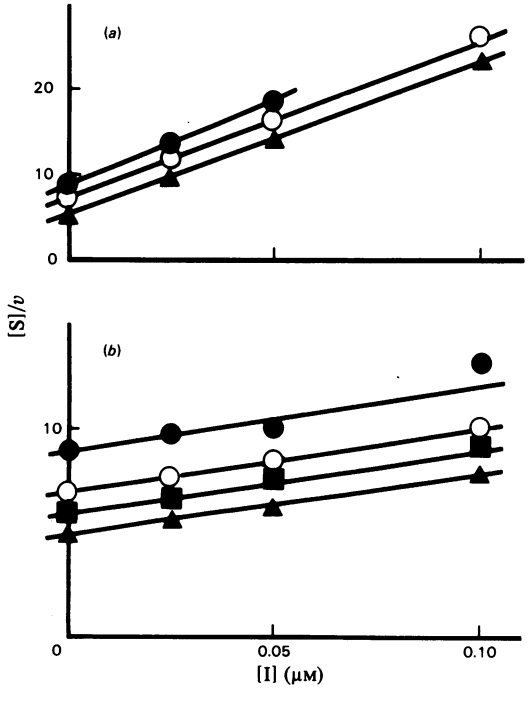

Fig. 4. *Cornish-Bowden (1974) plot for inhibition of pig renin by pepstatin and its homologues*

(*a*) Pepstatyl-Glu; (*b*) pepstatyl-Asp at various substrate concentrations (●, 27.6 μM; ○, 18.4 μM; ■, 13.8 μM; ▲, 9.2 μM). Pepstatin data were not represented because they gave superimposed lines.

Table 3. *K_i values of the pepstatin homologues for pig renin activity towards N-acetyl synthetic substrate at pH6.0*

K_i values were determined from: (1) weighted regressed Lineweaver–Burk plots; (2) the intersection point of the weighted regressed Dixon (1953) plots. Results are means ± S.E.M.

	$10^7 \times K_i$ (M)	
Plot ...	Lineweaver–Burk	Dixon
Inhibitor		
Pepstatin	1.4 ± 0.1	1.4 ± 0.1
Pepstatyl-Arg-OMe	4.7 ± 0.3	5.2 ± 0.5
Pepstatyl-Asp	0.70 ± 0.05	0.74 ± 0.1
Pepstatyl-Glu	0.14 ± 0.01	0.14 ± 0.04
Pepstatyl-Asp-Arg	1.5 ± 0.06	2.1 ± 0.1

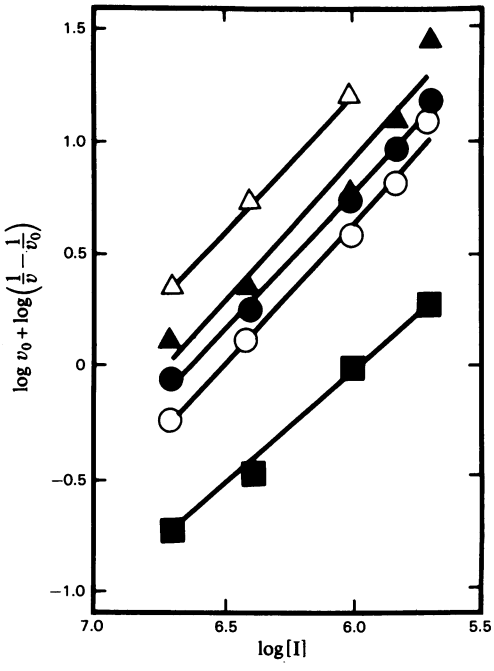

Fig. 5. *Lotfield & Eigner (1969) plot for inhibition of pig renin by pepstatin and its homologues*

■, Pepstatyl-Arg-OMe; ○, pepstatin; ●, pepstatyl-Asp-Arg; ▲, pepstatyl-Asp; △, pepstatyl-Glu.

The number of inhibitor molecules participating in inhibiting one molecule of enzyme was determined from the Lotfield plot (Fig. 5). We obtained straight lines with slope values ranging between 1.0 and 1.3. These data suggest that the interaction coefficient (n) equals one.

Inhibition of human renin

We checked that pepstatyl-Arg-OMe and pepstatyl-Glu also inhibited highly purified human renin. The results can be related to those obtained with pig renin, but a 50% inhibition of enzyme activity required the use of 50-fold more inhibitor than for pig renin (Table 2).

