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Novel renin inhibitors containing derivatives of N-alkylleucyl-β-hydroxy-γ-amino acids

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In search for new drugs lowering arterial blood pressure, which could be applied in anti-hypertensive therapy, research concerning agents blocking of renin-angiotensin-aldosteron system has been conducted. Despite many years of research conducted at many research centers around the world, aliskiren is the only one renin inhibitor, which is used up to now.

Four novel potential renin inhibitors, having structure based on the peptide fragment 8–13 of human angiotensinogen, a natural substrate for renin, were designed and synthesized. All these inhibitors contain unnatural moieties that are derivatives of N-methylleucyl-β-hydroxy-γ-amino acids at the P_2-P_1′ position: 4-[N-(N-methylleucyl)-amino]-3-hydroxy-7-(3-nitroguanidino)-heptanoic acid (AHGHA), 4-[N-(N-methylleucyl)-amino]-3-hydroxy-5-phenyl-pentanoic acid (AHPPA) or 4-[N-(N-methylleucyl)-amino]-8-benzyloxycarbonylamino-3-hydroxyoctanoic acid (AAHOA). The previously listed synthetic β-hydroxy-γ-amino acids constitute pseudodipeptidic units that correspond to the P_1-P_1′ position of the inhibitor molecule. An unnatural amino acid, 4-methoxyphenylalanin (Phe(4-OMe)), was introduced at the P_3 position of the obtained compounds. Three of these compounds contain isoamylamide of 6-aminohexanoic acid (ε-Ahx-Iaa) at the P_2′-P_3′ position. The proposed modifications of the selected human angiotensinogen fragment are intended to increase bioactivity, bioavailability, and stability of the inhibitor molecule in body fluids and tissues. The inhibitor Boc-Phe(4-OMe)-MeLeu-AHGHA-OEt was obtained in the form of an ethyl ester. The hydrophobicity coefficient, expressed as log P varied between 3.95 and 8.17. *In vitro* renin inhibitory activity of all obtained compounds was contained within the range 10^{-6}-10^{-9} M. The compound Boc-Phe(4-OMe)-MeLeu-AHPPA-Ahx-Iaa proved to be the most active ($IC_{50} = 1.05 \times 10^{-9}$ M). The compounds Boc-Phe(4-OMe)-MeLeu-AHGHA-Ahx-Iaa and Boc-Phe(4-OMe)-MeLeu-AHPPA-Ahx-Iaa are resistant to chymotrypsin. Copyright © 2016 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: hypertension treatment; renin inhibitors; amino acids; pseudodipeptidic unit

Introduction

Arterial hypertension is the major risk factor of cardiovascular diseases, such as cerebral stroke, myocardial infarction, cardiac insufficiency, or renal insufficiency. The most recent guidelines for handling arterial hypertension, published by European and American hypertensiology and cardiology societies, are classifying blockers of the renin-angiotensin system within the group of basic hypotensive drugs [1–4]. The angiotensin converting enzyme inhibitors and angiotensin II receptor blockers specified in these reports are also first-choice drugs also in diabetic patients with cardiovascular and renal complications [5,6]. Another promising strategy for blocking the renin-angiotensin-cascade at an early stage involves inhibition of renin. This mechanism prevents formation of angiotensin I from angiotensinogen that is a renin substrate. An indirect consequence of renin inhibition is prevention of converting of inactive angiotensin I to angiotensin II, an octapeptide having hypertensive activity. Until now, only one renin inhibitor, aliskiren, is available on the pharmaceutical market. It is marketed since 2007 [7]. First observations concerning efficacy and safety profile of aliskiren, after just several years of use, are satisfactory. Despite low bioavailability, not exceeding 2.7%, aliskiren effectively lowers blood pressure [8]. It is well tolerated by majority of patients. Because hepatobiliary elimination (90% of aliskiren is excreted in bile), it may be used in hypertensive patients with concomitant kidney diseases. Aliskiren has certain nephroprotective potential, unrelated to its hypotensive activity [9–11]. Adverse reactions and contraindications related to aliskiren are scarce. It must not be used in pregnant women or in patients with renal artery stenosis. The level of potassium in blood serum increases as a result of therapy with this drug. Therefore, it is necessary to continue development of this class of drugs. Poor bioavailability and instability of the molecule under physiological conditions are major obstacles that are preventing renin inhibitors from broader introduction into medicinal practice. Therefore, new-generation inhibitors that do not contain an unstable peptide linkage (-CONH-), which is susceptible to enzymatic degradation, were designed in order to overcome the aforementioned problems. Non-peptidic renin inhibitors include aliskiren and a novel class of inhibitors that are piperidine derivatives [12–14]. In our opinion, it is important that the inhibitors would have ability to form hydrogen bonds with the -CONH- moiety, which could result in increased inhibitory activity. Therefore, molecules of the designed compounds contain -CONH- moieties that, however, are not classic peptide bonds obtained by linking two α-amino acids. In the compounds synthesized by us, we combined unnatural

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derivatives of amino acids with pseudodipeptides. Such modified molecules that contain -CONH- moieties should be completely or at least significantly protected against enzymatic degradation. Results of concurrent studies on activity/structure relationship of renin inhibitors performed at various research centers suggest a great importance of hydrophobic properties of the inhibitors. The level of hydrophobicity has a decisive impact on biological activity and bioavailability of these compounds. Four novel compounds were designed and synthesized in order to determine the impact of interactions between the inhibitor's molecule and the hydrophobic active site of renin on their inhibitory activity, as shown in Figures 1–4 . The compounds were designed by modifying a moiety of angiotensinogen, that is, the natural renin substrate as it is depicted in Figure 5. Currently, it is known that the hydrophobic pocket S_3–S_1 is the key binding site for hydrophobic moieties of renin blockers. Hydrophobic interactions with the S_4 and S_2'–S_3' pockets [15] are also important for their inhibitory activity. Recent discovery of sub-pockets within the S_3 and S_1 sites [16,17] that could react with hydrophilic and polar moieties opens new possibility of designing inhibitors that are significantly modified at the P_1 position. By facilitating formation of additional hydrogen bonds, the modifications could lead to enhanced enzyme-inhibitor interactions as shown in Figure 6. Taking into consideration these discoveries and continuing our prior studies, we designed novel renin inhibitory molecules that contained hydrophobic moieties at all P_3–P_2–P_1–P_1'–P_2'–P_3' sites.

Experimental

Chemistry

The structures of inhibitors considered in the present work are shown in Figures 1–4. The inhibitors **7**, **11**, **18**, **25** (Table 1) as well as their intermediates were synthesized in a commonly used manner, by fragment condensation, according to schemes presented in Figures 7–10.

The applied methods are specified later in the syntheses section. Physicochemical properties of the inhibitors **7**, **11**, **18**, **25**, as well as newly synthesized intermediates **1**, **3**, **4**, **6**, **9**, **12**, **13**, **14**, **15**, **16**, **17**, **19**, **20**, **21**, **22**, **23**, **24**, are presented in Tables 2 and 3.

