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ANALYSIS

NEW RENIN INHIBITORS – STABILITY AND ACTIVITY DETERMINATION. PART I

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Abstract: A series of new six potential renin inhibitors containing pseudodipeptides were synthesized. Stability for all compounds (**1-6**) in homogenates of liver, kidney, lung and in serum, gastric, intestinal juice and in the presence of α-chymotrypsin was determined. Compound **5** was unstable, compound **6** was stable, other compounds were partly unstable, compound **2** was stable except kidney homogenate and compound **4** was stable except liver homogenate. Inhibitory activity of the compounds was measured *in vitro* by HPLC determination of lowering concentration of substrate (angiotensinogen) in the presence of renin and the potential renin inhibitor (compounds **1-6**). Compound **2, 4** and **6** showed inhibitory activity (1.4×10^{-6} , 5.2×10^{-6}, 1.5×10^{-7} M, respectively). Other compounds (**1, 3, 5**) showed no inhibitory activity up to 10^{-5} M.

Keywords: HPLC, activity of potential renin inhibitors, renin inhibitors

Renin, an aspartic proteinase, catalyzes a specific hydrolysis of the angiotensinogen to give the decapeptide angiotensin I. Angiotensin converting enzyme (ACE), converts it to the octapeptide angiotensin II, which is a very strong vasoconstrictor and it also stimulates aldosterone release and sodium retention. Renin is a specific enzyme that displays specificity for its only one known natural substrate – angiotensinogen. Therefore, the inhibition of renin, which action initiates the renin-angiotensin cascade, has been a highly attractive biological target for new antihypertensive drugs. Drugs that inhibit the renin-angiotensin system, like ACE inhibitors and angiotensin II receptor blockers, are very effective in hypertension treatment but these drugs are characterized by many side effects (they stimulate compensatory mechanism, which results in an increase of angiotensin II level). Therefore, the idea to treat hypertension through the renin inhibition has led to development of many potent renin inhibitors based on the peptide sequence of natural substrate – angiotensinogen.

Many trials to developed effective direct renin inhibitors were not successful (synthesized compounds, which were peptide substrate analogues, were not stable, they revealed low potency or poor pharmacological profiles). To avoid such problems, new substrates analogous to non-peptic amino acids, peptide-like inhibitors and fully nonpeptic inhibitors were developed (1, 2). Aliskiren is the first renin inhibitor registered at the FDA (3). The structure of aliskiren differed in 8–13 amino acids fragment from the structure of natural substrate – angiotensinogen. It shows high effectivnes and good pharmacokinetic profile.

Searching for new renin inhibitors, a series of dipeptide analogues of angiotensinogen have been prepared and they were all derived from renin substrate by replacing the scissile amide bond with a transition-state mimic structure and by incorporating bioisosteric replacements for the Val-10 amide bond. These derivatives showed high inhibiting activity (10^{-6} – 10^{-9} M) (4). Other transition-state renin inhibitors containing the dipeptide transition state mimic structure: (2*S*,4*S*,5*S*)-5-amino-4-hydroxy-2-isopropyl-7-methyloctanoic acid (Leu $\underline{\text{OH}}$ Val) and (2*S*,4*S*,5*S*)-5-amino-4-hydroxy-2-isopropyl-6-cyclohexylhexanoic acid (Cha $\underline{\text{OH}}$ Val) were synthesized (5, 6). The goal of such investigation was to lower the molecular weight, to minimize the number of peptide amide bonds and to enhance i*n vivo* stability. All derivatives showed high activi-

