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Synthesis and Structure–Activity Relationship of New Analogues of Antistasin

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Abstract: Intensive investigation connected with the development of new anticoagulant agents for the treatment of cardiovascular diseases was carried out. Direct and specific inhibition of thrombin and Factor Xa–like serine proteases in the coagulation cascade has been the focus of many efforts to design novel anticoagulants over the past decade. This work reports the synthesis and biological activity of new anticoagulant peptide analogues of natural isoforms 2 and 3 of antistasin. In addition they include different tripeptide sequences in their molecules, which are highly active inhibitors of different serine proteases such as plasmin, trypsin, thrombin and Factor Xa.

Keywords: antistasin; Factor Xa; phenylacetyl group (Pac); anticoagulant activity; peptido mimetic

INTRODUCTION

Factor Xa is a serine protease, which plays a crucial role in curtailing thrombin generation. Inhibitors of Factor Xa are a promising alternative to currently available anticoagulants for the treatment and prevention of thrombotic disorders.

Antistasin (ATS) is a slow-binding inhibitor of Factor Xa, which was isolated from the salivary gland of the Mexican leech *Haementeria officinalis* [1]. Condra *et al.* [2] reported on four closely related ATS isoforms, which have been identified in the glandular extract: isoform 1, isoform 2, isoform 3 and **rATS**. The basic *C*-terminal region of ATS (residues 109–119) is important for the interaction of ATS with Factor Xa.

In order to increase the anticoagulant activity of isoforms 2 and 3 and to investigate the role of aromatic and D-amino acids in P_3 position, peptides were designed and synthesized that represent a hybrid structure between isoforms 2 and 3 of antistasin and the active sequence D-Phe-Pro-Arg; D-Arg-Gly-Arg; Phe-Ile-Arg and Tyr-Ile-Arg, which are highly active inhibitors of serine proteases: H-D-Phe-Pro-Arg-Pro-Lys-Arg-OH **(6)**, H-D-Arg-Gly-Arg-Pro-Lys-Arg-OH **(12)**; H-D-Arg-Gly-Arg-Pro-Lys-Arg-Lys-OH **(17)**; H-Phe-Ile-Arg-Pro-Lys-Arg-OH **(20)** and H-Phe-Ile-Arg-Pro-Lys-Arg-Lys-OH **(24)**; H-Tyr-Ile-Arg-Pro-Lys-Arg-OH **(21)**; H-Tyr-Ile-Arg-Pro-Lys-Arg-Lys-OH **(25)** according to Schemes 1–5 [3–6]. On the other hand, the existence of D-amino acids in P_3 position can decrease enzymatic recognition of the new compounds.

RESULTS AND DISCUSSION

The synthesis of the peptides **(6), (12), (17), (20), (21), (24)** and **(25)** were realized by coupling the dipeptides D-Phe-Pro, D-Arg-Gly, Tyr-Ile and Phe-Ile to Arg residue in 109 position of isoforms 2 and 3 of antistasin.

The phenylacetyl group (Pac) was used for protection of the N^{ε}-amino function of lysine.

