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Active Site-directed Thrombin Inhibitors: α-Hydroxyacyl-prolyl-arginals. New Orally Active Stable Analogs of D-Phe-Pro-Arg-H

S. Bajusz,* E. Barabás, I. Fauszt, A. Fehér, Gy. Horváth, A. Juhász, A. G. Szabó and E. Széll
Institute for Drug Research, P.O. Box 82, Budapest, H-1325, Hungary

Abstract—D-α-Hydroxyacyl-prolyl-arginals have been designed and synthesized as orally active stable analogs of D-Phe-Pro-Arg-H, the active site-directed peptidyl thrombin inhibitor prototype. Many of the new analogs possess high *in vitro* anticoagulant activity while having little effect on fibrinolysis. Compounds GYKI-66104 (**2**), -66131 (**3**) and -66132 (**5**) effectively delay the clotting time in rabbits *ex vivo* and prevent thrombus formation in various thrombosis models in rabbits and rats when applied in a single oral dose of 5 mg kg^{-1}.

Introduction

Thrombin is the central mediator of thrombus formation in the pathogenesis of thrombotic diseases.

The pharmacological control of thrombosis is based on inhibition of thrombin, the serine protease that plays a key role in thrombus formation. A potent arginal tripeptide thrombin inhibitor, D-Phe-Pro-Arg-H,[1,2] could be derived from the thrombin cleavage sites of clotting factors as follows: (i) Pro–Arg in P_2–P_1 of factors II and XIII appeared to be of deciding importance for the affinity between the enzyme and the inhibitor; Val–Arg in P_2–P_1 of fibrinogen seemed to be of much less or no importance; (ii) the unnatural D-Phe residue was introduced into P_3 to provide an extra binding site for the enzyme.[3] Modifications of D-Phe-Pro-Arg[4] at the C- and/or N-terminus led to a number of potent irreversible and reversible inhibitors.[6,7]

D-Phe-Pro-Arg-H was prone to inactivation via intramolecular condensation.[8] The analog Boc-D-Phe-Pro-Arg-H[2,9] was stable but showed reduced thrombin selectivity.[10,11] Since the terminal NH_2 seemed to participate in the transformation, *N*-alkyl derivatives were prepared.[8] Of these D-MePhe-Pro-Arg-H (GYKI-14766/LY294468) possessed high and selective thrombin inhibiting activity, even *in vivo*, which makes it of potential clinical importance.

In our ongoing effort to develop new stable peptidyl arginals as oral anticoagulants, as well as to further understand the role of the P_3 α-substituent, we synthesized a series of D-Phe-Pro-Arg-H analogs having D-α-hydroxy acid residues (D-Hac) in P_3, e.g. mandelic acid (Man), 3,3-diphenyllactic acid (Dpl), 3-cyclohexyllactic acid, 3-hexahydrophenyllactic acid (Hpl), 3-phenyllactic acid (Pla), hexahydromandelic acid (Hma) and (2-naphthyl)glycolic acid [Nga(2)]. The structures discussed in this paper are presented in Figure 1.

Results

D-α-Hydroxyacyl-prolyl-arginals, D-Hac-Pro-Arg-H, were assembled by 2 + 1 coupling like the dipeptidyl arginals[1,2,8] but starting from α-hydroxy acids.

D-Hac (**a**) $^{i}\rightarrow$ D-Hac-OMe (**b**) $^{ii}\rightarrow$ THP-D-Hac-OMe (**c**)

c $^{iii}\rightarrow$ THP-D-Hac (**d**) $^{iv}\rightarrow$ THP-D-Hac-OTcp (**e**)

e + Pro $^{v}\rightarrow$ THP-D-Hac-Pro (**f**)