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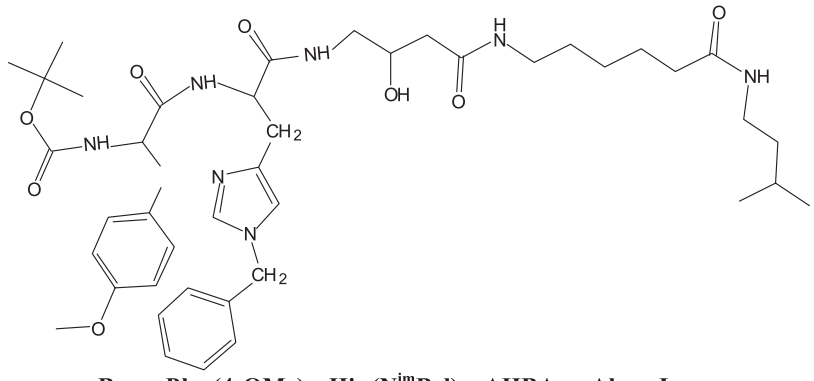
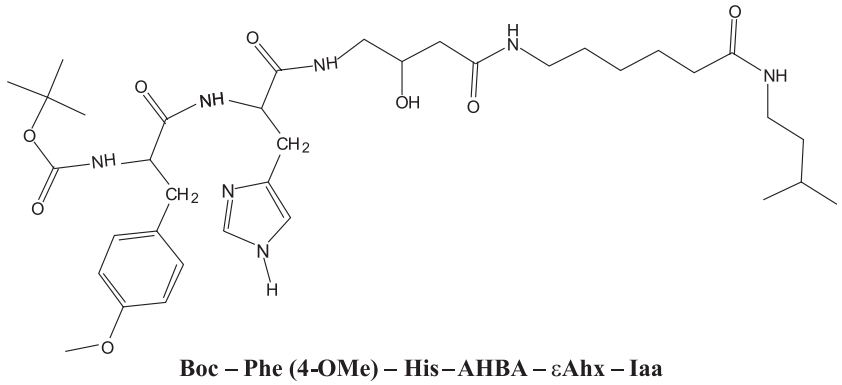


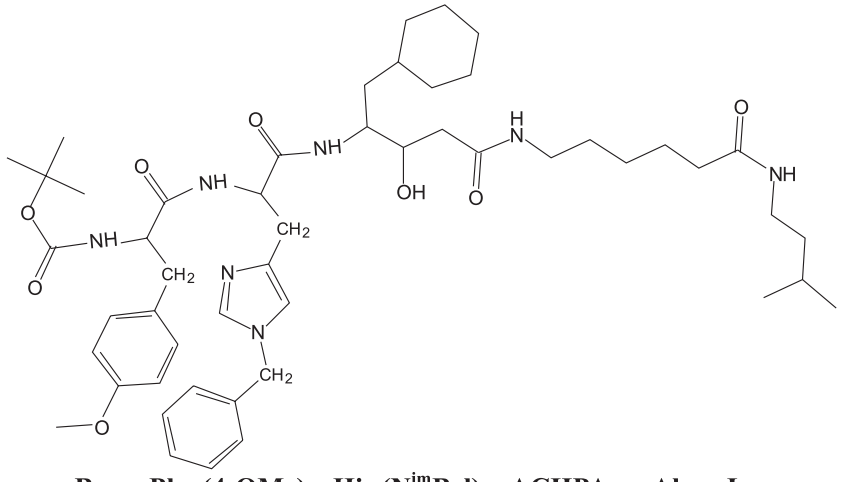
Boc – Phe (4-OMe) – His (N^{im}Bzl) – AHBA – εAhx – Iaa

Compound **1** – [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N^{im}benzylhistidil-(3S,4S)-4-amino-3-hydroxybutanoyl-ε-aminohexanoic acid isoamylamide



Boc – Phe (4-OMe) – His – AHBA – εAhx – Iaa

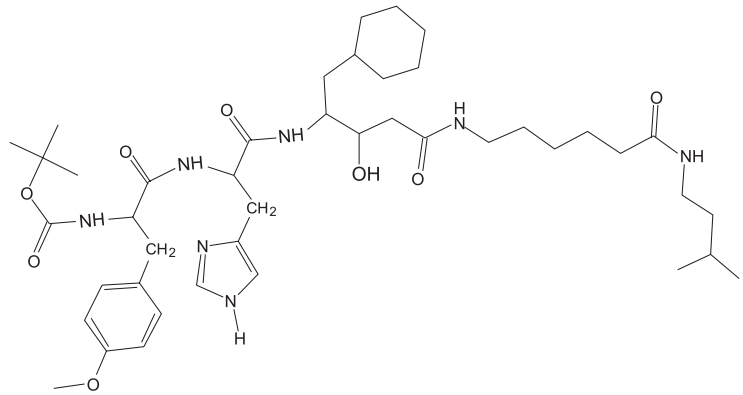
Compound **2** – [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-(3S,4S)-4-amino-3-hydroxybutanoyl-ε-aminohexanoic acid isoamylamide