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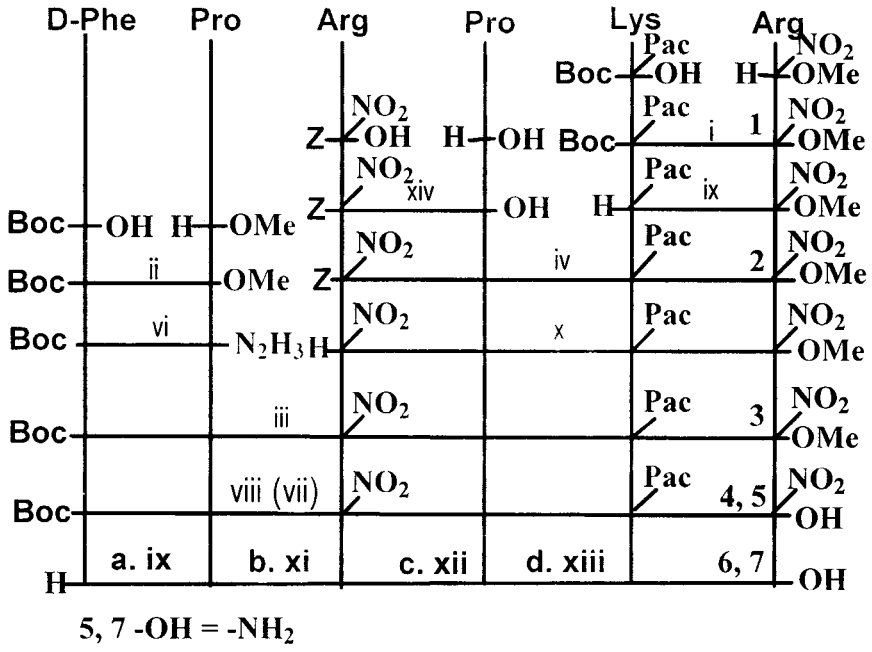
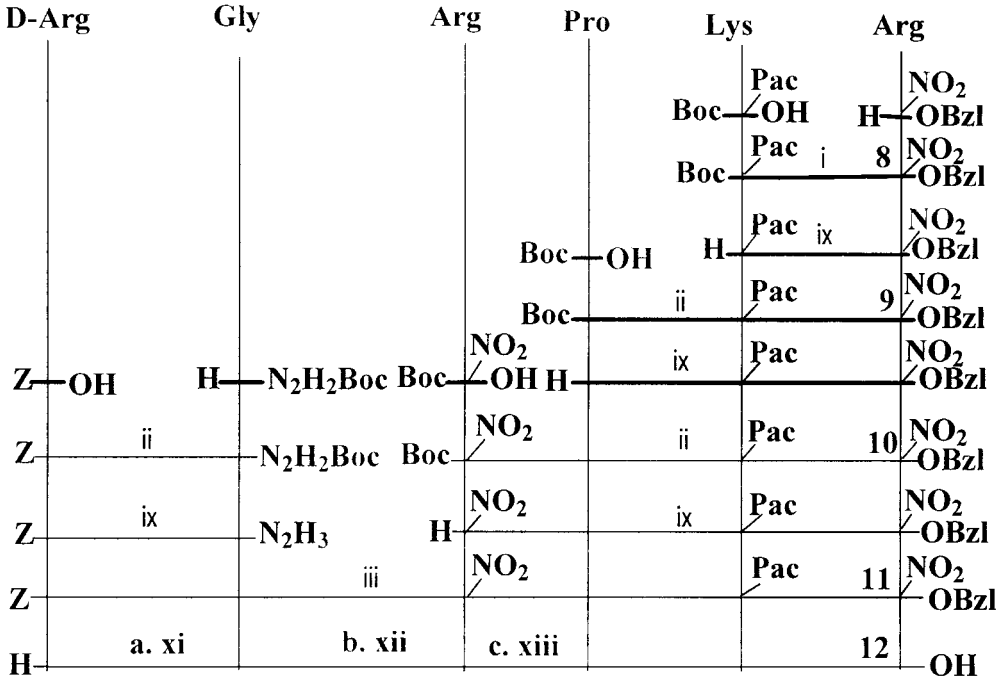


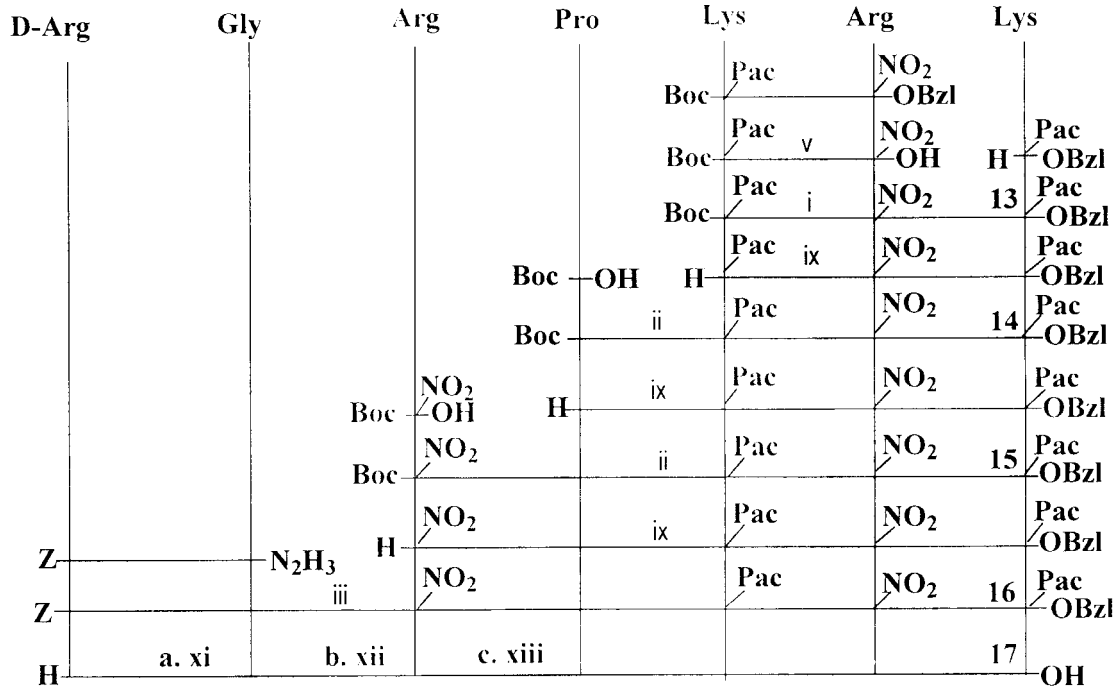
Scheme 1 Synthesis of analogues of isoform 3 of antistasin containing active sequence D-Phe-Pro-Arg.