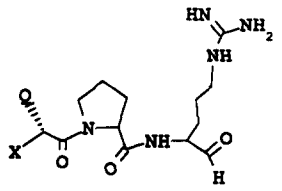
f + Arg (Z)> $^{vi}\rightarrow$ THP-D-Hac-Pro-Arg(Z)> (**g**)

g $^{vii}\rightarrow$ THP-D-Hac-Pro-Arg(Z)-H (**h**)

h $^{viii}\rightarrow$ THP-D-Hac-Pro-Arg-H (**i**) $^{ix}\rightarrow$ D-Hac-Pro-Arg-H (**1–5**)

*Hac = Man (**1**), Hpl (**3**), Pla (**4**), or Hma (**5**). Reagents: i, MeOH/0.05 equiv. H_2SO_4; ii, 1 equiv. dihydropyran/0.05 equiv. HCl/EtOAc in CH_2Cl_2; iii, NaOH/MeOH; iv, HOTcp/DCC in THF; v, coupling in DMF/1 equiv. NEt_3; vi, mixed anhydride coupling; vii, $LiAlH_4$/THF; viii, H_2/Pd in EtOH/0.5 M H_2SO_4 (1 equiv.); ix, catalyst removed, pH adjusted to 3.6, kept at 20 °C for 24 h.

Scheme 1. Synthesis of D-Hac-Pro-Arg-H.*



P_3-Pro-Arg-H

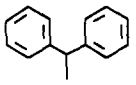
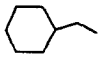
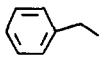
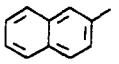
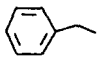
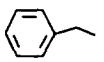
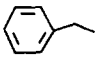
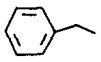
No.	X-	Q-	P_3 abbrev.
1	HO-		D-Man
2	HO-		D-Dpl
3	HO-		D-Hpl
4	HO-		D-Pla
5	HO-		D-Hma
6	HO-		D-Nga(2)
7	CH_3O-		D-MePla
R1	H_2N-		D-Phe
R2	CH_3-NH-		D-MePhe
R3	Boc-NH-		Boc-D-Phe

Figure 1.

Most of α-hydroxy acids were incorporated as 2,4,5-trichlorophenyl (Tcp) esters protected with O-2-tetrahydropyranyl (THP) at the α-OH, THP-D-Hac-OTcp (Scheme 1). These were coupled to Pro, then the ensuing THP-D-Hac-Pro to N^G–Z protected Arg lactam[8] [Arg(Z)>]. THP-D-Hac-Pro-Arg(Z)> was reduced by $LiAlH_4$ to the aldehyde, which was subjected to hydrogenolysis in aqueous ethanol or DMF containing H_2SO_4 to remove Z and THP. The free peptides were isolated by freeze-drying as hemisulfates.

For the synthesis of D-Dpl-Pro-Arg-H (**2**), the process was commenced from DL-Dpl, and THP-DL-Dpl-Pro-Arg(Z)> was subjected to chromatography on silica gel to give intermediate **2g**, THP-D-Dpl-Pro-Arg(Z)>.

D-Nga(2)-Pro-Arg-H (**6**) was prepared in a similar way but using *tert*-butyl (*t*Bu) for O-protection and starting from DL-Nga(2). Intermediate *t*Bu-D-Nga(2)-Pro (**6f**) was obtained from *t*Bu-DL-Nga(2)-Pro by ion exchange chromatography. The *t*Bu group was removed from the lactam, *t*Bu-D-Nga(2)-Pro-Arg(Z)>, because of the acid sensitivity of the Arg aldehyde moiety.[12,13]

Starting from O-methyl-3-phenyl-D-lactic acid (D-MePla) and proceeding through analogous intermediates, D-MePla-Pro-Arg-H (**7**) was also synthesized.

The compounds thus prepared (**1–7**) were evaluated for their *in vitro* anticoagulant activity in the thrombin time (TT), whole blood clotting time (WBCT), recalcification time (RCT) and activated partial thromboplastin time (APTT) assays.[15,16] Results are shown in Table 1; arginals **1–7** are listed in order of the activity determined in the TT. Compounds **R1–R3** are also included as reference compounds. According to these assays the three most active compounds were **1** (TT), **2** (RCT, APTT) and **3** (WBCT).