Boc – Phe (4-OMe) – His (N^{im}Bzl) – ACHPA – εAhx – Iaa

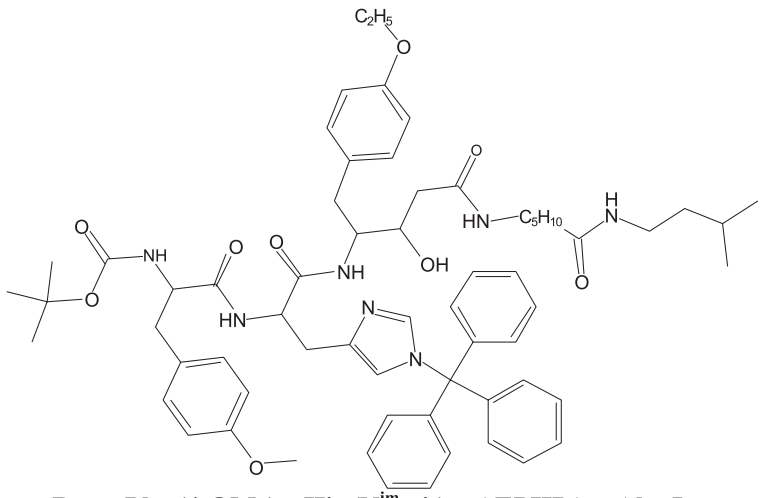
Compound **3** – [N-(*t*-butoxycarbonyl)-4-methoxyphenylalanyl]-N^{im}benzylhistidil-(3S,4S)-4-amino-5-cyclohexyl-3-hydroxypentanoyl-ε-aminohexanoic acid isoamylamide

Figure 1. Chemical structures of 6 new renin inhibitors



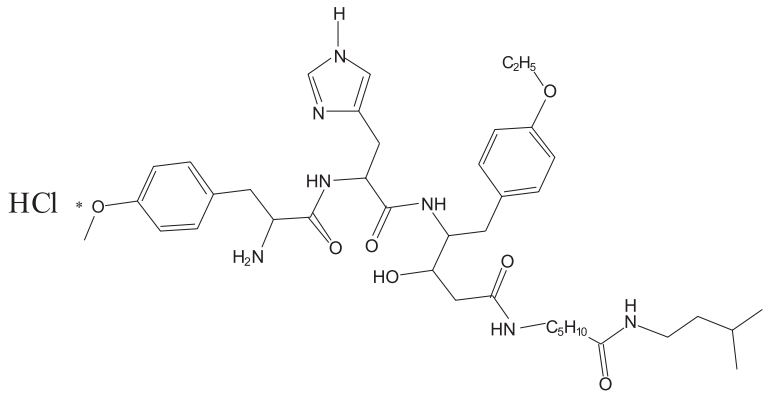
Boc – Phe (4-OMe) – His – ACHPA – εAhx – Iaa

Compound **4** – [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-(3S, 4S)-4-amino-5-cyclohexyl-3-hydroxypentanoyl-ε-aminohexanoic acid isoamylamide



Boc – Phe (4-OMe) – His (N^{im}trit) – AEPHPA- εAhx-Iaa

Compound **5** – [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N^{im}tritylhistidyl-(3S, 4S)-4-amino-5-(4-ethoxyphenyl)-3-hydroxypentanoyl-ε-aminohexanoic acid isoamylamide



Phe (4-OMe) – His – AEPHPA- Ahx-Iaa · HCl

Compound **6** – 4-methoxyphenylalanyl-histidyl-(3S, 4S)-4-amino-5-(4-ethoxyphenyl)-3-hydroxypentanoyl-ε-aminohexanoic acid isoamylamide hydrochloride

