DRUG SYNTHESIS

SYNTHESIS AND DOCKING STUDIES OF RENIN INHIBITORS CONTAINING ESTER AND AMIDE DERIVATIVES OF (3S, 4S)-4-AMINO-HYDROXY ACIDS WITH S₃-S₃' RENIN BINDING SITE

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Abstract: Seven novel potential renin inhibitors, having a structure based on the peptide fragment 8-13 of human angiotensinogen - a natural substrate for renin, have been designed and synthesized. All peptides were obtained by the carbodiimide method in solution and purified by chromatography on the SiO₂ column. In P₁-P₃' positions, they contain esters or N-alkyl amides of modified (3S, 4S)-4-amino-3-hydroxyacids. The achieved inhibitory activity IC₅₀ was of 10⁻⁶-10⁻⁷M. Inhibitory activity of the compounds was measured *in vitro* by high-performance liquid chromatography (HPLC) determination of lowering the concentration of substrate in the presence of renin and the potential renin inhibitor. Their resistance to enzymatic degradation was assayed by the determination of stability against chymotrypsin activity. Their hydrophobicity evaluated as a Log P-value was calculated by a computer method. The theoretical binding affinity of thus obtained inhibitors was elaborated based on docking to the S₃-S₃' pocket of the renin active site.

Keywords: amino acids, hypertension treatment, renin inhibitors, *in vitro* inhibitory activity determination, docking, pseudodipeptidic unit.

GENERAL

An active form of renin is formed when the N-terminal 43-amino acid fragment is removed from the zymogen. Renin is responsible for proteolytic cleavage of angiotensinogen to an inactive decapeptide, angiotensin I, and stimulates a (Pro)Renin Receptor (PRR) (1, 2). Angiotensin I is transformed by convertase enzyme into an active octapeptide angiotensin II (Figure 1). Angiotensin II directly causes blood pressure increase.

Stimulation of PRR by renin leads to proliferation and fibrosis of cardiac and vascular cells (3, 4). Pharmacological inhibition of renin bioactivity indirectly prevents angiotensin II from forming and reduces the effects of PRR stimulation by renin. Excessive biosynthesis of angiotensin II and enhanced PRR expression contributes to cardiovascular strain. This mechanism is the basis for the pathogenesis of numerous cardiovascular conditions, such as

arterial hypertension, cardiac failure, and ischaemic heart disease. Renin inhibitors, such as Aliskiren (5-7) and neprilysin inhibitors like Sacubitril or Entresto (8-12), have the most promising therapeutic efficacy amongst the newest family of hypotensives acting by inhibition of enzymatic activity. The low bioavailability of renin inhibitors is the main obstacle

Figure 1. Two-way mechanism of renin action; PRR – (Pro) Renin Receptor; 1, 2, 3 – main path of angiotensin II creation; 4 – (Pro) Renin activation; 5 – PRR stimulation by renin.

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in obtaining efficient treatment usability. Improved bioavailability can be achieved by enhancing the stability of sites susceptible to proteolysis and by establishing balanced hydrophobic-hydrophilic properties of the inhibitory molecule. On the other hand, it has to be assured that such modification will not decrease the affinity of the inhibitor to the respective enzyme. The molecule design of presently developed synthetic renin inhibitors is based on the structure of the substrate fragment. The applicable modification of this fragment serves to obtain non-peptide renin inhibitors.

Alterations of the core sites of inhibitory molecules can be carried out twofold. Firstly, by embedding a hydrophobic particle, containing lateral aliphatic, cycloaliphatic, or phenyl substituents, into the molecule. Secondly, by implementing a fragment containing more polar heterocyclic systems, such as piperidine (13-15) or piperazine (16, 17) ring.

The hydrophobic part of the renin inhibitor docks at the hydrophobic $S_3(S_3^{SP})$ - S_1 renin pocket (18). This is the key binding mechanism, but hydrophobic interactions of the inhibitor with the S_4 and S_2' - S_3' sites of the enzyme are also important.

The discovery of sub-pockets in S_3 and S_1 sites, which can interact with hydrophilic and polar groups, such as -OH, -NH₂, -OMe, and -OEt groups was of high significance. It enabled new ways of designing renin inhibitors, whose particles can undergo substantial structural changes in P_3 and P_1 positions.

Facilitated formation of additional hydrogen bonds may lead to increased enzyme-inhibitor interactions, and therefore to enhanced efficacy of potential renin inhibitors. Consequently, designing potential renin inhibitors based on the P_3 - P_1' fragment of human angiotensinogen, with the necessary modifications, should lead to improved stability and bioavailability of the molecule and enhanced binding affinity to the renin active site (19).

Previous work

The current study is a follow-up of our former research activities. For example, the previously synthesized renin inhibitor Boc-Phe(4-OMe)-MeLeu-AHPPA-Ahx-Iaa was taken as a starting point for the design of the inhibitors **12** and **25** presented here. This compound featured high *in vitro* inhibitory activity $IC_{50} = 1.05 \times 10^{-9}$ M, which is comparable to the inhibitory

activity of Aliskiren ($IC_{50} = 0.6 \times 10^{-9}$ M). It was also characterized by a significant hydrophobicity of log P 7.71 and stability in the chymotrypsin environment (19).

Design of the new inhibitors

The compound **12**, designed in the scope of the current project, differs from the previously (19) synthesized inhibitor Boc-Phe(4-OMe)-MeLeu-AHPPA-Ahx-Iaa by a pseudopeptide embedded in the P_1 - P_1' position, where AHPPA has been replaced by AEPHPA. Another type of modification to the basic molecule, implemented here, was substituting hydrophobic C-terminal with a more extensively branched one. The compound **25** was created by replacing C-terminal Ahx-Iaa with MeLeu-Iaa in this same basic molecule. The compound **20** was obtained by implementing this same C-terminal replacement, but applied to the Boc-Phe(4-OMe)-MeLeu-ACPHA-Ahx-Iaa inhibitor ($IC_{50} = 1 \times 10^{-6}$) synthesized earlier (20).

The structure of the new renin inhibitors, described in the present work, was based on the P_3 - P_1' fragment 8-11 of angiotensinogen (Figure 2) because this part of the substrate molecule plays a significant role in binding to the renin active center. Additionally, the C-terminal fragment corresponding to P_2' - P_3' position was extended and the effect of this modification on the inhibitory activity of the obtained compounds was investigated. The substituents

Figure 2. Comparison of general molecule structure of the derived renin inhibitors to the angiotensinogen 8-13 fragment (positions P_3 - P_3').

Table 1. Substituents used for the Synthesized Renin Inhibitors

No.	R ₁	R ₂	R ₃
7
12
20
25
31
36
39

R₁ to R₃, shown in Figure 2, are for all obtained compounds listed in Table 1.

The P₃-P₂ and P₁-P₁' sites, susceptible to proteolysis (Figure 3), were stabilized by substituting Phe-His and Leu-Val peptide fragments with unnaturally modified dipeptides and pseudodipeptides. In particular, Phe(4-OMe) in combination with various N-methyl amino acids was introduced into the P₃-P₂ fragment of the angiotensinogen molecule and non-peptide 4-amino-3-hydroxy acid derivatives were inserted in P₁-P₁' position (21-23).

Moreover, we made several changes to N- and C-terminal fragments of inhibitor molecules, considering their influence on the level of interactions with respective S₄ and S₂'-S₃' hydrophobic pockets of renin (22-24).

Seven new renin inhibitors were synthesized (Table 2). In four of them, C-terminal was extended with ethyl esters of the following amino

hydroxyacids: 4-amino-5-(4-ethoxyphenyl)-3-hydroxypentanoic acid (AEPHPA) (compound 7), 4-amino-5-phenyl-3-hydroxypentanoic acid (AHPPA) (compound 31), 4-amino-3-hydroxy-6-methylheptanoic acid (Sta) (compound 36), 4-amino-3-hydroxynonanoic acid (AHNA) (compound 39). In the other three compounds, N-isoamyl amino acid amides of the following acids were introduced in the P₂'-P₃' position: 6-aminohexanoic acid (Ahx-Iaa) associated with AEPHPA (compound 12) and

Figure 3. Decomposition scheme of the 8-11 (P₃-P₁') angiotensinogen fragment by proteolytic enzymes.

