New Renin Inhibitors with Pseudodipeptidic Units in P_1–$P_{1'}$ and $P_{2'}$–$P_{3'}$ Positions

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A series of four new potential renin inhibitors has been synthesized. The structure of the compounds was designed in such a way as to produce agents resistant to enzymatic degradation, metabolically stable, possibly potent and with improved oral absorption. All positions of the 8—13 fragment of the human angiotensinogen were occupied by unnatural units (two unnatural amino acids in positions P_3 and P_2 and two pseudodipeptides in positions P_1–$P_{1'}$ and $P_{2'}$–$P_{3'}$). Both N- and C-terminal functions of the inhibitors were blocked with *tert*-Boc and ethyl ester groups. Their hydrophobicity evaluated as a log P value, calculated by a computer method, was 6.57 and 6.08 respectively. All peptides were obtained by the carbodiimide method in solution and purified by chromatography on the SiO_2 column. Their resistance to enzymatic degradation was assayed by determination of stability against chymotrypsin activity. The potency was measured *in vitro* by a spectrofluorimetric method (assay of Leu-Val-Tyr-Ser released from the *N*-acetyltetradecapeptide substrate by renin in the presence of the inhibitor). All inhibitors were stable to chymotrypsin. Their IC_{50} (M/l) values were: 9.6×10^{-4} (12), 1.6×10^{-5} (17), 1.0×10^{-5} (22) and 1.0×10^{-5} (23) respectively.

Key words renin inhibitor; pseudodipeptide unit; spectrofluorimetric determination

So far a number of potent renin inhibitors have been obtained. Some of them were found to lower blood pressure after intravenous administration. However, only a moderate activity if any was observed when they were given orally. The likely reason was a poor absorption across the biological barriers, rapid metabolism before and after absorption as well as fast excretion with bile. Probably also the molecule size and peptidic character has limited the oral absorption. To improve the oral bioavailability many approaches have been attempted. Hashimoto *et al.*[1] examined intestinal absorption of 18 inhibitors after oral administration and interdependence of absorption upon molecular stucture of the compounds. In the result of extensive research they found some relationship, especially connected with solubility of the inhibitors. Samanen *et al.*[2] looking for possibility to improve the oral bioavailability of the inhibitors hypothesized, that intestinal permeability is increased by decreasing the desolvatation energy required to transfer a peptide from aqueous environment to the lipid one. They give as a example two inhibitors differing one from another by *N*-methyl group on amino function of His. Intestinal permeability of *N*-methyl analog was near twice higher. However, it is not always a rule. The most important courses look to be using nonpeptidic structures. Maibaum *et al.*[3] obtained two series of dipeptide transition state isosters containing a substituted P_3 moiety directly linked to the P_1 side chain and which lack the P_4–P_2 portion. These nonpeptidic inhibitors are highly potent *in vitro*. Some of them show long time of duration and considerable lower blood pressure after oral administration. It is necessary to cite here some nonpeptidic P_2–P_3 butanediamide orally active rennin inhibitors with high bioavailability discovered by Simoneau *et al.*[4] Also Rahuel *et al.*[5] discovered new group of nonpeptidic, orally active inhibitors which are binded to the S_1/S_3 hydrophobic pocket and in addition to subpocket extended from S_3 toward the hydrophobic core of the enzyme. The inhibitors orally administered to conscious unrestrained and Na^+ depleted marmosets produced the pronounced lasting to 24 h and dose dependent reduction of mean arterial pressur. We designed a series of four compounds, looking for an effective inhibitor which ought to be potent, metabolically stable and active after oral administration. Introduction of unnatural Phe(4-OMe) at P_3 position and MeLeu or MePhe at P_2 position was to protect the P_3–P_2 and P_2–P_1 bonds against enzymatic degradation. Substitution of His with MePhe or MeLeu could improve the oral bioavailability, because basic His side chain causes rapid biliary elimination of inhibitor.[6] Change in the P_2 position is possible because the hydrogen atom of the P_3–P_2 peptide bonding is not involved in hydrogen bonding.[7] Another aim of our study was to check the result of simultaneous introduction of aromatic or aliphatic hydrophobic residue in the position P_2 (MePhe or MeLeu) and aromatic or aliphatic, but with hydrophilic OH group, pseudodipeptide unit in the position P_1–P_1, [(*S*,*S*)-AHPPA: (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid or (SS)-Sta: (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid)]. It is well known that introduction of a pseudodipeptidic fragment at the P_1–$P_{1'}$ positions may produce compounds of improved activity, stable against the hydrolytic action of renin. Previously some active inhibitors with $P_3 \ldots P_{2'}$ sequence had been obtained in our laboratory.[8,9] Because it was found, that not only the $P_{2'}$ but also the $P_{3'}$, position was important for a high inhibitory potency,[10] in the present study a series of inhibitors of prolonged chain was designed. Replacement of Leu-Leu at the P_1–$P_{1'}$ and Val-Tyr at the $P_{2'}$–$P_{3'}$ positions with the transition-state mimetics: statine (Sta) or with AHPPA could presumably improve the metabolic stability of such inhibitors. Renin inhibitors with a free amino- and carboxyl function show poor activity because of their polarity and limited penetration across biological membranes. Moreover, positively charged compounds with a free amino group, well soluble in the gastric juice are precipitated in the small intestine at neutral pH. On the other hand, negatively charged compounds show a low intrinsic membrane permeability. Therefore the

