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Structure–activity relationship study between Ornithyl-Proline and Lysyl-Proline based tripeptidomimics as angiotensin-converting enzyme inhibitors

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Abstract—A designed library of tripeptidomimics of Ornithyl-Proline (Orn-Pro) and Lysyl-Proline (Lys-Pro) conjugated with various unnatural amino acids and carboxylic acid derived heterocyclics was synthesized and screened for possible inhibitors of angiotensin-converting enzyme (ACE). Among the tripeptidomimics 10[MTP-Orn-Pro], 11[HTP-Orn-Pro], 14[TA-Orn-Pro] and 20[BPA-Orn-Pro] showed prominent inhibition with IC_{50} values in micromolar concentrations. Structure–activity relationship study indicated that C_3 side chain of Orn as compared to C_4 side chain of Lys at $P_{1'}$ position was better suited to inhibit ACE, with propionic acid (C_3) derived heterocyclics and unnatural amino acids.

Angiotensin-converting enzyme (EC 3.4.15.1, ACE), a membrane anchored zinc metallopeptidase, plays a pivotal role in blood pressure regulation.[1–3] In hypertension and other related cardiovascular disorders like myocardial infarction, CHF and atherosclerosis increased ACE activity has been found. To reduce the activity of ACE by ACE inhibitors has been a preferred therapy to treat these disorders.[4–6]

Several ACE inhibitors have been developed during the last two decades.[7–15] The use of ACE inhibitors is fraught with many side effects like dry cough, skin irritation, angiodema and other problems particularly multiple dosing due to less bioavailability.[16] To overcome the problem of limited bioavailability, a new class of ACE inhibitors, termed as peptidomimics,[17,18] consisting of nonpeptide moieties like unnatural amino acids or carboxylic acid derived heterocyclics conjugated with peptide moiety, is being designed and synthesized. The peptidomimics containing particularly proline[19] may confer resistance against proteolysis[20] by digestive enzymes and hence result in enhanced bioavailability.

Our previous study demonstrated that propionic acid (C_3) derived heterocyclic containing tripeptidomimics BPA-Val-Trp, HTP-Val-Trp and BPA-Ile-Pro were suitable ACE inhibitors.[21] In the present work, based on our previous study and structural–activity relationship (SAR) studies of existing ACE inhibitors, two sets (set A and set B) of libraries containing Lys and Orn with Pro as a common moiety were designed and synthesized. In the designing of dipeptide motif, usual amino acid that is, Pro is put at $P_{2'}$ position, while $P_{1'}$ position is occupied by usual (e.g., Lys) or unusual (e.g., Orn) amino acids. At the $P_{2'}$ position, the $-COO^-$ group of Pro is likely to interact with a positively charged side chain of Arg (or Lys) in the $S_{2'}$ subsite of ACE.[22,23] The $S_{1'}$ subsite, being hydrophobic in nature, preferentially accepts linear amino acids like Lys or Orn for better interaction.[24] At the P_1 position, unnatural amino acids and carboxylic acid derived heterocyclic moieties are incorporated to interact with the S_1 subsite and Zn^{2+} ion located in the enzyme's active site (Fig. 1).

The various moieties selected for the P_1 position with above-discussed dipeptide motif (Lys-Pro or Orn-Pro)

