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Synthesis of novel tetrapeptides as potential ACE inhibitors†

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Pepsin hydrolysis of wheat gluten yields a tetrapeptide, Leu-Gln-Pro-Arg (**1**) which exhibits potent ACE inhibitory activity. Analogues (**2-7**) of this peptide have been synthesized and examined for ACE inhibitory activity *in vitro* and *in vivo*. None of the compounds synthesized showed improved *in vivo* activity. However, compound **6** has been found to be as active as captopril in *in vitro* assay.

Angiotensin converting enzyme (ACE) is a carboxydipeptidase which catalyses the hydrolysis of human prohormone, angiotensin I to the potent vasoconstrictor octapeptide Angiotensin II[1-3]. ACE has also been shown to degrade neuropeptides like enkephalins[4-6] and neurotensin[7,8], etc. whose properties include interaction with cardiovascular system. Development of potent inhibitors of ACE has been considered an important area to develop potent antihypertensive drugs. The first potent inhibitor reported by Ondetti *et al.*[9] was a nonapeptide teprotide. Since then a number of shorter peptides and their analogues have been synthesized and as a result highly potent and orally active compounds such as Captopril[10,11], enalapril[12-14] and isinopril[15,16], etc. are available in the market. These orally active ACE inhibitors have rapidly established themselves in the chemotherapy of hypertension and congestive heartfailure[17,18]. On the other hand interest in peptides related to teprotide as ACE inhibitors is also continuing. A number of pentapeptides and tripeptides related to teprotide with marked ACE inhibitory activity have been reported[19,20]. These peptides are having a modified peptide bond in order to improve metabolic stability.

Interestingly, for the last two decades all the research related to the designing of ACE inhibitors have been directed towards the design of peptides or non-peptidic compounds related to the snake venom peptide, teprotide. Recently, Yoshikawa *et al.*[21] have reported a very interesting tetrapeptide, Leu-Gln-Pro-Arg (**1**). This peptide has been shown to exhibit potent ACE inhibitory activity. Further, this peptide is derived from an edible protein, wheat gluten. The tetrapeptide was isolated from the pepsin digests of wheat gluten at *p*H 2 or trypsin and chymotrypsin digestion at *p*H 7.

We have examined the structure activity relationship of this tetrapeptide in view of the possibility of synthesising a more potent, metabolically stable and systemically active ACE inhibitor. Since ACE is a relatively non-specific enzyme, the inhibitory property of this tetrapeptide is also likely to unravel some important aspects for the site and mode of action of this peptide, possibly different from that of teprotide.

Modifications were incorporated in order to make analogues with different lipophilicity, enhanced duration of action (half life) and good interaction with the charged receptors, found on the surface of substrate. All the modifications were carried out keeping the overall backbone conformation of the resulting analogues similar to that of parent molecule. In the first instance, Leu at the N-terminus was replaced with Ala and Val to obtain peptides with altered lipophilicity. The second modification was to replace the N-terminal Leu with D-Leu with a view to achieving resistance to degradation with aminopeptidases. The C-terminal Arg was replaced with Lys in two analogues. This was done to see if the guanidine side chain of Arg plays a significant role in its interaction with the receptor or could be replaced by basic functionality of Lys. The third amino acid residue Pro was not altered, keeping in mind the structural requirement for the action of zinc containing carboxydipeptidase. The following analogues of **1** have been synthesized and examined for their ACE inhibitory activity *in vitro* and *in vivo*.

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1 Leu-Gln-Pro-Arg
2 Val-Gln-Pro-Arg
3 Ala-Gln-Pro-Arg
4 D-Leu-Gln-Pro-Arg
5 Leu-Gln-Pro-Lys
6 D-Leu-Gln-Pro-Lys
7 D-Leu-Asn-Pro-Arg

Synthesis of peptides. Peptides were assembled on *p*-benzyloxybenzyl alcohol (Wang) resin[23,24] (solid support) by manual solid phase synthesis[22]. Fmoc chemistry[25-27] was employed for the synthesis of all the peptides. The first N^{α}-Fmoc amino acid was loaded on the p-benzyloxybenzylalcohol resin by symmetrical anhydride using diisopropylcarbodiimide (DICI)[28,29] and 4-dimethylaminopyridine (DMAP)[30,31]. A consistent coupling protocol was employed for each coupling. Subsequent N^{α}-Fmoc amino acids were attached on the growing peptide chain by performing the following steps in each cycle:

(i) Cleavage of Fmoc group was carried out using 20% piperidine in DMF-CH_2Cl_2 (1:1) (5 min × 1 and 20 min × 1). (ii) Fmoc amino acids were activated and coupled using DICI and 1-hydroxybenzotriazole (HOBt) in DMF-CH_2Cl_2 (1:1). (iii) After each coupling (step 1) and deprotection (step 2), the resin was washed with DMF:CH_2Cl_2 (1:1) (1 min × 1) and CH_2Cl_2(1 min × 3).

