Highly Active and Selective Anticoagulants: D-Phe-Pro-Arg-H, a Free Tripeptide Aldehyde Prone to Spontaneous Inactivation, and Its Stable *N*-Methyl Derivative, D-MePhe-Pro-Arg-H

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D-Phe-Pro-Arg-H sulfate (GYKI-14166) is a highly active and selective inhibitor of thrombin both in vitro and in vivo. Recent studies on the stability of D-Phe-Pro-Arg-H in neutral aqueous solution at higher temperature have revealed that it is transformed into inactive 5,6,8,9,10,10a-hexahydro-2-(3'-guanidinopropyl)-5-benzyl-6-oxo-imidazo[1,2-*a*]pyrrolo[2,1-*c*]pyrazine. No such inactivation could be observed with Boc-D-Phe-Pro-Arg-H (GYKI-14451), but this compound was far less specific than the free peptide as it inhibited thrombin and, for instance, plasmin equally well. Assuming that the transformation of free tripeptide aldehyde, mentioned above, can only be initiated by a primary amino terminus, the *N*-alkyl derivatives of D-Phe-Pro-Arg-H were prepared. Of the new analogues, D-MePhe-Pro-Arg-H (GYKI-14766) proved to be as highly active and selective anticoagulant as its parent compound and was not inactivated by transformation into a heterocyclic compound.

The process of blood coagulation includes consecutive zymogen-activation reactions leading to the generation of thrombin and the conversion of fibrinogen, soluble in the blood plasma, into insoluble fibrin polymer. Thrombin regulates its own production, converts fibrinogen into fibrin gel, i.e. a polymer stabilized by hydrophobic and ionic bonds only, and generates factor XIIIa, which catalyzes the formation of covalently bound, insoluble fibrin polymer. The production of thrombin is also controlled by protein C, also called coagulation factor XIVa, and thrombin generated is inhibited by the plasma inhibitor antithrombin III.

The therapy and prevention of thrombosis is based on thrombin inhibition by using coumarin derivatives and heparins. Coumarins impede the generation of thrombin by blocking the posttranslational γ-carboxylation in the synthesis of prothrombin and other proteins of this type including, for example, the anticoagulant protein C. Thus, the effect of coumarins can only develop slowly, 6–24 h after administration, and they cannot be regarded as highly specific anticoagulants. Heparins accelerate the inhibition of thrombin by antithrombin III. However, they can only be applied parenterally and are totally inactive in the absence or deficiency of antithrombin III.

In view of the above, it is most desirable to develop a novel anticoagulant which (a) can act on thrombin independent of antithrombin III, (b) exerts its inhibitory action shortly after administration, and (c) permits the lysis of blood clots, the other regulatory process maintaining hemostasis, to proceed.

Our approach to obtain a thrombin inhibitory anticoagulant was based on a combination of two findings as follows: (i) Phe-Val-Arg-OMe[1] and Gly-Pro-Arg[2] derived from the thrombin-sensitive region of fibrinogen, i.e. -Gly-Val-Arg-↓-Gly-Pro-Arg-, delayed clotting in thrombin–fibrinogen systems. (ii) Peptide aldehydes, such as Ac-Leu-Leu-Arg-H,[3] Ac-Phe-Gly-H,[4] and Ac-Pro-Ala-Pro-Ala-H,[5] could inhibit certain proteinases, e.g. plasmin, papain, and elastase.

Table I. Intermediates of the Synthesis of Tripeptide Aldehydes D-Phe-Pro-Arg-H (1) and D-MePhe-Pro-Arg-H (2)

no.	intermediate[a]	formula	M_r	$[\alpha]_D$[b]	mp, C°
4	Z-D-Phe-OTCP	$C_{23}H_{18}O_4NCl_3$	478.75	+37[c]	139–141
5	Z-D-Phe-Pro	$C_{22}H_{24}O_5N_2$	396.43	−46.4[c]	132–133
6	Z-D-MePhe-Pro	$C_{23}H_{26}O_5N_2 \cdot C_6H_{13}N$	509.83	+12.1[d]	160–163
7	Boc-Arg(Z)	$C_{19}H_{28}O_6N_4 \cdot H_2O$	426.64	−20.7[e]	122–124
8	Boc-Arg-(Z)>	$C_{19}H_{26}O_5N_4$	390.43	−24[g]	164–166
9	Arg(Z)>·HCl	$C_{14}H_{18}O_3N_4 \cdot HCl$	326.78		
10	Z-D-Phe-Pro-Arg-(Z)>	$C_{36}H_{40}O_7N_6$	668.73	−59.8[f]	am[g]
11	Z-D-MePhe-Pro-Arg(Z)>	$C_{37}H_{42}O_7N_6$	682.75	+13.5[f]	am
12	Z-D-Phe-Pro-Arg-(Z)-H	$C_{36}H_{42}O_7N_6$	670.74	−43.6[f]	am
13	Z-D-MePhe-Pro-Arg(Z)-H	$C_{37}H_{44}O_7N_6$	768.93	+16.8[f]	am

[a] Arg(Z)> depicts Arg(Z)-lactam. [b] *c* = 1, at 20 °C. [c] In dimethylformamide. [d] In methanol. [e] In pyridine. [f] In tetrahydrofuran. [g] Am, amorphous.

The enzyme most probably recognizes and binds the side chain of the C-terminal residue whose aldehyde function reacts with the active OH (or SH) group of the enzyme to form a tetrahedral hemiacetal, an unproductive transition-state analogue.[4,5] The enzyme–peptide aldehyde complex thus formed is further stabilized by interactions mediated through the rest of the side chains.