Figure 1. cont

ty (10^{-9} M). Other report presented peptide backbone modifications that lead to greater resistance of the resulting peptides towards enzymatic degradation and some were potent inhibitors of human renin (e.g., Boc-Pro-Phe-N^{α}-MeHis-Leuψ-($CHOHCH_2$)-Val-Ile-Amp showed inhibiting activity – 2.6×10^{-10} M (7, 8). The synthesis of renin inhibitors (angiotensinogen analogues) having dehydrostatine, Leuψ[CH_2S]Val, or Leuψ[CH_2SO]Val at P_1-P_1' cleavage site was described (9). Activity of those compounds was between 10^{-6} to 10^{-8} M. Bock et al. obtained a series of statine containing tetrapeptides, modified at the C-terminal with various hydrophobic aromatic groups (10). The inhibiting activity of obtained compounds were between 10^{-6} to 10^{-8} M, e.g., for compound Boc-Phe-His-Sta-Leu-p-chlorobenzylamide it was 8.1×10^{-8} M. Other pepstatin analogues of general formula A-X-Y-Sta-Ala-Sta-R were synthesized (11). Various changes of A, X and Y groups were undertaken to improve the inhibitory activity against human plasma renin. The *tert*-butyloxycarbonyl group and the isovaleryl group were the most effective acyl groups (A). The replacement of Val with Phe residue (X) and incorporation of His or other amino acid with an aliphatic side chain in the position Y lead to an increase of inhibition activity against human plasma renin (10^{-8} M). Changes on C-terminal statine, like estrification or amidification, had no influence on inhibitory potency. Jones et al. (12) used solid-phase method to synthesize a series of peptides (analogues of angiotensinogen), in which statine or new analogues

Table 1. Characterization of new synthesized renin inhibitors.

No.	Compound	Formula	M_r
1	Boc-Phe(4-OMe)-His(BZL)-AHBA-Ahx-Iaa	$C_{43}H_{63}O_8N_7$	806.04
2	Boc-Phe(4-OMe)-His-AHBA-Ahx-Iaa	$C_{36}H_{57}O_8N_7$	715.92
3	Boc-Phe(4-OMe)-His(BZL)-ACHPA-Ahx-Iaa	$C_{50}H_{75}O_8N_7$	902.20
4	Boc-Phe(4-OMe)-His-ACHPA-Ahx-Iaa	$C_{43}H_{69}O_8N_7$	812.07
5	Boc-Phe(4-OMe)-His(trit)-AEPHPA-Ahx-Iaa	$C_{64}H_{81}N_7O_9$	1092.40
6	Phe-(4-OMe)-His-AEPHPA-Ahx-Iaa × HCl	$C_{40}H_{59}N_7O_7Cl$	784.94

Boc – *tert*-butoxycarbonyl; Iaa – isoamylamide; Ahx – 6-aminohexanoic acid; AHBA – 4-amino-3-hydroxybutanoic acid; ACHPA – 4-amino-5-cyclohexyl-3-hydroxypentanoic acid; Phe(4-OMe) – 4-methoxyphenylalanine; AEPHPA – 4-amino-5-(4-ethoxyphenyl)-3-hydroxypentanoic acid.

Table 2. Chromatographic and validation parameters.

Compd. no.	Column	Mobile phase (v/v/v)	r	Recovery (%)	CV (%)	Extraction
1	Beckman Ultrasphere Octyl (150 × 4.6 mm)	ACN–H_2O–H_3PO_4 (42 : 58 : 0.1)	0.9976	103.26 ± 8.25	4.93 ± 2.34	alkaline 71%
2	Discovery Wide Pore C_8 (150 × 4.6 mm)	ACN–H_2O–trichloroacetic acid (10 : 90 : 0.1)	0.9988	96.08 ± 10.59	5.13 ± 1.05	acidic 53%
3	Beckman Ultrasphere Octyl (150 × 4.6 mm)	MeOH–H_2O–H_3PO_4 (45 : 55 : 0.1)	0.9993	98.99 ± 3.31	5.66 ± 1.28	acidic 63%
4	Symmetry C_{18} (150 × 4.6 mm)	ACN–H_2O–H_3PO_4 (30 : 70 : 0.1)	0.9972	98.18 ± 6.00	4.48 ± 3.38	acidic 70%
5	Beckman Ultrasphere Octyl (150 × 4.6 mm)	ACN–H_2O–H_3PO_4 (70 : 35 : 0.1)	0.9996	103.21 ± 7.01	10.48 ± 2.85	acidic 81%
6	Symmetry C_{18} (150 ×4.6 mm)	ACN–acetate buffer pH 4.0 (40 : 60)	0.9990	100.80 ± 4.92	7.40 ± 4.28	alkaline 20%

