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Novel renin inhibitors containing a non-peptide aminoalkanoyl moiety at P_1-P_1' position

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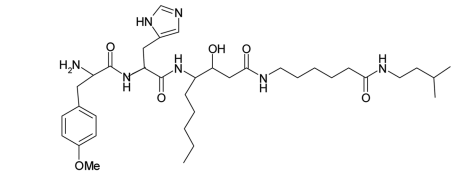
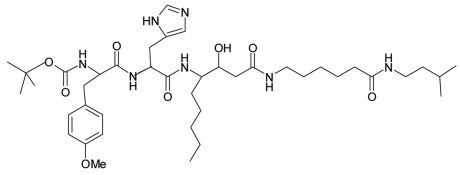
Six novel potential renin inhibitors have been designed and synthesized. All these inhibitors contained an unnatural aminoalkanoyl moiety at the central position P_1- P_1' of the molecule, which is attacked by renin. The moiety consists of pseudodipeptidic units, transition state analogues of a natural dipeptide of the parent substance: 4-amino-3-hydroxybutanoic acid (AHBA), 4-amino-5-(4-ethoxyphenyl)-3-hydroxypentanoic acid (AEPHPA), 4-amino-5-cyclohexyl-3-hydroxypentanoic acid (ACHPA) or 4-amino-3-hydroxynonanoic acid (AHNA). An unnatural moiety, 4-methoxyphenylalanylhistydyl (Phe(4-OMe)-His) has been introduced at the P_3-P_2 position of the obtained compounds. Five compounds contain isoamylamide of 6-aminohexanoic acid (ε-Ahx-Iaa) at the P_2'–P_3' position. One of designed inhibitors has been obtained in the form of an ethyl ester. The *in vitro* renin inhibitory activity of all synthesized compounds is contained within the range 10^{-6} – 10^{-8} M. The compound in the form of an ethyl ester has proven to be the most active ($IC_{50} = 1.3 \times 10^{-8}$ M) but also susceptible to enzymatic degradation. The other five inhibitors were stable to chymotrypsin.

1. Introduction

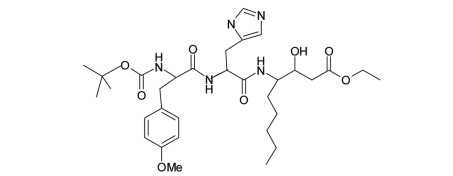
Proper functioning of the renin-angiotensin system (RAS) helps to maintain homeostasis of the body, in particular normalization of blood pressure. Renin, the proteolytic enzyme plays a key role in the first stage of the RAS cascade. By cleaving the peptide bond between Leu and Val (John et al. 2011) in the angiotensinogen molecule it catalyses formation of an inactive decapeptide angiotensin I (Ang I). The next step of octapeptide angiotensin II (Ang II) biosynthesis is catalyzed by the Ang I converting enzyme (ACE). By acting on the AT_1 receptor Ang II is narrowing blood vessels thus increasing blood pressure, stimulates aldosterone secretion and causes fibrosis and hypertrophy of arterial vessels and the ventricular cardiac muscle. Its vasopressive and cardiopressive action contributes to the development of diseases of the cardiovascular and renal systems. RAS-inhibiting drugs are useful in the treatment of arterial hypertension, congestive heart failure, ischemic heart disease and diabetic nephropathy (Carey 2008). The commonly used drugs include Ang I converting enzyme inhibitors (ACEI) and Ang II receptor antagonists (ARB). As by now, only one drug belonging to the group of renin inhibitors, i.e. aliskiren, has been introduced into clinical practice, despite a significant progress in studies on hormonal mechanisms for regulating blood pressure (De Mello et al. 2013; Jadhav et al. 2012; Jansen et al. 2008). This is related to difficulties in obtaining adequate correlation between the therapeutic utility parameters: inhibitory activity, bioavailability after oral administration, stability in extracellular fluids and tissues, duration of activity and solubility under physiological pH conditions. However, despite these difficulties, further studies on designing and synthesizing novel drugs from this group are well-founded. In comparison with ACEI and ARB, renin inhibition consists in blocking the RAS at an early stage of its functioning. Such an activity is better related to the causal mechanism of Ang II formation. Furthermore, administration of renin inhibitors does not result in increased plasma renin activity (PRA) (Bonanni and Dalla Vestra 2012; Ichihara et al. 2010), as in the case of administering ACEI and ARB. Aliskiren significantly reduces PRA in patients treated with ACEI and ARB (Kirimura et al. 2005; Yarows 2010; O'Brien et al. 2007; Stanton et al. 2009). Presumably it reduces expression of the prorenin (Feldman et al. 2008) receptor that upon activation enhances catalytic conversion of angiotensinogen (Nguyen et al. 2002). It seems also likely that renin-substrate specificity would allow for decreasing the risk of developing numerous adverse effects. Blocked formation of Ang I by the renin inhibitors prevents formation Ang II by alternative pathways, where conversion of Ang I into Ang II is not catalyzed by ACE but by other enzymes (Stanton et al. 2009). The alternative pathways of Ang II formation are not blocked by ACEI. At present, studies on the design and synthesis of novel renin inhibitors are focused on identifying compounds that could be transition state analogues of the natural substrate and on searching for compounds that contain heterocyclic moieties, such as derivatives of isoxazole (Fournier et al. 2012), piperidine (Chen et al. 2011; Ostermann et al. 2013), or pyrrolidine (Lorthiois et al. 2013). Introduction of aliskiren – the first drug from the generation of non-peptidic renin inhibitors – into the medical practice has proven advisability of designing transition state analogues that are modified moieties of the substrate. The structure of the aliskiren molecule resembles that of the P_3-P_2-P_1-P_1'-P_2' angiotensinogen moiety and therefore, one may



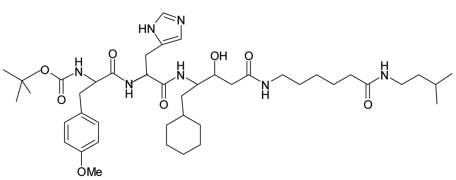
4-Methoxyphenylalanylhistidil-(3*S*, 4*S*)-4-amino-3-hydroxynonanoyl-ε-aminohexanoic acid isoamyloamide: H – Phe (4 – OMe) – His – AHNA – εAhx – Iaa (**13**)