2-(N-methylamino)-4-methylpentanoic acid (MeLeu) associated with 4-amino-5-cyclohexyl-3-hydroxypentanoic acid (ACHPA) (compound **20**) and AHPPA (compound **25**).

4-Methoxyphenylalanine (Phe(4-OMe)) is present in P₃ position of all the synthesized inhibitors, with its amine group substituted with *tert*-butoxycarbonyl group (t-Boc) corresponding to P₄ position. In P₂ position, compounds **7**, **12**, **20**, **25**, and **39** have 2-(N-methylamino)-4-methylpentanoic acid (MeLeu), while compounds **31** and **36** have 2-(N-methylamino)-4-methylhexanoic acid (MeIle). The studies were aimed at evaluating the bindability of the synthesized potential renin inhibitors, with particular respect to hydrophobic and hydrogen bonds of the compound's molecule to hydrophobic S₃^{sp}-P₁ pocket (18, 19). The focus was put on the effect of particular internal interactions between hydrophobic pharmacophores of the inhibitor, resulting from different molecule conformations, which in turn can lead to weakening or enhancing the inhibition

of enzymatic renin activity. Replacing the AHPPA amino acid at the position P₁-P₁' with AEPHPA or ACHPA shall allow for assessing bindability and affinity differences of the inhibitor fragment to the hydrophobic pocket S₁. Conformational properties of the more flexible cyclohexyl ring of ACHPA at the P₁ position in comparison to the planar and rigid phenyl ring of AHPPA can provide for a better fit to the hydrophobic pocket of the enzyme active center. The presence of a polar ethoxy substituent in position 4 of the phenyl ring of AEPHPA will probably enable the creation of an additional bound inside the sub-pocket S₁. The structural modifications introduced at the P₁ position were aiming at the possibly strong interaction between enzyme and the enzyme and inhibitor.

Hydrophobic groups in P₁, P₂, P₃, and P₄ positions (N-terminal), and P₂'-P₃' (C-terminal) seem to play a vital role in intramolecular interactions (25-27). It was assumed that possible, intramolecular interactions between P₁ side chains and C-terminal

Table 2. Structures of the Synthesized Renin Inhibitors

Compound No.	Compound	Compound Structure
7	Boc-Phe(4-OMe)-MeLeu-AEPHPA-OEt	
12	Boc-Phe(4-OMe)-MeLeu-AEPHPA-Ahx-Iaa	
20	Boc-Phe(4-OMe)-MeLeu-ACHPA-MeLeu-Iaa	

Table 2. Structures of the Synthesized Renin Inhibitors (cont.)

Compound No.	Compound	Compound Structure
25	Boc-Phe(4-OMe)-MeLeu-AHPPA-MeLeu-Iaa	
31	Boc-Phe(4-OMe)-MeIle-AHPPA-OEt	
36	Boc-Phe(4-OMe)-MeIle-Sta-OEt	
39	Boc-Phe(4-OMe)-MeLeu-AHNA-OEt	

tail $P_2'-P_3'$ can be particularly interesting. Therefore, the obtained molecules are strongly differentiated in structure, especially in the P_1 position and C-terminal tail of the molecule. Comparison between inhibitory concentrations, IC_{50} , and results of studies on docking of the obtained inhibitors to the renin active site can provide answers to the following questions:

- Is an extension of the inhibitor molecule by substituting the $P_2'-P_3'$ position with a longer, branched, hydrophobic aliphatic group (N-alkyl amide), as done in compounds **12**, **20**, **25**, advantageous when compared to shorter molecules of compounds **7**, **31**, **36**, **39**, which are terminated with a polar ester group in P_1 position?

- Is bindability of the renin active site influenced by hydrophobic interaction between vicinal molecular fragments, due to the elasticity of chains and ability to obtain various conformations, and if so, to what extent?

For all the obtained compounds, it is probable that intramolecular interactions can take place between substituents in P_1 or P_2 positions. Another issue is the effect of possible interactions between branched C-terminal (N-alkylamide fragment), containing isopentyl and isobutyl groups in $P_2'-P_3'$ positions, and various substituents in P_1 position on the inhibitory activity of the compound.

MATERIALS AND METHODS

Chemistry

The structures of inhibitors are presented in Table 2. The inhibitors **7**, **12**, **20**, **25**, **31**, **36**, **39**, as well as their intermediates, were synthesized by fragment condensation, according to schemes presented in Figure 4 to Figure 10.

The applied methods are specified below in the syntheses section. Physicochemical properties of the

Figure 4. Synthesis of Boc-Phe(4-OMe)-MeLeu-AEPHPA-OEt [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N-methylleucyl-(3S,4S)-4-amino-5-(4-ethoxyphenyl)-3-hydroxypentanoic-acid ethyl ester (compound **7**).

Figure 6. Synthesis of Boc-Phe(4-OMe)-MeLeu-ACHPA-MeLeu-laa [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N-methylleucyl-(3S,4S)-4-amino-5-cyclohexyl-3-hydroxypentanoyl-N-methylleucineisoamyloamide (compound **20**).

Figure 8. Synthesis of Boc-Phe(4-OMe)-MeIle-AHPPA-OEt [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N-methylisoleucyl-(3S,4S)-4-amino-5-phenyl-3-hydroxypentanoic-acid ethyl ester (compound **31**).

Figure 10. Synthesis of Boc-Phe(4-OMe)-MeLeu-AHNA-OEt [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N-methylleucyl-(3S,4S)-4-amino-3-hydroxynonanoic-acid ethyl ester (compound **39**).

inhibitors, as well as newly synthesized intermediates **3**, **4**, **6**, **10**, **18**, **19**, **23**, **24**, **28**, **29**, **30**, **33**, **34**, **35**, and **38**, are presented in Table 3 and Table 4. The numbers of final compounds in these tables and in the text below are written in bold.

Reagents Boc-Phe(4-OMe)-OH, Boc-N-Me-Leu-OH, Boc-N-Me-Ilu-OH, porcine kidney renin, and N-acetyl renin substrate tetradecapeptide were acquired from recognized vendors, listed in the materials section below. The AEPHPA, AHPPA, ACHPA,

Figure 5. Synthesis of Boc-Phe(4-OMe)-MeLeu-AEPHPA-Ahx-laa [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N-methylleucyl-(3S,4S)-4-amino-5-(4-ethoxyphenyl)-3-hydroxypentanoyl-amino hexanoic acid isoamylamide (compound **12**).

Figure 7. Synthesis of Boc-Phe(4-OMe)-MeLeu-AHPPA-MeLeu-laa [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N-methylleucyl-(3S,4S)-4-amino-5-phenyl-3-hydroxypentanoyl-N-methylleucineisoamyloamide (compound **25**).

Figure 9. Synthesis of Boc-Phe(4-OMe)-MeIle-Sta-OEt [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N-methylisoleucyl-(3S,4S)-4-amino-3-hydroxyheptanoic-acid ethyl ester (compound **36**).

Table 3. Physicochemical and analytical properties of the synthesized compounds.