Reagents Boc-Phe(4-OMe)-OH, Boc-N-Me-Leu-OH porcine kidney renin and N-acetylrenin substrate tetradecapeptide were acquired from recognized vendor. The AHGHA, AHPPA, and AAHOA (**1**, **12**, **19**) were synthesized according to the Maibaum protocol. Solvents were of analytical purity. Tetrahydrofuran (THF) was distilled from Na/benzophenone under N_2. Dichloromethane and dimethylformamide (DMF) were dried over 4-Å molecular sieves. The peptides were synthesized by the N,N-dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) method of fragment condensation in solution. Column Chromatography (CC) on silica gel (Merck, grade

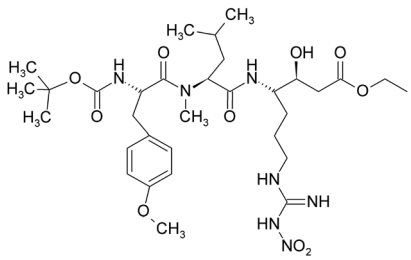


Figure 1. Ethyl 4-[N-[N-/N-(t-butoxycarbonyl)-4-methoxyphenylalanyl/-N-methylleucyl]-amino]-7-(3-nitroguanidino)-3-hydroxyheptanoate Boc-Phe(4-OMe)-MeLeu-AHGHA-OEt **7**.

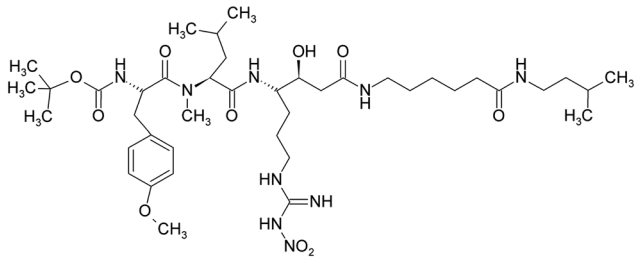


Figure 2. 6-{N-[4-[N-[N-/N-(t-butoxycarbonyl)-4-methoxyphenylalanyl/-N-methylleucyl]-amino]-7-(3-nitroguanidino)-3-hydroxyheptanoyl]-amino}-hexanoic acid isoamylamide Boc-Phe(4-OMe)-MeLeu-AHGHAAhx-Iaa **11**.

230 to 400 mesh) was used to separate and purify all synthesized compounds. Thin Layer Chromatography (TLC) was carried out on 0.25-mm thickness silica gel plates (Merck, silica gel 60 F_{254}). The solvent systems used in TLC and CC were $CHCl_3$/MeOH and hexane/EtOAc in various ratios. The spots were visualized with 0.3% ninhydrin in EtOH/AcOH (97: 3, v/v). Perkin–Elmer Microanalyser was used to carry out elemental analyses. Böetius apparatus was used to determine melting points. Bruker DM 300-MHz Avance 300-WB spectrometer was applied to record ^{1}H-NMR. Chemical shifts were measured relative to tetramethylsilane (TMS) as δ units (ppm). Optical rotations were measured at the Na-D line with 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 a LC-10AT pump, UV–vis SPD-10A detector and Chromax 2010 recorder. The peaks were recorded at the wavelength of 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 amino acid derivatives and angiotensinogen were obtained from Bachem AG (Switzerland). Alfa-chymotrypsin, dicyclohexylcarbodiimid, and isopropylmagnesium chloride solution were obtained from Sigma-Aldrich Chemie GmbH (Germany). Acetonitrile isocratic grade for liquid chromatography and methanol for liquid chromatography were acquired at LiChrosolv/Merck Millipore (Germany). Renin was purchased at Cayman Chemical Company (USA).

Synthesis of pseudopeptides

It is a two-step method.

In the first step, pseudopeptide Boc-ketoesters are obtained. Solution (A), containing 10 mmol of Boc-aminoacid (Boc-Arg

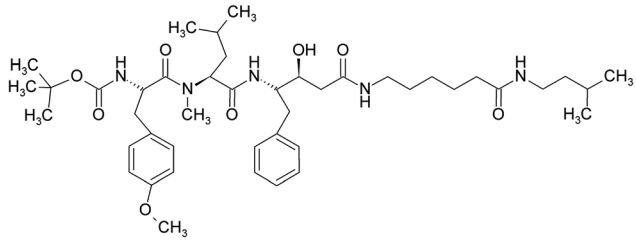


Figure 3. 6-{N-[4-[N-[N-/N-(t-butoxycarbonyl)-4-methoxyphenylalanyl/-N-methylleucyl]-amino]-3-hydroxy-5-phenyl-pentanoyl]-amino}-hexanoic acid isoamylamide Boc-Phe(4-OMe)-MeLeu-AHPPA-Ahx-Iaa**18**.

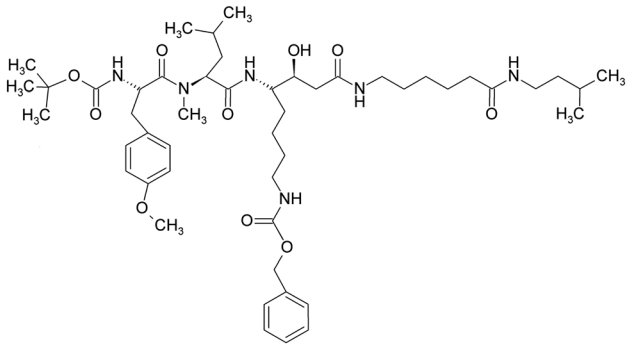


Figure 4. 6-{N-[4-[N-[N-/N-(t-butoxycarbonyl)-4-methoxyphenylalanyl/-N-methylleucyl]-amino]-8-benzyloxycarbonylamino-3-hydroxyoctanoyl]-amino}-hexanoic acid isoamylamide Boc-Phe(4-OMe)-MeLeu-AAHOA-Ahx-Iaa **25**.

(NO_2)-OH, Boc-Lys(Z)-OH, Boc-Phe-OH) in 30 mL of THF was activated with 12 mmol of N,N-carbonyldiimidazole. Solution (B), containing 15mmol of malonic acid monoethyl ester in 30 mL of THF was activated with 30 mmol of isopropyl magnesium chloride. Both solutions were mixed together in −20 °C and agitated. Boc-pseudopeptide esters were obtained in the second step. Ten 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 [19].