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potentially well-absorbed agents should be uncharged. It is known, that substitution at both the N and C terminals is essential for absorption from the small intestine.[1] The *N*-butyloxycarbonyl (Boc) group and *C*-ethyl ester group to block N- and C- termini of the inhibitors were used. The designed inhibitors are supposed to penetrate well across the biological barriers because of their unpolar character and moderate hydrophobicity. Such blocked peptides are poorly soluble. However, it is possible to some extent to improve the solubility of the new inhibitors obtained by increasing hydrophilicity as a result of introduction of the Sta or AHPPA hydroxyl group into the molecule. Hydrophobicity of Boc-[(SS)-Sta]-[(SS)-AHPPA]-OEt and Boc-[(SS)-AHPPA]-[(SS)-Sta]-OEt is low (log P = 3.94 and 3.5). As a result the hydrophobicity of the inhibitors designed is reduced (log P = 6.57 and 6.08).

Chemistry The inhibitors (**12**, **17**, **22**, **23**) as well as their intermediates were synthesized as shown in Charts 1—4. General methods are given in the experimental part. Physicochemical and analytical data of the new synthesized compounds (**9**, **12**, **17**, **20**, **22**, **23**) are presented in Tables 1 and 2. Properties of derivatives (**6**, **7**, **11**, **18**, **19**, **21**) obtained by removal of the substituents (N-*tert*-Boc or ester) blocking functional groups of the parent compounds are not given. Data of the other synthesized compounds are consistent with those described elsewhere: **2**,[11] **3**,[12] **1**, **8**, **13**, **15**,[13] **4**.[14,15]

Biochemical Assays Stability of the inhibitors was determined against chymotrypsin in a solution of ammonium carbonate (pH 6.9) after incubation for 4 h at 37 °C. Analysis of incubates was carried out by HPLC. Activity was assayed *in vitro* by a spectrofluorimetric determination of Leu-Val-Tyr-Ser released from *N*-acetyl-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Tyr-Ser under action of rennin in the presence of the inhibitor tested. Hydrophobicity of the inhibitors was calculated as log P value by a computer method.

Conclusion

Four synthesized renin inhibitors are stable against chymotrypsin as a result of replacing natural amino acids with unnatural units. Inhibitory activity of all four compounds is moderate and very similar. This moderate activity might result from poor solubility during the *in vitro* activity determination. It is necessary to mention, that sometimes there is no simple relation between *in vitro* and *in vivo* activity. Similar activity of the inhibitors irrespective of the structure of the unit in P_1–$P_{1'}$ position (Sta or AHPPA) as well C-terminus (Sta or AHPPA in $P_{2'}$–$P_{3'}$ position) shows that aliphatic or