No.	Compound	Formula m.w.	Yield (%)	M.p. (°C)	(c, MeOH)	TLC, R _f (m.ph.) [*]	HPLC (% of purity)
3	Boc-Phe(4-OMe)-MeLeu-OMe	C ₂₃ H ₃₆ N ₂ O ₆ 436.56	53.64	Semi-solid	-42.2 (1.03)	0.33 (B)	
4	Boc-Phe(4-OMe)-MeLeu-OH	C ₂₂ H ₃₄ N ₂ O ₅ 422.53	14.39	Semi-solid	-35.00 (1.08)	0.11 (B)	
5	Boc-AEPHPA-OEt	C ₂₀ H ₃₁ NO ₆ 381.48	37.50	83-85	-22.00 (1.0)	0.18 (A)	
6	HCl×H-AEPHPA-OEt	C ₁₅ H ₂₄ NO ₄ Cl 317.80	89.80	Semi-solid	-	0.44 (F)	
7	Boc-Phe(4-OMe)-MeLeu-AEPHPA-OEt	C ₃₇ H ₅₅ N ₃ O ₉ 685.85	53.3	Semi-solid	-18.00 (0.6)	0.86 (B)	99.18
10	Boc-Phe(4-OMe)-MeLeu-AEPHPA-OH	C ₃₅ H ₅₁ N ₃ O ₉ 661.83	81.40	Semi-solid	-178.00 (0.8)	0.27 (E)	
12	Boc-Phe(4-OMe)-MeLeu-AEPHPA-Ahx-Iaa	C ₄₆ H ₇₃ N ₅ O ₉ 840.10	81.50	Semi-solid	-79.01 (1.7)	0.39 (C)	99.67
13	Boc-ACHPA-OEt	C ₁₈ H ₃₃ NO ₅ 343.44	13.27	Semi-solid	-34.0 (1.2)	0.42 (A)	
17	Boc-ACHPA-OH	C ₁₆ H ₂₉ NO ₅ 317.44	65.02	Semi-solid	-28 (1.1)	0.62 (B)	
18	Boc-ACHPA-MeLeu-Iaa	C ₂₈ H ₅₃ N ₃ O ₅ 511.76	27.91	Semi-solid	-45 (1.04)	0.67 (B)	
19	HCl×H-ACHPA-MeLeu-Iaa	C ₂₃ H ₄₅ N ₃ O ₃ Cl 447.09	83.33	Semi-solid	-	0.38 (F)	
20	Boc-Phe(4-OMe)-MeLeu-ACHPA-MeLeu-Iaa	C ₄₅ H ₇₇ N ₅ O ₈ 816.15	93.75	Semi-solid	-52 (0.98)	0.28 (B)	97.45
21	Boc-AHPPA-OEt	C ₁₈ H ₂₆ NO ₅ 336.41	35.30	84-86	-30.10 (1.0)	0.34 (A)	
22	Boc-AHPPA-OH	C ₁₆ H ₂₃ NO ₅ 309.37	62.35	Semi-solid	-24.10 (1.0)	0.60 (C)	
23	Boc-AHPPA-MeLeu-Iaa	C ₂₈ H ₄₇ N ₃ O ₅ 505.70	76.24	Semi-solid	-58.00 (1.2)	0.89 (A)	
24	HCl×H-AHPPA-MeLeu-Iaa	C ₂₃ H ₄₀ N ₃ O ₂ Cl 442.10	96.67	Semi-solid	-	0.42 (F)	
25	Boc-Phe(4-OMe)-MeLeu-AHPPA-MeLeu-Iaa	C ₄₅ H ₇₁ N ₅ O ₇ 798.08	34.8	Semi-solid	+69.17 (1.0)	0.34 (D)	96.98
28	HCl×H-AHPPA-OEt	C ₁₃ H ₂₀ NO ₃ Cl 272.72	93.0	Semi-solid	-	0.20 (F)	
29	Boc-Melle-AHPPA-OEt	C ₂₅ H ₄₀ N ₂ O ₆ 464.59	49	Semi-solid	-16.11 (1.2)	0.97 (A)	
30	HCl×H-Melle-AHPPA-OEt	C ₂₀ H ₃₃ N ₂ O ₄ Cl 400.94	77.8	Semi-solid	-	0.21 (F)	
31	Boc-Phe(4-OMe)-Melle-AHPPA-OEt	C ₃₅ H ₅₁ N ₃ O ₈ 641.79	39.5	Semi-solid	-26.42 (1.1)	0.93 (E)	99.87
32	Boc-Sta-OEt	C ₁₅ H ₂₉ NO ₅ 303.41	20.8	Semi-solid	-39.68 (1.06)	0.33 (A)	
33	HCl×H-Sta-OEt	C ₁₀ H ₂₂ NO ₃ Cl 239.74	86.00	Semi-solid	-	0.12 (B)	
34	Boc-Melle-Sta-OEt	C ₂₂ H ₄₂ N ₂ O ₆ 430.58	65	Semi-solid	-17.8 (1.1)	0.95 (A)	
35	HCl×H-Melle-Sta-OEt	C ₁₇ H ₃₅ N ₂ O ₄ Cl 366.92	90.6	Semi-solid	-	0.31 (F)	

Table 3. Physicochemical and analytical properties of the synthesized compounds (cont.).

No.	Compound	Formula m.w.	Yield (%)	M.p. (°C)	(c, MeOH)	TLC, R _f (m.ph.) ^a	HPLC (% of purity)
36	Boc-Phe(4-OMe)-Melle-Sta-OEt	C ₃₂ H ₅₃ N ₃ O ₈ 607.78	45	Semi-solid	-111.10 (1.1)	0.95 (A)	98.96
37	Boc-AHNA-OEt	C ₁₆ H ₃₂ NO ₅ 317.44	25.34	52.4-53.6	+4.5 (1.2)	0.60 (D)	
38	HCl×H-AHNA-OEt	C ₁₁ H ₂₅ NO ₃ Cl 253.77	90.58	Semi-solid	-	0.47 (F)	
39	Boc-Phe(4-OMe)-MeLeu-AHNA-OEt	C ₃₃ H ₅₅ N ₃ O ₆ 621.83	57.03	Semi-solid	-140.62 (1.1)	0.65 (A)	96.97

Mobile phase systems (v/v), were: hexane-EtOAc 80 : 20 (A), CH₃Cl-MeOH 90 : 10 (B), BAW (C), CH₃Cl-MeOH 99 : 1 (D), CH₃Cl-MeOH 95 : 5 (E), BPW (F).