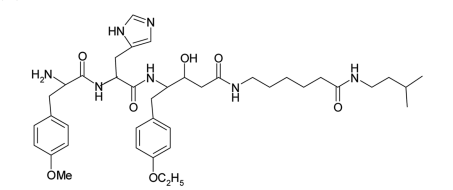
[*N*-(*t*-Butoxycarbonyl)-4-methoxyphenylalanyl]-histidil-(3*S*, 4*S*)-4-amino-3-hydroxynonanoyl-ε-aminohexanoic acid isoamyloamide: Boc – Phe (4 – OMe) – His – AHNA – εAhx – Iaa (**20**)



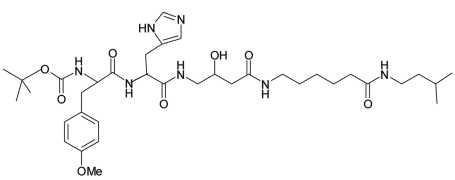
[*N*-(*t*-Butoxycarbonyl)-4-methoxyphenylalanyl]-histidil-(3*S*, 4*S*)-4-amino-3-hydroxynonanoic-acid ethyl ester: Boc – Phe (4 – OMe) – His – AHNA – OEt (**21**)



[*N*-(*t*-Butoxycarbonyl)-4-methoxyphenylalanyl]-histidil-(3*S*, 4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoyl-ε-aminohexanoic acid isoamyloamide: Boc – Phe (4 – OMe) – His – ACHPA – εAhx – Iaa (**29**)



4-Methoxyphenylalanylhistidil-(3*S*, 4*S*)-4-amino-5-(4-ethoxyphenyl)-3-hydroxypentanoyl-ε-aminohexanoic acid isoamyloamide: H – Phe (4 – OMe) – His – AEPHPA – εAhx – Iaa (**35**)



[*N*-(*t*-Butoxycarbonyl)-4-methoxyphenylalanyl]-histidil-(3*S*, 4*S*)-4-amino-3-hydroxybutanoyl-ε-aminohexanoic acid isoamyloamide: Boc – Phe (4 – OMe) – His – AHBA – εAhx – Iaa (**43**)

Fig. 1: Structures of the compounds synthesized

suppose that the search for renin inhibitors that contain pseudodipeptide fragments could be a route that offers new prospects for contemporary hypertensiology. In continuation of our earlier studies, we used the previously obtained active transition state analogue that contained 4-amino-3-hydroxy-6-methylheptanoic acid (Sta) at P_1-P_1' position as the starting point for our further research, e.g.: Boc-Phe(4-OMe)-His-Sta-Ahx-Iaa featuring IC_{50} 7×10^{-8}M (Paruszewski et al. 1994).

We have designed and synthesized six novel compounds that are potential renin inhibitors (**13**, **20**, **21**, **29**, **35**, **43**), as shown in Fig. 1.

The compounds have been designed on the basis of the structure of 8–13 angiotensinogen moiety (see the Fig. 2) that binds to renin under physiological conditions.

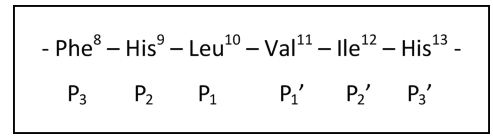


Fig. 2: The 8–13 angiotensinogen moiety

Instead of Leu-Val, all these compounds contain a stable aminoalkanoyl moiety at the P_1-P_1' position that is susceptible to proteolytic activity of renin. The moieties consist of pseudodipeptide transition state analogues that are derivatives of β-hydroxy-γ-amino acids:

4-Amino-3-hydroxynonanoic acid (AHNA) (**13**, **20**, **21**),
4-Amino-5-cyclohexyl-3-hydroxypentanoic acid (ACHPA) (**29**),
4-Amino-5-(4-ethoxyphenyl)-3-hydroxypentanoic acid (AEPHPA) (**35**),
4-Amino-3-hydroxybutanoic acid (AHBA) (**43**).

We have assumed that the proposed structural diversification of hydrophobic groups in the used pseudodipeptides: AHNA, ACHPA, AEPHPA, AHBA would allow to determine the optimal interaction of these moieties with the hydrophobic pocket S_1 of the renin active site. This is supported by reports (e.g. Rahuel et al. 2000) concerning the complex structure of the large S_3-S_1 hydrophobic pocket of renin. The pocket contains sub-pockets capable of binding to aliphatic chains and cycloaliphatic or aromatic rings, including those bearing small polar groups (Winiecka et al. 2014). Assuming that our studies would contribute to the discussion concerning the impact of various structures of substituents at P_1 on the inhibitory activity, we have designed compounds bearing aliphatic (**13**, **20**, **21**)**,** cycloaliphatic (**29**) and aromatic (**35**) substituents as well as compound lacking a substituent at P_1 (**43**)**.** The compounds (**13**, **20**, **21**) contain an aliphatic, unbranched side chain (the C_5-C_9 moiety in the AHNA molecule) at P_1. Presumably, a long, flexible chain adapts well to the hydrophobic pocket S_1 after assuming an adequate suitable conformation, similarly as a cyclohexyl substituent at position 5 of the ACHPA molecule of (**29**). This is confirmed by a high inhibitory activity of previously obtained inhibitors that contained this pseudodipeptide (Boger et al. 1985; Dellaria et al. 1987). The renin inhibitors bearing a cyclohexyl or phenyl substituent at this position have shown high inhibitory activity toward rennin: Iva-His-Pro-Phe-His-ACHPA-Leu-Phe-NH_2 (IC_{50} 10^{-10}M) and Iva-His-Pro-Phe-His-APHPA-Leu-Phe-NH_2 (IC_{50} 10^{-9}M).