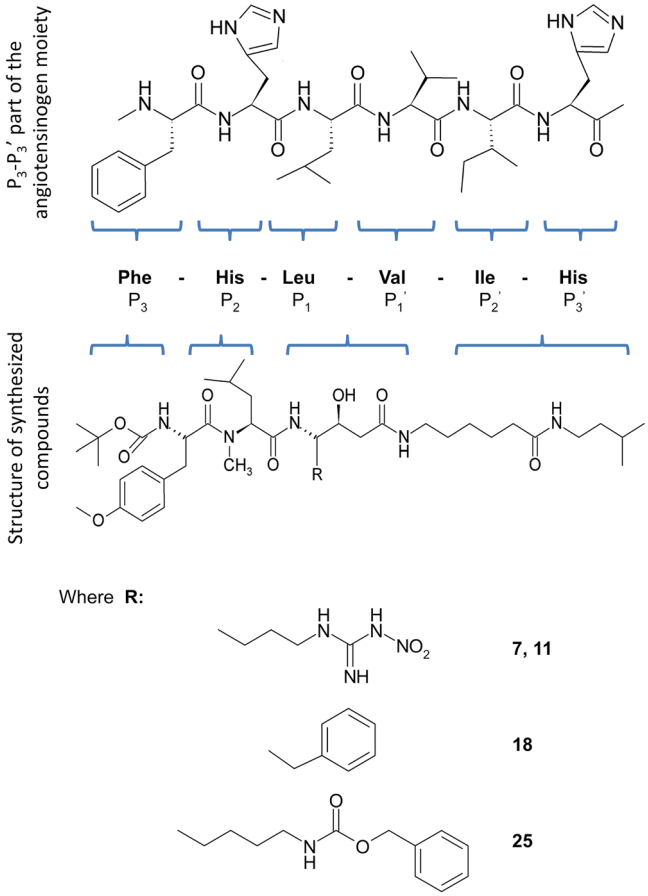
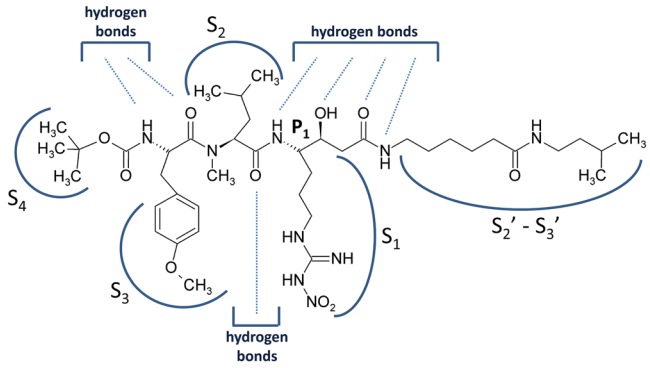


Figure 5. The comparison of the derived renin inhibitor molecules **7**, **11**, **18**, **25** to the angiotensinogen 8-13fragment (positions P_3-P_3').



Schematic chart for the Compound **11**

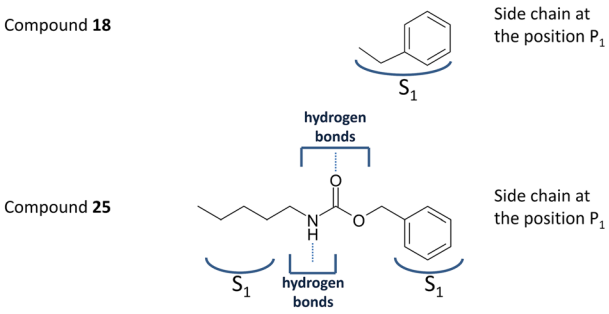


Figure 6. The expected binding sites of the potential new renin inhibitors with the active center of the renninmolecule.

Identity of diastereoisomers was confirmed in analysis of a specific rotation, $[\alpha]_D^{20}$. According to the literature, diastereoisomers with S,S conformation of the hydroxyethylated pseudodipeptides exhibit higher specific rotation when compared with the R,S diastereoisomers [20–22], which is as follows: (S,S) AHGHA $[\alpha]_D^{20} = -10.5$, (R,S) AHGHA $[\alpha]_D^{20} = -6.8$; (S,S) AAHOA $[\alpha]_D^{20} = -15.6$, (R,S) AAHOA $[\alpha]_D^{20} = -8.43$, and (S,S) AHPPA $[\alpha]_D^{20} = -30.0$, (R,S) AHPPA $[\alpha]_D^{20} = -10.5$.

Table 1. Biochemical Properties of the Synthesized Compounds, Some Intermediates and the Reference Compounds

Compound	Compound NR	IC_{50} M	Stability [%]*	Log P**
Boc-Phe(4-OMe)-MeLeu-AHGHA-OEt	**7**	1.7×10^{-6}	46.45	3.96
Boc-Phe(4-OMe)-MeLeu-AHGHA-Ahx-Iaa	**11**	0.96×10^{-8}	100 (stable)	5.44
Boc-Phe(4-OMe)-MeLeu-AHPPA-Ahx-Iaa	**18**	1.05×10^{-9}	100 (stable)	7.71
Boc-Phe(4-OMe)-MeLeu-AAHOA-Ahx-Iaa	**25**	1.31×10^{-7}	42.09	8.17

*Fraction of inhibitor amount remaining after 2 h incubation time in presence of chymotrypsin at 37 °C [18]

**Hydrophobicity of the compounds expressed as log P value was calculated numerically.

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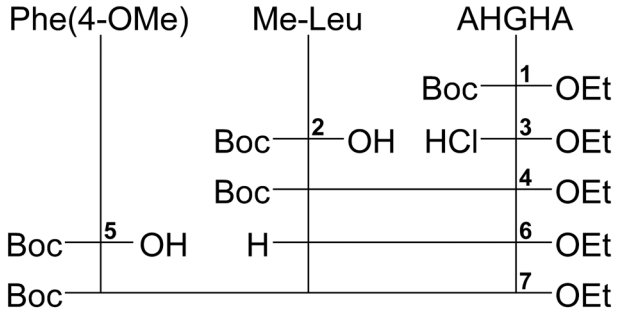

Figure 7. Ethyl 4-[N-[N-/N-(t-butoxycarbonyl)-4-methoxyphenylalanyl/-N-methylleucyl]-amino]-7-(3-nitroguanidino)-3-hydroxyheptanoate Boc-Phe (4-OMe)-MeLeu-AHGHA-OEt **7**.

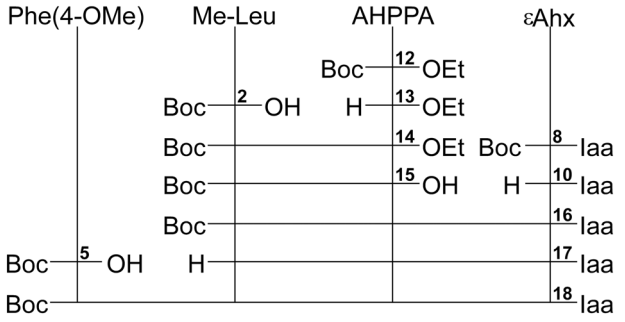

Figure 9. 6-{N-[4-[N-[N-/N-(t-butoxycarbonyl)-4-methoxyphenylalanyl/-N-methylleucyl]-amino]-3-hydroxy-5-phenyl-pentanoyl]-amino}-hexanoic acid isoamylamide Boc-Phe(4-OMe)-MeLeu-AHPPA-Ahx-Iaa **18**.

Introduction of the N-tert-Boc group

This group was introduced in a commonly used manner [19].

Removal of the N-tert-Boc group

Boc-amino acid or Boc-peptide (1 mmol) in a solution of 4 M HCl in dioxane (3 n 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* [23].

Esterification and hydrolysis

Boc-AHGHA-OEt, Boc-AHPPA-OEt, and Boc-AAHOA-OEt were formed from mono-ethyl malonate used to prepare these compounds [24]. Alkaline hydrolysis of ester group was carried out as described earlier [25].

Coupling reaction with DCC/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 HOBt (1.5 mmol) were added followed by a solution of DCC (1.1 mmol) in CH_2Cl_2 (5 mL). The reaction mixture was stirred at 0 °C for 2–4 h and left at RT 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 anh. $MgSO_4$, and conc. *in vacuo*. The peptide was purified by silica gel CC to yield the pure product [26].