Table 4. ¹H NMR spectra of the synthesized compounds

Compd. No	Solvent	Chemical shifts δ, ppm
3	CDCl ₃	0.76-1.84 (m, 9H, 2 × CH ₃ MeLeu, 2HC _β MeLeu, HC _γ MeLeu); 1.429 (s, 9H, C ₄ H ₉); 2.80-3.24 (9m, 3H, N-CH ₃); 3.24-3.48 (m, 3H, OCH ₃ ester); 3.78 (s, 3H, OCH ₃); 4.51 (s, 1H, HC _α MeLeu); 4.99 (m, 1H, HC _α Phe(4-OMe)); 6.82, 7.11 (dd, 4H, C ₆ H ₄); 7.26 (s, 1H, NH).
4	CDCl ₃	0.80-1.84 (m, 8H, 2 × CH ₃ MeLeu, 2HC _β MeLeu); 1.40 (s, 9H, C ₄ H ₉); 2.30-2.40 (m, HC _γ MeLeu); 2.82 (s, 1H, OH); 2.90-3.65 (m, 5H, N-CH ₃ , HC _β Phe(4-OMe)); 3.79 (s, 3H, OCH ₃); 4.55 (d, 1H, HC _α MeLeu); 4.93 (d, 1H, HC _α Phe(4-OMe)); 6.68, 7.10 (dd, 4H, C ₆ H ₄); 7.26 (s, 1H, NH).
5	CDCl ₃	1.25 (t, 3H, CH ₃ ester); 1.39 (t, 3H, CH ₃ ester); 1.41 (s, 9H, C ₄ H ₉); 2.33-2.62 (m, 2H, 2HC _β); 2.84 (d, 2H, 2HC _β); 3.43 (s, 1H, OH); 3.66 (q, 1H, HC _γ); 3.95-4.04 (m, 3H, OCH ₂ ester, 1HC ₃); 4.13 (q, 2H, OCH ₂ ester); 4.91 (d, 1H, NH); 6.82 (d, 2H, C ₆ H ₄); 7.15 (d, 2H, C ₆ H ₄).
7	CDCl ₃	0.80-0.94 (m, 6H, 2 × CH ₃ MeLeu); 1.12-1.30 (m, 3H, CH ₃ ester); 1.34-1.50 (m, 3H, CH ₃ ester); 1.38 (s, 9H, C ₄ H ₉); 1.50-1.93 (m, 3H, 2HC _β MeLeu, HC _γ MeLeu); 2.32-3.07 (m, 6H, 2HC ₂ , 2HC ₅ , 2HC _β Phe(4-OMe)); 2.35 (s, 1H, OH); 2.59 (s, 3H, N-CH ₃); 3.77 (d, 3H, OCH ₃); 3.90-4.20 (m, 6H, OCH ₂ ester, OCH ₂ eter, HC ₃ , HC ₄); 4.60-4.78 (m, 1H, HC _α MeLeu); 5.07-5.24 (m, 1H, HC _α Phe(4-OMe)); 6.08 (d, 2H, NHAEPHPA, NHPhe(4-OMe)); 6.73-6.90 (m, 4H, C ₆ H ₄); 7.01-7.17 (m, 4H, C ₆ H ₄).
10	CDCl ₃	0.80-0.94 (m, 6H, 2 × CH ₃ MeLeu); 1.24-1.47 (m, 3H, CH ₃ eter); 1.37 (s, 9H, C ₄ H ₉); 1.50-1.69 (m, 3H, 2HC _β MeLeu, HC _γ MeLeu); 2.30-3.06 (6H, 2HC ₂ , 2HC ₅ , 2HC _β Phe(4-OMe)); 2.37 (s, 1H, OH); 2.73 (s, 3H, N-CH ₃); 3.73 (s, 3H, OCH ₃); 3.78 (q, 2H, OCH ₂ eter); 4.08-4.25 (m, 2H, HC ₃ , HC ₄); 4.70 (q, 1H, HC _α MeLeu); 5.14 (t, 1H, HC _α Phe(4-OMe)); 6.70, 6.81 (dd, 4H, C ₆ H ₄); 7.06, 7.12 (dd, 4H, C ₆ H ₄).
12	CDCl ₃	0.80-0.96 (m, 12H, 2 × CH ₃ MeLeu, 2 × CH ₃ Iaa); 1.20-1.74 (m, 15H, 3 × CH ₂ Ahx, 2HC _β Iaa, HC _γ Iaa, CH ₃ eter, 2HC _β MeLeu, HC _γ MeLeu); 1.38 (s, 9H, C ₄ H ₉); 2.33 (s, 1H, OH); 2.55 (s, 3H, N-CH ₃); 2.62-2.98 (m, 8H, 2HC ₂ , 2HC ₅ , 2HC _β Phe(4-OMe), 2HC _α Ahx); 3.27 (s, 4H, 2HC _α Ahx, 2HC _α Iaa); 3.76 (s, 3H, OCH ₃); 3.90-4.10 (m, 4H, OCH ₂ eter, HC ₃ , HC ₄); 4.70 (q, 1H, HC _α MeLeu); 5.05-5.27 (m, 1H, HC _α Phe(4-OMe)); 5.86 (s, 1H, NH ₂ Iaa); 6.14 (d, 2H, NHAEPHPA, NHPhe(4-OMe)); 6.44 (s, 1H, NHAhx); 6.75, 6.88 (dd, 4H, C ₆ H ₄); 7.02, 7.13 (dd, 4H, C ₆ H ₄).
13	CDCl ₃	0.78-1.88 (m, 13H, C ₆ H ₁₁ , 2HC ₅); 1.27 (t, <i>J</i> = 9 Hz, 3H, CH ₃); 1.44 (s, 9H, C ₄ H ₉); 2.40-2.62 (m, 2H, 2HC ₂); 3.64 (q, <i>J</i> = 7 Hz, 1H, HC ₄); 4.00 (d, <i>J</i> = 8 Hz, 1H, HC ₃); 4.17 (q, <i>J</i> = 8 Hz, 2H, OCH ₂ ester); 4.96 (d, <i>J</i> = 12 Hz, 1H, NH).
17	CDCl ₃	0.78-1.88 (m, 13H, C ₆ H ₁₁ , 2HC ₅); 1.44 (s, 9H, C ₄ H ₉); 2.40-2.66 (m, 2H, 2HC ₂); 3.65 (q, <i>J</i> = 7 Hz, 1H, HC ₄); 4.00 (d, <i>J</i> = 8 Hz, 1H, HC ₃); 4.82 (d, <i>J</i> = 12 Hz, 1H, NH).
18	CDCl ₃	0.70-1.00 (m, 6H, 2 × CH ₃ MeLeu); 1.00-2.00 (m, 18H, C ₆ H ₁₁ , 2HC ₅ , 2HC _β Iaa, HC _γ Iaa); 1.26 (s, 9H, C ₄ H ₉); 1.93 (q, 2H, 2HC _β Iaa, HC _γ MeLeu); 2.20-2.60 (m, 2H, 2HC ₂); 3.17-3.80 (m, 4H, N-CH ₃ , HC ₄); 3.98 (d, 1H, OH); 4.12-4.24 (m, 1H, HC ₃); 4.96 (sbr, 1H, NH); 5.35 (t, 1H, 2HC _α MeLeu); 6.24 (s, 1H, NH).
20	CDCl ₃	0.78-1.01 (m, 12H, 4 × CH ₃ MeLeu); 1.06-1.50 (m, 13H, 2HC ₅ , C ₆ H ₁₁); 1.42 (s, 9H, C ₄ H ₉); 1.50-1.76 (m, 6H, 2 × 2HC _β MeLeu, 2 × HC _γ MeLeu); 1.93 (q, 2H, 2HC _β Iaa); 2.70 (s, 2H, 2HC ₂); 2.81 (s, 2H, 2HC _β Phe(4-OMe)); 2.84-3.36 (m, 6H, 2 × N-CH ₃); 3.40-3.62 (m, 2H, HC ₃); 3.68 (s, 1H, OH); 3.71 (s, 1H, NH); 3.79 (s, 3H, OCH ₃); 4.74-4.86 (m, 1H, NH); 5.03 (q, 1H, HC _α Phe(4-OMe)); 5.18-5.40 (m, 2H, 2HC _α MeLeu); 5.52 (t, 1H, NH); 6.78-7.17 (m, 4H, C ₆ H ₄ , 1H, NH).

Table 4. ¹H NMR spectra of the synthesized compounds (cont.).