Of the aldehyde analogues of the two thrombin inhibitory tripeptides prepared, Phe-Val-Arg-H and Gly-Pro-Arg-H, the Pro-containing compound proved to be the more active. Systematic replacements at the N-terminus of Gly-Pro-Arg-H led to D-Phe-Pro-Arg-H, which possessed the expected high and selective thrombin inhibiting activity.[6-8] In the presence of this tripeptide aldehyde, depending on its concentration, either no fibrin clot was formed in the thrombin–fibrinogen reaction or a loose

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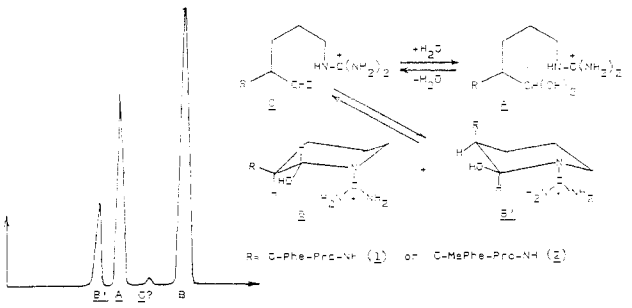

Figure 1.

fibrin gel easily lysed by plasmin was produced.

The acetate form of D-Phe-Pro-Arg-H, as prepared first, was subject to spontaneous inactivation in neutral aqueous solution ($t_{1/2} \sim 15$ days at 5 °C and ~3 days at 40 °C), and only D-Phe-Pro-Arg-H sulfate (GYKI-14166) possessed satisfactory stability in cool solution (5 °C) for a prolonged period (~6 months). As indicated by TLC, the process of inactivation was associated with the formation of a less polar compound. No inactivation could be observed with Boc-D-Phe-Pro-Arg-H (GYKI-14451),[8,9c] but this compound was far less specific than the free peptide, as it inhibited the thrombin-fibrinogen and plasmin-fibrin reactions equally well.[8]

We report here on the anticoagulant properties of D-Phe-Pro-Arg-H and its transformation into the inactive 5,6,8,9,10,10a-hexahydro-2-(3′-guanidinopropyl)-5-benzyl-6-oxoimidazo[1,2-*a*]pyrrolo[2,1-*c*]pyrazine, as well as the synthesis and biological evaluation of D-MePhe-Pro-Arg-H (GYKI-14766), which is as active and selective an inhibitor of thrombin as its parent compound but is completely stable.

Chemistry.[9] D-Phe-Pro-Arg-H (1) and its *N*-methyl analogue (2) were prepared by standard procedures using intermediates listed in Table I. Acylation of Pro with Z-D-Phe-OTCP (4) afforded Z-D-Phe-Pro (5). Z-D-MePhe-Pro (6) was obtained by alkylation of 5 with methyl iodide in the presence of NaH (cf. ref 10) and isolated as the cyclohexylamine salt. Arg-(Z)-lactam (9) was made from Boc-Arg(Z)-OH (7) via the mixed anhydride method followed by partial deprotection with HCl in ethyl acetate. Coupling of 9 with the N-terminal dipeptide moiety as carboxyl component (5, 6) afforded the protected tripeptide lactam (10, 11), which was reduced by $LiAlH_4$ to the protected tripeptide aldehyde (12, 13) and, finally, the protecting groups were removed by catalytic hydrogenation in the presence of an equivalent amount of an acid.

It has been known that peptidyl arginine aldehydes exist in equilibrium structures in aqueous solutions, i.e. aldehyde hydrate (A) and two amino cyclols (B, B′), and so they appear as mixtures of three or four components by HPLC.[11,12] Compounds 1 and 2 also showed this characteristic elution pattern in HPLC (Figure 1). The three main peaks could be assigned to structures B′, A, and B, respectively, whereas the minor peak may represent the aldehyde form C or may arise from D-MePhe-Pro-D-Arg-H being present as an impurity. The NMR spectra of 1 and 2 in D_2O reflected an even more complicated system. In addition to forms A, B, and B′ of Arg-H (with an approx ratio of 35:45:15), two rotamers of Pro (approx ratio of 75:25), and, in 2, two rotamers of MePhe (approx ratio of 55:45) are simultaneously present. Most of the signals of the ^1H NMR spectra remain unresolved even at 400 MHz and can be tentatively assigned only with the aid of ^1H–^1H and ^1H–^{13}C correlation spectra. Therefore, only some characteristic peaks of the ^1H NMR spectrum of 2 are given in Table II, while the data of the ^{13}C NMR spectrum are given in more detail in Table III. Nevertheless some peaks are not resolved and cannot unambiguously be assigned in the ^{13}C NMR spectra either.

For obtaining the inactive transformation product 14, 1 and sodium acetate were dissolved in water and heated at 80 °C for 5 h and, after evaporation, 14 was isolated by column chromatography on silica gel. MS of 14 showed a composition comprising the original amino acid side chains unchanged and lacking for two molecules of water as compared to the structure of 1. On the basis of these data a tricyclic structure could be suggested for 14. This structure of 14, including the relative configuration of its ring system 5,6,9,10,10a-hexahydro-2-(3′-guanidinopropyl)-5-benzyl-6-oxoimidazo[1,2-*a*]pyrrolo[2,1-*c*]pyrazine

Table II. ^1H NMR Spectral Data of 2

	chemical shifts,[a] ppm			
assignment	common signals	A	B	B′
MePhe αH	4.50[b,c]			
MePhe N-CH_3	2.72			
Pro αH	4.31 (4.37)[b]			
Arg-H 1H[d]		4.96	5.44	5.37
Arg-H αH		3.82	3.95	4.04

[a] Reference, ethanol-CH_3: 1.18 ppm. [b] Rotamers. [c] Signals of the two rotamers collapse at 343 K. [d] Traces of free aldehyde form of 2 gives rise to a singlet at 9.50 ppm, i.e. the aldehyde proton.

Table III. ^{13}C NMR Data of 2

	chemical shifts,[a] ppm			
assignment	common signals	A	B	B′
	MePhe			
1-C		167.47	167.54	167.35
2-C	61.68 (61.62)[b]			
3-C	36.74 (36.77)[b]			
1′-C	133.84[c]			
2′,6′-C_2	129.91			
3′,5′-C_2	130.30			
4′-C	129.00			
N-CH_3	32.39 (32.35)[b]			
	Pro			
1-C		174.46	173.71	174.09
2-C	61.42 (61.13)[b]			
3-C	30.27[c]			
4-C	24.84			
5-C	48.70			
	Arg-H			
1-C[d]		91.18	77.12	77.94
2-C		54.77	50.34	49.13
3-C		25.20	26.40	22.22
4-C		23.59	23.38	19.45
5-C		41.55	40.64	40.84
6-C (guanidino)		157.47	158.15[e]	

[a] Reference, dioxane: 67.3 ppm. [b] Rotamers. [c] Mean value of an unresolved doublet of rotamers. [d] aldehyde O=CH: 203.06 ppm (from DEPT spectra). [e] The signal of B practically coincides with that of form B′.