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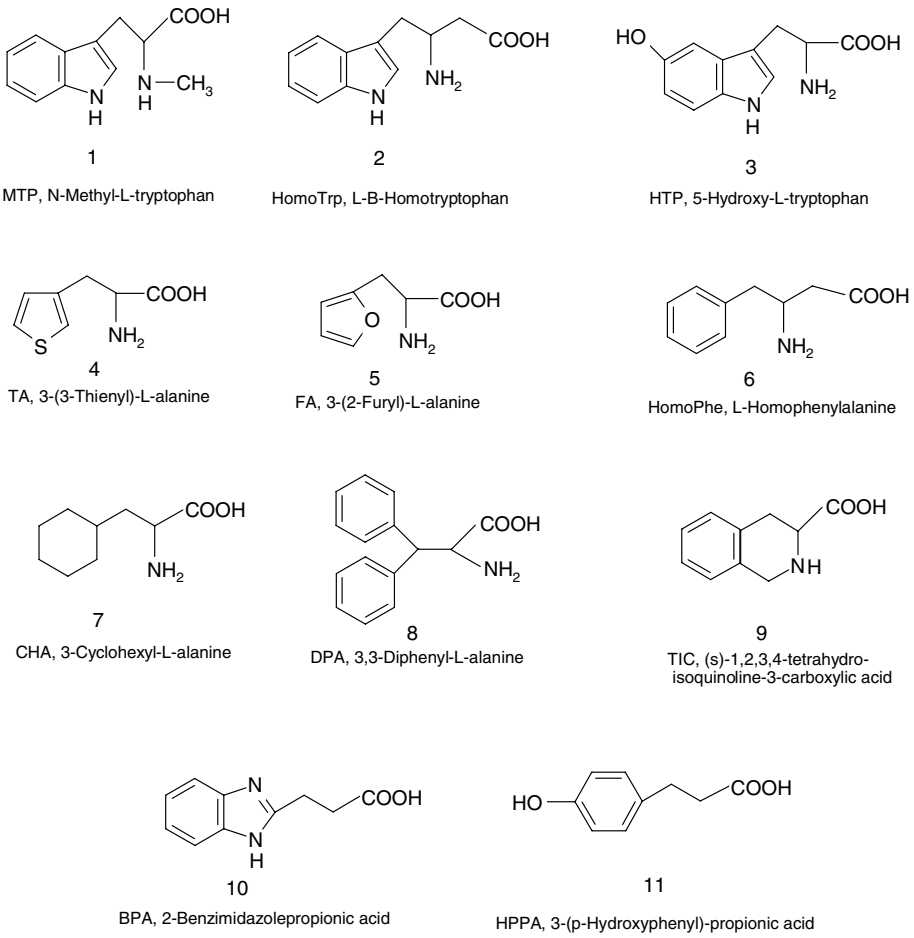

Figure 1. Unnatural amino acids and carboxylic acid derived heterocyclic moieties.

can be divided into two categories. First category contains—unnatural amino acids derived from L-tryptophan and L-alanine like *N*-methyl-L-tryptophan (MTP, **1**), L-β-homotryptophan (HomoTrp, **2**), 5-hydroxy-L-tryptophan (HTP, **3**), 3-(3-thienyl)-L-alanine (TA, **4**), 3-(2-furyl)-L-alanine (FA, **5**), L-homophenylalanine (HomoPhe, **6**), 3-cyclohexyl-L-alanine (CHA, **7**) and 3,3-diphenyl-L-alanine (DPA, **8**). While the second category contains—carboxylic acid derived heterocyclics like (*S*)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (TIC, **9**), 2-benzimidazolepropionic acid (BPA, **10**) and 3-(*p*-hydroxyphenyl)-propionic acid (HPPA, **11**) (Fig. 1).

All solvents and reagents used in the synthesis of tripeptidomimics were of peptide synthesis grade. The reagents were purchased from Sigma chemical Co. *N*,*N*-Dimethylformamide (DMF) and dichloromethane (DCM) were from MERCK. Fmoc-protected L-amino acids and 2-chlorotrityl chloride resin were obtained from Novabiochem. Unnatural amino acids and carboxylic acid derived heterocyclic moieties were purchased from Aldrich Chemical Co.