Compd. No	Solvent	Chemical shifts δ , ppm
21	CDCl ₃	1.28 (t, 3H, CH ₃ ester); 1.37 (s, 9H, C ₄ H ₉); 2.42-2.71 (m, 2H, 2HC ₅); 3.55-4.30 (m, 2H, HC ₃ , HC ₄); 3.90 (d, J = 6Hz, 2H, 2HC ₂); 4.15 (q, J = 9Hz, 2H, OCH ₂ ester); 4.60 (d, J = 8Hz, 1H, NH); 7.15-7.31 (m, 5H, C ₆ H ₅)
22	CDCl ₃	1.29 (t, J = 7.0 Hz, 3H, CH ₃ ester); 1.56-1.64 (m, 5H, 2 $\times$ CH ₂ , 1 $\times$ CH); 2.48-2.57 (m, 2H, CH ₂); 3.15-3.25 (m, 2H, CH ₂); 3.57 (sbr, 1H, CH); 3.98 (sbr, 1H, OH); 4.26 (q, J = 12 Hz, 2H, OCH ₂); 4.83 (d, J = 9 Hz, 1H, NH); 5.01 (sbr, 1H, NH); 5.19 (s, 2H, CH ₂); 7.51 (s, 5H, C ₆ H ₅).
23	CDCl ₃	1.02-1.19 (m, 6H, 2 $\times$ CH ₃ MeLeu); 1.22 (t, J = 4.0 Hz, 3H, CH ₃ ester); 1.28-1.49 (m, 1H, CH ₂); 1.55 (s, 9H, C ₄ H ₉); 1.58-1.84 (m, 7H, 2HC ₆ MeLeu, HC ₅ MeLeu, 2HC ₅ , 2HC ₆); 1.93 (dbr, J = 6 Hz, 2H, 2HC ₆ MeLeu); 2.75 (t, J = 4.0 Hz, 2H, CH ₂); 3.22 (d, J = 4 Hz, 3H, N-CH ₃); 3.48 (s br, 1H, CH ₂); 3.83 (s, 1H, OH); 4.15 (q, J = 8 Hz, 2H, OCH ₂ ester); 4.96 (s br, 1H, NH); 5.09 (s, 2H, CH ₂); 7.26 (s, 1H, NH); 7.34 (s, 5H, C ₆ H ₅).
25	CDCl ₃	1.16-1.38 (m, 8H, 2HC ₆ Iaa, HC ₅ Iaa, 2 $\times$ CH ₂ Ahx, HC ₃); 1.42 (s, 9H, C ₄ H ₉); 1.52-1.86 (m, 6H, 2 $\times$ CH ₂ , 2HC ₆ Iaa); 1.86-2.00 (m, 4H, 2 $\times$ CH ₂ Ahx); 3.11 (d, J = 9.0 Hz, 2H, 2HC ₆ Phe(4-OMe)); 3.15 (d, J = 4.0 Hz, 3H, N-CH ₃); 3.40 (s, 1H, CH ₂); 3.78 (s, 3H, OCH ₃); 4.53 (s br, 1H, HC ₅ MeLeu); 4.96 (d, J = 9.0 Hz, 1H, NH); 5.09 (s, 2H, CH ₂ Bzl); 6.83-7.10 (dd, J = 6.5 Hz, J = 6.5 Hz, 4H, C ₆ H ₄); 7.26 (sbr, 1H, NH); 7.34 (s, 5H, C ₆ H ₅).
29	CDCl ₃	0.81-0.90 (m, 6H, 2 $\times$ CH ₃ Ile); 1.20-1.29 (m, 5H, CH ₃ ester, CH ₃ Ile); 1.42-1.53 (m, 9H, C ₄ H ₉); 2.38-2.58 (m, 2H, 2HC ₅); 2.62 (s, 3H, NCH ₃); 2.82-3.02 (m, 2H, 2HC ₅); 4.00-4.20 (m, 2H, OCH ₂ ester); 6.1-6.18 (sbr, 1H, NH); 6.58-6.70 (d, J = 9Hz, 1H, NH); 7.15-7.38 (m, 5H, C ₆ H ₅).
31	CDCl ₃	0.78-1.00 (m, 6H, 2 $\times$ CH ₃ Ile); 1.20-1.58 (m, 16H, C ₄ H ₉ , CH ₃ ester, CH ₃ Ile, 2HC ₆ Ile); 1.80-2.00 (m, 2H, 2HC ₅); 2.60 (s, 3H, NCH ₃); 2.78-3.12 (m, 2H, 2HC ₆ Phe); 3.65-3.80 (m, 3H, OCH ₃); 4.00-4.20 (m, 2H, OCH ₂ ester); 5.00 (d, J = 9.3Hz, 1H, NH); 6.78-7.22 (m, 9H, C ₆ H ₅ , C ₆ H ₄).
32	CDCl ₃	0.95 (q, 6H, 2 $\times$ CH ₃ Sta); 1.24-1.28 (sbr, 3H, CH ₃ ester); 1.46 (s, 9H, C ₄ H ₉); 1.52-1.74 (m, 3H, HC ₆ , 2HC ₅); 2.48-2.70 (m, 2H, 2HC ₂); 3.67 (s, 1H, HC ₃); 4.013 (s, 1H, HC ₃); 4.20 (q, 2H, OCH ₂ ester); 5.00-5.20 (m, 1H, NH).
34	CDCl ₃	0.82-0.99 (m, 12H, 2 $\times$ CH ₃ Sta, 2 $\times$ CH ₃ Ile); 1.20-1.30 (m, 3H, CH ₃ ester); 1.42-1.52 (sbr, 9H, C ₄ H ₉); 1.92-2.16 (m, 3H, HC ₆ , 2HC ₅); 2.70-2.90 (d, J = 9Hz, 2HC ₂); 3.60-3.80 (m, 3H, N-CH ₃); 3.00-4.25 (m, 4H, HC ₃ , HC ₄ , OCH ₂ ester); 5.00-5.20 (m, 1H, NH); 6.10-6.30 (m, 1H, NH).
36	CDCl ₃	0.85-1.00 (m, 12H, 2 $\times$ CH ₃ Sta, 2 $\times$ CH ₃ Ile); 1.19-1.25 (m, 3H, CH ₃ ester); 1.41 (s, 9H, C ₄ H ₉); 1.59 (s, 3H, HC ₆ , 2HC ₅); 2.74-3.10 (m, 2H, CH ₂ Phe); 3.69-3.80 (m, 8H, OCH ₃ , N-CH ₃ , OCH ₂ ester); 4.50-4.60 (m, 1H, HC ₂ Ile); 6.80-6.86 (d, J = 7Hz, 2H, C ₆ H ₄); 7.00-7.03 (d, J = 7.7Hz, 2H, C ₆ H ₄).
37	CDCl ₃	0.84-0.92 (m, 3H, CH ₃); 1.20-1.36 (m, 7H, CH ₃ ester, 2 $\times$ CH ₂); 1.443 (s, 9H, C ₄ H ₉); 2.40-2.60 (m, 2H, CH ₂); 3.40-3.55 (m, 1H, NH); 4.0-4.1 (d, J = 2Hz, 1H, CH); 4.18 (q, 2H, OCH ₂); 4.7 (d, J = 2Hz, 1H, NH).
39	CDCl ₃	0.76-1.00 (m, 3H, CH ₃ ester); 1.10-1.50 (m, 19H, 2 $\times$ CH ₃ , 2 $\times$ CH ₂ , C ₄ H ₉); 1.5-2.0 (m, 5H, CH ₂); 3.00-3.45 (m, 6H, OCH ₃ , N-CH ₃); 3.70 (s, 1H, NH); 4.20-4.40 (m, 2H, OCH ₂); 5.30 (m, 1H, NH); 7.50-7.80 (m, 4H, C ₆ H ₄).

Sta, AHNA, and intermediate compounds 5, 13, 21, 32, 37 were synthesized according to the Maibaum protocol (28). Solvents were of analytical purity. Tetrahydrofuran (THF) was distilled from Na/benzophenone under N₂. Dichloromethane and dimethylformamide (DMF) were dried over 4 Å molecular sieves. The peptides were synthesized by the N,N-dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCCl/HOBt) method of fragment condensation in solution. Column chromatography (CC) on silica gel (Merck, grade 230 to 400 mesh) was used to separate and purify all synthesized compounds. TLC was carried out on 0.25 mm thickness silica gel plates (Merck, silica gel 60 F₂₅₄). The solvent systems used in TLC and CC were CHCl₃/MeOH and hexane/EtOAc

in various ratios. The spots were visualized with 0.3% ninhydrin in EtOH/AcOH (97: 3, v/v). Böetius apparatus was used to determine melting points. Bruker DM 300 MHz Avance 300 WB spectrometer was applied to record ¹H NMR. Chemical shifts were measured relative to tetramethylsilane (TMS) as δ units (ppm). Optical rotations were measured at the Na-D line with the use of AP-300 (Atago) polarimeter in a 5 cm polarimeter cell. HPLC analyses of purity and activity of synthesized inhibitors were performed on a Shimadzu apparatus equipped with an LC-10AT pump, UV-Vis SPD-10A detector, and Chromax 2010 recorder. The peaks were recorded at 213 nm. The separation was carried out in the reverse phase system (Wide Pore C8, Symmetry C18) with various mobile phases.