Discussion

Although several chemical modifications of pepstatin have been reported (Rich *et al.*, 1980), only two peptidic derivatives have been prepared, and they were not studied as renin inhibitors (Knight & Barrett, 1976; Workman *et al.*, 1979).

The aim of coupling charged amino acids to the C-terminus of pepstatin was to increase the solubility of the proteinase inhibitor. By attaching the dipeptide Asp-Arg, the (residues 1–2) *N*-terminal sequence of angiotensinogen (the natural renin substrate) we hoped also to increase the specificity against renin.

The peptide coupling reagent we used, BOP-PF_6 was known to give fast quantitative reactions (Castro *et al.*, 1975). By using two equivalents of amino acid residue and two equivalents of BOP-PF_6, we effectively obtained quantitative coupling to pepstatin, whereas Knight & Barrett (1976) reported incomplete coupling of glycine to pepstatin. Compared with the commonly used dicyclohexylcarbodi-imide, BOP-PF_6 does not produce any insoluble by-product that would render the isolation and purification of the desired compound difficult. This is particularly important in the case of pepstatin derivatives, which have a relatively poor solubility in any organic solvent, as well as in aqueous media. As shown in Table 1, pure products were isolated in high yields by precipitation followed by washings and deprotection.

The water-solubility of pepstatin was effectively increased by one order of magnitude by coupling charged amino acids; pepstatyl-Glu and pepstatyl-Arg-OMe were found to be the most soluble. The solubility of the free-carboxy-group containing compounds was further increased by preparing the homologues in the form of arginine salts, as previously reported for pepstatin (Evin *et al.*, 1979). Prepared as a diarginine salt, pepstatyl-Asp had a solubility as high as 12.5 mM.

All the modifications in the structure of pepstatin that have been reported were accompanied by an important loss of activity against renin (Marciniszyn, 1976; Rich *et al.*, 1980). We showed that coupling amino acid residues to the *C*-terminus of pepstatin did not greatly affect the inhibitory activity towards renin. Furthermore, we found that this could increase the affinity of pepstatin for renin with the two compounds pepstatyl-Asp and pepstatyl-Glu.

The type of inhibition of renin by pepstatin has been a matter of controversy. Most authors have been in favour of competitive inhibition (Miller *et al.*, 1972; Corvol *et al.*, 1973; Kokubu *et al.*, 1974; Hackenthal *et al.*, 1978; Gardes *et al.*, 1980), but others found a non-competitive type (Orth *et al.*, 1974; McKown *et al.*, 1974). By using highly purified enzyme and pure substrate, we have clearly shown by three different graphical methods that pepstatin and our four homologues acted as competitive inhibitors for pig renin acting on the *N*-acetyltetradecapeptide.

It seems that McKown *et al.* (1974) obtained different results because they did not use pure enzyme. Their renin preparation had a specific activity of 0.13 G.U./mg and was only 0.02% pure compared with the human renin in our study (860 G.U./mg). Moreover these authors reported recently that their renin preparation contained significant amounts of 'pseudorenin' and that both renin and 'pseudorenin' attacked the polymeric substrate they used (Chou *et al.*, 1978). The explanation of the inhibition pattern they observed could be due to 'pseudorenin', which has now been identified as cathepsin D. The inhibition of purified cathepsin D by pepstatin has been studied and found to be of a non-competitive type (Johnson & Poisner, 1977).

It was hoped that coupling of Asp–Arg to the *C*-terminus of pepstatin would increase the affinity for renin, because this dipeptide represents the *N*-terminus of natural substrate. But unexpectedly, two other pepstatin homologues were more potent renin inhibitors than was pepstatin. In particular, it seems that the presence of glutamic acid at the sixth position induced a notable affinity for renin and may contribute to the binding at the enzyme active site. We should emphasize that, by coupling glutamic acid to pepstatin, we increased the ratio solubility/K_i by two orders of magnitude. The data obtained with human renin, being related to those obtained with pig renin, make it worthwhile to test the antihypertensive properties of this compound *in vivo*. Pepstatyl-Arg-OMe has already been tested *in vivo* in the rat. It was found to prevent the blood-pressure increase caused by exogenous renin administration in binephrectomized rats (Gardes *et al.*, 1980) and to lower the blood pressure produced in renovascular hypertension by complete ligature of the aorta between the two renal arteries (C. Kreft, unpublished work). More specific inhibitors towards human renin need to be developed.

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