The designed library of tripeptidomimics was synthesized by employing the technique of multiple parallel synthesis on a solid support of 2-chlorotrityl chloride resin by employing standard solid-phase peptide synthesis using diisopropylethylamine (DIPEA) method[25] and Hydroxybenzotriazole (HOBt)/diisopropyl carbodiimide (DIPCDI) activation method. Synthesized tripeptidomimics were cleaved from the resin by using triflouroacetic acid and DCM (95%:5%) with required scavengers and were purified by gel filtration on LH-20 using methanol as eluent. The purity of tripeptidomimics was assessed by reverse-phase HPLC on C-18 columns (Table 1) and their masses were characterized by MALDI-Tof (Kratos Analytical) and liquid chromatography-mass spectrometry (LC-MS) (Table 1).

In vitro inhibition of ACE for these tripeptidomimic compounds was evaluated by a synthetic substrate of ACE, Hip-His-Leu, using the spectrophotometric method.[26] The following assay components in a final volume of 0.25 mL were incubated for 30 min at 37 °C: 100 mM potassium phosphate buffer (pH 8.3), 5 mM Hip-His-Leu, 300 mM NaCl, 2.5 μL of ACE (13.46 ± 0.03 mU/mL) and 2.5 μL of 25 μM tripeptidomimics. The rate of hydrolysis of Hip-His-Leu was determined by measuring the absorbance of hippuric acid after extracting into ethyl acetate, evaporating the solvent at 120 °C and then redissolution into water. The extracted hippuric acid was then measured by reading the absorbance at 228 nm to determine ACE inhibition potency of tripeptidomimics.

Table 1. In vitro activity of tripeptidomimics as angiotensin-converting enzyme inhibitors

S. No.	Compounds	*m/z* [b]	HPLC profile [d] (rt, %)	ACE activity[a] (mU/mL)	ACE inhibition (%)	*p*-Value
Set A						
1	MTP-Lys-Pro	444.03	5.6 (93.1)	8.20 ± 0.06	39.07	<0.0001
2	HomoTrp-Lys-Pro	443.8	5.5 (99.4)	9.40 ± 0.11	30.20	<0.0001
3	HomoPhe-Lys-Pro	404.8	5.6 (99.8)	10.42 ± 0.15	22.63	<0.05
4	TA-Lys-pro	418.3[c]	6.1 (82.1)	11.67 ± 0.13	13.33	<0.05
5	FA-Lys-Pro	381.30	5.5 (98.6)	12.11 ± 0.11	10.09	<0.05
6	CHA-Lys-Pro	397.3	5.5 (100)	13.11 ± 0.23	3.71	NS[e]
7	DPA-Lys-Pro	467.7	5.5 (99.6)	8.91 ± 0.06	33.82	<0.0001
8	TIC-Lys-Pro	403.0	5.6 (98.4)	10.85 ± 0.07	19.43	<0.0001
9	HPPA-Lys-Pro	394.6	5.7 (99.7)	9.52 ± 0.17	29.32	<0.05
SetB						
10	MTP-Orn-Pro	430.01	5.6 (100)	4.88 ± 0.09	63.62	<0.0001
11	HTP-Orn-Pro	453[c]	5.4 (93.3)	4.2 ± 0.07	68.80	<0.0001
12	HomoTrp-Orn-Pro	429.2	5.8 (91.3)	12.23 ± 0.16	9.13	<0.05
13	HomoPhe-Orn-Pro	411.5	6.0 (100)	9.21 ± 0.07	31.72	<0.0001
14	TA-Orn-pro	382.96	5.4 (99.1)	3.41 ± 0.12	74.68	<0.0001
15	FA-Orn-Pro	366.98	5.5 (98.5)	8.67 ± 0.08	35.61	<0.0001
16	CHA-Orn-Pro	403.8[c]	5.6 (96.7)	9.32 ± 0.05	30.78	<0.0001
17	DPA-Orn-Pro	452.4	5.5 (99.30)	9.74 ± 0.15	27.67	<0.05
18	TIC-Orn-Pro	389.5	6.9 (100)	10.1 ± 0.06	25.01	<0.0001
19	HPPA-Orn-Pro	380.9	5.7 (99.4)	10.26 ± 0.07	23.76	<0.0001
20	BPA-Orn-Pro	402.3	6.1 (100)	4.70 ± 0.10	65.45	<0.0001

[a] Tripeptidomimics were assayed against bovine kidney extract of angiotensin-converting enzyme with activity 13.46 ± 0.03 mU/mL of assay volume with synthetic substrate Hip-His-Leu.
[b] Mass analysed and confirmed by MALDI-Tof and LC-MS.
[c] Molecular mass + sodium ion.
[d] HPLC: C_{18} isocratic water/acetonitrile (70:30) 0.05% TFA.
[e] Non-significant ACE inhibition.