(9) These and some related compounds possessing anticoagulant activities are protected by US Patents: (a) 4,316,889 (1982), (b) 4,399,065 (1983), and (c) 4,478,745 (1984) to S. Bajusz, E. Szell, E. Barabas, and D. Bagdy; US Patent (d) 4,346,078 (1982) to S. Bajusz, E. Szell, E. Barabas, D. Bagdy, and Z. Mohai; and US Patent (e) 4,703,039 (1987) to S. Bajusz, E. Szell, D. Bagdy, E. Barabas, M. Dioszegi, Z. Fittler, f. Jozsa, G. Horvath, and E. Tomori.

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Scheme I

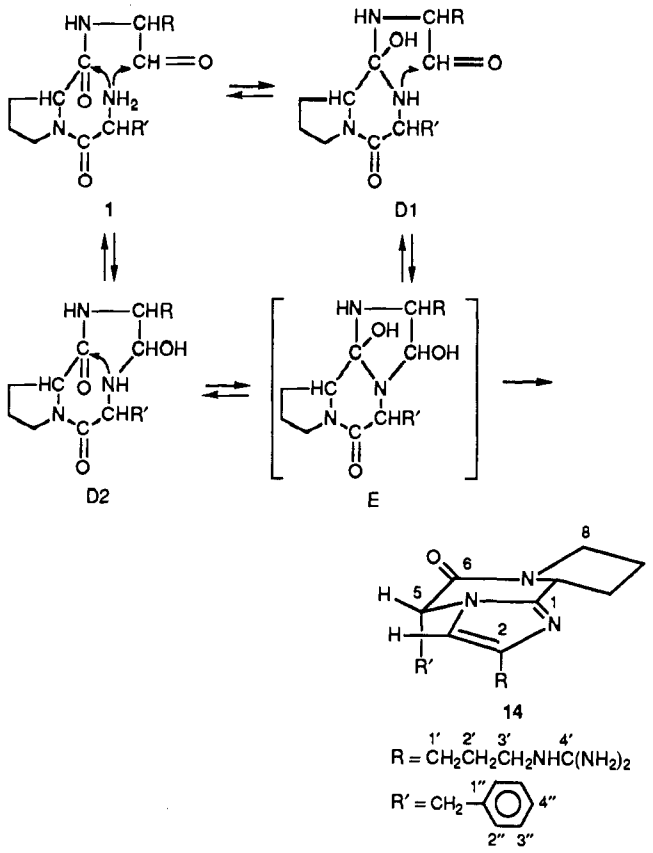

Table IV. ^1H and ^{13}C NMR Data of 14

assignment[a]	δ,[b] ppm ^1H	δ,[b] ppm ^{13}C	assignment[a]	δ,[b] ppm ^1H	δ,[b] ppm ^{13}C
C-2	–	135.8	C-5,CH_2	3.38	41.3
C-3	7.32	118.7	C-1″(Ph)		143.6
C-5	5.25	63.8	C-2″,6″	6.89	131.7
C-6	–	167.2	C-3″,5″	7.2–7.4	131.7
C-8	e3.54	47.7	C-4″	7.2–7.4	131.1
	a3.47		C-2,C-1′	2.76	23.9
C-9	e2.03	24.2	C-2′	1.93	29.4
	a1.88		C-3′	3.22	42.7
C-10	e1.75	31.5	C-4′	–	159.4
	a2.36		(guanidino)		
C-10a	3.25	55.3			
C-10b	–	137.3			

[a] Assignments are based mostly on ^{13}C–^1H and ^1H–^1H correlation, spin decoupling, and NOE difference measurements. [b] In D_2O; e, equatorial; a, axial; reference, 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt.

(see Scheme I), was further confirmed by NMR measurements (NOE difference, spin decoupling, ^1H–^{13}C correlation spectrum). Assignments for ^1H and ^{13}C NMR spectra are given in Table IV.

Anticoagulant and Antithrombotic Assays. The peptide aldehydes were tested in various assay systems in vitro, in vivo, and ex vivo.

The inhibition of the thrombin–fibrinogen reaction in vitro can be directly measured in *the thrombin time assay (TT)*.[13,14] Anticoagulant and antithrombotic effects in vivo were mainly studied in rabbit and dog after sc, im, and oral application, respectively, as well as during and after iv infusion. To assess the anticoagulant effect *the activated partial thromboplastin time (APTT)*[15,16] and *the whole blood clotting time (WBCT)*[17] assays were used, i.e. two assays which involve the process of thrombin generation in addition to the thrombin–fibrinogen reaction. Antithrombotic effects of the peptides were characterized by their action on platelet functions, i.e. changes of *platelet count*[18] and *platelet aggregation (PA)*[19,20] and *inhibition of thrombus* formation in an arteriovenous shunt model[21] in rabbit.[22]

Table V. Change in the Antithrombin Activity As Determined by the TT Assay of Aqueous Solution of X,Y-D-Phe-Pro-Arg-H Sulfates 1–3 Stored at 40 °C (pH = 6.0)

no.	X,Y	initial activity: I_{80},[a] μM (zero day)		% activity day 1	% activity day 3	% activity day 5
1	H,H	0.25	(100%)	100	50	40
2	Me,H	0.25	(100%)	100	100	100
3	Boc,H	0.27	(100%)	100	100	100

[a] Peptide concentration in the reaction mixture inducing a 5-fold prolongation of clotting time in the system compared to that of the control.

The in vitro effects of the peptides on plasmin, the key enzyme of the fibrinolytic system, were studied in *the fibrin plate lysis method*.[23,24] The effect of peptides on the plasmin lysis of fibrin formed in their presence was studied in systems containing bovine fibrinogen, thrombin, plasmin, and various amounts of peptides.

Details of the biological experiments will be published elsewhere.