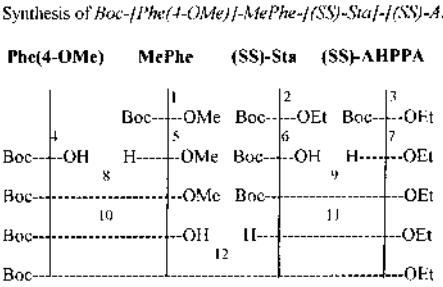

Chart 1

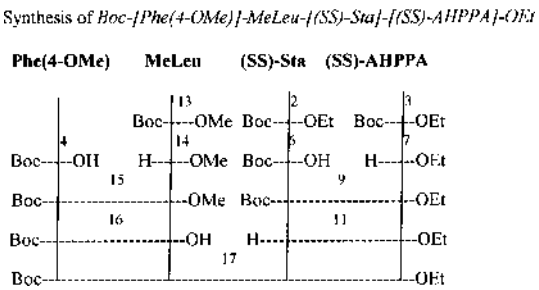

Chart 2

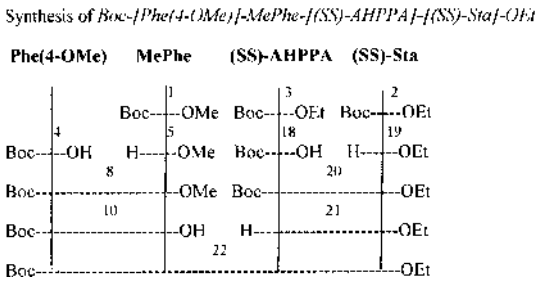

Chart 3

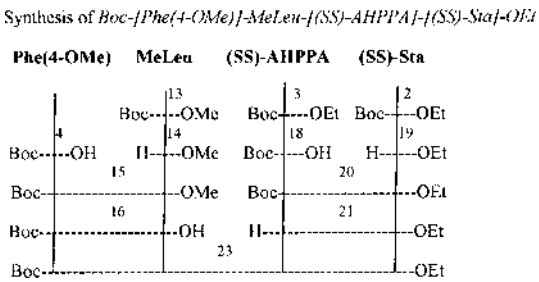

Chart 4

Table 1. Physicochemical and Analytical Data of the Synthesized Compounds

Compd.		Formula	MW	Yield (%)	mp (°C)	$[\alpha]_D^{20}$ (*c*, MeOH)	TLC, *Rf* (solv. syst.)[a]	HPLC (% of purity)
9	Boc-[(SS)-Sta]-[(SS)-AHPPA]-OEt	$C_{26}H_{42}N_2O_7$	494.62	70	Semisolid	−56.5 (1.7)	0.25 (B)	nd
12	Boc-[Phe(4-OMe)]-MePhe-[(SS)-Sta]-[(SS)-AHPPA]-OEt	$C_{46}H_{64}N_4O_{10}$	833.00	34	80—83	−50.4 (1.5)	0.33 (A)	100
17	Boc-[Phe(4-OMe)]-MeLeu-[(SS)-Sta]-[(SS)-AHPPA]-OEt	$C_{43}H_{66}N_4O_{10}$	798.99	25	80—85	−28.2 (1.6)	0.35 (A)	100
20	Boc-[(SS)-AHPPA]-[(SS)-Sta]-OEt	$C_{26}H_{42}N_2O_7$	494.62	62	Semisolid	−55.5 (1.8)	0.23 (B)	nd
22	Boc-[Phe(4-OMe)]-MePhe-[(SS)-AHPPA]-[(SS)-Sta]-OEt	$C_{46}H_{64}N_4O_{10}$	833.00	30	76—78	−44.1 (1.7)	0.30 (A)	98
23	Boc-[Phe(4-OMe)]-MeLeu-[(SS)-AHPPA]-[(SS)-Sta]-OEt	$C_{43}H_{66}N_4O_{10}$	798.99	32	75—79	−18.2 (1.5)	0.38 (A)	100

The elemental analyses were within ± 0.4% of the theoretic value. *a*) A = hexane/EtOAc 50 : 50, B = hexane/EtOAc 60/40.