The experimental data of the ACE inhibition studies were compared by two-tailed Student's *t*-test. A *p*-value $\leqslant 0.05$ was considered significant (Table 1).

On the basis of in vitro screening, the tripeptidomimics, which contain Lysyl-Proline dipeptide motif (set A), were categorized into two groups based on their inhibition potency. First group consisting of—MTP-Lys-Pro (**1**), HomoTrp-Lys-Pro (**2**) and DPA-Lys-Pro (**7**) showed 30–40% ACE inhibition (39.07%, $p < 0.0001$; 30.20%, $p < 0.0001$ and 33.82%, $p < 0.0001$, respectively). Second group consisting of least potent tripeptidomimics—HomoPhe-Lys-Pro (**3**), TA-Lys-Pro (**4**), FA-Lys-Pro (**5**), CHA-Lys-Pro (**6**), TIC-Lys-Pro (**8**) and HPPA-Lys-Pro (**9**) showed less than 30% ACE inhibition (22.63%, $p < 0.05$; 13.33%, $p < 0.05$, 10.09%, $p < 0.05$; 3.71%, *p*-NS; 19.43%, $p < 0.0001$ and 29.32%, $p < 0.05$, respectively).

The tripeptidomimics containing Ornithyl-Proline (set B) dipeptide motif were categorized into three groups according to their inhibition potency. First group consisting of—MTP-Orn-Pro (**10**), HTP-Orn-Pro (**11**), TA-Orn-Pro (**14**) and BPA-Orn-Pro (**20**) showed 60–75% ACE inhibition (63.62%, $p < 0.0001$; 68.80%, $p < 0.0001$; 74.68%, $p < 0.0001$; and 65.45%, $p < 0.0001$, respectively). Second group consisting of the lesser potent tripeptidomimics—HomoPhe-Orn-Pro (**13**), FA-Orn-Pro (**15**) and CHA-Orn-Pro (**16**) exhibited 30–40% ACE inhibition (31.72%, $p < 0.0001$; 35.61%, $p < 0.0001$ and 30.78%, $p < 0.0001$, respectively). Third group consisting of least potent tripeptidomimics—HomoTrp-Orn-Pro (**12**), DPA-Orn-Pro (**17**), TIC-Orn-Pro (**18**) and HPPA-Orn-Pro (**19**) showed only less than 30% ACE inhibition (9.13%, $p < 0.05$; 27.67%, $p < 0.05$; 25.01%, $p < 0.0001$ and 23.76%, $p < 0.0001$, respectively).

The results were more encouraging when propionic acid (C_3) derived heterocyclic ring containing compounds, that is, MTP, HTP, TA, FA and BPA, were introduced with Ornithyl-Proline (set B) dipeptide motif as compared to the Lysyl-Proline (set A) dipeptide motif at P_1 position. Further screening of four promising tripeptidomimics **10**, **11**, **14**, and **20** (Fig. 2) had shown an IC_{50} of 10×10^{-6} M, 4×10^{-6} M, 6×10^{-7} M and 10×10^{-6} M (Table 2). Among these four ACE inhibitors, TA-Orn-pro (**14**) found to be the most potent.