(3*S*,4*S*)-3,4-diamino- or (3*S*,4*S*)-3,4-diamino-6-methylheptanoic acid and (3*S*,4*S*)-4-amino-3-aminomethyl- or (3*S*,4*S*)-4-amino-3-aminomethyl-6-methylheptanoic acid replaced either residue 10 or both residues 10, 11 at the P_1-P_1' cleavage site. Peptide Boc-His-Pro-Phe-His-Sta-Val-Ile-His-NH_2 showed highest inhibiting activity (10^{-9} M) against human renin among all new synthesized compounds. Chen et al. described a novel series of spirocyclic renin inhibitors (13). These inhibitors were potent but the bioavailability was poor in animal model.

Our intention was to search for new active inhibitors with simple structure, high bioavailability and easy synthesis. These compounds should be resistant to enzymatic degradation, metabolically stable and with improved oral absorption. Several compounds with unnatural amino acids in position P_3 and P_2 and pseudopeptides in position P_1-P_1 and P_2-P_3 were synthesized (14). All compounds were stable against chymotrypsin, inhibitory activity was moderate but solubility was poor. Other obtained inhibitors with εAhx ethylamide or εAhx-isoamylamide at the P_2'- P_3' positions showed inhibitory activity at the concentration of 10^{-5} mL^{-1} (14, 15). The next renin inhibitors obtained in our department contained nonpeptic units: (3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoic acid (ACHPA), (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA), (3*S*,4*S*)-4-amino-3-hydoxy-6-methylheptanoic acid (Sta) and unnatural dipeptide Phe(4-OMe)-MeLeu (16). All compounds were stable against chymotrypsin and the inhibitory activity was measured *in vitro* and it was < 10^{-3}, 1.0×10^{-6}, 4.0×10^{-4} and 1.0×10^{-6} M, respectively.

To continue the search for the effective renin inhibitors, 6 new compounds of potential inhibiting activity were synthesized. Their chemical structures were presented in Table 1 and Figure 1.

The purpose of the study was to determine the stability of the newly synthesized compounds in homogenate of body organs and body fluids (*in vitro*) and to check the inhibiting activity of all synthesized potential renin inhibitors (*in vitro*).

EXPERIMENTAL

Materials and reagents

New renin inhibitors synthesized in the Department of Drug Chemistry, Medical University.of Warsaw are presented in Table 1 and Figure 1.

Renin human and α-chymotrypsin from bovine pancreas were purchased from Sigma. Angiotensinogen was purchased from Bachem.

Apparatus and methods

A Shimadzu HPLC apparatus that consisted of an LC-10AT pump and SPD-10A spectrophotometer was used with Chroma computer recorder (POL-LAB, Poland) and the Chromax 2001 software (POL-LAB, Poland).

The HPLC method for determination of compounds was developed. The separation was carried out in the reverse phase system, the flow rate was 1 mL/min. The concentration range was between

Table 3. Evaluation of stability of compounds **1–6**

Compd. no.	α-Chymotrypsin	Serum	Gastric juice	Intestinal juice	Kidney homogenate	Lung homogenate	Liver homogenate
1	first-order reaction $t_{0.5}$ = 5 h	stable	first-order reaction $t_{0.5}$ = 78 min	stable	stable	stable	stable
2	stable	stable	stable	stable	unstable	stable	stable
3	stable	first-order reaction $t_{0.5}$ = 198 min	stable	stable	first-order reaction $t_{0.5}$ = 73 min	first-order reaction $t_{0.5}$ = 140 min	first-order reaction $t_{0.5}$ = 5 min
4	stable	stable	stable	stable	stable	stable	first-order reaction $t_{0.5}$ = 57 min
5	unstable	unstable	unstable	unstable	first-order reaction $t_{0.5}$ = 73 min	first-order reaction $t_{0.5}$ = 114 min	unstable
6	stable	stable	stable	stable	stable	stable	stable