Table 2. ^{1}H-NMR Spectra of the Synthesized Compounds

Compound	Chemical shifts δ (ppm), $CDCl_3$:
9	0.91 (6H, d, J=6.3 Hz), 1.20—1.30 (6H, m), 1.45 (9H, s), 3.60 (1H, q, J=6.2 Hz), 3.91—4.10 (2H, m), 4.12 (2H, q, J=6.9 Hz), 4.84 (1H, d, J=9.4 Hz), 6.54 (1H, d, J=9.4 Hz), 7.24 (5H, s).
12	0.84—0.96 (6H, m), 1.19—1.40 (15H, m), 2.93, 2.97 (3H total, s, s), 3.81 (3H, s), 4.13 (2H, q, J=7.1 Hz), 6.88, 7.18 (4H total, d, d, J=8.6, 8.6 Hz), 7.26 (10H, s).
17	0.83—0.97 (12H, m), 1.19—1.43 (15H, m, s at 1.39 ppm), 2.75, 2.87 (3H total, s, s), 3.78 (3H, s), 4.15 (2H, q, J=7.1 Hz), 6.81, 7.13 (4H total, d, d, J=8.6, 8.6 Hz), 7.26 (5H, s).
20	0.90 (6H, d, J=6.1 Hz), 1.20—1.60 (15H, m, s at 1.39 ppm), 3.72 (1H, q, J=7.0 Hz), 3.90—4.08 (2H, m), 4.15 (2H, q, J=7.1 Hz), 5.04 (1H, d, J=8.9 Hz), 6.00 (1H, d, J=8.9 Hz), 7.25 (5H, s).
22	0.80—0.94 (6H m), 1.18—1.44 (15H, m), 2.86, 2.91 (3H total, s, s), 3.77 (3H, s), 4.12 (2H, q, J=6.9 Hz), 6.81, 7.12 (4H total, d, d, J=8.2, 8.2 Hz), 7.26 (10H, s).
23	0.81—0.97 (12H, m), 1.20—1.42 (15H, m), 2.77, 2.83 (3H total, s, s), 3.77 (3H, s), 4.12 (2H, q, J=7.0 Hz), 6.81, 7.12 (4H total, d, d, J=8.4, 8.4 Hz), 7.26 (5H, s).

Table 3. Biochemical Properties of the Synthsized Compounds, Some Intermediates and the Reference Compounds

Compound	Stability[a], $t_{1/2}$ min. (chymotrypsin)	IC_{50} (M/l)	log P[b]
[(SS)-Sta]-[(SS)-AHPPA]-OEt	—	—	2.32
[(SS)-AHPPA]-[(SS)-Sta]-OEt	—	—	1.89
12	Stable	9.6×10^{-4}	6.57
17	Stable	1.6×10^{-5}	6.08
22	Stable	1.0×10^{-5}	6.57
23	Stable	1.0×10^{-5}	6.08
Boc-Phe-His-OMe	<10	—	—
Boc-Val-Tyr-OMe	<10	—	—

a) Stable means that no detectable degradation was found at 4 h. *b*) log P=value calculated by computer method.

aromatic character of this fragment has no effect on the potency of the inhibitor. Activity of the truncated structure deprived of $P_{2'}$–$P_{3'}$ fragment was not determined. But comparing activity of the inhibitors substituted and not substituted at this fragment obtained by Hui *et al.*[10] is possible conclude, that pseudodipeptidic unit unsusceptible to enzymatic degradation at C terminus of our compounds could increase their inhibitory activity. In this study inspection *in vitro* of the obtained compounds was designed. Examination in isolated tissues and in animals is planned for the most promising inhibitors obtained to date and just lately synthesized.

Experimental

The reagents were purchased from Aldrich. Phe(4-OMe) was obtained according to the method of Behr.[14] Boc-MePhe and Boc-MeLeu were prepared using the method of Cheung *et al.*[16] Sta and AHPPA were synthesized according to the Maibaum protocol.[11] Renin from porcine kidney, *N*-acetyl renin substrate tetradecapeptide and chymotrypsin type I S from bovine pancreas were obtained from Sigma. Solvents were of analytical purity. THF was distilled from Na/bezophenone under N_2. Dichloromethane and DMF were dried over molecular sieves (4 Å). The peptides were synthesized by the *N*,*N'*-dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCCI/HOBt) method of the amino acid or fragment derivatives condensation in solution.[17] All synthesized compounds were separated and purified by column chromatography (CC) on silica gel (Merck, grade 230 to 400 mesh). TLC was carried out on a 0.25 mm thickness silica gel plates (Merck, Kieselgel 60 F-254). The solvent systems used in TLC and CC were: $CHCl_3$/MeOH or hexane/EtOAc in various ratios. The spots were visualized with 0.3% ninhydrin in EtOH/AcOH 97 : 3. Elemental analyses were performed on a Perkin-Elmer Microanalyser. Melting points were determined in a Böetius apparatus. ^{1}H-NMR spectra were recorded on a Varian, Unity 200 or 500 spectrometer. Chemical shifts were measured as δ units (ppm) relative to tetramethylsilane. Optical rotations were measured at the Na-D line with a Polamat (Carl Zeiss, Jena) polarimeter in a 5 cm polarimeter cell. HPLC was performed on a Techma-Robot Type 302 apparatus equipped with a UV detector LCD 2040 (Laboratorni Pristroje, Praha) and a computer registrator/recorder CHROMA (POLLAB Warsaw). The peaks were recorded at 210 nm. Spectrofluorimetric determination of Leu-Val-Tyr-Ser released from the substrate tetradecapeptide was performed on a Shimadzu apparatus with Fluram solution. The fluorescence was detected at 395—495 nm.