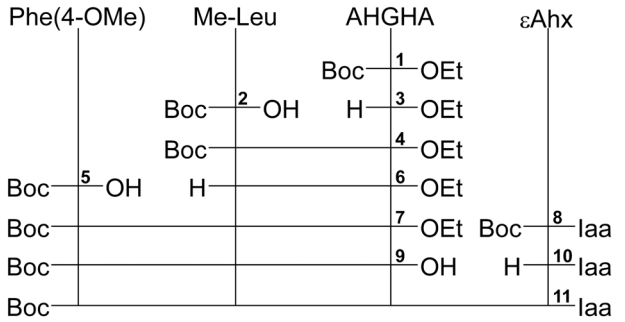

Figure 8. 6-{N-[4-[N-[N-/N-(t-butoxycarbonyl)-4-methoxyphenylalanyl/-N-methylleucyl]-amino]-7-(3-nitroguanidino)-3-hydroxyheptanoyl]-amino}-hexanoic acid isoamylamide Boc-Phe(4-OMe)-MeLeu-AHGHAAhx-Iaa **11**.

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. The activity of the compounds was tested in the range 10^{-6}–10^{-9} M. Inhibition is expressed as IC_{50} value, that is, the molecular concentration of the synthesized inhibitors causing 50% inhibition of the control renin activity [18].

Stability in the presence of α-chymotrypsin

α-Chymotrypsin was dissolved in phosphatate buffer 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 [18].

Log P value calculation

Because of 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 use of HyperChem 4.5 software, and the semi-empirical MP3 method was used for single-point calculation. Geometry optimization was performed by the Polak–Ribier method. Calculation of log P in HyperChem was carried out using atomic parameters derived by Ghose *et al.* [27].

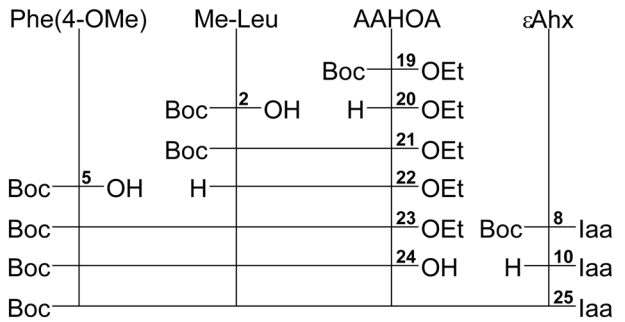

Figure 10. 6-{N-[4-[N-[N-/N-(t-butoxycarbonyl)-4-methoxyphenylalanyl/-N-methylleucyl]-amino]-8-benzyloxycarbonylamino-3-hydroxyoctanoyl]-amino}-hexanoic acid isoamylamide Boc-Phe(4-OMe) MeLeu- AAHOA-Ahx-Iaa **25**.

Table 2. Physicochemical and analytical properties of the synthesized compounds

Compd. No.	Structure	Formula m.w.	Yield (%)	M.p. (°C)	$[\alpha]_D^{20}$ (c,MeOH)	TLC,R_f (m.ph.)*	HPLC (% of purity)
1	Boc-AHGHA-OEt	$C_{15}H_{27}O_7N_5$ 389.41	31.4	Semisolid	−10.5(1.0)	0.23(A)	—
3	H-AHGHA-OEt x HCl	$C_{10}H_{19}O_3N_5Cl$289.34	87.0	Semisolid	−8.7(1.1)	0.38(D)	—
4	Boc-MeLeu-AHGHA-OEt	$C_{22}H_{42}O_8N_6$ 518.62	47.0	Oil	+28.0(1.2)	0.64(A)	—
6	H-MeLeu-AHGHA-OEt x HCl	$C_{17}H_{34}O_6N_6Cl$468.10	100.0	Oil	+23.0(1.0)	0.68(D)	—
7	Boc-Phe(4-OMe)- MeLeu-AHGHA-OEt	$C_{32}H_{53}O_{10}N_7$695.80	89.0	Oil	+50(1.0)	0.37(E)	96.98
9	Boc-Phe(4-OMe)- MeLeu-AHGHA-OH	$C_{32}H_{45}O_8N_5$627.77	34.0	Semisolid	−29.4(1.0)	0.67(C)	—
11	Boc-Phe(4-OMe)- MeLeu-AHGHA-Ahx-Iaa	$C_{41}H_{71}O_{10}N_9$850.05	67.0	Semisolid	−28.0(1.0)	0.46(A)	98.18
12	Boc-AHPPA-OEt	$C_{18}H_{25}O_5N$336.41	63.0	80-84	−30.0(1.3)	0.34(F)	—
13	H-AHPPA-OEt x HCl	$C_{13}H_{17}O_3NCl$ 285.89	97.0	Semisolid	−27(1.0)	0.65(G)	—
14	Boc-MeLeu-AHPPA-OEt	$C_{25}H_{38}O_6N_2$462.58	43.9	Oil	−61(1.0)	0.62(A)0.43(B)	—
15	Boc-MeLeu-AHPPA-OH	$C_{23}H_{41}O_6N_2$ 454.65	74.0	Semisolid	−58(1.0)	0.54(B)	—
16	Boc-MeLeu-AHPPA-Ahx-Iaa	$C_{34}H_{58}O_6N_4$ 618.34	37.2	Oil	−100(1.1)	0.37(A)	—
17	H-MeLeu-AHPPA-Ahx-Iaa x HCl	$C_{29}H_{51}O_4N_4Cl$ 554.79	100.0	Semisolid	−98(1.0)	0.46(A)	—
18	Boc-Phe(4-OMe)- MeLeu-AHPPA-Ahx-Iaa	$C_{44}H_{69}O_8N_5$ 795.00	14.1	53-57	−94.0(1.0)	0.9(A)0.75(C)	99.78
19	Boc-AAHOA-OEt	$C_{23}H_{36}O_7N_2$452.55	9.4	Semisolid	−15.6(1.01)	0.18(B)	—
20	H-AAHOA x HCl	$C_{18}H_{29}O_4N_2Cl$388.9	99.7	Semisolid	−8.9(1.0)	0.47(D)	—
21	Boc-MeLeu-AAHOA-OEt	$C_{30}H_{49}O_8N_3$ 579.75	60.0	Semisolid	−35(1.67)	0.84(A)	—
22	H-MeLeu-AAHOA-OEt x HCl	$C_{25}H_{42}O_6N_3Cl$516.09	99.0	Semisolid	−29(1.0)	0.42(D)	—
23	Boc-Phe(4-OMe)- MeLeu-AAHOA-OEt	$C_{40}H_{60}O_{10}N_4$756.95	35.2	Semisolid	−12.6(0.32)	0.86(A)	—
24	Boc-Phe(4-OMe)- MeLeu-AAHOA -OH	$C_{38}H_{56}O_{10}N_4$728.9	52.0	Semisolid	−16.7(0.16)	0.48(A)	—
25	Boc-Phe(4-OMe)- MeLeu-AAHOA -Ahx-Iaa	$C_{49}H_{78}O_{10}N_6$911.21	60.8	Semisolid	−52.6(0.04)	0.72(A)	93.5

*Mobile phase systems (v/v) were: CH_3Cl-MeOH 95:5 (A), CH_3Cl-MeOH 98:2 (B), Hexane-AcOEt 60:40 (C), BAW (D), CH_3Cl-MeOH 90:10 (E), Hexane-AcOEt 80:20 (F), BPW(G)

The elemental analysis results were within ±0.4% of theoretical values.