The ACHPA containing inhibitor has shown fifteen-fold higher inhibitory activity toward the human plasma renin than an APHPA-containing inhibitor (Plattner et al. 1988). This favourable biological effect has resulted from the possibility of rotating a C-C bond around the C_5-carbon atom and assuming a "chair" conformation by the cyclohexane ring in ACHPA. This results in a better match to hydrocarbon radicals in the hydrophobic pocket as compared to a flat phenyl ring in APHPA. A comparison of biological activity of an inhibitor (**29**) bearing a cycloaliphatic part to that of a compound (**35**) that contained an aromatic 4-ethoxyphenyl substituent at position 5 of the AEPHPA molecule has seemed interesting. Theoretically, a lower affinity of the flat, rigid aromatic ring to the hydrophobic pocket could be balanced by the presence of a small aliphatic ethoxy substituent at position 4 of the phenyl ring, due to the possible existence of specific sub-pockets within the S_1 region. Due to its probable participation in a hydrogen bond, the presence of a moderately polar ethoxy group in the side chain may impact not only activity but also pharmacokinetic parameters such as

Table 1: Stability and activity of the synthesized compounds

Compound	IC_{50} [M]	% (chymotrypsin remaining after 2h at 37 °C)
Phe (4-OMe)-His-AHNA-Ahx-Iaa **(13)**	0.9×10^{-6}	Stable
Boc- Phe (4-OMe)-His-AHNA-Ahx-Iaa **(20)**	2.2×10^{-6}	Stable
Boc-Phe (4-OMe)-His-AHNA- OEt **(21)**	1.3×10^{-8}	3.1
Boc- Phe (4-OMe)-His-ACHPA-Ahx-Iaa **(29)**	5.2×10^{-6}	Stable
Phe (4-OMe)-His-AEPHPA-Ahx-Iaa **(35)**	1.5×10^{-7}	Stable
Boc- Phe (4-OMe)-His-AHBA-Ahx-Iaa **(43)**	1.4×10^{-6}	Stable

Table 2: Physicochemical and analytical properties of the synthesized compounds

Compd.	Structure	Formula m.w.	Yield (%)	M.p. (°C)	$[\alpha]_D^{20}$ (c,MeOH)	TLC,$R_{f\ (m.ph.)*}$	HPLC (% of purity)
(3)	Boc-Phe(4-OMe)-His(N^{im}Trt)-OMe	$C_{41}H_{44}O_6N_4$ 688.84	80.0	73 - 76	+ 18.2 (1.1)	0.64 (A) 0.21 (E)	–
(4)	Boc-AHNA-OEt	$C_{16}H_{32}O_5$ N 317.41	85.0	49 - 50	−4.7 (1.4)	0.38 (B)	–
(8)	Boc-Phe(4-OMe)-His(N^{im}-Trt)-AHNA-OEt	$C_{51}H_{63}O_8N_5$ 873.60	47.0	Oil	+ 28.0 (1.2)	0.64 (A)	–
(12)	Boc-Phe(4-OMe)-His(N^{im}Trt)-AHNA-Ahx-Iaa	$C_{60}H_{81}O_8N_7$ 1028.36	23.0	Oil	+ 23.0 (1.0)	0.68 (A)	99.04
(13)	H-Phe(4-OMe)-His-AHNA-Ahx-Iaa x HCl	$C_{36}H_{60}O_6N_7Cl$ 722.48	89.0	Oil	+ 50 (1.0)	0.37 (F)	90.07
(15)	Boc-His(N^{im}Bzl)-AHNA-OEt	$C_{29}H_{44}O_6N_4$ 662.82	61.0	97 - 98	−16.0 (1.0)	0.42 (E)	–
(17)	Boc-Phe(4-OMe)-His(N^{im}Bz)l-AHBA-OEt	$C_{32}H_{45}O_8N_5$ 627.77	34.0	Semisolid	−29.4 (1.0)	0.67 (C)	–
(19)	Boc-Phe(4-OMe)-His(N^{im}Bzl)-AHNA-Ahx-Iaa	$C_{48}H_{71}O_8N_7$ 874.12	19.0	Semisolid	−26.4 (1.2)	0.71 (D)	96.97
(20)	Boc-Phe(4-OMe)-His-AHNA-Ahx-Iaa	$C_{21}H_{67}O_8N_7$ 786.15	82.0	Semisolid	−28.0 (1.0)	0.46 (C)	95.42
(21)	Boc-Phe(4-OMe)-His-AHNA-OEt	$C_{32}H_{49}O_8N_5$ 631.86	78.0	Semisolid	+ 30.0 (1.3)	0.64 (B)	93.60
(24)	Boc-His(N^{im}Bzl)-ACHPA-OEt	$C_{31}H_{46}O_7N_4$ 586.71	67.0	144 - 145	−34.8 (1.0)	0.41 (A)	-
(26)	Boc-Phe(4-OMe)-His-(N^{im}Bzl)-ACHPA-OEt	$C_{39}H_{57}O_8N_5$ 747.44	48.0	Semisolid	−36.0 (1.2)	0.38 (A)	-
(28)	Boc-Phe(4-OMe)-His(N^{im}Bzl)-ACHPA-Ahx-Iaa	$C_{50}H_{75}O_8N_7$ 902.20	26.0	Semisolid	−25.0 (1.0)	0.35 (A)	99.57
(29)	Boc-Phe(4-OMe)-His-ACHPA-Ahx-Iaa	$C_{43}H_{69}O_8N_7$ 812.19	49.0	Semisolid	−20 (1.1)	0.26 (B)	97.63
(30)	Boc-AEPHPA-OEt	$C_{20}H_{31}O_6N$ 381.48	78.0	83 - 85	−22.0 (1.0)	0.16 (B)	-
(32)	Boc-Phe(4-OMe)-His(N^{im}Trt)-AEPHPA-OEt	$C_{55}H_{63}O_8N_5$ 933.65	39.0	94 - 96	−20.0 (1.0)	0.62 (A)	-
(34)	Boc-Phe(4-OMe)-His(N^{im}Trt)-AEPHPA-Ahx-Iaa	$C_{64}H_{81}O_9N_7$ 1091.41	28.0	Semisolid	−15.0 (1.1)	0.71 (A)	99.78
(35)	H-Phe(4-OMe)-His-AEPHPA-Ahx-Iaa xHCl	$C_{40}H_{60}O_7N_7Cl$ 786.52	64.0	Semisolid	−8.9 (1.0)	0.64 (F)	99.53
(36)	Boc-AHBA-OEt	$C_{11}H_{21}O_5N$ 247.30	60.0	Semisolid	−14.7 (1.0)	0.10 (B)	-
(38)	Boc-His(N^{im}Bzl)-AHBA-OEt	$C_{22}H_{34}O_6N_4$ 450.56	8.0	117 - 123	−21.5 (1.1)	0.63 (D)	-
(40)	Boc-Phe(4-OMe)-His(N^{im}Bzl)-AHNA-OEt	$C_{36}H_{53}O_8N_5$ 707.36	32.0	Oil	−31.3 (1.0)	0.57 (C)	-
(42)	Boc-Phe(4-OMe)-His(N^{im}Bzl)-AHBA-Ahx-Iaa	$C_{43}H_{63}O_8N_7$ 806.04	21.0	Semisolid	−22.0 (1.2)	0.75 (A)	95.96
(43)	Boc-Phe(4-OMe)-His-AHBA-Ahx-Iaa	$C_{36}H_{57}O_8N_7$ 716.00	39.0	Semisolid	−27.0 (1.0)	0.52 (B)	95.45