Scheme 2 Synthesis of new analogue of isoform 3 of antistasin containing the active sequence D-Arg-Gly-Arg.

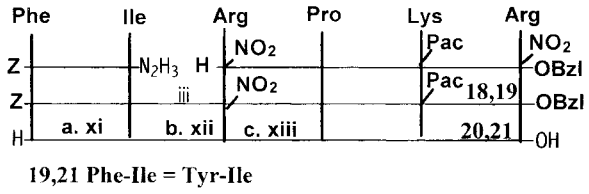
The DCC/1-HOBt, azide and TBTU/DIEA methods were used for synthesis of new compounds. Most reactions ran with high yields and good purity. Some difficulties were met with the synthesis of several peptides:

— the attempts to synthesize the hexapeptide **(6)** by consecutive attachment of Boc-Pro-OH and Boc-D-Phe-OH to the tetrapeptide ester H-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OMe by the TBTU/DIEA method were not successful,

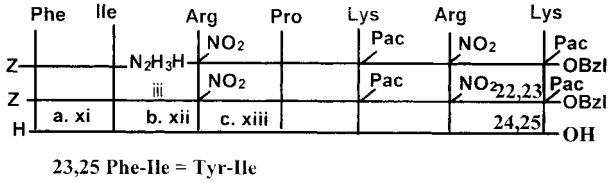


Scheme 3 Synthesis of new analogue of isoform 2 of antistasin containing the active sequence D-Arg-Gly-Arg.

because of a low yield of the first stage. A fragment condensation was achieved of Boc-D-Phe-Pro-N_2H_3 and H-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OMe in the presence of t-BuONO as a coupling reagent;



Scheme 4 Synthesis of new analogues of isoform 3 of antistasin containing the active sequences Phe-Ile-Arg and Tyr-Ile-Arg.



Scheme 5 Synthesis of new analogues of isoform 2 of antistasin containing the active sequences Phe-Ile-Arg and Tyr-Ile-Arg.

— the methyl group of the protected hexapeptide ester Boc-D-Phe-Pro-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OMe was removed by enzymatic hydrolysis in the presence of trypsin because of the difficulties involved in the use of alkali hydrolysis by 2N and 4N NaOH;
— the tetrapeptide methyl ester **(2)** from Z-Arg(NO_2)-Pro-OH and H-Lys(Pac)-Arg(NO_2)-OMe were synthesized by both DCC/1-HOBt and TBTU/DIEA methods. The TBTU/DIEA method gave better results in respect of yield, purity and decreased reaction time;
— the needed tetrapeptide ester **(10)** for scheme 2 was obtained by staged attachment of Boc-Pro-OH and Boc-Arg(NO_2)-OH to H-Lys-(Pac)-Arg(NO_2)-OBzl by using the DCC/1-HOBt method in DMF, and yields of 55.4% and 57.0%, respectively, were achieved because of difficulties met with the hydrolysis of the dipeptide ester Boc-Arg(NO_2)-Pro-OMe.

The data for synthetic methods, yields and physical constants of the new peptides are shown in Table 1.

The new compounds were finally proved by elemental and amino acid analyses, and the angle of optical rotation. The blocking groups of protected peptides were removed by treatment with H_2/Pd and

Table 1 Methods for Obtaining, Yields and Physical Constants of the New Compounds