Results and Discussion

In vitro renin inhibitory activity of all compounds obtained in this study was within the range 10^{-6}–10^{-9} M (Table 1). The most active compound was Boc-Phe(4-OMe)-MeLeu-AHPPA-Ahx-Iaa **18**, featuring $IC_{50} = 1.3 \times 10^{-9}$ M. For comparison, activity of aliskiren amounts to $IC_{50} = 0.6 \times 10^{-9}$ M [28]. Structure of the compounds we obtained was designed on the basis of the fragment of human angiotensinogen and considerably differs from the aliskiren's structure [15,29,30]. Renin inhibition activity, expressed as IC_{50}, of inhibitors obtained by our team ranged from 10^{-3} M to 10^{-7} M. Structure of the said compounds was developed on the basis of the structure of the renin substrate's fragment.

In the compounds **7**, **11**, and **18**, we accounted for possible interaction of the substituent at P_1 with the hydrophilic sub-pockets within the S_1 area. In particular, we focused our efforts on designing a hydrophobic moiety P_2–P_1'. Our aim was to find out whether a probable intra-molecular relationship between the side chains at P_2 and P_1, that are located closely to each other, could reduce or enhance hydrophobic interactions of the inhibitor with the enzyme. The compounds synthesized by us previously [29,31,32], which contained flexible and branched isobutyl side chains at P_2 and P_1, have only moderate inhibitory activity, for example, Boc-Phe(4-OMe)-MeLeu-Sta-Ahx-EA $IC_{50} = 2.5 \times 10^{-4}$ M [33]. In this case, however, moderate activity could result from the presence of hydrophilic ethanolamine (EA) at the P_3' position of the C-terminus. On the other hand, the isobutyl substituent MeLeu and Sta (statin: 4-amino-3-hydroxy-6-methylheptanoic acid), which are adjacent to each other at P_2–P_1, do not disturb interaction with the hydrophobic pockets S_2 and S_1. We hope to obtain even more active inhibitors in the future. This paper concerns the compounds **11**, **18**, and **25** that contain a strongly hydrophobic system Ahx–Iaa at the P_2'–P_3' position [25]. The compound **7** was designed in the form of an ester in order to compare it with the compound **11** that has an amide structure. It should allow for verifying results of the studies on bioactivity and bioavailability as a function of the structure of the C-terminus of the renin inhibitor molecules [34]. In contrast to the previously [35] obtained compounds, instead of His, we introduced a much more hydrophobic MeLeu at P_2. Currently, we returned to an earlier concept, in which histidine was not necessary at the P_2 position and may be replaced by synthetic amino acids. Replacement of His at P_2 by N-methyl amino acids with a branched alkyl side chains increases activity of the inhibitors [15,33]. Presumably, it is caused by hydrophobic interaction of the side chains in these amino acids with the S_2 pocket of the enzyme. In our previous studies, we had assumed that the enzyme-inhibitor interactions could be enhanced as a result of introducing hydrophobic aromatic groups at P_2. Poor *in vitro* inhibitory activity of these inhibitors gives reason to suppose that the presence of arylhistidine derivatives at P_2 hinders binding between the inhibitor's molecule and rennin [30]. No hydrogen bond is formed between the enzyme active site and the hydrogen atom that is a constituent of the NH group belonging to the amino acid at the P_2 position of the P_3–P_2 peptide bond. Therefore, the hydrogen atom could be substituted for a short alkyl, for example, the methyl group [36–39]. Introduction of an N-methyl amino acid, such as MeLeu, MeVal, or MeHis, increases resistance of the P_3–P_2 bond to proteolytic enzymes, while their affinity to renin remains unaffected [40,41]. All compounds obtained by us contain an unnatural Phe(4-OMe)-MeLeu moiety at the P_3–P_2 position. Introduction of 4-methoxyphenylalanine at the P_3 position should secure the peptide bond against proteolytic action of chymotrypsin in the gastrointestinal tract. An aromatic ring [15,42] that binds to the hydrophobic pocket S_{3sp} [16,17,43] is