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

* Mobile phase systems (v/v) were: CH_3Cl-MeOH 95:5 (A), CH_3Cl-MeOH 50:50 (C), CH_3Cl-MeOH 90:10 (D), CH_3Cl-MeOH 98:2 (E), Hexane-AcOEt 80:20 (B), BAW (F)

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

Compd.	Solvent	Chemical shifts δ, ppm
(3)	$CDCl_3$	1.34(s, 9H, C_4H_9); 2.86–3.12(m, 4H, 2xCH_2); 3.59(s, 3H, OCH_3ester); 3.72(s, 3H, OCH_3eter); 4.11(q, 1H, HC_2); 4.39(d, J = 5 Hz, 1H, NH); 4.77(q, 1H, CH), 5.23(d, J = 6 Hz, 1H, NH); 6.53(s, 1H,CH^{im}); 7.02–7.38(m, 20H, C_6H_4, 3xC_6H_5, CH^{im})
(4)	$CDCl_3$	0.88(t, 3H, CH_3); 1.24–1.40(m, 7H, CH_3ester, 2xCH_2); 1.44(s, 9H, C_4H_9); 2.43–2.60(m, 2H, CH_2); 3.50(s, 1H, NH); 4.06(s, 1H, CH); 4.16(q, 2H, OCH_2); 4.77(d, J = 9.6, 1H, NH)
(8)	$CDCl_3$	0.87(t, 3H, CH_3ester); 1.10–1.38(m, 7H, CH_3, 2xCH_2) 1.41(s, 9H, C_4H_9); 2.96–3.01(m, 2H, CH_2His); 3.58–3.65 (m, 2H, CH_2 Phe(4-OMe)); 3.77(s, 3H, OCH_3); 4.13(q, 2H, OCH_2); 4.80–4.87(m, 1H, CH); 6.03(d, J = 9.6 Hz, 1H, NH); 6.19(d, J = 9.6 Hz, 1H, NH); 6.81(d, J = 8.4 Hz, 1H, NH); 7.10–7.50(m, 21H, C_6H_4, 3xC_6H_5, 2xCH^{im})
(12)	$CDCl_3$	0.82–1.56(m, 26H, C_4H_9, 2HC_5, 2xCH_3Iaa, 2HC_βIaa, HCγIaa, 3xCH_2Ahx); 1.92(d, J = 10.2 Hz, 2H CH_2); 3.4–3.6(m, 2H, CH_2His); 3.8(q, 2H, 2xCH); 4.02–4.40(m, 5H, CH_2Phe(4-OMe), OCH_3); 6.20–6.22(dd, J = 9.6 Hz, J = 9.6 Hz, 1H, NH); 6.66(d, J = 8.4 Hz, 2H, 2HC^{im}); 7.24–8.20(m, 21H, C_6H_4, 3xC_6H_5, 2xHC^{im}); 7.10–7.16(m, 1H, NH).
(13)	$CDCl_3$	0.82–1.56(m, 17H, 2HC_5, 2xCH_3 Iaa, 2HC_βIaa, HCγIaa, 3xCH_2Ahx); 1.92(d, J = 10.2 Hz, 2H, CH_2); 3.4–3.6(m, 2H, CH_2His); 3.8(q, 2H, 2xCH); 4.02–4.40(m, 5H, CH_2Phe(4-OMe), OCH_3); 6.20–6.22(dd, J = 9.6 Hz, J = 9.6 Hz, 1H, NH); 6.66(d, J = 8.4 Hz, 2H, 2HC^{im}); 7.24–8.20(m, 6 H, C_6H_4, 2xHC^{im}); 7.10–7.16(m, 1H, NH).
(15)	$CDCl_3$	0.85(t, 3H, CH_3), 1.18–1.38(m, 16H, C_4H_9, CH_3ester, 2xCH_2); 2.32–2.52(m, 2H, 2HC_2); 2.88–3.22(m, 2H, 2HC_β); 4.5(s, 1H, HC_3); 5.11(d, J = 5.2 Hz, 2H, CH_2Bzl); 5.4–5.73(m, 4H, HC_αHis, CH_3); 5.92(s, 1H, NH); 6.16(s, 1H, NH); 6.83(d, J = 8.7 Hz, 1H, CH^{im}); 7.03–7.90(m, 8H, C_6H_5, CH, 2CH); 7.68(d, J = 10.2 Hz, 1H, HC^{im})
(17)	$CDCl_3$	1.25–1.42(m, 12H, C_4H_9, CH_3ester); 1.9(d, J = 9 Hz, 2H, 2HC_2); 3.05(d, J = 5.4 Hz, 2H, 2HC_βHis); 3.4–3.8(m, 9H, CH_2Phe, CH_2Bzl, HCαHis, HC_3, OCH_3); 4.95–4.38(m, 2H, OCH_2); 4.55(d, J = 7.2 Hz, 1H, HC); 4.95(d, J = 7.2 Hz, 1H, HC); 6.78- 7.13(m, 7H, C_6H_5, 2HC^{im})
(19)	$CDCl_3$	0.76–0.96(m,6H, 2xCH_3Iaa); 1.02–1.76(m, 26H, 2HC_αIaa, HCγIaa, 2HC_3, C_4H_9, 2HC_2, 2HC_5, 2HC_βIaa, 3xCH_2Ahx); 2.43(s, 2H, 2HC_β); 2.9–3.1(m, 2H, CH_2His); 3.31–3.50(s, br, 1H, CH); 3.72(s, 3H, OCH_3); 3.60–3.75(m, 5H, CH_2Phe, CHPhe, CH_2Bzl); 3.90(d, J = 9 Hz, 1H, CH); 4.48(s, 1H, NH); 4.95(s, 1H, NH); 6.72(d, J = 7.8 Hz, 1H, HC^{im}); 7.20–7.32(m, 11H, C_6H_4, C_6H_5, 2HC); 7.33(d, J = 4,8 Hz, 1H, HC^{im})
(20)	$CDCl_3$	0.76–0.96(m,6H, 2xCH_3Iaa); 1.02–1.76(m, 26H, 2HC_αIaa, HCγIaa, 2HC_3, C_4H_9, 2HC_2, 2HC_5, 2HC_βIaa, 3xCH_2Ahx); 2.43(s, 2H, 2HC_β); 2.90–3.10(m, 2H, CH_2His); 3.31–3.50(s, br, 1H, CH); 3.72(s, 3H, OCH_3); 3.60–3.75(m, 3H, CH_2Phe, CHPhe); 3.90(d, J = 9 Hz, 1H, CH); 4.48(s, 1H, NH); 4.95(s, 1H, NH); 6.34(d, J = 9 Hz, 1H, HC^{im}); 7.26(s, 2HC); 7.50–7.73(m, 1H, HC^{im})