Product	Method for synthesis	Yield (%)	M.p. (°C)	$[\alpha]_{546}^{22}$ (°)	Solvent for recrystallization
(1) Boc-Lys(Pac)-Arg(NO_2)-OMe[a]	i	79.4	56–60	—	EtOAc
(2) Z-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OMe	iv	97.2	Oil	—	—
(3) Boc-D-Phe-Pro-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OMe	iii	51.2	63–66	—	DMF/H_2O
(4) Boc-D-Phe-Pro-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OH	viii	63.3	Oil	—	—
(5) Boc-D-Phe-Pro-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-NH_2	vii	99.8	Oil	—	—
(8) Boc-Lys(Pac)-Arg(NO_2)-OBzl	i	80.1	Oil	—	—
(9) Boc-Pro-Lys(Pac)-Arg(NO_2)-OBzl	ii	55.4	52–55	−27.8 (c 1 MeOH)	DMF/H_2O
(10) Boc-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OBzl	ii	57.0	Oil	−20.0 (c 1 MeOH)	—
(11) Z-D-Arg-Gly-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OBzl	iii	53.7	Oil	—	—
(13) Boc-Lys(Pac)-Arg(NO_2)-Lys(Pac)-OBzl	i	99.7	Oil	—	—
(14) Boc-Pro-Lys(Pac)-Arg(NO_2)-Lys(Pac)-OBzl	ii	73.7	Oil	—	—
(15) Boc-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-Lys(Pac)-OBzl	ii	61.4	65–67	−16.0 (c 1 MeOH)	DMF/H_2O
(16) Z-D-Arg-Gly-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-Lys(Pac)-OBzl	iii	32.0	Oil	−8.0 (c 1 MeOH)	—
(18) Z-Phe-Ile-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OBzl	iii	54.6	92–95	−27.6 (c 1 MeOH)	EtOAc/ petroleum ether
(19) Z-Tyr-Ile-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OBzl	iii	55.7	85–89	—	DMF/H_2O
(22) Z-Phe-Ile-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-Lys(Pac)-OBzl	iii	70.3	94–96	−7.9 (c 1 MeOH)	DMF/H_2O
(23) Z-Tyr-Ile-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-Lys(Pac)-OBzl	iii	63.3	Oil	—	—

[a] IR; (KBr) ν, δ [cm^{-1}] 3318; 3106; 2935; 2868; 1743; 1702; 1696; 1653; 1648; 1602; 1540; 1532; 1454; 1391 and 1366; 1259; 727; 696.

subsequent enzymatic hydrolysis in the presence of penicillin amidohydrolase. The purifying of newly synthesized compounds was achieved by column gel chromatography on Sephadex G15; 50% and 0.2N acetic acid was used as an eluent. The data from amino acid and elemental analyses of the new compounds are shown in Table 2.

The anticoagulant activity of all compounds was measured in respect to APTT (activated partial thromboplastin time), PT (prothrombin time), TT (thrombin time) and the level of fibrinogen in the human poor platelet plasma (PPP). IC_{50} values (the concentrations for doubling APTT clotting times for human plasma) were determined. The data for anticoagulant activity of the new compounds are shown in Tables 3–6. The results show that the peptide H-D-Phe-Pro-Arg-Pro-Lys-Arg-OH has IC_{50} = 2628 nmol, i.e. it is lower in comparison with the activity of isoform 3, of antistasin (IC_{50} = 740 nmol). On the assumption that replacement of COOH