Syntheses

Materials

All standard protected Boc-aminoacid derivatives and angiotensinogen were obtained from Bachem AG (Switzerland). α -Chymotrypsin, dicyclohexylcarbodiimide, and isopropyl-magnesium chloride solution were obtained from Sigma-Aldrich Chemie GmbH (Germany). Acetonitrile isocratic grade and methanol for liquid chromatography were acquired at LiChrosolv/Merck Millipore (Germany). Renin was purchased at Cayman Chemical Company (USA).

Synthesis of pseudopeptides

A two-step method was applied. In the first step, pseudopeptide Boc-ketoesters are obtained. Solution (A), containing 10 mmol of Boc-aminoacid (Boc-Cha-OH, Boc-Phe-OEt, Boc-Phe(4-OEt)-OH, Boc-Leu-OH, Boc-Hep-OH) in 30 mL of THF was activated with 12 mmol of *N,N*-carbonyldiimidazole (DCI). Solution (B), containing 15 mmol of malonic acid monoethyl ester in 30 mL of THF was activated with 30 mmol of isopropyl magnesium chloride. Both solutions were mixed in -20°C and agitated. Boc-pseudopeptide esters were obtained in the second step. 10 mmol of pseudopeptide Boc-ketoester was dissolved in 100 mL of THF-MeOH mixture (98: 2) and then reduced with 2.5 mmol of NaBH_4 in -78°C (28). In this way, a mixture of diastereomers was obtained.

The identity of diastereoisomers was confirmed in the analysis of a specific rotation. According to the literature, diastereoisomers with *S,S* conformation of the hydroxyethylated pseudodipeptides exhibit higher specific rotation when compared with the *R,S* diastereoisomers (29-31), which is as follows: (*S,S*) Boc-AEPHPA-OEt = -22.00 , (*R,S*) Boc-AEPHPA-OEt = -5.00 ; (*S,S*) Boc-ACHPA-OEt = -34.70 , (*R,S*) Boc-ACHPA-OEt = -20.04 ; (*S,S*) Boc-AHPPA-OEt = -33.0 , (*R,S*) Boc-AHPPA-OEt = -13.40 ; (*S,S*) Boc-Sta-OEt = -39.60 , (*R,S*) Boc-Sta-OEt = -10.40 ; (*S,S*) Boc-AHNA-OEt = $+24.39$, (*R,S*) Boc-AHNA-OEt = $+4.50$.

Introduction of the *N*-tert-Boc-Group

This group was introduced in a commonly used manner (28).

Removal of the *N*-tert-Boc group

Boc-aminoacid or Boc-peptide (1 mmol) in a solution of 4 M HCl in dioxane (3-5 mL) was stirred at room temperature for 30 min. The solution was conc. *in vacuo*, then the residue was evaporated twice with ethyl ether and dried *in vacuo* (32).

Esterification and hydrolysis

Boc-AEPHPA-OEt, Boc-AHPPA-OEt, Boc-ACHPA-OEt, Boc-Sta-OEt, and Boc-AHNA-OEt were formed from mono-ethyl malonate used to prepare these compounds (33). Alkaline hydrolysis of the ester group was carried out as described (34).

Coupling reaction with DCCI/HOBt

The amino acid or peptide ester hydrochloride (1 mmol) was dissolved in CH_2Cl_2 (5 mL) and neutralized at 0°C with THF (1 mmol). Boc-amino acid or Boc-peptide (1 mmol) and 1-hydroxybenzotriazole (HOBt) (1.5 mmol) were added followed by a solution of *N,N'*-dicyclohexylcarbodiimide (DCCI) (1.1 mmol) in CH_2Cl_2 (5 mL). The reaction mixture was stirred at 0°C for 2-4 h and left at room temperature overnight. Dicyclohexylurea (DCU) was filtered off and the filtrate was evaporated *in vacuo*. The residue was dissolved in CHCl_3 , washed successively with 5% HCl, 5% NaHCO_3 , saturated NaCl solution, dried with anhydrous MgSO_4 and conc. *in vacuo*. The peptide was purified by silica gel CC to yield the pure product (35).

Biochemical assay

Determination of inhibition of renin activity

Renin inhibiting activity of the synthesized potential inhibitors was determined *in vitro*. HPLC method was used to determine the concentration of renin substrate (36, 37). 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. The inhibition of human renin was determined after its incubation with angiotensinogen and with each of compounds 1-7. The activity of the compounds was tested in the range 10^{-6} - 10^{-7} M.

Stability in the presence of α -chymotrypsin

α -Chymotrypsin was dissolved in a phosphate buffer solution pH 7.8. The solution was incubated at 37°C and the samples were collected at the time points 0, 30, 60, 90, and 120 min. Then, the samples were isolated from the solution with diethyl ether, evaporated to dryness, dissolved in the mobile phase, and determined by HPLC (37).

Log P-value calculation

Because of the poor solubility of the compounds in water and in *n*-octanol the Log P values were calculated by a computer program. Structures of the compounds were modeled with the use of HyperChem Professional 8.0 software and the semi-empirical method MP3 was used for single-point

calculation. Geometry optimization was performed with the Polak-Ribier method. Calculation of Log P in HyperChem was carried out using atomic parameters derived by Ghose (38).

The molecular docking

The molecular docking studies were performed using BIOVIA Discovery Studio software (39) in which the Flexible Docking workflow was applied to obtain ligand-protein complexes. This approach allows some receptor flexibility during the docking of flexible ligands. Here, low-energy ligands conformations are docked into the active site of each receptor side-chain conformation. Moreover, a side-chain residues optimization around docked ligand positions was carried out. It should be emphasized that protein flexibility may play an important role in the mechanism of ligand docking and should not be ignored during this kind of study.

RESULTS AND DISCUSSION

All the obtained renin inhibitors have an inhibitory activity of IC_{50} between 10^{-6} and 10^{-7} M (Table 5). Compounds **25**, **36**, **39** turned out to be the most active (IC_{50} of 10^{-7} M), from which the most prominent blocker was compound **25**, i.e. Boc-Phe(4-OMe)-MeLeu-AHPPA-MeLeu-Iaa with $IC_{50} = 1.58 \times 10^{-7}$ M. Inhibitor compounds **36** and **39** contain hydrophobic, aliphatic groups in the P_1 position, and polar C-terminal tail of an alkyl ester structure. Inhibitor **25** contains AHPPA with a cyclic, aromatic group in position P_1 and a branched C-terminal chain in P_2 - P_3 position, unlike inhibitor compounds **36** and **39**.

Compounds **36** and **39** have only slightly lower inhibitory activity in comparison to compound **25**. It allows assuming that the absence of hydrophobic interaction between C-terminus and S_2 - S_3 site is compensated by an elastic aliphatic chain in

the P_1 position, easily fitting to the S_1 hydrophobic pocket.