(21)	$CDCl_3$	0.95–1.25(m, 12H, CH_3ester, C_4H_9); 2.43(s, 2H, 2HC_β); 2.90–3.10(m, 2H, CH_2His); 3.30–3.50(s, br, 1H, CH); 3.72(s, 3H, OCH_3); 3.60–3.75(m, 3H, CH_2Phe, CHPhe); 3.90(d, J = 9 Hz, 1H, CH); 4.12(q, J = 7, 2H, OCH_2); 4.48(s, 1H, NH); 4.95(s, 1H, NH); 6.72(d, J = 7.8 Hz, 1H, HC^{im}); 7.2–7.32(s, 2H, 2HC); 7.45–7.76(m, 1H, HC^{im})
(24)	$CDCl_3$	0.72–1.84(m, 13H, C_6H_{11}, 2HC_5); 1.24(t, 3H, CH_3); 1.42(s, 9H, C_4H_9); 2.28–2.56(m, 2H, 2HC_2); 2.92–3.12(m, 2H, 2HCβ); 4.05–4.20(m, 2H, OCH_2); 4.26–4.36(m,1H, 1HC_3); 5.03(d, J = 2 Hz, 2H, CH_{2Bzl}); 6.26(d, J = 4 Hz, 1H, NH); 6.59(d, J = 10 Hz, 1H, NH); 6.75(s, 1H, H C^{im}); 7.26(s, 5HC_6H_5); 7.5(s, 1H, HC^{im}).
(26)	$CDCl_3$	0.72–1.86(m, 15H, C_6H_{11}, 2HC_2, 2HC_5); 1.25(t, 3H, CH_3); 1.36(s, 9H, C_4H_9); 2.73–3.24(m, 4H, 2HCβHis, HCαPhe, HC_4); 3.78(s, 3H, OCH_3); 4.14(q, 2H, OCH_2); 4.20–4.73(m, 4H, 2HC_βPhe, HCαHis, HC_3); 5.01(s, 2H, CH_2Bzl); 6.64–6.91(m, 3H, CH^{im}, 2xNH); 6.92–7.20(m, 4H, C_6H_4); 7.27(s, 5H, C_6H_5); 7.33(s, 1H, CH^{im}); 8.25(d, J = 12 Hz, 1H, NH)
(28)	$CDCl_3$	0.72–1.85(m, 28H, C_6H_{11}, 2HC_5, 2xCH_3Iaa, 2HC_βIaa, HCγIaa, 3xCH_2Ahx), 1.36(s, 9H, C_4H_9); 1.86–2.00(d, J = 12 Hz, 2H, 2HC_2); 3.47(s, br, 2H, 2HC_βHis); 4.08(s, 3H, OCH_3); 4.24–4.51(m, 10H, CH_2Phe, HC α His, HC_3, 2HCαIaa, 2xCH_2Ahx); 4.76(s, 2H, CH_2 Bzl); 6.64(d, J = 8 Hz, 4H, C_6H_4); 6.82(d, J = 8 Hz, 1H, NH); 7.78–7.94(m, 4H, 2x HC^{im}, 2xNH); 8.08(d, J = 8 Hz, 1H, NH)
(29)	$CDCl_3$	0.72–1.85(m, 28H, C_6H_{11}, 2HC_5, 2xCH_3Iaa, 2HC_βIaa, HCγIaa, 3xCH_2Ahx), 1.36(s, 9H, C_4H_9); 1.86–2.00(d, J = 12 Hz, 2H, 2HC_2); 3.47(s, br, 2H, 2HC_βHis); 4.08(s, 3H, OCH_3); 4.24–4.51(m, 10H, CH_2Phe, HCαHis, HC_3, 2HCαIaa, 2xCH_2Ahx); 6.64(d, J = 8 Hz, 4H, C_6H_4); 6.82(d, J = 8 Hz, 1H, NH); 7.78–7.94(m, 4H, 2x HC^{im}, 2xNH); 8.08(d, J = 8 Hz, 1H, NH)
(30)	$CDCl_3$	1.24(t, 3H, CH_3ester); 1.39(t, 3H, CH_3eter); 1.41(s, 9H, C_4H_9); 2.32–2.35(m, 1H, CH); 2.53–2.62(m, 1H, CH); 2.84(d, J = 9 Hz, 2H, 2HC_5); 3.62–3.96(m, 2H, CH_2); 4.00(q, 2H, OCH_2ester); 4.13(q, 2H, OCH_2ester); 4.92(d, J = 10 Hz, 1H, NH); 6.81; 7.14(dd, J = 8 Hz, 8 = Hz, 4H, C_6H_4)
(32)	$CDCl_3$	1.99(t, 3H, CH_3); 1.33(s, 9H, C_4H_9); 1.35(t, 3H, CH_3); 2.39–2.57(m, 2H, CH_2); 2.75–3.02(m, 5H, 2xCH_2, CH); 3.41(d, J = 3,41 Hz, 1H, CH); 3.76(s, 3H, OCH_3); 3.94–4.12(m, 6H, 2xOCH_2, CH_2); 4.30(q, 1H, CH); 4.66–4.78(m, 1H, CH); 5.41(s, br, 1H, NH); 6.78–7.45(m, 21H, 3xC_6H_5, C_6H_4, 2xCH^{im}); 7.72(s br, 1H, NH); 7.87(s, br, 1H, NH)
(34)	$CDCl_3$	0.87(d, J = 6 Hz, 6H, 2xCH_3Iaa); 1.12–1.46(m, 8H, 2HC_αIaa, HCγIaa, HC_3, 2xCH_2Ahx); 1.24(s, 9H, C_4H_9); 1.47–1.83(m, 12H, 2HC_2, 2HC_5, 2HC_βIaa, 3xCH_2Ahx); 2.16(t, 3H, CH_3); 2.62–3.00(m, 2H, 2xHC); 3.79(s, 3H, OCH_3); 3.89(s, 2H, CH_2); 3.99(q, 2H, OCH_2); 4.18(q, 1H, HC_4); 4.51(d, J = 5 Hz, 2H, CH_2); 5.08(s, 1H, NH); 5.93(s, 1H, NH); 6.65(s, 1H, NH); 6.75–6.94(m, 5H, C_6H_4, NH); 6.95–7.50(m, 21H, 3xC_6H_5, C_6H_4, 2xCH^{im}); 9.39(s, br, 1H, NH)