Table 3. ^{1}H NMR spectra of the synthesized compounds

Compd. No	Solvent	Chemical shifts δ, ppm
1	$CDCl_3$	1.25(t, 3H, CH_3ester), 1.43(s, 9H, C_4H_9), 1.64–1.80(m, 2HC_6), 2.48–2.62(m, 2H, CH_2), 3.24–3.36(m, 1H, CH_2), 3.40–3.50(m, 2H, CH_2), 3.54–3.60(m, 2H, CH_2), 4.06–4.10(m, 1H, CH_2), 4.15 (q, J = 7.0 Hz, 2H, OCH_2), 5.24(d, J = 8.4 Hz, 1H, NH), 7.70(s, br, 2H, 2NHguan), 8.68(s, br, 1H, NHguan)
3	$CDCl_3$	1.31(m, 3H, CH_3ester), 1.71–1.80(m, 2H, CH_2), 2.48–2.62(m, 2H, CH_2), 3.22–3.37(m, 1H, CH_2), 3.40–3.50(m, 2H, CH_2), 3.60–3.62(m, 2H, CH_2), 4.06–4.10(m, 1H, CH_2), 4.15, (q, J = 7.0 Hz, 2H, OCH_2), 5.24(d, J = 8.4 Hz, 1H, NH), 7.75(s, br, 2H, 2 × NHguan), 8.05(s, br, 1H, NHguan)
4	$CDCl_3$	0.91–1.20(m, 6H, 2 × CH_3MeLeu), 1.24–1.32(m, 3H, CH_3estr., 1H, CH_2), 1.47(s, 9H, C_4H_9), 1.56–1.78(m, 7H, 2HC_βMeLeu, HCγMeLeu, 2HC_5, 2HC_6), 1.90–2.00(d, J = 9.0 Hz, 1H, CH_2,), 3.00(s, 3H, N-CH_3), 3.30–3.45(m, 1H, CH_2), 4.10–4.20(m, 2H, OCH_2ester), 4.60–4.70(m, 1H, NH), 4.75–4.97(m, 1H, NH), 6.75–6.90(m, 2H, 2 × NHguan), 7.25(s, 1H, NHguan)
6	$CDCl_3$	0.81–1.20(m, 6H, 2 × CH_3MeLeu), 1.24–1.45(m, 3H, CH_3estr., 1H, CH_2), 1.50–1.78(m, 7H, 2HC_χMeLeu, HC_γMeLeu, 2HC_5, 2HC_6), 1.90–2.00(d, J = 9.0 Hz, 1H, CH_2,), 3.26(s, 3H, N-CH_3), 3.30–3.45(m, 1H, CH_2), 4.10–4.20(m, 2H OCH_2ester), 4.60–4.70(m, 1H, NH), 4.75–4.97(m 1H, NH), 6.75–6. 90(m, 2H, 2 × NHguan), 7.34(s, 1H, NHguan)
7	$CDCl_3$	2.96–1.00(m, 3H, CH_3ester), 1.25–1.31(m, 6H, 2 × CH_3MeLeu), 1.42(s, 9H, C_4H_9), 2.76–2.83(m, 2H, 2HC_βPhe(4-OMe), 3.00(s, 3H, N-CH_3), 3.71(s, 3H, OCH_3ester), 3.78(s, 3H, OCH_3ester), 4.34–4.36(m, 2H, OCH_2ester), 4.52(d, J = 6.6 Hz, 1H, NH), 4.94(s, 1H, NH), 5.28–5.33(m, 2H, HC(Phe-4OMe), HCMeLeu), 6.81–7.05(m, 6H, 4H C_6H_4, 2H, 2 × NHguan,), 7.26(s, 1H, NHguan)
9	$CDCl_3$	1.27–1.32(m, 6H, 2 × CH_3MeLeu), 1.46(s, 9H, C_4H_9), 2.76–2.83(m, 2H, 2HC_βPhe(4-OMe)), 3.00(s, 3H, N-CH_3), 3.71(s, 3H, OCH_3ester), 3.78(s, 3H, OCH_3ester), 4.52(d, J = 6.6 Hz, 1H, NH), 4.98(s, 1H, NH), 5.25–5.37(m, 2H, HC(Phe-4OMe), HCMeLeu), 6.81–7.05(m 6H, 4H C_6H_4, 2H, NHguan,), 7.26(s, 1H, NHguan)
11	$CDCl_3$	0.86–0.96(m, 12H, 4 × CH_3), 1.03–1.37(m, 21H, 10 × CH_2 $CH(CH_3)_2$), 1.42(s, 9H, C_4H_9), 1.59–1.75(m, 5H, 2 × CH_2Ahx, 3H$C_{\beta Iaa}$), 2.10(t, J = 7.6 Hz, 2H, CH_2), 2.94–3.09(m, 5H, 3H, N-CH_3, 2HC(Phe-4OMe)), 3.79(s, 3H, OCH_3ester), 4.02(d, J = 6.6 Hz, 2HOCH_3ester), 5.07(s, 1H, NH), 5.45(s, 1H, NH), 5.74(s, 1H, NH), 6.83, 7.11(dd, J = 9.2 Hz, 4H, C_6H_4), 7.26(s, 2H, 2 × NHguan), 7.5(br. s, 1H, NHguan).
12	$CDCl_3$	1.23(t, 3H, CH_3estr.), 1.40(s, 9H, C_4H_9), 2.38(d, J = 9 Hz, 2H, CH_2), 2.90(d, J = 8 Hz, 2H, CH_2), 3.70(q, J = 7 Hz, 1H, CH_2), 4.00(d, J = 8 Hz, 1H, CH_2), 4.13(q, J = 8 Hz, 2H, OCH_2estr.), 4.96(d, J = 12 Hz, 1H, NH), 7.26(s, 5H, C_6H_5)
13	$CDCl_3$	1.23(t, 3H, CH_3estr.), 2.38(d, J = 9 Hz, 2H, CH_2), 2.94(s, 2H, CH_2), 3.70–4.00(m, 2H, CH_2), 4.15(q, J = 8 Hz, 2H, OCH_2estr.), 4.63(d, J = 9 Hz, 1H, NH), 7.28(s, 5H, C_6H_5)
14	$CDCl_3$	0.8–1.0(m, 6H, 2 × CH_3), 1.25(t, J = 9.0 Hz, 3H, CH_3estr.), 1.47(s, 9H, C_4H_9), 2.51(s, 3H, N-CH_3), 2.29–2.43(m, 2H, 2HC_5AHPPA), 2.75–3.00(m, 2H, 2HC_2AHPPA), 3.37(t, J = 6.6 Hz, HC_3AHPPA), 4.04–4.17(m, 4H, HC_3AHPPA, HC_4AHPPA, CH_2estr.), 4.66(d, J = 8.6 Hz, 1H, NH), 7.1–7.4(m, 5H, C_6H_5).
15	$CDCl_3$	0.8–1.0(m, 6H, 2 × CH_3), 1.47(s, 9H, C_4H_9), 2.51(s, 3H, N-CH_3), 2.29–2.43(m, 2H, 2HC_5AHPPA), 2.75–3.00(m, 2H, 2HC_2AHPPA), 3.37(t, J = 6.6 Hz, HC_3AHPPA), 4.04–4.17(m, 2H, HC_3AHPPA, HC_4AHPPA,), 4.66(d, J = 8.6 Hz, 1H, NH), 7.1–7.4(m, 5H, C_6H_5).
16	$CDCl_3$	0.85–0.98(m,12H,4 × CH_3), 1.0–1.72(m, 11H, $CH(CH_3)_2$, 5× CH_2), 1.52(s, 9H, C_4H_9), 2.15(t, J = 1.5 Hz, 2H, CH_2Ahx), 2.7(s, 3H, N-CH_3), 3.15–3.4(m, 4H, 2 × CH_2), 4.6(s, br, 1H, NH),5.75(s, br, 1H, NH), 7.25(br, s, 5H, C_6H_5).
17	$CDCl_3$	0.85–0.98(m, 12H, 4 × CH_3), 1.0–1.72(m, 11H, $CH(CH_3)_2$, 5× CH_2), 2.15(t, J = 1.5 Hz, 2H, CH_2Ahx), 2.7(s, 3H, N-CH_3), 3.15–3.4(m, 4H, 2 × CH_2), 4.6(s, br, 1H, NH),5.75(s, br, 1H, NH), 7.25(br, s, 5H, C_6H_5).
18	$CDCl_3$	0.82–0.98(m, 12 H, 4 × CH_3), 1.07–1.73(m, 11 H, $CH(CH_3)_2$, 5 × CH_2), 1.25 (s, 9H, C_4H_9), 2.14(t, J = 1 Hz, 2H, CH_2), 2.69(s, 3H, N-CH_3), 3.43–3.50(m, 4H, 2 × CH_2), 3.78(d, J = 6 Hz, 3H, OCH_3), 6.9(s, br, NH), 7.26 (br, s, 5H, C_6H_5), 7.53, 7,58(dd, J = 6.8 Hz, 4H, C_6H_4), 7.60 (br, s, 1H, NH).
19	$CDCl_3$	1.27(t, J = 8.0 Hz, 3H, CH_3estr.), 1.43(s, 9H, C_4H_9), 1.48–1.64(m, 5H, 2 × CH_2, 1 × CH), 2.40–2.53(m, 2H, CH_2), 3.15–3.25(m, 2H, CH_2), 3.57(s br, 1H, CH), 3.98(s br, 1H, OH), 4.16 (q, J = 4.0 Hz, 2H, OCH_2), 4.71(s, br, 1H, NH), 4.83(s, br, 1H, NH), 5.09(s, 2H, CH_2Bzl), 7.35(s, 5H, C_6H_5Bzl)
20	$CDCl_3$	1.29(t, J = 7.0 Hz, 3H, CH_3estr.), 1.56–1.64(m, 5H, 2 × CH_2, 1 × CH), 2.48–2.57(m, 2H, CH_2), 3.15–3.25(m, 2H, CH_2), 3.57(s, br, 1H, CH), 3.98(s, br, 1H, OH), 4.26 (q, J = 12 Hz, 2H, OCH_2), 4.83(d, J = 9 Hz, 1H, NH), 5.01(s, br, 1H, NH), 5.19(s, 2H, CH_2Bzl), 7.51(s, 5H, C_6H_5Bzl)
21	$CDCl_3$	1.02–1.19(m, 6H, 2 × CH_3MeLeu), 1.22(t, J = 4.0 Hz, 3H, CH_{3ester}), 1.28–1.49(m, 1H, CH_2), 1.55(s, 9H, C_4H_9), 1.58–1.84(m, 7H, 2HC_βMeLeu, HC_γMeLeu, 2HC_5, 2HC_6), 1.93(d, br, J = 6 Hz, 2H, 2HC_αMeLeu), 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_2ester), 4.96(s, br, 1H, NH), 5.09(s, 2H, CH_2Bzl), 7.26(s, 1H, NH), 7.34(s, 5H, C_6H_5).
22	$CDCl_3$	1.22–1.31(m, 6H, 2 × CH_3MeLeu), 1.37(t, J = 4.0 Hz, 3H, CH_3ester), 1.39–1.49(m, 1H, CH_2), 1.59–1.94(m, 7H, 2HC_βMeLeu, HC_γMeLeu, 2HC_5, 2HC_6), 1.99(d, br, J = 4.0 Hz, 2H, 2HC_αMeLeu), 2.75(t, J = 9.0 Hz, 2H, CH_2), 3.24(d, J = 4.0 Hz, 3H, N-CH_3), 3.52(s, br, 1H, CH_2), 3.93(s, 1H, OH), 4.15(q, J = 8.0 Hz, 2H, OCH_2ester), 4.96(s, br, 1H, NH), 5.09(s, 2H, CH_2Bzl), 7.34(s, 5H, C_6H_5).