Table 2 Amino Acids and Elemental Analyses of the New Compounds

Product	Elemental analysis		Amino acid analysis	
	Calculated (%)	Found (%)	Calculated	Found
(11) Z-D-Arg-Gly-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OBzl $C_{62}H_{82}O_{15}N_{18}$ (1318)	C 56.45 H 6,22 N 19.12	C 56.44 H 6,24 N 19.14	—	—
(16) Z-D-Arg-Gly-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-Lys(Pac)-OBzl $C_{68}H_{94}O_{16}N_{20}$ (1446)	C 56.43 H 6,50 N 19.36	C 56.44 H 6,48 N 19.40	—	—
(18) Z-Phe-Ile-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OBzl $C_{69}H_{87}O_{15}N_{15}$ (1365)	C 60.66 H 6,37 N 15.38	C 60.64 H 6,40 N 15.40	—	—
(22) Z-Phe-Ile-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-Lys(Pac)-OBzl $C_{75}H_{99}O_{16}N_{17}$ (1493)	C 60,28 H 6,63 N 15.94	C 60,30 H 6,65 N 15.98	—	—
(19) Z-Tyr-Ile-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OBzl $C_{68}H_{85}O_{16}N_{15}$ (1367)	C 59.69 H 6,22 N 15.36	C 59.69 H 6,25 N 15.40	—	—
(23) Z-Tyr-Ile-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-Lys(Pac)-OBzl $C_{74}H_{97}O_{17}N_{17}$ (1495)	C 59.40 H 6,49 N 15.92	C 59.44 H 6,50 N 15.95	—	—
(6) H-D-Phe-Pro-Arg-Pro-Lys-Arg-OH $C_{37}H_{61}N_{13}O_7$ (799)[a]	C 55.57 H 7.64 N 22.78	C 55.55 H 7.60 N 22.80	Phe 1 Pro 2 Lys 1 Arg 2	0.9 2 1.2 1.9
(7) H-D-Phe-Pro-Arg-Pro-Lys-Arg-NH_2 $C_{37}H_{62}N_{14}O_6$ (798)[a]	C 55.64 H 7.77 N 24.56	C 55.65 H 7.77 N 24.55	Phe 1 Pro 2 Lys 1 Arg 2	1 2.1 1 1.9
(12) H-D-Arg-Gly-Arg-Pro-Lys-Arg-OH $C_{31}H_{60}N_{16}O_7$ (830)[b]	C 44.82 H 7.23 N 26.99	C 44.79 H 7.23 N 27.03	Gly 1 Pro 1 Lys 1 Arg 3	1 1.1 1.05 2.9
(17) H-D-Arg-Gly-Arg-Pro-Lys-Arg-Lys-OH $C_{37}H_{72}N_{18}O_8$ (896)[b]	C 49.55 H 8.04 N 28.13	C 49.53 H 8.02 N 28.10	Gly 1 Pro 1 Lys 2 Arg 3	1 1.08 1.85 3
(20) H-Phe-Ile-Arg-Pro-Lys-Arg-OH $C_{38}H_{65}N_{13}O_7$ (815)[b]	C 55.95 H 7.98 N 22.33	C 55.91 H 8.00 N 22.35	Phe 1 Ile 1 Pro 1 Lys 1 Arg 2	0.9 0.9 1 1.2 1.85
(24) H-Phe-Ile-Arg-Pro-Lys-Arg-Lys-OH $C_{44}H_{77}N_{15}O_8$ (943)[b]	C 55.99 H 8.17 N 22.27	C 56.02 H 8.19 N 22.25	Phe 1 Ile 1 Pro 1 Lys 2 Arg 2	1 0.9 1.2 2.2 1.9
(21) H-Tyr-Ile Arg-Pro-Lys-Arg-OH	C 54.35	C 54.35	Tyr 1	1

(*continued overleaf*)

Table 2 (*Continued*)

Product	Elemental analysis		Amino acid analysis	
	Calculated (%)	Found (%)	Calculated	Found
$C_{37}H_{63}N_{13}O_8$ (817)[b]	H 7.71	H 7.68	Ile 1	1.1
	N 22.28	N 22.27	Pro 1	1
			Lys 1	0.8
			Arg 2	1.95
(25) H-Tyr-Ile Arg-Pro-Lys-Arg-Lys-OH	C 54.60	C 54.58	Tyr 1	1
$C_{43}H_{75}N_{15}O_9$ (945)[b]	H 7.94	H 7.95	Ile 1	0.95
	N 22.22	N 22.19	Pro 1	1.05
			Lys 2	2.1
			Arg 2	2

[a] The products was obtained according to method E after deblocking of Boc-group by treatment with 10 fold excess of TFA and —NO_2 group according to method D.
[b] The products was obtained according to method E after deblocking of Z-, -NO_2 and —OBzl groups according to method D.

Table 3 Data for Anticoagulant Activity of the Compound H-D-Phe-Pro-Arg-Pro-Lys-Arg-OH and its Amide

Concentration (nmol) Parameters of anticoagulant activity	1314.0[a]	1971.0[a]	2628.0[a]	37.8[b]	60.6[b]	303.0[b]	Ref.T(s)
APTT(s)	7,2	22,6	29,8	4.2	32.1	Out of range	31 Ref.T
TT(s)	—	—	—	2.6	11.2	49.1	12.8 Ref.T
PT(s)	3,9	7,9	12,8	0,8	13.0	75.0	17.5 Ref.T
Level of fibrinogen (mg/dl)	189	180	176	—	—	<18	200–400

[a] The data are for the compound H-D-Phe-Pro-Arg-Pro-Lys-Arg-OH, IC_{50} = 2628 nmol.
[b] The data are for the compound H-D-Phe-Pro-Arg-Pro-Lys-Arg-NH_2, IC_{50} = 60.6 nmol.
' — ' in limits.