Table 3: (*Continued*)

Compd.	Solvent	Chemical shifts δ, ppm
(35)	$CDCl_3$	0.87(d, J = 6 Hz, 6H, 2xCH_3Iaa); 1.12–1.46(m, 8H, 2HC_αIaa, HCγIaa, HC_3, 2xCH_2Ahx); 1.47–1.83(m, 12H, 2HC_2, 2HC_5, 2HC_βIaa, 3xCH_2Ahx); 2.16(t, 3H, CH_3); 2.62–3.00(m, 2H, 2xHC); 3.79(s, 3H, OCH_3); 3.89(s, 2H, CH_2); 3.99(q, 2H, OCH_2); 4.18(q, 1H,HC_4); 4.51(d, J = 5 Hz, 2H, CH_2); 5.08(s, 1H, NH); 5.93(s, 1H, NH); 6.65(s, 1H, NH); 6.75–6.94(m, 5H, C_6H_4, NH); 7.62(s, 6H, C_6H_4, 2xCH^{im}); 9.39(s, br, 1H, NH)
(36)	$CDCl_3$	1.20–1.31(m, 3H, CH_3ester); 1.45(s, 9H, C_4H_9); 2.41–2.58(m, 2H, CH_2); 3.03–3.39(m, 2H, CH_2); 4.06–4.28(m, 3H, OCH_2, CH); 5.04(s, br, 1H, NH)
(38)	$CDCl_3$	1.25(s, 3H, CH_3ester); 1.41(s, 9H, C_4H_9);1.90–1.94(m, 2H, CH_2); 3.11(d, 2H, CH_2); 4.07–4.20(m, 3H, HCβ, OCH_2); 4.45(s, br, 1H, CHαHis); 5.09(s, 2H, CH_2Bzl); 5.85(s, br, 1H, NH); 5.88(s, br, 1H, NH); 6.74(s, 1H, HC^{im}); 7.15–7.37(m, 5H, C_6H_5), 7.74(s, 1H, HC^{im}).
(40)	$CDCl_3$	0.85(t, 3H, CH_3ester); 1.2–1.34(m, 16H, C_4H_9, 2HC_2, 2HC_5, CH_3); 1.50–16(m, 2H, 2HC_2); 2.26–2.44(m, 2H, 2HC_β); 2.78–3.18(m, 5H, HC_αHis, HC_3, CH_2Bzl, HC_αPhe); 3.74(s, 3H, OCH_3); 4.12(q, 2H, OCH_2); 4.30(d, J = 6 Hz, 1H, NH); 4.76(q, 1H, HC_3); 5.06(d, J = 12 Hz, 2H, CH_2Bzl); 5.36(d, J = 6,4 Hz, 1H, NH); 5.60–5.90(s,br, 4H, HC_αHis, CH_3); 7.06(d, J = 8,6 Hz, 1H, HC^{im}); 7.14–7.40(m, 12H, C_6H_4, C_6H_5, CH, 2HC); 7.75(d, J = 4 Hz, 1H, HC^{im}); 8.04(d, J = 6,6 Hz, 1H, NH).
(42)	$CDCl_3$	0.91–1.92(m, 26H, C_4H_9, 2HC_5, 2xCH_3Iaa, 2HC_βIaa, HCγIaa, 3xCH_2Ahx); 1.93–2.15(m, 4H, 2HC_2 Ahx, 2HC_3(Phe-4OMe); 3.01(s, br, 2H, 2HC_βHis); 3.41–3.59(m, 1H, HC); 3.67–3.83(m, 5H, OCH_3, CH_2Phe); 4.12–4.19(m, 4H, CH_2Bzl, 2HCPhe); 4.50(d, J = 6,3 Hz,1H, HC), 4.90(d, J = 7,2 Hz, 1H, HC); 5.12–5.38(m, 7H, C_6H_5, $2HC^{im}$).
(43)	$CDCl_3$	0.91–1.92(m, 26H, C_4H_9, 2HC_5, 2xCH_3Iaa, 2HC_βIaa, HCγIaa, 3xCH_2Ahx); 1.93–2.15(m, 4H, 2HC_2 Ahx, 2HC_3(Phe-4OMe); 3.01(s, br, 2H, 2HC_βHis); 3.41–3.59(m, 1H, HC); 3.67–3.83(m, 5H, OCH_3, CH_2Phe); 4.00–4.38(m, 2H, 2HCPhe); 4.51(d, J = 6.3 Hz, 1H, HC), 4.9(d, J = 7.2 Hz, 1H, HC); 4.85–5.12 (m, $2HC^{im}$).