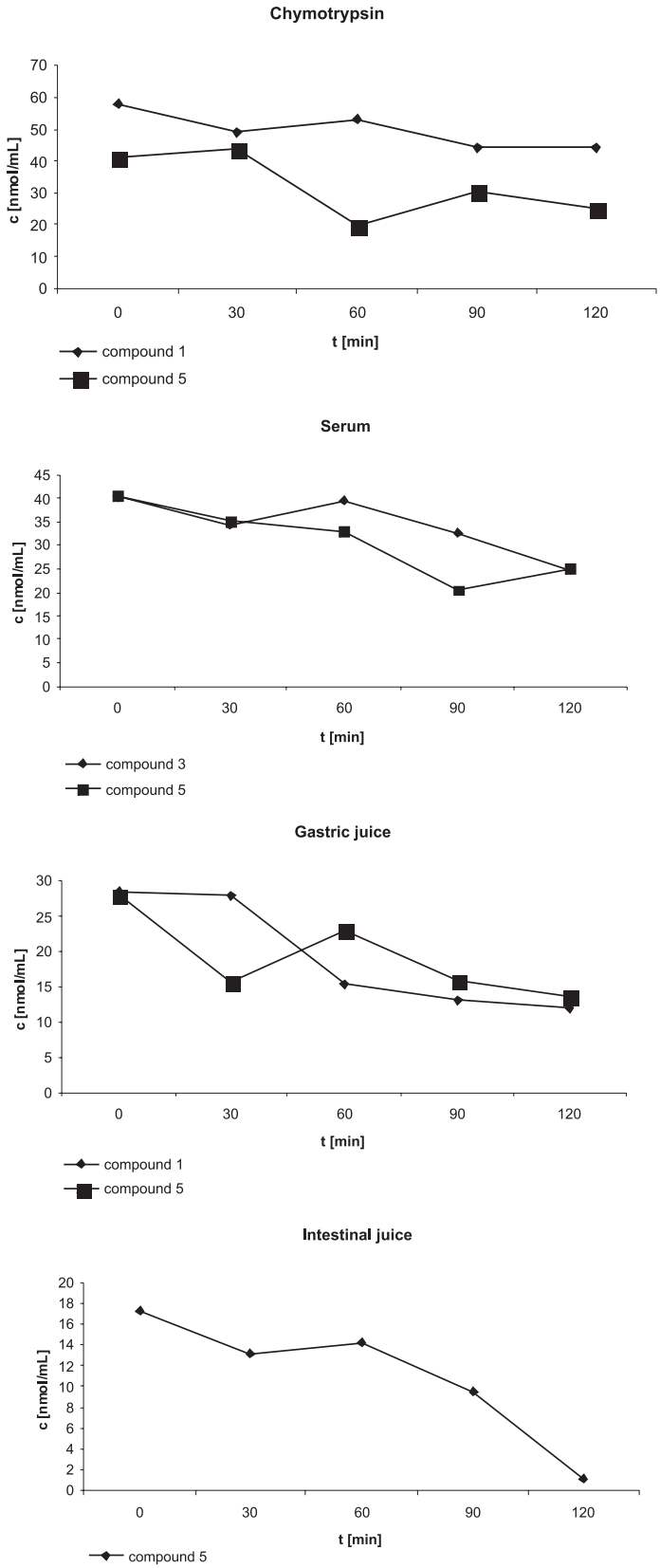


Figure 2. Stability of compounds **1**–**6** in body fluids and organs (the plots of concentration *vs*. time)