Results and Discussion

As discussed above, the acetate salt of D-Phe-Pro-Arg-H was rather unstable in neutral aqueous solution, rapidly loosing its initial high enzyme inhibitory activity, and only the sulfate salt possessed significant stability in cool solution (5 °C). However, even this salt of D-Phe-Pro-Arg-H (1) was deactivated and transformed into an inactive compound (14) at higher temperatures within h. Data of mass spectrometry and NMR spectroscopy revealed that 14 had the structure of 5,6,8,9,10,10a-hexahydro-2-(3′-guanidinopropyl)-5-benzyl-6-oxoimidazo[1,2-*a*]pyrrolo-[2,1-*c*]pyrazine. On the basis of this structure and the fact that Boc-D-Phe-Pro-Arg-H[8,9c] (3) was stable under similar conditions, we assumed that the inactivation of the free tripeptide aldehyde can only be initiated by a primary amino terminus. Presumably the terminal H_2N group of 1 gives in an equilibrium process cyclol carbinolamine E–via cyclol aldehyde D1 and/or carbinolamine D2. Intermediate E spontaneously and irreversibly loses two molecules of water, yielding the stable, biologically inactive end product 14 (Scheme I). This speculation led to the conclusion that N-alkylation might result in stable tri-

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Table VI. Inhibitory Action of 1–3, X,Y-D-Phe-Pro-Arg-H, on the Lysis of Fibrin by Plasmin and on the Activation of Plasminogen by Urokinase and Tissue Plasminogen Activator As Determined by the Fibrin Plate Assay[a]

		ID_{50},[b] μg (A) and potency relative to 3 (B)					
		PL[c]		UK		tPA	
no.	X,Y	A	B	A	B	A	B
1	H,H	6.6	0.6	7.0	0.28	4.0	2.75
2	Me,H	0.7	5.7	0.6	3.33	3.2	3.44
3	Boc,H	0.4	100	0.02	100	0.11	100

[a] Reaction mixtures dropped onto fibrin plate contain 0.1 CTU plasmin (PL), 1 unit of UK (urokinase), or 0.15 μg of tPA (tissue plasminogen activator). [b] Amount of peptide (μg/reaction mixture) required for the reduction of lysed area to 50% of that of the control. [c] Heat-treated plates were used in order to deactivate plasminogen present in fibrin polymer.

Table VII. Effect of Tripeptide Aldehydes X,Y-D-Phe-Pro-Arg-H (1–3) on the Lysis of Fibrin Clot Formed in Their Presence

		lysis time[a] of fibrin clot, min, at peptide concentrations		
no.	X,Y	0.18 μM	0.36 μM	0.72 μM
1	H,H	10	*	no clot formed
2	Me,H	10	*	no clot formed
3	Boc,H	10	20	40

[a] Lysis time of fibrin gel formed in peptide-free system is 10 min, * denotes no measurable lysis time due to clot of rather loose structure.

Table VIII. Anticoagulant and Antiplatelet Effects of 1, 2, and Heparin in Various in Vitro Assays

	I_{50},[b] μg		
assay[a]	1	2	heparin[c]
APTT	0.24	0.44	0.38
TT, #1 normal plasma	0.032	0.04	0.10
TT, #2 antithrombin III deficient patient plasma	0.032	0.04	no activity
WBCT	0.085	0.090	0.38
PA inhibition	0.010	0.013	0.50

[a] APTT, activated partial thromboplastin time; TT, thrombin time; WBCT, whole blood clotting time; PA, platelet aggregation. Fresh human blood (WBCT) and citrated plasma (platelet poor in APTT, normal in TT, platelet rich in PA) were used. [b] Amount of agents (μg/reaction mixture) required for doubling the clotting time (WBCT, APTT, TT) and for inducing 50% inhibition of PA, respectively. [c] Commercial preparation with a specific activity of 142 USP units/mg.

peptide aldehydes because an HN< terminus could not lead to intermediate E, which was prone to irreversible dehydration.

To assess the stability of the *N*-alkyl derivative D-MePhe-Pro-Arg-H (2), as compared to that of the parent compound D-Phe-Pro-Arg-H (1) and the Boc analogue[8,9c] (3), peptides 1–3, as sulfates, were stored in a buffer (pH 6) at 40 °C for 5 days. The activity of the peptides in solution, assayed on the 1st, 3rd, and 5th day, was expressed as a percentage of the initial activity (0 day). Table V shows that each peptide had about the same initial potency and no deactivation could be detected after 1 day of storage (activity 100% on day 1). By the 5th day, however, 1 lost more than 50% of its original activity while the *N*-methyl derivative (2) as well as the Boc-peptide (3) retained their initial potencies. Apparently not only acylation but also alkylation could convey stability to D-Phe-Pro-Arg-H.

The stability of 1 and 2 in various biological fluids, such as whole blood, plasma, gastric juice, and bile, was also examined. The antithrombin activity of both tripeptide aldehydes, measured by the TT assay, remained practically unchanged for 6 h,[22,25] suggesting that neither irreversible binding nor structural alteration of the compounds occurred in such media.[26]