High hydrophobicity of the C-terminal tail, advantageous -long and branched-chain, and its interaction with S_2 - S_3 hydrophobic pocket of compound **25** seems to be affinity-determining upon strong binding to renin. Benzene ring in P_1 position seems to be of a less significant effect, as a compound of an ester structure **31**, containing AHPPA pseudopeptide in P_1 position, but devoid of a hydrophobic C-terminal tail has a lower activity (IC_{50} of 0.95×10^{-6} M). The above-described considerations have given rise to a conclusion that compounds with a C-terminal tail of ester structure have higher inhibitory activity towards renin when P_1 position is substituted with non-branched **39** or branched **36** aliphatic group. Compounds with C-terminal amide structure specifically branched N-alkyl amides, have higher activity when P_1 position contains an unsubstituted aromatic ring **25**. Potential inhibitor compounds **25** and **20** differ only by P_1 - P_1' fragment structure (phenyl and cyclohexyl ring) and have an inhibitory activity of IC_{50} of 1.58×10^{-7} and 1.19×10^{-6} M, respectively. Inhibitors with an ester structure, with an aliphatic group at P_1 position, i.e. compounds **36** and **39**, are an order of magnitude more active than inhibitors with ester structure of C-terminal tail containing aromatic ring groups at P_1 position **7** and **31**. It can eventually be concluded that the highest inhibitory activity towards renin is exhibited by inhibitors with long, aliphatic, and branched hydrophobic chains at P_1 and P_2 - P_3 positions. A minor difference in the structure of the side chains at P_2 position of inhibitors **36** and **39**, respectively containing sec-butyl and isobutyl groups, seems to have the same minor effect on the activity of these inhibitors due to similar space occupancy of those groups. Our studies revealed that a hydrophobic N-terminal tail is not necessary for obtaining inhibitory activity in synthesized compounds.

Table 5. Stability and activity of the synthesized compounds.

Compound No.	Compound	IC_{50} [M]	Degree of stability against chymotrypsin**	*Log P
7	Boc-Phe(4-OMe)-MeLeu-AEPHPA-OEt	1.6×10^{-6}	Unstable	4.38
12	Boc-Phe(4-OMe)-MeLeu-AEPHPA-Ahx-Iaa	1.3×10^{-6}	Stable	5.08
20	Boc-Phe(4-OMe)-MeLeu-ACHPA-MeLeu-Iaa	1.19×10^{-6}	Stable	5.94
25	Boc-Phe(4-OMe)-MeLeu-AHPPA-MeLeu-Iaa	1.58×10^{-7}	Unstable	5.99
31	Boc-Phe(4-OMe)-Melle-AHPPA-OEt	0.96×10^{-6}	Unstable	4.37
36	Boc-Phe(4-OMe)-Melle-Sta-OEt	1.12×10^{-7}	Unstable	3.88
39	Boc-Phe(4-OMe)-MeLeu-AHNA-OEt	0.96×10^{-7}	Stable	3.93

*Hydrophobicity of the compounds expressed as Log P-value was calculated by a computational method (38).

**Fraction of inhibitor amount remaining after 2h incubation time in presence of chymotrypsin at 37°C (36).

t-Boc group removal increases the solubility of compounds, as a polar N-terminal tail is formed. Due to a small difference in inhibitory activity towards renin, as determined in *in vitro* studies, evaluation of effects on binding inhibitors with renin, hydrophobic interactions between P_2 and P_1 position-substituted groups, and between groups at P_1 and P_2' - P_3' positions is difficult to establish. Mutual interaction between side chains at positions P_2 and P_1 has probably no significant effect on the bioactivity of inhibitors.

The IC_{50} values for compounds of similar structures, differentiated only by C-terminal tail structure, either ester or N-alkyl amide, are similar. This suggests that hydrophobic interactions of branched chains within P_2' - P_3' and S_2' - S_3' renin site can be reduced due to steric interactions of N-isopentyl and N-isobutyl chains with hydrophobic groups at P_1 position within the inhibitor molecules. This, in turn, allows for a conclusion that hydrogen bonds play a vital role in binding inhibitors to the renin active site.

The results of *in vitro* activity and stability assessment for the obtained compounds indicate that modifications implemented to obtain compounds **12**, **20** and **25**, did not increase their inhibition capability of renin activity. They also did not significantly affect their stability against chymotrypsin (Table 5).

Pre-sampled conformations of each of the eight compounds were docked to the binding site of the renin model, which was developed on the basis of the crystal structure of renin-indole-piperazin inhibitor complexes (PDB: 3OOT) reported by Scheiper et al. (40). Taking into consideration the mode of action of renin inhibitors, it can be assumed that, in the case of compounds regarded here, the following residues play an essential role: Gln13, Val30, Thr77, Asp32, Val120, Asp215, Gly217, Ala218, Ser219, and His287. Hence, the binding pocket was defined within these residues. It should be emphasized that binding to the catalytic aspartate residues is vital for all protease inhibitors. This may lead to the conclusion that potential renin inhibitors should interact with Asp32 and/or Asp215 residues in the renin's active site (41-43).

Analysis of the docking results, binding orientations, and intermolecular interactions of compounds **7**, **12**, **20**, **25**, **31**, **36**, **38** with the active site revealed that all studied molecules fit well the active site of renin. A large number of degrees of freedom and, thus, many conformations of studied compounds make the analysis difficult. The most discriminative parameters, which distinctly classify the quality of docking, are number and strength (equivalently length and geometry) of the hydrogen bonds formed between ligand and the corresponding amino acids inside the receptor-binding pocket but also, although

to a less degree, intermolecular interactions of other types, e.g. hydrophobic and edge-to-face.

A closer inspection of the obtained results revealed that different binding modes can be distinguished inside the set of studied compounds. Apparently, the orientation of the docked ligand inside the active pocket is the most important factor. Here, at least two scenarios can be delineated – studied compounds can be oriented in the way that either the P_4 and P_3 fragments or P_2' and P_1 - P_1' fragments are faced to the inside of the renin's active site. The former configuration can be attributed to compound **31** and the latter is characteristic especially for compounds **7**, **25**, **36**, and **39**. Compounds **12** and **20** approach the active site mainly with the P_2' fragment, whereas the P_1 - P_1' fragment is placed rather on the verge of it. Therefore, a hypothesis can be made that the binding mode, affinity, and most likely, also the activity of the studied compounds strongly depends on the structure of the P_2' and P_1 - P_1' fragments. It is noteworthy that the P_1 - P_1' fragment is engaged into the vast majority of interactions between ligands and renin's active site residues.

According to *in vitro* studies, in which the affinity of analyzed compounds to renin was measured, one can assume that molecules **25**, **36**, and **39** may be the most potent renin's ligands. Indeed, this hypothesis may be strengthened by docking results as compounds **25**, **36**, and **39** seem to have the same binding mode in which P_2' and P_1 - P_1' fragments are faced to the inside of the renin's active site. The obtained complexes are stabilized by a vast array of intermolecular interactions such as conventional hydrogen bonds, carbon-hydrogen bonds, or hydrophobic interactions. The latter seems to play a pivotal role as a few characteristic hydrophobic surfaces can be distinguished within the renin's active site. This particular binding mode, characteristic for compounds **7**, **25**, **36**, **39** (as well as **12** and **20**, to a certain extent) can be illustrated by interactions of compound **39** with the renin active site residues (Figure 11).

Molecule **39** fits the renin's active site very well. It is worth noting that the docked ligand is oriented in the way that the AHNA (P_1 - P_1' fragment), OEt (P_2' fragment), and MeLeu (P_2 fragment) face the center of the binding pocket whereas the Phe(4-OMe) (P_3 fragment) slightly extends beyond an analyzed active site and the Boc group (P_4 fragment) is on the verge of it. Even though there are no conventional hydrogen bonds between the studied molecule and Asp215, this particular amino acid is engaged in carbon-hydrogen bonding interactions via its oxygen atom towards hydrogen atoms of the OEt moiety of the P_2' fragment (~2.3 Å). Another carbon-hydrogen

Figure 11. Compound **39** inside the renin's active pocket. Figure 12. Compound **31** inside the renin's active pocket.

bonds, yet less important, are formed between C=O of the P_3 fragment and hydrogen atom of the Ser219 (~ 3.0 Å) and between the C=O of the P_1 - P_1' fragment and Ser222 (~ 2.4 Å). The analyzed complex is stabilized by two hydrogen bonds, which are formed between the -OH group of Thr77 and C=O of the P_2 fragment (~ 2.2 Å) as well as between the -NH group of Ser219 and the oxygen atom of the -OH group of the P_1 - P_1' fragment (~ 3.0 Å); the latter one seems to be of less importance. Moreover, hydrophobic interactions can be distinguished between P_2 fragment of the molecule **39** and Val30 (alkyl-alkyl), the P_1 - P_1' fragment's side chain and Phe(4-OMe) of Tyr75 (pi-alkyl) and Boc residue of the studied ligand (P_4' fragment) and both the imidazole ring of His287 (pi-alkyl) and the phenyl ring of the Phe243 (pi-alkyl). Moreover, the -CH₃ group of C-terminal fragment of the molecule interacts with Ala300 (alkyl interaction) and with Ile291 (alkyl interaction). Yet other hydrophobic interactions can be distinguished between the P_2 fragment of the molecule (MeLeu's-CH₃ terminal groups) and phenyl ring of Phe117 (pi-alkyl) and between the phenyl ring of the P_3 fragment of the molecule and Leu114 (pi-alkyl).