Prior to deprotection, the peptide couplings were monitored by the Kaiser's test and were allowed to proceed until completion (generally within 120 min). After assembly of the desired peptide chain, washing of the peptide resin was carried out with CH_2Cl_2, DMF-CH_2Cl_2 (1:1), DMF, DMF-CH_2Cl_2 (1:1), CH_2Cl_2, MeOH-CH_2CL_2 (1:1), MeOH, MeOH-CH_2Cl_2 (1:1), CH_2Cl_2 and ether (each 2 times for 1 min). Removal of the side chain protecting groups of the protected tetrapeptide as well as its concomitant cleavage from the resin was achieved using TFA[25-27] containing ethanedithiol (EDT) and thioanisole as scavengers. The crude peptides were purified by RP-HPLC on Waters LC system. Homogeneity of purified peptides was further established by analytical TLC and RP-HPLC and all the peptides were found to be > 95% pure.

***In vivo* ACE inhibitory activity.** *In vivo* ACE inhibitory activity was evaluated according to the procedure reported by Smith *et al.*[33] The male Sprague Dawley rats (150-180 g) were anaesthetized with pentobarbitone sodium (50 mg/kg, ip). Blood pressure was recorded from carotid artery through a pressure transducer (Statham P23DC, Gross Instruments Co., USA) on a Grass model 7 polygraph (Grass Instruments Co., USA). External jugular vein was cannulated for intravenous injections. Following 20-30 min of stabilization period, Angiotensin I(1 μg/kg in normal saline, i.v.) induced increase in blood pressure (BP) was recorded. Captopril or test compounds were administered (i.v.) slowly at a dose of 1 mg/kg and after 5 min Ang I response on BP was recorded. Inhibition of Ang I response after the administration of captopril or test compound was calculated and the results are summarized in Table I.

***In vitro* ACE inhibitory activity.** Table II summarizes the effect of various synthetic peptides on ACE activity. Compounds (100 n*M* concentration) were incubated for 5 min with male S/D rat serum (which contained ACE activity), and ACE catalyzed hydrolysis of 2-furanacryloyl-L-phenylalanylglycylglycine (FAPGG) in 0.05 *M* Tris (*p*H 7.5) was followed at 37°C on the spectrophotometer at 340 nm[34]. Captopril was used as a standard drug which inhibited the ACE activity by 100% at 100 n*M* concentration.

It was observed that by altering the lipophilicity of the peptide **1** at the N-terminus did not affect the ACE activity. However, replacement of Leu

Table I—Inhibitory effect of captopril and test compounds on angiotensin I induced pressor response on rat blood pressure

Compound	% Inhibition of Ang I response (mean ± SE)*
Captopril	59.67 ± 4.40
Leu-Gln-Pro-Arg	35.33 ± 9.86
Val-Gln-Pro-Arg	9.67 ± 2.67
Ala-Gln-Pro-Arg	12.67 ± 7.22
D-Leu-Gln-Pro-Arg	0.0
Leu-Gln-Pro-Lys	0.0
D-Leu-Gln-Pro-Lys	24.80 ± 5.64
D-Leu-Asn-Pro-Arg	13.67 ± 2.03

*3-5 experiments were done for each compound)

Table II—Effect of captopril and peptides on rat serum ACE inhibitory activity *in vitro*

Compound (Concn 100 n*M*)	% Inhibition
Captopril	100
Leu-Gln-Pro-Arg	10
Val-Gln-Pro-Arg	20
Ala-Gln-Pro-Arg	12
D-Leu-Gln-Pro-Arg	42
Leu-Gln-Pro-Lys	0
D-Leu-Gln-Pro-Lys	100
D-Leu-Asn-Pro-Arg	31

with D-Leu at the N-terminus resulted in significant inhibition of ACE in the presence of this peptide. In peptide **5** the basic functionality at the C-terminus was replaced with Lys which has no effect on ACE activity. Interestingly, replacement of Leu with D-Leu at the N terminus and Arg with Lys at the C-terminus, led to 100% inhibition of ACE activity. At 30 n*M* concentration these peptides had no effect on ACE activity except **6** which produced 60% inhibition of ACE activity.