A good anticoagulant applicable in therapy is obviously expected to inhibit the blood coagulation process while permitting clot lysis to proceed. Table VI shows the inhibitory action of 1–3 on the plasmin lysis of fibrin and on the preceding event, i.e. generation of plasmin (PL) by urokinase (UK) or tissue plasminogen activator (tPA), as determined by the fibrin plate assay.[23,24] The potency order of 1 < 2 ≪ 3 obtained in the inhibition of PL and UK indicated that introduction of the *N*-methyl group into 1 increased the inhibiting activities on these enzymes, although far less so than the Boc substitution. Inhibition of tPA (last column) also favored the presence of N-Boc, but it was practically not effected by N-methylation. Thereafter the effect of peptides on the plasmin lysis of the clot formed in their presence was studied. A system consisting of fibrinogen, peptide, and plasmin was coagulated with thrombin, then the time required for the lysis of clot formed was recorded. Data of Table VII demonstrate that the properties of 1 are similar to those of its *N*-methyl derivative (2). In the presence of small amounts of peptides the clot formed is lysed within the control period (10 min) while in the presence of larger amounts either only a gel of rather loose structure is formed or no clot is produced at all. However, the plasmin lysis time of the fibrin clot increased dose-dependently in the case of Boc-peptide 3. These results are in accord with our previous findings[6] that the free tripeptide aldehydes H-X-Pro-Arg-H can interfere with the aggregation of fibrin monomer while the corresponding acyl derivatives, e.g. Bz-D-Phe-Pro-Arg-H, as well as the analogous free peptides having a residue other than Pro in position 2, e.g. H-D-Phe-Val-Arg-H and H-D-Phe-Gly-Arg-H, do not show such reaction. This phenomenon has been explained by the ability of H-X-Pro-Arg-H to block a Pro-Arg-binding site of thrombin which is to mediate the transposition of the nascent Gly-Pro-Arg terminal of fibrin α chain required for its aggregation. Some related peptides, such as Gly-Pro-Arg and Gly-Pro-Arg-Pro, were found to prevent the polymerization of fibrin monomers through binding to fibrinogen at 0.16–0.33 mM.[28] Binding of H-X-Pro-Arg-H to fibrinogen was not indicated at 0.25–2.5 μM used in the clotting assay.

The anticoagulant activities and inhibition of thrombin-mediated platelet aggregation by the tripeptide al-

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(26) The analogous chloromethyl ketone, D-Phe-Pro-Arg-CH_2Cl,[27] the irreversible inhibitor of thrombin, in contrast, showed a significant loss (50–100%) of its initial activity, in particular in whole blood, plasma, and bile.[22]

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Table IX. Characteristics of the in Vivo Antithrombin Effects of **2** upon Sc, Im, Iv, and Oral Application in Rabbits (n = 5–7 Each Dose), As Measured by the Whole Blood Clotting Time Assay

dose, mg/kg	peak concentration in plasma, μg/mL[a]				maximum intensity[b]				duration of action, min			
	sc	im	iv[c]	oral	sc	im	iv[c]	oral	sc	im	iv[c]	oral
6	0.60	0.40	0.37		3.2	2.7	2.5		270	170	220	
9	0.88	0.51			3.8	3.1			295	244		
12	1.70	0.85	0.55		5.4	3.8	2.0		295	250	290	
15	2.00	1.20		0.22	5.8	4.5		2.0	300	290		270
20				0.66				3.4				>300

[a] Estimated by a dose–response calibration curve. [b] Maximum delay of clotting time relative to the control (=1). [c] Doses of 2 and 4 mg/kg per h infused for 3 h correspond to total doses of 6 mg/kg and 12 mg/kg, respectively.

Table X. Correlation between Anticoagulant and Antithrombotic Effects of **2** at a Single Oral Dose of 10 mg/kg in Rabbits (n = 5–7)[a]

time, min	WBCT[b]	TT[b]	thrombus formation weight, mg (%)	inhibition of PA (%)	platelet count/μL
0	1.0	1.0	106.1 ± 14.1 (100)	0	617 280 ±53 810
40	1.1	2.7		42 ± 15	522 930 ±73 940
60	1.6	12.6	34.6 ± 4.7 (32.6)	76 ± 13	584 160 ±4 448
240	1.3	10.2	5.7 ± 2.3 (5.4)	64 ± 16	555 780 ±44 300
300	1.0	1.3	23.3 ± 4.4 (22)	38 ± 14	495 350

[a] Anticoagulant activity is assessed by the WBCT and TT assays; antithrombotic effect is evaluated by measuring the inhibition of PA and thrombus formation. [b] Clotting times relative to the controls, i.e. 11 min in WBCT and 22.4 s in TT assays, respectively.

dehydes **1** and **2**, as compared to that of commercial heparin, in various in vitro assays are presented in Table VIII. In the systems containing platelet-poor or normal plasma (APTT and TT),[33] more or less similar data were obtained for the peptides and heparin having an activity of 142 USP units/mg. The fact that the clotting of antithrombin III deficient plasma (TT #2) could only be delayed by **1** and **2** is obvious and points out the advantage of peptide anticoagulants over heparin. Apparently the anticoagulant and antiplatelet activities of heparin measured in whole blood (WBCT) and in platelet-rich plasma (PC), respectively, were significantly lower (1/4–1/50) than those of the peptides. This phenomeon may be explained by the ability of this polyanion to combine with certain plasma components,[29] such as lypoproteins and platelets. Due to such interactions, less than the expected amount of heparin associates with thrombin and antithrombin III to yield the inactive complex.

The anticoagulant activities of **1** and **2** could be proved in vivo upon both parenteral and oral applications. Table IX presents the characteristics of actions observed after sc, im, iv, and oral administration of **2** at various doses in rabbits. Regarding parenteral applications, the highest potency and longest duration of action were obtained upon sc application. When given orally, the so-called therapeutic anticoagulant effect[30] of the peptides (1.5–2.5-fold delay in WBCT assay) persisted for 3 and >5 h at doses of 10 and 20 mg/kg, respectively.

In an arteriovenous shunt model[21] in rabbits,[22] it could be demonstrated that thrombus formation was markedly reduced by both **1** and **2** when given by iv infusion, sc, or orally. One hour after iv infusion of the peptides (1 mg/kg per h), the weight of thrombus was 18% of that formed at zero time (i.e. during 20 min extracorporeal circulation in the shunt) in untreated animals. Upon sc (10 mg/kg) and oral (20 mg/kg) administration, thrombus weight was reduced to about 20% after the 1st h, and even higher effects, reduction to 2–5%, could be measured after 4 h which persisted for at least 2 h more.

Table X presents data on the correlation between the anticoagulant and antithrombotic effects of **2** given in a single oral dose of 10 mg/kg to rabbits. The anticoagulant effects, detected by the WBCT and TT assays, appeared at 40 min and persisted up to 240 min after application. The antithrombotic effects, such as inhibition of thrombus formation and platelet aggregation (PA), seemed to run in parallel. As indicated in the last column, no significant reduction in platelet count occurred, indicating that the peptide was completely harmless to platelets.[31]

Acute toxicity tests in mice and rabbits showed LD_{50} values of 40–45 mg/kg iv (bolus) and >1500 mg/kg orally for both **1** and **2**. It is also of great importance that the tripeptide aldehydes did not cause overt bleeding in the acute toxicity studies, even in toxic doses, and no excessive bleeding was noted in the in vivo models, e.g. in the surgery of the rabbit arteriovenous shunt model.