Table 4 Data for Anticoagulant Activity of the Compounds H-D-Arg-Gly-Arg-Pro-Lys-Arg-OH and H-D-Arg-Gly-Arg-Pro-Lys-Arg-Lys-OH

Concentration (nmol) Parameters of anticoagulant activity	5141.0[a]	97324.0[a]	723.0[b]	1033.0[b]	1400.0[b]	Ref.T.(s)
APTT(s)	—	32.3	1.9	5.7	30.8	31 Ref.T
TT(s)	—	9.7	—	4.1	15.7	12.8 Ref.T
PT(s)	—	17.5	4.2	5.3	21.8	17.5 Ref.T
Level of fibrinogen (mg/dl)	196	174	—	93	140	200–400

[a] The data are for the compound H-D-Arg-Gly-Arg-Pro-Lys-Arg-OH, IC_{50} = 97324 nmol.
[b] The data are for the compound H-D-Arg-Gly-Arg-Pro-Lys-Arg-Lys-OH, IC_{50} = 1400 nmol.
' — ' in limits.

Table 5 The Data for Anticoagulant Activity of the Compounds H-Phe-Ile-Arg-Pro-Lys-Arg-OH and H-Phe-Ile-Arg-Pro-Lys-Arg-Lys-OH

Concentration (nmol) Parameters of anticoagulant activity	1540.0[a]	2050.0[a]	3593.0[a]	186.9[b]	280.4[b]	373.8[b]	Ref.T.(s)
APTT(s)	8.9	9.2	29.4	11.0	30.5	82.7	31 Ref.T
TT(s)	2.1	2.2	6.0	7.9	10.6	25.2	12.8 Ref.T
PT(s)	3.3	3.0	13.7	5.7	23.5	81.5	17.5 Ref.T
Level of fibrinogen (mg/dl)	163	155	140	425	—	—	200–400

[a] The data are for the compound H-Phe-Ile-Arg-Pro-Lys-Arg-OH, IC_{50} = 3593.0 nmol.
[b] The data are for the compound H-Phe-Ile-Arg-Pro-Lys-Arg-Lys-OH, IC_{50} = 233.6 nmol.
' — ' in limits.

Table 6 Data for Anticoagulant Activity of the Compounds H-Tyr-Ile-Arg-Pro-Lys-Arg-OH and H-Tyr-Ile-Arg-Pro-Lys-Arg-Lys-OH

Concentration (nmol) Parameters of anticoagulant activity	1475.0[a]	4986.2[a]	93.3[b]	148.2[b]	186.7[b]	373.0[b]	Ref.T.(s)
APTT(s)	—	30.7	1.4	31.8	51.8	Out of range	31 Ref.T
TT(s)	—	12.5	—	19.5	27.2	33.6	12.8 Ref.T
PT(s)	—	18.1	2.0	10.4	16.9	18.2	17.5 Ref.T
Level of fibrinogen (mg/dl)	—	—	—	—	—	—	200–400

[a] The data are for the compound H-Tyr-Ile-Arg-Pro-Lys-Arg-OH, IC_{50} = 4986.2 nmol.
[b] The data are for the compound H-Tyr-Ile-Arg-Pro-Lys-Arg-Lys-OH, IC_{50} = 148.2 nmol.
' — ' in limits.

function with $CONH_2$ would lead to an increased stability of peptides against enzymatic hydrolysis, another analogue of isoform 3 of antistasin H-D-Phe-Pro-Arg-Pro-Lys-Arg-NH_2 was synthesized. The results show that the analogue with amide function in the *C*-terminus is 12 times more active than the natural isoform 3, and 40 times more active than the peptide H-D-Phe-Pro-Arg-Pro-Lys-Arg-OH.

Both analogues containing the active sequence D-Arg-Gly-Arg: H-D-Arg-Gly-Arg-Pro-Lys-Arg-OH **(12)** and H-D-Arg-Gly-Arg-Pro-Lys-Arg-Lys-OH **(17)** revealed no increase in activity in comparison with the natural isoforms 2 and 3 of antistasin. The lack of better activity of the analogues **(6)**, **(12)** and **(17)** containing D-amino acid in P_3 position shows that it probably not significant for anticoagulant activity, which is in accordance with data obtained in other studies [7]. The analysis of the data of anticoagulant activity for another four analogues: H-Phe-Ile-Arg-Pro-Lys-Arg-OH **(20),** H-Tyr-Ile-Arg-Pro-Lys-Arg-OH **(21),** H-Phe-Ile-Arg-Pro-Lys-Arg-Lys-OH **(24)** and H-Tyr-Ile-Arg-Pro-Lys-Arg-Lys-OH **(25)** showed that the analogues **(24)** and **(25)** have a significantly increased activity in comparison with natural isoform 2 (IC_{50} = 500 nM), but analogues **(20)** and **(21)** have no increasing of activity. This fact shows that existence of aromatic amino acid in P_3 position does not lead to an increase in activity, but the lysine residue plays a crucial role in anticoagulant activity (Tables 4–6).