Table 3. *(Continued)*

Compd. No	Solvent	Chemical shifts δ, ppm
23	$CDCl_3$	1.22(t, J = 7.0 Hz, 3H, CH_3ester), 1.39(s, 9H, C_4H_9), 1.48–1.88(m, 6H, $2HC_5$, $2HC_6$, $2HC_\beta$MeLeu), 2.75(t, J = 1.5 Hz, 2H, CH_2), 3.21(d, J = 4.0 Hz, 3H, N-CH_3), 3.83(s, 3H, OCH_3), 4.15 (q, J = 7.0 Hz, 2H, OCH_2ester), 4.93(s, br, 1H, NH), 5.09(s, 2H, CH_2Bzl), 7.26(s, 1H, NH), 7.34(s, 5H, C_6H_5).
24	$CDCl_3$	1.00–2.10(m, 16H, 3 × CH_2, $2HC_\alpha$MeLeu, $2HC_\beta$MeLeu, HC_γMeLeu, 2 × CH_3MeLeu), 1.26(s, 9H, C_4H_9), 2.75(t, J = 2.0 Hz, 2H, CH_2), 3.24(d, J = 4.0 Hz, 3H, N-CH_3), 3.76(s, 3H, OCH_3), 3.84(s, 1H, OH), 5.03(s, br, 1H, NH), 5.09(s, 2H, CH_2Bzl), 6.81–7.10(d, J = 6.0 Hz, 4H, C_6H_4), 7.26(s, 1H, NH), 7.34(s, 5H, C_6H_5).
25	$CDCl_3$	1.16–1.38(m, 8H, $2HC_\alpha$laa, HC_γlaa, 2 × CH_2Ahx, HC_3), 1.42(s, 9H, C_4H_9), 1.52–1.86(m, 6H, 2 × CH_2, $2HC_\beta$laa), 1.86–2.00(m, 4H, 2 × CH_2Ahx), 3.11(d, J = 9.0 Hz, 2H, $2HC_\beta$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_αMeLeu), 4.96(d, J = 9.0 Hz, 1H, NH), 5.09(s, 2H, CH_2Bzl), 6.83–7.10(dd, J = 6.5 Hz, J = 6.5 Hz, 4H, C_6H_4), 7.26(s, br, 1H, NH), 7.34(s, 5H, C_6H_5)

necessary at the P_3 position. The amino group in Phe was substituted by t-butoxycarbonyl (Boc) group that corresponds to a branched alkyl at the P_4 position. This hydrophobic substituent presumably interacts with the hydrophobic site S_4 of renin. In all compounds synthesized by us, synthetic pseudodipeptides were placed at the P_1–P_1′ position that corresponds with the Leu–Val bond, which is cleaved by renin (Fig. 5). They are transition-state analogs of the natural dipeptide Leu–Val. The pseudodipeptide moiety ensures a good affinity to renin and simultaneously resistance to its proteolytic activity. The pseudodipeptides used are derivatives of 4-amino-3-hydroxyacids. With renin, the pseudodipeptides form hydrogen bonds involving the NH and OH groups. Their side chains interact with the hydrophobic pocket S_1. Except for the hydrophobic part, the side chain at P_1 of the pseudodipeptides AHGHA and AAHOA contains polar moieties that could form hydrogen bonds (Fig. 6). We decided to carry out a comparative test of inhibitory activity of the compound **18** that contains a purely hydrophobic benzyl substituent at the P_1 position with that of other compounds that contain pseudodipeptides AHGHA and AAHOA. The compound **18** contains pseudodipeptide AHPPA; its P_1 moiety is a phenylalanine analog. Accordingly, the P_1 moiety of the pseudodipeptide AHGHA **7**, **11** is an analog of L-arginine. Similarly to Arg, it has a basic guanidine structure. S_1 sub-pockets, occurring in the large, hydrophobic pocket, contain fragments, which are hydrogen bond acceptors [44]. Polar character of the sub-pocket can be explained by the charge distribution because of the electronic displacement. Computer-aided molecular charge distribution simulation was carried out with use of 'Gaussian 03' computer program for the compound **7** (Fig. 11). The B3LYP/6-31G(d) method with corresponding databasis was applied for structure geometry optimization and energy computation of the optimized structure. Results of this simulation indicate that N^2 and N^3 nitrogen atoms in nitroguanidine have alcaline properties. On the basis of these observations, we hypothesize the polar nitro group not removed from the obtained inhibitor will facilitate an interaction between the nitroguanidine residue present at P_1 position and the polar sub-pocket at the S_1 site. This additional effect can increase the enzyme-inhibitor interaction.

In order to obtain drugs with potent hypotensive activity, we considered further renin inhibitors, based on **7** and **11**, which contain a modified L-arginine analog without the protective NO_2 group. We expect dual mechanism of action of these drugs—besides of the inhibitory activity described earlier, the L-arginine is expected to be a source of endogenous nitrogen monoxide (NO)—a strong vasodilatory agent [45]. Such a novel class of hybrid drugs, which in addition to their principal hypotensive activity, release also nitrogen monoxide, has been developed recently [46–49]. We assume that because of the presence of guanidine residues, besides their renin inhibitory activity, the compounds **7** and **11**, after removing the protective NO_2 group, may also be NO donors [50]. The compound **25** contains the pseudodipeptide AAHOA; an expanded moiety of the pseudodipeptide at P_1 is an analog of the lysine side chain. The amino group of the lysine side chain was substituted by a group comprising a phenyl ring and an ester linkage, forming urethane (-NH-CO-O-). Such modification allows for a hydrophobic interaction between the phenyl ring and a four-carbon aliphatic chain at the S_1 pocket. The polar urethane moiety presumably forms a hydrogen bond with the renin active site. Because of the possibility of forming additional bonds in sub-pockets of the enzyme's hydrophobic active site by the P_1 moieties of the inhibitor's molecule, the presence of the polar moiety -NH-CO-O- in the side chain, can affect not only activity of the inhibitor but also its bioavailability.