The above experiments clearly demonstrate that D-MePhe-Pro-Arg-H is a stable tripeptide aldehyde and D-Phe-Pro-Arg-H as sulfate also shows significant stability in neutral aqueous solution. Both tripeptide aldehydes exert direct action on thrombin shortly after parenteral or oral application while hardly or not interfering with the plasmin–fibrin reaction, in particular when fibrin to be lysed by plasmin was formed in their presence.

Experimental Section

Ascending TLC was performed on DC-Alufolien Kieselgel-60 F_{254} (Merck, Darmstadt, BRG) plates in solvent systems (A) ethyl acetate and (B–F) mixtures of ethyl acetate/pyridine/acetic acid/water (v/v) as follows: (B) 480:20:6:11, (C) 240:20:6:11, (D) 120:20:6:11, (E) 60:20:6:11, (F) 30:20:6:11.

Analytical HPLC was performed on an LKB liquid chromatograph (LKB pump, Model 2150; a controller, Model 2152, Rheodyne injector, Model 970A) with a multiwavelength UV–visible detector, LKB Model 2151 (at 210 nm), an LKB recorder

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(31) According to our experiences, reduction of platelet count to 80–85% of that of control should not be regarded significant mainly due to sampling inaccuracy.

10 mV, Model 2210, and a Polygosil-60 C_{18} 10 μm, 250 × 4 mm column, with eluent system 0.1 M NaH_2PO_4–H_3PO_4 (pH 2.2)/CH_3CN (93:7 v/v) under isocratic condition at a flow rate of 1 mL/min. ^1H NMR spectra of 1 and 2 as well as an ^{13}C NMR spectrum of 1 were taken at 400 MHz and 100 MHz, respectively, on a Varian XL-400 instrument in D_2O. ^1H–^1H correlation spectra (COSY 45) were done on the same instrument. ^1H NMR spectrum of 14 in both D_2O and DMSO-d_6 and ^{13}C NMR spectra of 2 and 14 in D_2O were recorded at 250 and 63 MHz, respectively, on a Bruker AC 250 instrument. DEPT spectra and ^1H–^{13}C correlation spectra of 1, 2, and 14 were obtained on the same instrument. Mass spectra were recorded on a MAT MS-1 spectrometer: R = 1250 vs 10000; ion accelerating voltage, 8 kV; electron energy, 70 eV; electron currant, 300 μA; ion-source temperature, 250 °C; evaporation temperature, 190 °C; reference substance for mass measurement, PFK. Positive FAB mass spectrum of 2 was obtained on a Finnigan MAT 8430 mass spectrometer: matrix, glycerol with 5% H_3PO_4. (FAB conditions: reagent gas, Xe; saddle field gun voltage, 9 kV; trap current, 1 mA. Mass spectrum conditions: R = 1250; ion-source temperature, 40 °C.)

Z-D-Phe-OTCP (4). Z-D-Phe (60.0 g, 200 mmol) and 2,4,5-trichlorophenol (9.4 g, 200 mmol) dissolved in THF (200 mL) were condensed by DCC (41.2 g, 200 mmol) in the usual manner. After workup and crystallization from ethanol, 72 g (75%) of 4 was obtained. Anal. ($C_{23}H_{18}NO_4Cl_3$) C, H, N, Cl.

Z-D-Phe-Pro (5). Acylation of Pro (17.3 g, 150 mmol) with 4 (71.8 g, 150 mmol) in pyridine (180 mL) containing triethylamine (21.0 mL, 150 mmol) gave after crystallization from diethyl ether 50.4 g (85%) of 5. Anal. ($C_{22}H_{24}N_2O_5$) C, H, N.

Z-D-MePhe-Pro·CHA (6). Treatment of 6 (39.6 g, 100 mmol) with methyl iodide (50 mL, 800 mmol) in the manner suggested by McDermott and Benoiton[10] gave, after workup and crystallization as the cyclohexylamine salt from benzene, 38.2 g (75%) of 6. Anal. ($C_{29}H_{39}N_3O_5$) C, H, N.

Boc-Arg(Z)·H_2O (7). Boc-Arg·HCl·H_2O[32] (65.8 g, 200 mmol) dissolved in 4 N NaOH (100 mL) was simultaneously treated with benzyl chloroformate (80 mL, 500 mmol) and 4 N NaOH (150 mL) at pH ≥ 12 in the usual manner. The crude product obtained was crystallized from a mixture of benzene and water to afford 60 g (70%) of 7. Anal. ($C_{19}H_{30}N_4O_7$) C, H, N.

Boc-Arg(Z)>[33] (8). Compound 7 (59.7 g, 140 mmol) dissolved in THF (200 mL) was converted into a mixed anhydride by means of isobutyl chloroformate (18.5 mL, 140 mmol) and triethylamine (19.6 mL, 140 mmol) and then treated with triethylamine (19.6 mL, 140 mmol) to give after workup 44 g (81%) of crystalline 8. Anal. ($C_{19}H_{26}N_4O_5$) C, H, N.

Z-D-Phe-Pro-Arg(Z)> (10) (a). Arg(Z)>·HCl (9) was prepared by treatment of 8 (8.59 g, 22 mmol) in $CHCl_3$ (22 mL) with 3–4 N hydrochloric acid in ethyl acetate (55 mL). The product, after drying for about 1 h, was dissolved in DMF (20 mL), cooled to −15 °C, and treated with triethylamine to liberate the lactam base. The resulting suspension was used for coupling. **(b) Coupling.** Compound 5 (8 g, 20 mmol) dissolved in DMF (15 mL) was converted into a mixed anhydride by means of isobutyl chloroformate (2.64 mL, 20 mmol) and *N*-methylmorpholine (2.24 mL, 20 mmol) in the usual manner and then combined with the above suspension containing lactam base 9. The crude product obtained after the usual workup was submitted to column chromatography with Kieselgel-60 (150 g) in a 1:4 mixture of THF/benzene. The fractions containing the pure product were pooled and evaporated, then the residue was triturated with diisopropyl ether to give 9.4 g (70%) of 10: TLC R_f 0.60 (A). Anal. ($C_{36}H_{40}N_6O_7$) C, H, N.