EXPERIMENTAL

Chemical Analysis

The melting points were determined on a Kofler apparatus and are uncorrected. The optical

rotation was measured on a Quick Russel-Jouan Type SL1D polarimeter. The infrared (IR) spectra were recorded on a Perkin-Elmer Model 1600 Series FTIR instrument. Amino acid analysis was done with a Biotronic LC 6001 apparatus. The elemental analysis was performed on a Perkin-Elmer M.240 apparatus. The purity of the products was checked by TLC on precoated plates of Silica gel 60 F_{254} (Merck) with the following solvent systems: $CHCl_3$: AcOH 9 : 1 (S_1); *n*-BuOH : AcOH : H_2O 3 : 1 : 1 (S_2) and *n*-BuOH : AcOH : pyridine : H_2O 60 : 6 : 24 : 20 (S_3). Spots on TLC chromatograms were detected by ninhydrin and chlorine/*o*-tolidine reaction. The following methods were used to synthesize new compounds: (i) DPPA (diphenyl phosphoryl azide method), (ii) DCC (N,N′-dicyclohexylcarbodiimide method)/1-HOBt, (iii) azide method, (iv) TBTU/DIEA method, (v) treatment with 2N NaOH, (vi) treatment with $N_2H_4.H_2O$, (vii) treatment with MeOH/NH_3, (viii) treatment with trypsin, (ix) treatment with TFA, (x) treatment with HBr/CH_3COOH, (xi) treatment with H_2/Pd, (xii) treatment with PAH(penicillin amido hydrolase)/10% DMF, (xiii) purifying by column gel chromatography on Sephadex, (xiv) MA (mixed anhydrides method).

Pharmacological Analysis

The APTT, PT, TT and the level of fibrinogen in human poor platelet plasma was measured on the automatic Stago apparatus by incubation of 450 µl citrated PPP at different concentrations of the peptides. The values for APTT, PT and TT in the tables are the prolonged times above Ref.t.(s).

PPP (pure platelet plasma): Blood samples were collected in 1/10 volume of 3.8% sodium citrate by venipuncture from healthy volunteers in vacuumtainers with collection tubes. Whole blood was centrifuged at 300 × **g** for 5 min to separate the platelet-rich plasma. The remainder was centrifuged at 3400 × **g** for 10 min and PPP was collected.

General procedure for preparation by the DCC (N,N′-dicyclohexylcarbodiimide)/1-HOBt (1-hydroxybenzotriazol) method. 1.00 mmol of the peptide (obtained from Boc-peptide ester by treatment with 10-fold excess of TFA) was dissolved in DMF (10 ml) and, after cooling to −5 °C, neutralized to pH 7–7.5 with Et_3N. 1.20 mmol of Z- or Boc- amino acid; 1.20 mmol of DCC and 1.40 mmol of 1-HOBt were added. The reaction mixture was stirred for 24 h at 0° to −5 °C and for another 24 h at room temperature. The obtained DC-urea was removed by filtration and then 30 ml of water was added. The product was extracted into EtOAc (3 × 10 ml) and the organic layer was washed with 5% $NaHCO_3$ (3 × 10 ml), H_2O (2 × 10 ml), 10% citric acid (3 × 10 ml) and H_2O to pH 7. The solvent was dried with Na_2SO_4 and removed *in vacuo* followed by recrystallization.

General procedure for preparation by the diphenylphosphorylazide (DPPA) method. 1.00 mmol of an amino acid ester hydrochloride was dissolved in DMF (10 ml) and cooled on ice bath to 0 °C to −5 °C. 1.00 mmol of Et_3N and 1.20 mmol of Boc-amino acid were added to a stirred solution. The reaction mixture was stirred for 20–30 min at 0 °C to −5 °C, and 1.50 mmol of DPPA and 1.35 mmol of Et_3N were added at the same time. The mixture was stirred for 3 h at 0 °C to −5 °C and 24 h at room temperature and then water (30 ml) was added. The product was extracted into EtOAc (3 × 30 ml) and the organic layer was washed in the same way as described in method A.