Experimental Section

General. All the optically active amino acids used in this study were of L-configuration unless stated otherwise. The protected amino acid derivatives were purchased from Novabiochem, Switzerland. The deblocking reagent was freshly prepared. Homogeneity and purity of the final peptides were determined by TLC on silica gel-G plates using the solvent systems: (A) *n*-BuOH-pyridine-AcOH-H_2O (15:10:3:12), (B) *n*-BuOH-pyridine-AcOH-H_2O (45:15:10:20).

Final peptides were detected by spraying the TLC plates with 0.2% ninhydrin solution and heating the plates at 100°C in a hot-air oven. Final purification and confirmation of their homogeneity was done by HPLC on a Waters or Applied Biosystem instrument. The solvent system used for the analytical HPLC was a binary gradient system: water containing 0.1% TFA (*p*H 2) and acetonitrile as the organic modifier, and the solvent programme involved a linear gradient of 0-35% acetonitrile over 45 min at a flow rate of 1 mL/min. A μ-Bondapak C_{18}-column (3 × 300 mm) was used for this purpose. Preparative (50 mg) low pressure (100 psi) chromatographic purification was accomplished on a Shimpak Prep-ODS column (20 × 250 mm) using a binary gradient system. The solvent programme involved 0-25% acetonitrile over 30 min, then 25-100% acetonitrile over 30-40 min at a flow rate of 9 mL/min.

Characterization of the final purified peptides was done with the help of spectroscopic methods. ^{1}H NMR spectra were taken on a Bruker 300 MHz Avance DRX spectrometer (chemical shifts in δ, ppm downfield from TMS as internal standard). The FAB mass spectra (FAB-MS) were recorded on a JEOL SX-102 mass spectrometer. Capillary melting points were determined on a Buchi 550 melting point apparatus and are uncorrected. Optical rotations were recorded on a Perkin-Elmer 241 automatic polarimeter.

Synthesis of analogues 1-4

Fmoc-Gln-Pro-Arg(Mtr)-Resin (I). Wang resin (2.608 g, 2.0 m moles) was taken in a reaction vessel and swelled in DCM under nitrogen current for 30 min. A four-fold excess of C-terminal amino acid, Fmoc-Arg (Mtr) (5.66 g, 8 mmoles), diisopropylcarbodiimide (DICI) (0.8 mL; 4 mmoles) and 4-dimethylaminopyridine (0.244 g; 2.0 mmoles) were added to this and the resin beads stirred for 4 hr under nitrogen current. Loading was repeated taking two-fold excess of Fmoc-Arg(Mtr) (2.83 g, 4 mmoles). The resin was washed properly with DMF and DCM. The tripeptide was assembled on the resin by successive addition of Fmoc-Pro (0.421 g, 1.25 mmoles) and using DICI (0.2 mL; 1.0 mmoles) as a coupling reagent according to the protocol mentioned earlier. Completion of the coupling was monitored by the Kaiser's qualitative ninhydrin test[32]. After assembly of the tripeptide on the resin, it was washed properly with ether and dried over P_2O_5 *in vacuo*. The peptide resin (3.05 g) was divided into four parts and each part was further used separately to get the tetrapeptide by the addition of N-terminal Fmoc-amino acid.

Leu-Gln-Pro-Arg 1. Fmoc-Gln-Pro-Arg(Mtr)-Resin (0.760 g, 0.5 mmoles) was taken in a reaction vessel and swelled in DCM. Fmoc-Leu (0.441 g, 1.25 mmoles) was coupled to tripeptide according to the protocol mentioned earlier. The Fmoc group was finally removed and the peptide resin washed successively with a series of solvents as mentioned earlier. The peptide resin was then dried *in vacuo* over P_2O_5.

The protected peptide resin (0.830 g) was treated with TFA (22.5 mL) in the presence of ethanedithiol (1.25 mL) and thioanisole (1.25 mL) for 90 min at room temperature. The resulting crude peptide was purified by RP-HPLC under the conditions mentioned earlier. The fractions containing the desired compound were lyophilized. The product was found to be pure by TLC and analytical RP-HPLC, yield 0.165 g (65%); R_fA (0.59); R_f B (0.57) m.p. 108° (d); $[\alpha]_D^{25}$-40° (*c* = 0.11, DMF); K′ 1.93; FAB-MS: $[M+H]^+$ (Found) 513, $[M+H]^+$ (Calcd) m/z 513; ^{1}H NMR (DMSO-d_6) δ 0.86 (m, 6H, Leu C^{δ} H^S), 1.5-2.2 (bm, 13H, Leu $C^{\beta,\gamma}H^S$, Arg $C^{\beta,\gamma}H^S$, Pro $C^{\beta,\gamma}H^S$, Gln $C^{\beta}H^S$), 3.1 (bs, 2H, Arg $C^{\delta}H^S$), 3.4-4.53 ($C^{\alpha}H^S$), 6.9-7.4 (s, 2H, N^{δ} H^S), 7.7-8.7 (NH^S).