Z-D-MePhe-Pro-Arg(Z)> (11). Compound 6 (10.2 g, 20 mmol) was dissolved in diethyl ether (40 mL) and 1 N $KHSO_4$ (24 mL). The diethyl ether layer was dried and evaporated. The resulting Z-D-MePhe-Pro was converted to a mixed anhydride and reacted with 9, prepared from 8 (8.59 g 22 mmol), and then worked up as described above to yield 9.0 g of 11: TLC R_f 0.73 (B). Anal. ($C_{37}H_{42}N_6O_7$) C, H, N.

Z-D-Phe-Pro-Arg(Z)-H (12). To a stirred solution of 10 (10.8 g, 15 mmol) in THF (90 mL) cooled to −20 °C was added $LiAlH_4$ (0.428 g, 11.25 mmol) dissolved in THF (about 20 mL). The progress of the reduction was monitored by TLC in system C (R_f values for the lactam and aldehyde were about 0.8 and 0.5, respectively). If required, further portions of $LiAlH_4$ were added, then the reaction mixture was acidified with 1 N sulfuric acid to pH 2 with cooling and stirring. The solution was diluted with water until it turned opaque, then it was extracted twice with *n*-hexane (20 mL). Afterward the aqueous THF layer was extracted twice with CH_2Cl_2. The combined CH_2Cl_2 solutions were washed with water and sodium hydrogen carbonate solution and then, after drying, concentrated. The residue was dissolved in benzene, evaporated, and diluted with cyclohexane to precipitate the desired compound, 12: Yield 9.9 g (88%); TLC R_f 0.5 (C). Anal. ($C_{36}H_{42}N_6O_7$) C, H, N.

Z-D-MePhe-Pro-Arg(Z)-H (13) was prepared as described for 12 but with Z-D-MePhe-Pro-Arg(Z)> (11, 10.25 g, 15 mmol) as starting material. The yield of 13 was 8.1 g (70%): TLC R_f 0.57 (C). Anal. ($C_{37}H_{44}N_6O_7$) C, H, N.

D-Phe-Pro-Arg-H·H_2SO_4 (1) and D-MePhe-Pro-Arg-H·H_2SO_4 (2). The protected tripeptide aldehyde (6.7 g of 12, 7.7 g of 13) dissolved in a 3:2 mixture of ethanol–0.5 N sulfuric acid (85 mL) was hydrogenated in the presence of 1 g of 10% Pd/C catalyst at ambient temperature and pressure. After the reaction was completed, the catalyst was filtered off; the filtrate was concentrated to about 30 mL and was diluted with water to about 60 mL. The pH was adjusted to 6.5 with 0.1 N sulfuric acid or an ion-exchange resin in an OH^- cycle, then the solution was freeze-dried to yield 3.0–3.2 g of both 1 and 2: TLC R_f 0.37 and 0.43 (D), respectively. Anal. 1 ($C_{20}H_{30}N_6O_3·H_2SO_4·H_2O$) C, H, N; 2 ($C_{21}H_{32}N_6O_3·H_2SO_4·H_2O$) C, H, N. ^1H NMR data of 1 are identical with those of 2 given in Table II except for the NCH_3 signal of 2. ^{13}C NMR data of 2 are presented in Table III. Compound 1 has similar chemical shifts (<±0.45 ppm) for all carbon atoms with the exception of those for Phe 1-C (+1.36 ppm for form A and +1.29 ppm for forms B and B′) and Phe C-2 (−7.9 ppm) due to lack of rotamers in the absence of the N^{α}-Me group. MS of 2 (FAB) is as follows: m/z (relative intensity) 417 (30) $[M + H]^+$, 134 (100) $[CH_3\text{-}^+HN{=}CH\text{-Bzl}]$, 91 (20) $[Bzl]^+$, 70 (23) [1-pyrrolinium]$^+$.

Transformation Product of 1 (14, 5,6,8,9,10,10a-Hexahydro-2-(3′-guanidinopropyl)-5-benzyl-6-oxoimidazo[1,2-*a*]pyrrolo[2,1-*c*]pyrazine). A solution of 1 (2.5 g, 50 mmol) in water (20 mL) containing sodium acetate (0.9 g, 11 mmol) was heated at 80 °C for 6 h. After evaporation under reduced pressure the residue was purified by chromatography on Kieselgel-60 (40 g) with solvent system D and then converted into a hydrochloride with HCl/methanol and precipitated with ethyl ether to yield 1.2 g of 14: TLC R_f 0.60 (D). Anal. ($C_{20}H_{26}N_6O·HCl$) C, H, N, Cl. ^1H and ^{13}C NMR data given in Table IV were obtained from the following measurements: (a) ^1H NMR spectra both in DMSO-d_6 and D_2O at 250 MHz, (b) ^{13}C NMR spectra in D_2O at 63 MHz, (c) ^1H–^{13}C correlation spectrum in D_2O, (d) spin decoupling experiments, and (e) NOE difference measurement in D_2O. Data for spin decoupling in DMSO-d_6: irradiating at 8.15 ppm (N*H*), CH_2 at 3.15 ppm transformed from qa to t; irradiating in D_2O at 5.25 ppm (1-H), CH_2(Ph) at 3.38 ppm transformed from AA′X to AA′ qa, irradiating at 1.95 ppm (2′-CH_2), both 1′-CH_2 at 2.76 ppm and 3′-CH_2 at 3.22 ppm transformed from t to s. In the NOE difference measurement, while the signal of 9-H at 7.38 ppm was saturated, the intensity of the signal of 1-H at 5.25 ppm was enhanced by 5.8%. MS (EI) is as follows: m/z (relative intensity) 366 (7) $[M]^{\bullet+}$ ($C_{20}H_{26}N_6O$), 324 (15) $[M - H_2NCN]^{\bullet+}$ ($C_{19}H_{24}N_4O$); 294 (100) $[M - {}^{\bullet}CH_2NH\text{-}C(=NH)NH_2]^+$ ($C_{18}H_{20}N_3O$), 281 (30) $[M - CH_2{=}CHNH\text{-}C(=NH)NH_2]^{\bullet+}$ ($C_{17}H_{19}N_3O$), 190 (9) $[281 - {}^{\bullet}CH_2Ph]^+$ ($C_{10}H_{12}N_3O$), 91 (16) $[CH_2Ph]^+$, 86 (17) [*N*,*N*′-ethyleneguanidinium]$^+$ ($C_3H_8N_3$), 70 (18) [1-pyrrolinium]$^+$ (C_4H_8N).