The new arginals **1–3** and **5** were also evaluated for their ability to inhibit plasmin (PL) and plasmin generation by tissue plasminogen activator (tPA), urokinase (UK) and streptokinase (SK) by using the fibrin plate assay.[15,18] Controls were included: **R2**, a specific thrombin inhibitor; and **R3**, which was known to inhibit

Table 1. Anticoagulant Activity of Xaa-Pro-Arg-H in the thrombin time (TT), activated partial thromboplastin time (APTT), recalcification time (RCT) and whole blood clotting time (WBCT) assays and the WBCT:TT, APTT and RCT IC_{50} ratios*

		IC_{50}†				WBCT	WBCT	WBCT
No.	Xaa	TT	WBCT	RCT	APTT	TT	RCT	APTT
1	D-Man	0.092	1.026	0.274	0.342	11.15	3.74	3.00
2	D-Dpl	0.146	0.983	0.208	0.226	6.73	4.73	4.35
3	D-Hpl	0.157	0.697	0.240	0.436	4.44	2.90	1.60
4	D-Pla	0.192	1.392	0.332	0.375	7.25	4.19	3.71
5	D-Hma	0.225	1.620	0.449	0.788	7.20	2.81	1.60
6	D-Nga(2)	0.307	2.128	0.358	1.064	6.93	5.94	2.00
7	D-MePla	0.322	2.184	1.911	1.794	6.78	1.14	1.22
R1	D-Phe	0.104	0.699	0.639	0.608	6.72	1.09	1.15
R2	D-MePhe	0.107	0.758	0.544	0.622	7.08	1.44	1.22
R3	Boc-D-Phe	0.236	2.357	1.414	0.743	9.65	0.89	3.14

*See Ref 15. †IC_{50}, the concentration (μM) required to double the clotting time of human plasma or whole blood.

both thrombin and other enzymes of the fibrinolytic system.[10,11]

The results are presented in Table 2. Data of columns B indicated **1–3** and **5** exert lower inhibitory actions on these enzymes than **R2**, which is 3.2–23.5 less potent than **R3**.

The anticoagulant effect of **2**, **3** and **5** in an oral dose of 5 mg kg^{-1} was determined by *ex vivo* clotting time measurements in conscious New Zealand white rabbits[19] as described in Ref. 20. Results are shown in Table 3. As indicated by the values in bold face the so-called therapeutic effect[21] of the peptides appeared 30 min after application and persisted for > 1 h and for 2.0–2.5 h with **2**.

The antithrombotic effect of **2**, **3** and **5** applied orally in a dose of 5 mg kg^{-1} was studied in a venous, an arterial and an arterio–venous shunt model of thrombosis as described in Ref. 22.

Results obtained in the venous thrombosis model in rats[23] (Table 4) demonstrated that each peptide induced a significant decrease in thrombus weight.

In the arterial thrombosis model in rats[24] the effect of peptides on the occlusion of the vessel can be measured by recording the decrease of vessel-surface temperature. Table 5 shows that each compound prevented the occlusion significantly.

Table 2. Inhibitory actions of Xaa-Pro-Arg-H on plasmin (PL) and plasmin generation by tissue plasminogen activator (tPA), urokinase (UK) and streptokinase (SK) as determined by the fibrin plate assay*