Val-Gln-Pro-Arg 2. Fmoc-Gln-Pro-Arg(Mtr)-Resin (0.760 g, 0.5 mmoles) was taken in a reaction vessel. The Fmoc group was removed and Fmoc-Val (0.423 g, 1.25 mmoles) coupled to the tripeptide on the resin. The title peptide was obtained in a manner similar to that mentioned above for peptide **1**. Yield 0.166 g (67%); R_f A

(0.57); R_f B(0.54); m.p. 174-76° (d); $[\alpha]_D^{25} - 38.3°$ (*C*, 01; DMF); K′ 1.36; FAB-MS: $[M+H]^+$ (Found) 4.99; $[M+H]^+$ (Calcd) 499; ^1H NMR (DMSO-d_6): δ 1.0 (m, 6H, Val $C^{\gamma}H^S$), 1.66-2.38 (bm, 10 H, $Arg^{\beta,\gamma}H^S$, Pro $C^{\beta,\gamma}H^S$, Gln $C^{\beta}H^S$), 3.15 (t, 2H, Arg $C^{\delta}H^S$), 3.5-4.8 ($C^{\alpha}H^S$), 7.3-8.6 (NH^S).

Ala-Gln-Pro-Arg 3. Fmoc-Gln-Pro-Arg(Mtr)-Resin (0.760 g; 0.5 mmoles) was taken in a reaction vessel. The Fmoc group was removed and Fmoc-Ala (0.388 g; 1.25 mmoles) was coupled to the tripeptide resin **I**. The title peptide was obtained in a manner similar to that mentioned for peptide **1**. Yield 0.145 g (62%); R_f A (0.51); R_f B (0.49); m.p. 180-83° (d); $[\alpha]_D^{25} - 41.6°$ (*c*, 0.11, DMF); K′ 1.28; FAB-MS: $[M+H]^+$] (Found) m/z 471; $[M+H]^+$ (Calcd)m/z 471; ^1H NMR (DMSO-d_6): δ 1.30 (d, 3H, Ala $C^{\beta,\gamma}H^S$), 1.6-2.2 (bm, 10H, Arg $C^{\beta,\gamma}H^S$, Pro $C^{\beta,\gamma}H^S$, Gln $C^{\beta}H^S$), 3.1 (d, 2H, Arg $C^{\delta}H^S$), 3.4-4.5 (C^{α}, H^S), 6.8-8.62 (NH^S).

D-Leu-Gln-Pro-Arg 4. Fmoc-D-Leu (0.442 g; 1.25 mmoles) was coupled to the tripeptide I (0.760 g, 0.5 mmoles) on the solid support according to the standard coupling protocol. The title peptide was obtained in a manner similar to that adopted for peptide **1**. Yield 0.175 g (69%); R_f A (0.62); R_f B (0.61); m.p. 210(d); $[\alpha]_D^{25} - 46.6°$(*c* 0.1; DMF); K′ 1.81; FAB-MS: $[M+H]^+$ (Found) 513; $[M+H]^+$ (Calcd) 513; ^1H NMR (DMSO-d_6): δ 0.83 (m, 6H, Leu $C^{\delta}H^S$), 1.6-2.2 (bm, 13H, Leu $C^{\beta,\gamma}H^S$, Arg $C^{\beta,\gamma}H^S$), 3.2-4.61 ($C^{\alpha}H^S$), 6.9-7.4 (s, 2H, $N^{\delta}H^S$), 7.7-8.7 (NH^S).

Synthesis of analogues 5-6

Fmoc-Gln-Pro-Lys (Boc)-Resin II. Fmoc Lys (Boc) (1.872 g, 4 mmoles) was used for loading on the wang resin(1.306 g; 1 mmoles) by symmetrical anhydride method. The tripeptide **II** was assembled on the resin in a manner similar to that mentioned for **I**. Yield 1.416 g. The peptide resin (1.416 g) was divided into two parts to be used further for synthesizing the compounds **5** and **6**.