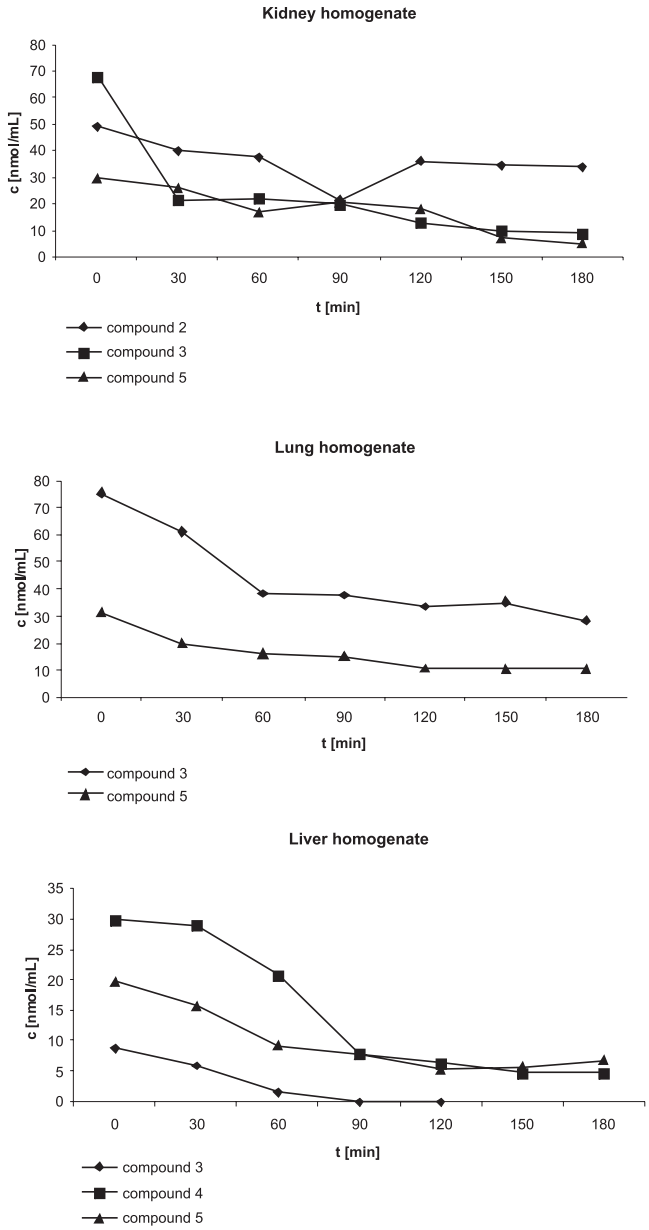


Figure 2. cont.

Table 4. Inhibiting activity of compounds **1-6**.

Compd. no.	Human renin IC_{50} (M)
1	inactive > 10^{-5}
2	1.4×10^{-6}
3	inactive > 10^{-5}
4	5.2×10^{-6}
5	inactive > 10^{-5}
6	1.5×10^{-7}

10–50 nmol/mL. The wavelength was 213 nm. The columns, mobile phases and validation parameters have been presented in Table 2.

The method of compounds **1–6** determination was validated. Recovery, accuracy and linearity of the analytical procedure are presented in Table 2.

Stock solutions of determined compounds were prepared by dissolving each compound in methanol. The final working concentration for the examined substances was 100 nmol/mL. The liquid-

liquid extraction method was used for all biological material.

Determination of enzymatic stability of compounds 1–6 *in vitro*

The stability of all compounds in body fluids and organ homogenates was examined. The concentrations of compounds were measured at different time points during incubation in biological material. The developed HPLC method was used to determine the concentration of compounds **1–6** isolated from biological material by liquid-liquid extraction with the use of diethyl ether.

Stability determination of compounds 1–6 in liver, kidney and lung enzymes

The homogenates of body organs (40%) in 0.1 mole/L TRIS solution (pH = 8.4) were prepared. Each homogenate was spiked with each of 6 compounds and incubated at 37°C. The samples were collected at the time points 0, 30, 60, 90, 120, 150, 180 min and then isolated from homogenates with diethyl ether, evaporated to dryness, dissolved in mobile phase and determined by HPLC. Results are presented in Table 3 and Figure 2.