General procedure for preparation by the azide method. 1.00 mmol of the peptide hydrazide were dissolved in a minimal amount of DMF. The solution was cooled to −30 °C and 4.00 mmol of 1N HCl/THF and 1.40 mmol of tert- butylnitrite (t-BuONO) were added. The reaction mixture was stirred at −20 °C to −30 °C for 40 min and then cooled to −35 °C, followed by the addition of 5.26 mmol of Et_3N (pH 7–7.5) and peptide ester (obtained by treatment of 0.83 mmol of Boc- or Z- amino acid with 10-fold excess of TFA or HBr/AcOH, and neutralized with Et_3N to pH 7–7.5), dissolved in DMF (minimal amount). The reaction mixture was stirred for 24 h at 0 °C and for another 48 h at room temperature and then water (30 ml) was added. The product was extracted into EtOAc (3 × 10 ml) and the organic layer was washed in the same way as described in method A.

Deblocking of Z-,-OBzl and -NO_2 groups by catalytic hydrogenolysis in the presence of Pd/C. 1.00 mmol of the protected peptide was dissolved in MeOH and then Pd/C and 1.00 mmol of HCl or AcOH were added. Hydrogen was passed through the reaction mixture at 40 °C. The

deblocking of the protecting groups was watched on TLC and, after finishing the reaction, Pd/C was filtered out and MeOH was evaporated *in vacuo*. The formed oil was subjected to the next deblocking.

Deblocking of the Pac group by enzymatic hydrolysis in the presence of penicillin amidohydrolase. 1.00 mmol of the protected peptide was dissolved in 10% DMF/0.1 M KCl(40 ml). The reaction mixture at pH 8–8.5 (0.01 M KOH) was stirred in argon atmosphere at 37°C and then penicillin amidohydrolase was added. The reaction was monitored on TLC and after the end of the deblocking the solvent was removed *in vacuo*. The obtained product was subjected to column chromatography on Sephadex, with the subsequent use of 50% and 0.2N AcOH as eluents.

Boc-Lys(Pac)-Arg(NO_2)-OH

1.41 g (2.15 mmol) of Boc-Lys(Pac)-Arg(NO_2)-OMe was dissolved in MeOH (10 ml) and 12.01 ml (8.61 mmol) of 2N NaOH was added. The reaction was monitored with TLC in the S_2 system. After completion of the reaction, the pH was reduced to 7 with 10% citric acid, and a part of the solvent was removed *in vacuo*. Afterwards the pH was reduced to 2–3 with 10% citric acid and the product was extracted with EtOAc (3 × 10 ml). The following extraction of the product from the organic phase was by 5% $NaHCO_3$ (3 × 10 ml), and after reducing the pH to 2–3 with 10% citric acid, it was extracted into EtOAc (3 × 10 ml). Finally, the organic layer was washed with H_2O (3 × 10 ml), dried with Na_2SO_4 and evaporated *in vacuo*. White crystals were obtained (1.17 g 95.9%).

IR: (KBr) ν,δ [cm^{-1}] 3412-2620; 3069; 2988; 2936; 2870; 1730; 1692; 1668; 1634; 1538; 1455; 1394; 1368; 1269; 1163; 727; 696.

Boc-D-Phe-Pro-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OH (4)

0.2 g Boc-D-Phe-Pro-Arg(NO_2)-Pro-Lys(Pac)-Arg(NO_2)-OMe was dissolved in 8 ml DMF and 6 ml 0.1M Tris-buffer with pH 7.8 was added by stirring stepwise. After that 2 mg trypsin was added and the reaction mixture was stirred at room temperature. The reaction was monitored by TLC in the S_2 system. After finishing the reaction, the pH was reduced to 2–3 with 10% citric acid and the product was extracted with EtOAc. The product was extracted from the organic solvent into 5% $NaHCO_3$ and, after reducing the pH to 2–3 with 10% citric acid repeatedly into EtOAc, the organic layer being washed with H_2O, dried over Na_2SO_4 and removed *in vacuo*. The performed procedure gave 0.13 g of oil (63.3%).

CONCLUSIONS

Our investigation on the anticoagulant activity of the series of new analogues of isoforms 2 and 3 of antistasin showed that:

1. The availability of the lysine residue in position 113 in natural antistasin plays a significant role on increasing anticoagulant activity.
2. The creation of the amide function in the *C*-terminus of some analogues of isoform 3 of antistasin leads to a significant increase of biological activity.
3. Replacement of L- with D-amino acids in P_3 position did not increase the anticoagulant activity of newly synthesized compounds, which confirms the investigations of Marlowe *et al.* [7].
4. The availability of the aromatic amino acid on P_3 position is not significant for anticoagulant activity.
5. The availability of D-amino acids at higher concentration leads to a significant decrease of the level of fibrinogen as seen in Tables 1 and 2.

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