Introduction of the N-*tert*-Boc-Group This group was introduced in a commonly used manner.[15]

Esterification Reaction Boc-amino acids were esterified with CH_3I as described earlier.[18] Boc-Sta-OEt and Boc-AHPPA-OEt were formed from mono-ethyl malonate used to prepare these pseudodipeptide units.[11]

Coupling Reaction with DCCI/HOBt The amino acid or peptide ester hydrochloride (1 mmol) was dissolved in dichloroethane (5 ml) and neutralized at 0 °C with triethylamine (TEA). Boc-amino acid or Boc-peptide (1 mmol) and HOBt (1.5 mmol) were added followed by a solution of DCCI (1 mmol) in dichloromertane (5 ml). The reaction mixture was stirred at 0 °C for 4 h and left at room temperature overnight. Dicyclohexylurea (DCU) was filtered off and the filtrate was evaporated *in vacuo*. The residue was dissolved in EtOAc, left for 2 h and filtered again. The filtrate was washed successively with 5% HCl solution, 5% $NaHCO_3$ solution, saturated NaCl solution, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The raw compound was purified by silica gel CC to yield the final product.

Removal of the Boc-Group Boc-amino acid or peptide (1 mmol) in solution of 4 M HCl in dioxane (3—5 ml) was stirred at room temperature for 30 min. The solution was concentrated *in vacuo*, the residue was re-evaporated twice with ethyl ether and then dried *in vacuo*.[19]

Alkaline Hydrolysis of Amino Acid or Peptide Ester Hydrolysis was carried out as described earlier.[18]

Inhibition of Renin Activity Inhibitory renin activity was determined according to the method of Galen *et al.*[20] The course of determination is described in detail in a previous paper.[18] The activity is designed in terms of IC_{50}, *i.e.* the molecular concentration of the tested inhibitor causing 50% inhibition of the control renin activity.

Stability Determination Chymotrypsin (1 mg) was dissolved in 0.5 M NH_4HCO_3 solution (2.5 ml) adjusted with AcOH to pH 6.9. The inhibitor (5 μm) was dissolved in MeOH (0.3—0.5 ml) and diluted with 0.5 M NH_4HCO_3 solution to a volume of 2.5 ml. Both solutions were mixed and incubated at 37 °C for 4 h. Aliquots were withdrawn after 2 and 4 h. The reaction in each sample was stopped by addition of CH_3CN (5 ml). Each solution was evaporated *in vacuo*, the residue was dissolved in 0.5 M pH 6.9 NH_4HCO_3 solution (2.5 ml). Analyses of incubates were carried out by HPLC. Peak areas of inhibitors in incubates were compared with peak areas of standard solutions containing 5 μm of inhibitor dissolved in MeOH (0.3—0.5 ml) and diluted with 0.5 M pH 6.9 NH_4HCO_3 solution to a volume of 2.5 ml. The values were determined in 3 separate assays. The differences between the peak areas in limits ±5% were recognized as a determination error. Stability of Boc-Phe-His-OMe and Boc-Val-Tyr-OMe was determined in similar way. The differences were as follows: all reagents were used in tenfold quantities, incubation was performed for 1 h, aliquots were withdrawn at intervals of 5 min., standard solutions contained the reference compounds dissolved in MeOH (1 ml) and diluted as above. Results of the experiments are given in Table 3.

Log P Value Calculation Log P values were calculated by a computer

method because of poor solubility of the compounds in water and in *n*-octanol. Structures of the compounds were built within the HyperChem 4.5 programme. The semiempirical method PM 3 was used for single point calculation. Geometry optimization was performed by the Polak-Ribier method. Log P as a measure of hydrophobicity of optimized structure was calculated using the QSAR Properties programme, Chem Plus, extension for HyperChem (Hypercube, Inc.). Calculation of log P in this programme was carried out using atomic parameters derived by Ghose *et al*.[21)]

Acknowledgement This investigation was supported in part by the Warsaw Medical University (Grant FW-22/W/99-02).

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