Our previous study had shown that interaction of dipeptidomimics is confined to $S_{1'}$ and $S_{2'}$ subsites of the enzyme only, while tripeptidomimics have an additional interaction with S_1 subsite of enzyme and also help to chelate Zn^{2+} ion.[21] In the present study, we, therefore, focused only on tripeptidomimics. Two sets of tripeptidomimics were designed and synthesized in which at $P_{2'}$ position Pro was kept as a common moiety with exchangeable amino acids Orn or Lys at the $P_{1'}$ position.

Our studies found that Orn containing tripeptidomimics MTP-Orn-Pro (**10**), HTP-Orn-Pro (**11**), TA-Orn-pro

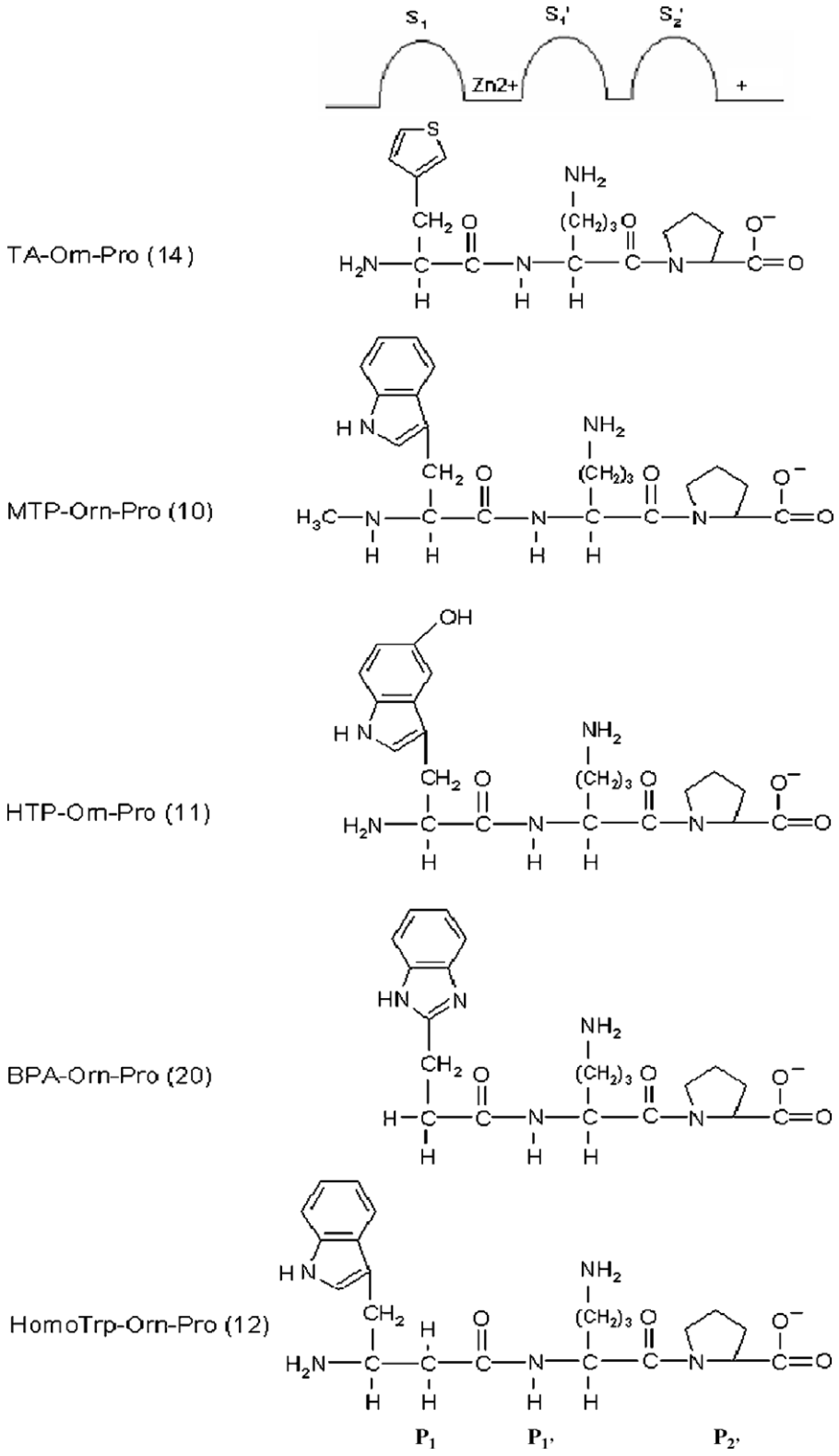

Figure 2. Binding of tripeptidomimics to the hypothetical active site of ACE.