Leu-Gln-Pro-Lys 5. Fmoc-Gln-Pro-Lys(Boc)-Resin (0.708 g; 0.5 mmoles) was taken in a reaction vessel and swelled in DCM. Fmoc-Leu (0.440 g; 1.25 mmoles) was coupled to the tripeptide according to the protocol mentioned earlier. The Fmoc group was removed and the title peptide obtained in a manner similar to that mentioned for peptide **1**. Yield 0.166 g (69%); R_f A (0.56); R_f B (0.55); m.p. 195° (d); $[\alpha]_D^{25} - 25.8°$ (*c* 0.11, DMF); K′ 2.01; FAB-MS: $[M+H]^+$ (Found) 485; $[M+H]^+$ (Calcd) 485; ^1H NMR (DMSO-d_6): 0.9 (m, 6H, Leu $C^{\delta}H^S$), 1.30 (t, 2H, Lys $C^{\gamma}H^S$), 1.59 (m, 3H, Leu $C^{\beta,\gamma}H^S$), 1.8-1.9 (m, 4H, Lys $C^{\beta,\gamma}H^S$), 2.0-2.2 (bm, 8H, Gln $C^{\beta,\gamma}H^S$, Pro $C^{\beta,\gamma}H^S$), 2.8 (t, 2H. Lys $C^{\varepsilon}H^S$),3.74 (t, 2H, Pro $C^{\delta}H^S$), 4.0-4.8 ($C^{\alpha}H^S$), 6.82, 7.35 (ds, 2H, Gln $N^{\delta}H^S$), 8.0-8.9 (NH^S).

D-Leu-Gln-Pro-Lys 6. Fmoc-D-Leu (0.440 g; 1.25 mmoles) was coupled to the tripeptide loaded resin **II** (0.708 g; 0.5 mmoles) according to the standard coupling protocol. The peptide **6** was obtained in a manner similar to that mentioned for peptide **1**. Yield 0.163 g (68%); R_f A (0.56); R_f B (0.54); m.p. 179° (d); $[\alpha]_D^{25} - 41.6°$ (*c* 0.10, DMF), K′ 1.98; FAB-MS: $[M+H]^+$ (Found) 485; $[M+H]^+$ (Calcd) 485; ^1H NMR (DMSO-d_6): δ 0.9 (m, 6H, Leu $C^{\delta}H^S$), 1.35 (t, 2H, Lys $C^{\gamma}H^S$)1.55 (m, 3H, Leu $C^{\beta,\gamma}H^S$), 1.8-1.9 (m, 4H Lys $C^{\beta,\delta}H^S$), 2.0-2.2 (bm, 8H, Gln $C^{\beta,\gamma}H^S$, Pro $C^{\beta,\gamma}H^S$), 2.8 (t, 2H, Lys $C^{\varepsilon}H^S$), 3.72 (t, 2H, Pro $C^{\delta}H^S$), 4.1-4.6 ($C^{\alpha}H^S$), 6.87, 7.35 (ds, 2H, Gln $N^{\delta}H^S$, 8.2-8.9 (NH^S).

Synthesis of D-Leu-Asn-Pro-Arg 7. The tetrapeptide **7** was obtained by sequential addition of Fmoc amino acids on the wang resin (0.654 g; 0.5 mmoles) in a manner similar to that described for the analogue **4**. However, Fmoc-Asn (0.439 g, 1.25 mmoles) was used at the dipeptide stage in place of Fmoc-Gln. The crude peptide obtained by the cleavage from the solid support was purified by HPLC. Yield 0.150 g (60%); R_f A (0.56); R_f B (0.55); m.p. 192° (d); $[\alpha]_D^{25} - 46.6°$ (*c* 0.1, DMF); K′ 1.66; FAB-MS: $[M+H]^+$ (Found) 499; $[M+H]^+$ (Calcd) 499; ^1H NMR (DMSO-d_6: δ 0.88 (bs, 6H, Leu $C^{\delta}H^S$, 1.5-1.75(bm, 7H, Leu $C^{\beta,\gamma}H^S$, Arg $C^{\beta,\gamma}H^S$) 1.89-2.1 (m, 4H, Pro $C^{\beta,\gamma}H^S$), 3.08 (d, H, Asn $C^{\beta}H^S$), 3.4 (t, 2H, Arg $C^{\delta}H^S$), 3.80 (t, 2H, Pro $C^{\delta}H^S$), 4.1-4.9 ($C^{\alpha}H^S$, 7.02,7.52 (ds, 2H, Asn $N^{\gamma}H^S$), 7.75-8.92 (NH^S).

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