solubility at physiological pH value and bioavailability. Designing the compound (**43**) without any side chain at P_1 (AHBA) should provide an answer to the issue of the impact of the lack of a substituent on the strength of enzyme-ligand interaction. All compounds obtained by us have an unnatural Phe(4-OMe)-His moiety at the P_3-P_2 position. In the gastrointestinal tract, the natural dipeptide Phe-His at P_3-P_2 undergoes chymotrypsin-catalyzed proteolysis. In order to increase stability and improve bioavailability, we have modified the P_3-P_2 moiety by substituting the phenyl ring of Phe with a methoxy group at position 4. Such modification significantly reduces the affinity of the inhibitor to chymotrypsin, with no adverse effect on binding to the active site of renin (Boger et al. 1985). The presence of a rigid, flat aromatic ring at position P_3 is required for obtaining active compounds. This is due to the fact that the most important structural element of the active position S_3 within the renin active site is a sub-pocket S_{3sp} specific for the phenyl ring (Chen et al. 2010; Sund et al. 2011). This has been confirmed by the biological activity of inhibitors containing a 4-methoxyphenyl ring (Paruszewski et al. 2002) at this position, which had been synthesized by us previously. On the other hand, as it has been proven by experiments carried out previously, lack of a ring substituent at position P_3 is resulting in a lack of inhibitory activity of potential renin inhibitors (Winiecka et al. 2010). The compounds discussed in the present paper (**13, 20, 21, 29, 35, 43**) contain histidine at position P_2. The inhibitors, recently synthesized by us contained aryl derivatives of histidine (Winiecka et al. 2014) at this position. In our previous study we have assumed that substitution of a nitrogen atom in the imidazole ring of His for a hydrophobic group, e.g. benzyl or trityl group, could contribute to enhancing the enzyme-inhibitor interactions. We have made this assumption due to the presence of a large hydrophobic pocket that includes also the S_2 site of the enzyme. Lack of inhibitory activity of these inhibitors in the *in vitro* studies allowed us to suppose that the presence of arylhistidine derivatives at P_2 makes binding the inhibitor's molecule to renin difficult (Winiecka et al. 2014). In order to verify this hypothesis, we decided to build-in non-substituted His at the position P_2 in the currently tested compounds. In these compounds (**20, 21, 29, 43**), a t-butoxycarbonyl (Boc) substituent has been provided at the N-terminus of the molecule. This is due to the assumption that a hydrophobic moiety having a branched alkyl chain may improve lipophilicity and absorption from the gastrointestinal tract. Lack of the Boc substituent in compounds (**13, 35**) should explain the impact of this substituent on the binding strength of renin inhibitors to the enzyme and on their inhibitory activity. Within the scope of the studies carried out by our team, a number of inhibitors has been synthesized, in which 6-aminohexanoic acid (εAhx), a unnatural amino acid of a linear and flexible structure has been placed directly after P_1-P_1' position. The branched alkylamide of an unnatural amino acid (Ahx-Iaa), placed at the P_2'-P_3' position, protects the C-terminus of the molecule against enzymatic degradation. Furthermore, it could affect the binding strength between the inhibitor and renin due to a hydrophobic interaction with the S_2'- S_3' position of the renin's active site. This hypothesis has been confirmed by a number of active and stable inhibitors that contained isoamylamide of 6-aminohexanoic acid (Ahx-Iaa) at the C-terminus, which had been obtained previously (Paruszewski et al. 1993, 1994, 1997, 2005). Therefore, we designed five compounds (**13, 20, 29, 35, 43**) containing Ahx-Iaa at the C-terminus. However, opinions of various researchers regarding structural requirements for renin inhibitors at the C-terminus are divided. In order to verify whether the presence of a hydrophobic structure at the P_2'-P_3' position is necessary, compound (**21**) has been deprived of this part of the molecule. The size of molecule (**21**) has been restricted to the P_3-P_2-P_1-P_1' chain in the form of an ethyl ester at the C-terminus.

2. Investigations, results and discussion

In vitro renin inhibitory activity of all compounds obtained in this study was within the range 10^{-6}-10^{-8} M. Boc-Phe(4-OMe)-His-AHNA-OEt (**21**) has proven to be the most potent compound (IC_{50} 1.3×10^{-8}M).

Activity of compound (**21**), which is larger by two orders of magnitude than that of compound (**20**) differing only by the structure