(**14**) and BPA-Orn-Pro (**20**) showed more than 60% inhibition, whereas tripeptidomimics having Lys in place of Orn, that is, MTP-Lys-Pro (**1**), HTP-Lys-Pro,[21] TA-Lys-pro (**4**) and BPA-Lys-Pro[21] showed less than 40% inhibition. At $P_{1'}$ position, it seems that C_4 side chain of Lys with the selective nonpeptide moieties used in this study is causing a hindrance in efficient binding with the active site of ACE. On the other hand, the C_3 side chain of Orn acts as a desirable conformational constraint as compared to the C_4 side chain of Lys.

Table 2. IC_{50} of potent Orn-Pro based tripeptidomimics

Potent tripeptidomimics	ACE activity (mU/mL)	ACE inhibition (%)	*p*-Value	IC_{50} (M)
MTP-Orn-Pro (10)	4.88 ± 0.09	63.62	<0.0001	10×10^{-6}
HTP-Orn-Pro (11)	4.2 ± 0.07	68.80	<0.0001	4×10^{-6}
TA-Orn-pro (14)	3.41 ± 0.12	74.68	<0.0001	6×10^{-7}
BPA-Orn-Pro (20)	4.70 ± 0.10	65.45	<0.0001	10×10^{-6}

Among the various nonpeptide moieties selected, 3-(3-thienyl)-L-alanine (TA) is found to be most suitable for P_1 position with Orn-Pro dipeptide motif. TA-Orn-Pro (**14**) showed better inhibition of ACE activity than other tripeptidomimics. TA contains sulfur atom in its five-membered aromatic ring of side chain, which has high affinity for the divalent Zn^{2+} ion (Fig. 2). In case of captopril, its –SH group is very prone to oxidation,[27] while sulfur-containing aromatic ring of TA is not likely to undergo oxidation or disulfide bond formation reactions in physiological system. The replacement of TA with FA as in tripeptidomimic **15** resulted in a two time decrease in ACE inhibition potency. This decrease in potency may be attributed to the fact that oxygen atom, being more electronegative and smaller in size than sulfur atom, cannot make a strong co-ordinate bond with Zn^{2+}.

ACE inhibition potency of the other three tripeptidomimics **10**, **11** and **20** containing heterocyclic moieties with indole or imidazole ring such as HTP, MTP and BPA was significant. On the other hand, there was substantial loss in the inhibitory potency in case of tripeptidomimic **12** having HomoTrp at P_1 position. The possible explanation for the lack of significant activity might be the presence of the indole ring at the β-carbon atom of the carboxyl group that increases the chain length of the tripeptidomimic molecule by one carbon atom, which results in the shifting of the hydrophobic imidazole ring far from the S_1 subsite (Fig. 2).

In conclusion, the results from the present study show the valuable role of Orn in the tripeptidomimics to effectively inhibit the activity of ACE. Propionic acid (C_3) derived heterocyclic ring containing unnatural amino acids and carboxylic acid derived heterocyclics at P_1 position linked to Ornithyl-Proline dipeptide motif may have better interaction with the S_1 subsite and Zn^{2+} ion than with Lysyl-Proline dipeptide motif.

The structural approach of the ACE inhibitors developed in the present work can be used with further suitable modifications in the heterocyclic moieties and unnatural amino acids with dipeptide Orn-Pro to obtain leads to potentiate ACE activity inhibition in basic as well as clinical research applications.

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