Stability determination of compounds 1–6 in serum, gastric and intestinal juice

Each compound was dissolved in certain amount of serum or freshly prepared gastric or intestinal juice (according to USP). The solutions were incubated at 37°C and the samples were collected at the time points 0, 30, 60, 90, 120 min. The samples were isolated from body fluids with diethyl ether, evaporated to dryness, dissolved in mobile phase and determined by HPLC. Results are presented in Table 3 and Figure 2.

Stability determination of compounds 1–6 in the presence of α-chymotrypsin

α-Chymotrypsin was dissolved in phosphatate buffor solution pH 7.8. The solution was incubated at 37°C and the samples were collected at the time points 0, 30, 60, 120 min. Then, the samples were isolated from the solution with diethyl ether, evaporated to dryness, dissolved in mobile phase and determined by HPLC. Results are presented in Table 3 and Figure 2.

Determination of inhibition activity of compounds 1–6 *vs.* human renin

Renin inhibiting activity of the synthesized potential inhibitors was determined *in vitro*. The modified HPLC method of determination of angiotensinogen (substrate) concentration was used (column: Wild Pore C_8 15 cm × 4.6 mm, mobile phase MeOH : H_2O : H_3PO_4 40 : 60 : 0.1 (v/v/v), wavelength 213 nm). The HPLC method was modified in comparison with other method (17), used to determine the renin inhibitor activity. The modification includes change of chromatographic conditions and change of the method of inhibiting activity determination. Instead of assay of concentration of tetrapeptide (which is the product of reaction), the concentration of angiotensinogen (substrate) was measured. To check if modified method was reliable in activity determination, we compared results of activity measurements obtained by the method described in this paper with the results obtained with our modified method for renin inhibitor (Boc-Phe-His-Sta-εAhx-OMe) (18). The results of inhibiting activity for method described in the paper and modified method were $IC_{50} = 5.0 \times 10^{-9}$ M and $IC_{50} = 5.3 \times 10^{-9}$ M, respectively (18). These results confirmed that the modified method was reliable.

The inhibition of human renin was determined after its incubation with angiotensinogen and with each of compounds **1**–**6**. Human renin 9 mU.G. was incubated with 6 mM angiotensinogen in 30 mM citrate-phosphate buffer (pH 7.4) for 2 h at 37°C with lovering amount (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M) of each renin inhibitor prepared in phosphoric-citric buffer pH 7.4. To stop the reaction after 2 h, the sample was immersed in boiling water for 2 min. To control the activity assay, the sample contained only human renin and angiotensinogen was prepared and the activity was measured in time 0 and after 2 h of incubation.

The renin inhibitory activity was designed in terms of the IC_{50}, which is the molar concentration of the examined inhibitor causing 50% inhibition of the control renin activity.

Results are presented in Table 4.

RESULTS AND DISCUSSION

Determination of enzymatic stability of 6 new compounds have been performed in test *in vitro*. The results showed that compound **6** was stable, compound **5** was totally unstable and all other compounds were partly stable in body fluids and organ homogenates. Compound **2** was unstable in kidney homogenate, compound **4** in liver homogenate, compound **1** in gastric juice and in the presence of α-chymotrypsin and compound **3** in serum, kidney, lung and liver homogenates.

Determination of inhibiting activity of compounds **1–6** against human renin had been performed *in vitro*.

The results showed that the removal of substituents of His was necessary to obtain biologically active compound and made compound more stable. Simultaneous removal of Boc group caused that compound was stable in body fluids and organ homogenates and showed the highest inhibitory activity (1.5×10^{-7} M). Compound with AHBA group showed activity 10^{-6} and was rather stable (decomposed only in kidney homogenate). Compound **4** had four times lower activity than compound **2** and was rather stable (decomposed only in liver homogenate), the half-time was about 60 min. The presence of trityl group caused compound unstable in all homogenates and body fluids. The removal of Boc group increased metabolic stability and increased solubility in water.

The search for other compounds with higher potency is under way.

Acknowledgments

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