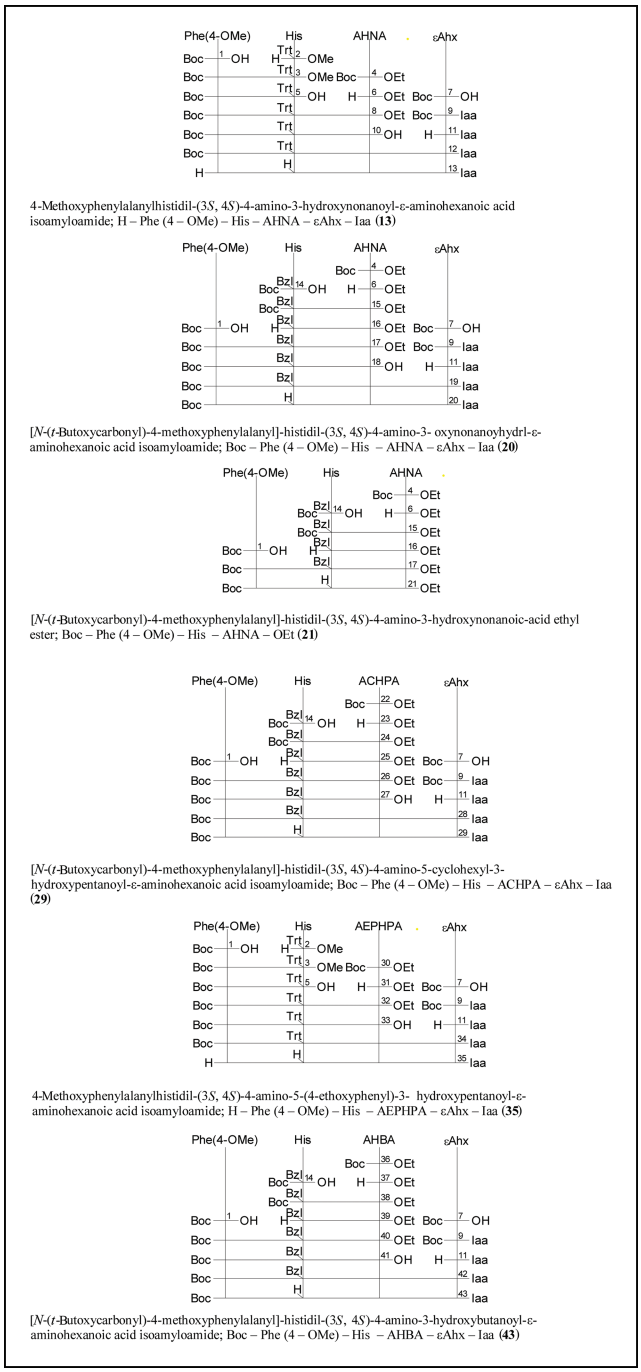


Fig. 3: Synthesis of the compounds

of its C-terminus proves that there is no necessity of extending the major chain P_3-P_2-P_1-P_1' by the branched, aliphatic and hydrophobic moiety Ahx-Iaa. Hydrophobic influence of the P_2'-P_3' moiety is not substantial for exhibiting inhibitory activity. It seems likely that spatial arrangement of the flexible, long and branched Ahx-Iaa chain is hindering hydrophobic interaction of the P_1 moiety with the hydrophobic pocket S_1 that is essential for activity of the inhibitor. Lack of the hydrophobic Boc substituent at the N-terminus of the molecule (**13**) is causing only minor, i.e. a two-fold decrease of inhibitory activity of this compound (**20**). On comparing activity of the inhibitors (**29**) and (**35**) that contain cyclic pseudodipeptides at P_1-P_1', it should be pointed out that (in contrast to the results of previous studies) (Boger et al. 1985) a compound bearing an aromatic ring (**35**) is a more potent renin inhibitor despite lacking Boc group at the N-terminus. This result presumably confirms the assumed significant interaction of the ethoxy substituent with adequate sub-pocket at the S_1 position. The inhibitor (**29**) containing a cycloalkane ring (6 carbon atoms) at P_1 has proven to be two-fold more active than that with an aliphatic chain (5 carbon atoms) (**20**) at P_1. Unexpectedly, activities of the compounds (**20**) and (**43**) are comparable. It indicates the significance of a hydrogen bond that is formed with the OH group in all inhibitors. The presence of a hydrophobic C_5-C_9 side chain in AHNA (**20**) does not increase distinctively inhibitory activity as compared to that of compound (**43**) lacking a side chain in AHBA. The compound AHBA contains a hydrophobic moiety in the form of a backbone chain C_1-C_4. The results of activity studies indicate that a hydrophobic structure (not necessarily a side substituent) that interacts with the pocket at S_1-S_1' is required in order to inhibit the enzyme.

The most important result of our studies is the conclusion that the imidazole ring of His located at P_2 must not be substituted. Large hydrophobic aromatic substituents present at that position preclude binding the inhibitor to renin, which results in lack of biological activity (Winiecka et al. 2014). Another significant achievement of this study is the finding that inhibitory activity does not require the presence of a side chain at the P_1 position of the pseudodipeptide structure (**43**).

3. Experimental

3.1. Chemistry

The structures of inhibitors considered in the present work are shown in Fig. 1. The inhibitors (**13, 20, 21, 29, 35, 43**) as well as their intermediates were synthesized in a commonly used manner, by fragment condensation, according to schemes presented in Fig. 3.

The applied methods are specified below in the syntheses section. Physicochemical properties of the inhibitors, as well as newly synthesized intermediates (**3, 4, 8, 12, 15, 17, 19, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42**) are presented in Table 2 and Table 3.

Reagents Boc- Phe (4- OMe)- OH, His (N^{im}Bzl)- OEt, His (N^{im}Trt)-OEt, porcine kidney renin and N-acetylrenin substrate tetradecapeptide were acquired from a recognized vendor. ACHPA, AHNA, AHBA and AEPHPA (**4, 30, 36**) 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 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 $CHCl_3$/MeOH in various ratios. The spots were visualized with 0.3% ninhydrin in EtOH/AcOH (97: 3, v/v). A Perkin-Elmer Microanalyser was used to carry out elemental analyses. A 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 2001 recorder. The peaks were recorded at 213 nm. The separation was carried out in the reverse phase system (Ultrasphere C8, Wide Pore C8, Symmetry C18) with various mobile phases.

3.2. Syntheses

3.2.1. Introduction of the N-tert-Boc-group

This group was introduced in a commonly used manner (Maibaum and Feldman 2009).

3.2.2. 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 concentrated *in vacuo*, then the residue was evaporated twice with ethyl ether and dried *in vacuo* (Anderson and McGregor 1957).

3.2.3. Esterification and hydrolysis

Boc-amino acids were esterified with CH_3I or C_2H_5I as described earlier (Paruszewski and Strzałkowska 1990). Boc – ACHPA - OEt, Boc – AHNA - OEt, Boc – AEPHPA - OEt and Boc – AHBA - OEt were formed from mono-ethyl malonate used to prepare these compounds (Paruszewski et al. 1997). Alkaline hydrolysis of ester group was carried out as described in literature (Maibaum and Rich 1988).

3.2.4. Catalytic hydrogenolysis

Removal of His-N-imidazole benzyl and Trt group by palladium-based catalytic hydrogenation.

3.2.5. 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.

3.3. Biochemical assay

3.3.1. 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^{-5} – 10^{-11}M. Inhibition is expressed as IC_{50} value *i.e.* the molecular concentration of the synthesized inhibitors causing 50% inhibition of the control renin activity. The *in vitro* stability in presence of chymotrypsin is a result of unnatural amino acid moiety Phe(4-OMe) placed at the P_3 position. Due to the fact that compound **(21)** is an ester it is more susceptible to enzymatic degradation than amide structures at the C-terminus (see the Table 1).

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