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(33) Abbreviations: APTT, activated partial thromboplastin time; Arg(Z)>, N^G-Z-Arg-lactam; Boc, *tert*-butoxycarbonyl; CHA, cyclohexylamine; DCC, *N*,*N*′-dicyclohexyl carbodiimide; DCU, *N*,*N*′-dicyclohexylurea; DMF, dimethylformamide; Et, ethyl; Me, methyl; OTCP, 2,4,5-trichlorophenoxy; PA, platelet aggregation; PL, plasmin; THF, tetrahydrofuran; TLC, thin-layer chromatography; tPA, tissue plasminogen activator; TT, thrombin time; WBCT, whole blood clotting time; UK, urokinase; Z, benzyloxycarbonyl.

Registry No. 1 (free base), 60503-05-1; 1·H_2SO_4, 83997-16-4; 2 (free base), 105806-65-3; 2·H_2SO_4, 126721-07-1; 3, 69201-89-4; 4, 63358-25-8; 5, 17460-56-9; 6, 105806-76-6; 7, 51219-18-2; 8, 51219-20-6; 9, 81344-50-5; 10, 61635-50-5; 11, 105806-73-3; 12, 77433-27-3; 13, 105806-74-4; 14, 126644-60-8; Z-D-Phe-OH, 2448-45-5; H-Pro-OH, 147-85-3; MeI, 74-88-4; BOC-Arg-OH·HCl, 35897-34-8; Z-Cl, 501-53-1; Z-D-MePhe-Pro-OH, 105806-75-5; thrombin, 9002-04-4.

1,4-Bis(3-oxo-2,3-dihydropyridazin-6-yl)benzene Analogues: Potent Phosphodiesterase Inhibitors and Inodilators

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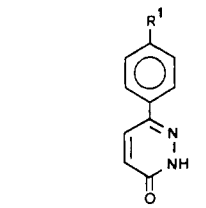
1,4-Bis(3-oxo-2,3-dihydropyridazin-6-yl)benzene and a series of related bis(azinone) compounds were synthesized. These novel compounds were evaluated for inhibition of the low K_m, cAMP-selective, cGMP-inhibited phosphodiesterase (PDE III) derived from cat heart and hemodynamic activity in the ganglion- and β-blocked anesthetized cat. The most potent PDE III inhibitor of the series was 6-[4-(5-methyl-3-oxo-2,3,4,5-tetrahydropyridazin-6-yl)-phenyl]pyridazin-3(2*H*)-one (IC_{50} = 0.07 μM), which also retained the greatest inotrope and vasodilator (inodilator) potency (ED_{50} for first derivative of left ventricular pressure (dLVP/dt(max)) = 0.02 μmol/kg, ED_{15} for 15% fall in perfusion pressure = 0.01 μmol/kg). The structure–activity relationships observed within the bis(azinone) series were consistent with those reported for formally analogous 6-(4-substituted-phenyl)pyridazin-3(2*H*)-one-based PDE III-inhibiting inodilators with less-extended phenyl substituents (see e.g. Sircar et al. *J. Med. Chem.* **1987**, *30*, 1955, Moos et al. *J. Med. Chem.* **1987**, *30*, 1963). PDE III inhibitory potency is associated with overall planar topology of the phenylpyridazinone moiety and the presence of two critically separated electronegative centers. A methyl group at the 5-position of a dihydropyridazinone ring leads to enhanced potency. However, the generally higher levels of PDE III inhibitory potency shown by compounds in the bis(azinone) series relative to earlier 6-(4-substituted-phenyl)pyridazin-3(2*H*)-one derivatives appears to derive from a closer to optimal separation of two interacting points in the inhibitor molecule achieved through the more extended bis(azinone) structure. Correlation between the pharmacological and PDE III inhibitory activities of compounds in the bis(azinone) series provides additional evidence for PDE III being an important mediator of inodilator action.

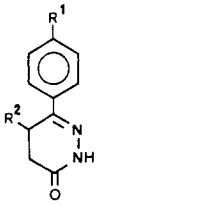
The inotropic and vasodilator (inodilator) properties of substituted 6-phenylpyridazin-3(2*H*)-ones and 4,5-dihydro-6-phenylpyridazin-3(2*H*)-ones are well-recognized.[1-14] Activity is retained by the phenylpyridazinone derivatives 1 and 2 where R^1 is a small, heteroatom-containing substituent and compounds in which R^1 is acetamido (e.g. **1a**, **2a**,**b**) or 1H-imidazol-1-yl (e.g. **1b**, **2c**,**d**) are particularly potent. There is substantial evidence that the inodilator activity of 6-(4-substituted)phenylpyridazin-3(2*H*)-ones is strongly associated with their ability to inhibit the low K_m, cAMP-selective, cGMP-inhibited cyclic nu-



1

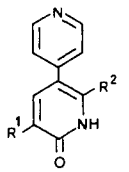
a) R^1 = NHAc

b) R^1 = 1-imidazolyl



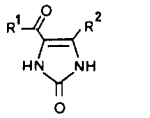
2

	R^1	R^2	
a)	NHAc	H	
b)	NHAc	CH_3	
c)	1-imidazolyl	H	(CI-914)
d)	1-imidazolyl	CH_3	(CI-930)



3

	R^1	R^2	
a)	NH_2	H	(amrinone)
b)	CN	CH_3	(milrinone)



4

	R^1	R^2	
a)	4-SCH_3-phenyl	CH_3	(enoximone)
b)	3-pyridyl	C_2H_5	(piroximone)

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