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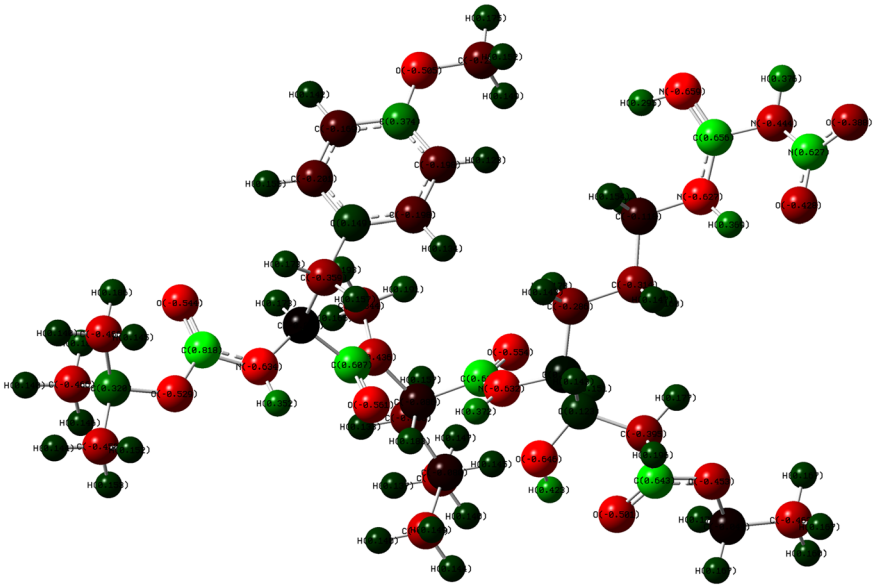

Figure 11. Molecular charge distribution simulation for the compound **7**.

In vitro, the compounds **11** and **18** have been resistant to chymotrypsin. The compounds **7** and **25** were unstable in the presence of chymotrypsin. On the basis of the obtained results of *in vitro* bioactivity and biostability studies, we can verify assumptions concerning diversified structure of the side chain at P_1 position in the synthesized renin inhibitors. Similarly, we can assess the impact of various structure of the C-terminus on activity and stability of the inhibitors **7** and **11**. The thesis that substitution of His at P_2 for MeLeu does not result in lowering inhibitory activity was confirmed. Moreover, such substitution may even increase the activity. It proves the significance of the hydrophobic sub-pocket at the site S_2 for establishing a strong ligand-enzyme bond. The high values of inhibitory activities determined for the renin inhibitors **11**, **18**, and **25** allow for ascertaining lack of adverse intra-molecular interactions between the side chains at P_2 and P_1. Therefore, it could be concluded that there could be a synergistic effect of the hydrophobic substituents P_2 and P_1 at the sub-pockets S_2-S_1'. In two compounds, **18** and **25**, the aromatic benzyl substituent was present at the P_1 position in the side chain. Despite the presence of a polar urethane moiety at this position in the compound **25**, hydrophobicity of the compounds, expressed as log P 8.17, was the highest. It could be explained by the presence of an additional hydrophobic aliphatic chain consisting of four carbon atoms. A slightly less hydrophobic compound **18** (log P 7.71) showed the renin inhibitory activity higher by two orders of magnitude. Higher activity of the compound **18** at comparable hydrophobicity shows significance of the position of an aromatic ring within the region of the P_1 substituent. For the compounds that contain a phenyl ring, the presence of an additional aliphatic chain **25** is not required for increasing their activity. A too large substituent or inadequate location of a polar moiety presumably precludes fitting hydrophobic moieties and the group -HN-CO-O- to suitable sub-pockets of the S_1 site. A drop of activity of the inhibitor **7** by two orders of magnitude as compared with activity of the inhibitor **11** results from a various structure of the C-terminal moiety P_2'–P_3' for these compounds. The presence of a polar ester group at the C-terminus of the inhibitor molecule **7** causes a decrease of hydrophobicity log P to 3.96 and in consequence a lower activity. This observation suggests that the best approach would be to incorporate a non-polar, and most preferably a branched moiety, for example, Ahx–Iaa at the C-terminus, which could enter in hydrophobic interactions at the S_2'–S_3' site. This is inconsistent with the observations based on our previous studies, in which an inhibitor having an ester structure had shown a slightly higher inhibitory activity as compared with an analogous inhibitor provided with Ahx–Iaa at the C-terminus. This inconsistency could be explained by possible difficulty in essential for the activity interaction of the P_1 moiety with the hydrophobic pocket S_1, which is caused by spatial arrangement of the long, flexible, and branched Ahx-Iaa chain. The renin inhibitors **7** and **25** were unstable in the presence of chymotrypsin. Susceptibility of these compounds to hydrolytic degradation could presumably result from the presence of unstable ester bonds at the C-terminus of the molecule **7** and in the urethane moiety of the substituent P_1 **25**. Under the same conditions, the inhibitors **11** and **18**, which do not contain an ester bond, have been stable in the *in vitro* studies. Activity of proteolytic enzymes toward the −CO–O- and −HN–CO–O- bonds that are present in unnatural, synthetic moieties of the molecule, remains hard to explain. It seems likely that hydrolysis of these bonds, −CO-O- in particular, could occur under the influence of weakly basic aquatic environment, in which the analysis was carried out. It remains an open issue for further investigation. It could be assumed that probable degradation of the ester linkage in the urethane moiety of the side chain P_1 in the inhibitor molecule **25** under physiological conditions will have an impact on bioavailability. Removal of the aromatic benzyloxycarbonyl substituent will transform the side chain of the pseudodipeptide AAHOH in the butylamino substituent, as in endogenous lysine. A decrease of hydrophobicity of the inhibitor and an increase of its polarity after ionization of the NH_2 group in the butyloamino substituent can decrease bioavailability. However, the presence of the NH_2 group creates the possibility of forming an additional hydrogen bond within the sub-pocket S_1. Presumably, formation of a similar bond at the same site of the inhibitor molecule **18** has allowed for obtaining a compound with increased renin inhibitory activity as compared with that of the compound **25**.

Conclusion

Two novel, active and stable renin inhibitors Boc-Phe(4-OMe)-MeLeu-AHPPA-Ahx-Iaa (**18**) and Boc-Phe(4-OMe)-MeLeu-AHGHA-Ahx-Iaa (**11**), were developed. Furthermore, the second one, after removing its protective NO_2 group, could be considered as a potential exogenous NO donor. This fact opens up a very interesting prospect for further studies on pharmacotherapy of arterial hypertension.

Conflict of interest

The authors declare that they have no competing financial interests

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