As already mentioned, compound **31** represents a different binding mode (Figure 12), in which the docked ligand is oriented in the way that the Boc residue (P_4 fragment), the Phe(4-OMe) of P_3 fragment, and to a much lesser degree, the phenyl group of the AHPPA (P_1 - P_1' fragment) face the center of the binding pocket and the P_2' fragment of the studied molecule slightly extends beyond the analyzed active site. This particular complex is stabilized by a few hydrogen bonds, carbon-hydrogen bonds, and some hydrophobic interactions. Here, the hydrogen bond formed between the -NH- group of the P_3 fragment and the oxygen atom of Asp215 (~ 2.4 Å) should be emphasized. Moreover, a hydrogen bonding interaction can be found between the -OH group of Thr77 and the C=O of the peptide bond of the P_2 fragment (~ 2.3 Å). Another hydrogen bond can be identified between the C=O group of P_3 fragment as well as between the C=O group of P_4' fragment of the molecule **31** and -NH- group of Ser76 (~ 2.7 Å and ~ 2.5 Å, respectively). Furthermore, carbon-hydrogen bonds are also formed between the C=O of the P_1 - P_1' fragment of the molecule and Tyr75 hydrogen atom (~ 2.9 Å), the C=O of the P_3 fragment and the

Table. 6. Details of interactions between inhibitors and renin active center

Ligand No.	Orientation of the docked ligand	Interacting Residues	Category	Type
39	Ligand is oriented in the way that the AHNA (P ₁ -P ₁ ' fragment), OEt (P ₂ ' fragment), and MeLeu (P ₂ fragment) face the center of the binding pocket whereas the Phe(4-OMe) (P ₃ fragment) slightly extends beyond an analyzed active site and the Boc group (P ₄ fragment) is on the verge of it.	Thr77	H-Bond ~2.2 Å	Conventional
		Ser219	H-Bond ~3.0 Å	Conventional
		Asp215	H-Bond ~2.3 Å	Carbon hydrogen bond
		Ser219	H-Bond ~3.0 Å	Carbon hydrogen bond
		Ser222	H-Bond ~2.4 Å	Carbon hydrogen bond
		Val30	Hydrophobic	Alkyl
		Ala300	Hydrophobic	Alkyl
		Ile291	Hydrophobic	Alkyl
		Tyr75	Hydrophobic	Pi-Alkyl
		His287	Hydrophobic	Pi-Alkyl
		Phe243	Hydrophobic	Pi-Alkyl
31	Ligand is oriented in the way that the Boc residue (P ₄ fragment), the Phe(4-OMe) of P ₃ fragment, and to a much lesser degree, the phenyl group of the AHPPA (P ₁ -P ₁ ' fragment) face the center of the binding pocket and the P ₂ ' fragment of the studied molecule slightly extends beyond the analyzed active site.	Asp215	H-Bond ~2.4 Å	Conventional
		Thr77	H-Bond ~2.3 Å	Conventional
		Ser76	H-Bond ~2.5 Å	Conventional
			H-Bond ~2.7 Å	Conventional
			H-Bond ~2.5 Å	Carbon hydrogen bond
		Tyr75	H-Bond ~2.9 Å	Carbon hydrogen bond
		Ser219	H-Bond ~2.7 Å	Carbon hydrogen bond
		Ala218	Hydrophobic	Alkyl
		Met289	Hydrophobic	Alkyl
		Ile291	Hydrophobic	Alkyl
		Leu213	Hydrophobic	Alkyl
		Val30	Hydrophobic	Pi-Alkyl
		His287	Hydrophobic	Pi-Alkyl

hydrogen atom of Ser76 (~2.5 Å), and between the -OH group of Ser219 and a hydrogen atom attached to the C₄ of the P₁-P₁' peptide bond. Additionally, the molecule **31** complex with renin active site is stabilized by hydrophobic interactions, in which the -CH₃ group of Ala218 is engaged into alkyl interactions with the N-CH₃ group of the P₂ fragment as well as with the -CH₃ group attached to C₃ of the P₂ fragment. Moreover, a pi-alkyl hydrophobic interaction can be distinguished between the

-CH₃ group of the C-terminal fragment of the molecule and the imidazole ring of His287. The same imidazole ring of His287 interacts with molecule **31** via its -CH₃ group attached to C₃ of the P₂ fragment (pi-alkyl). The same fragment of molecule **31** (C₅ of the Ile chain) is stabilized by alkyl hydrophobic interactions with Met289 and Ile291. Furthermore, the Boc group of the P₄ fragment interacts with the Leu213 and Ile291 residues within the renin's active site via alkyl hydrophobic interactions. Yet other

hydrophobic interactions (pi-alkyl) can be found between the phenyl ring of the P₁-P₁' fragment of the molecule and Val30.

A comprehensive summary of docking results including a description of the most relevant interactions between ligands **31**, **39**, and the renin active center can be found in Table 6.

Taking into consideration all the above, it can be assumed that there are a few important features, which determine studied ligands affinity (and most likely activity) towards renin:

1. Binding mode in which P₂' and P₁-P₁' fragments are faced to the inside of the renin's active site.
2. Structure of the P₂' and P₁-P₁' fragments:
 - a. It seems that aliphatic hydrocarbon chains are preferred (branched or not); they are important in the case of hydrophobic interactions where the flexibility and, hence, the ease of fitting hydrophobic surfaces inside the active site is an asset.
 - b. The phenyl ring (substituted or not) of AHPPA may trigger some steric hindrances and, therefore, impede the proper emplacement of the ligand in the active site. This observation is quite relative but becomes evident upon the comparison of compounds with AHPPA or ACHPA in their structure with compounds without those fragments or with hydrocarbon chains (branched or not).
3. Branching of P₂' and P₁-P₁' fragments with hydrocarbon chains may improve the ligand's adaptation to the active site's cavity.

CONCLUSIONS

Compounds without a hydrophobic C-terminal tail, i.e., ester-structure compounds **7**, **31**, **36**, **39** of Log P = 4, exhibit weaker hydrophobic interactions with respective pockets (Table 5). Compounds **20** and **25**, as well as compound **12**, have the most prominent hydrophobic interaction capacity due to the high renin binding affinity of Log P = 6 and Log P = 5, respectively. Those compounds have a branched C-terminus. Higher hydrophobicity is not a direct estimation of higher *in vitro* inhibitory activity in each and every case. Compounds **36** and **39**, with an ester structure, and without P₂'-P₃' fragment, for which hydrophobic constant amounts to Log P = 3.88 for compound **36** and Log P = 3.93 for compound **39**, revealed only slightly decreased inhibitory activity of IC₅₀ of 1.12 × 10⁻⁷ M for compound **36** and 0.96 ×

10⁻⁷ M for compound **39** when compared to the most active inhibitor **25** with a branched aliphatic chain at the C-terminus, of Log P = 5.99, and IC₅₀ = 1.58 × 10⁻⁷ M. This suggests a significant contribution of other interactions, particularly hydrogen bonds between the inhibitor molecule and renin active site, which is confirmed by docking studies.

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Conflicts of interest

The authors declare no conflict of interest.

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