		IC_{50},† (A) and Relative Potencies‡ (B)							
		PL§		tPA‖		UK¶		SK**	
No.	Xaa	A	B	A	B	A	B	A	B
1	D-Man	153.3	0.4	205.9	0.6	105.1	0.7	1139	0.6
2	D-Dpl	311.0	0.2	395.1	0.3	283.6	0.3	> 1800	0.4
3	D-Hpl	104.9	0.5	157.4	0.8	130.1	0.6	703	0.9
5	D-Hma	237.8	0.2	328.6	0.4	270.2	0.3	1384	0.5
R2	D-MePhe	56.3	1.0	127.7	1.0	75.1	1.0	667	1.0
R3	Boc-D-Phe	17.6	3.2	7.9	16.2	3.2	23.5	137	4.9

*See Ref. 15. †The concentration (μM) required for the reduction of lysed area to 50% of the control. ‡Referred to the enzyme inhibiting activities of **R2**. §33 μg mL^{-1} plasmin. ‖5 μg mL^{-1} actilyse. ¶25 Plough U mL^{-1} urokinase. **250 U mL^{-1} streptase.

Table 3. Changes in thrombin time (TT), activated partial thromboplastin time (APTT) and whole blood clotting time (WBCT) after a single oral dose of 5 mg kg^{-1} of **2**, **3** and **5**, D-Hac-Pro-Arg-H, administered to rabbits (n = 5)*†‡

	2, Dpl			**3**, Hpl			**5**, Hma		
Time (min)	TT	APTT	WBCT	TT	APTT	WBCT	TT	APTT	WBCT
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
30	**12.74**	**1.70**	**2.01**	**12.41**	**2.12**	**1.68**	**14.22**	**2.26**	**1.68**
45	**13.65**	**1.55**	**1.69**	**9.05**	**1.96**	**1.58**	**19.25**	**2.25**	**1.83**
60	**18.39**	**1.62**	**1.58**	**9.42**	**1.79**	**1.54**	**20.99**	**2.00**	**1.87**
90	**16.86**	**1.67**	**1.52**	**6.26**	**1.90**	1.43	**14.59**	**1.84**	**1.74**
120	**9.21**	**1.56**	**1.50**	2.24	**1.72**	1.39	**8.02**	**1.61**	1.35
150	**7.09**	1.47	1.34	1.55	1.30	1.21	3.23	1.31	1.25
180	2.03	1.45	1.18	1.27	1.29	1.07	1.44	1.20	1.16

*See Ref. 19. †Therapeutic values are emboldened. ‡Clotting times relative to the respective untreated controls, were as follows. TT (s): 17.2 ± 0.6 (**2**), 14.6 ± 0.6 (**3**), 17.5 ± 0.6 (**5**); APTT (s): 34.8 ± 3.5 (**2**), 31.9 ± 1.5 (**3**), 31.4 ± 1.4 (**5**); WBCT (min): 12.5 ± 0.6 (**2**), 13.1 ± 0.3 (**3**); 12.7 ± 0.3 (**5**).

Table 4. Inhibitory effect of a single oral dose of 5 mg kg^{-1} of **2**, **3** and **5**, D-Hac-Pro-Arg-H, on thrombus formation in a venous thrombosis model in rats*

Peptide No.	Hac	(n)	Wet weight of thrombus (mg)	Inhibition (%)
Control		(5)	23.24 ± 2.26	-
2	Dpl	(7)	11.07 ± 1.20 ($p < 0.001$)	52.4
3	Hpl	(5)	9.74 ± 1.57 ($p < 0.001$)	58.1
Control		(5)	24.30 ± 1.80	-
5	Hma	(8)	9.70 ± 0.82 ($p < 0.001$)	60.1

*See Refs 19 and 23.

Table 5. Inhibitory effect of a single oral dose of 5 mg kg^{-1} of **2**, **3** and **5**, D-Hac-Pro-Arg-H, on experimental arterial thrombosis in rats*

Peptide No. Hac	(n)	Decrease in vessel-surface temperature (°C)	Inhibition (%)
Control	(11)	1.52 ± 0.039 ($p < 0.001$)	-
2 Dpl	(6)	0.57 ± 0.05 ($p < 0.001$)	62.5
3 Hpl	(6)	0.72 ± 0.04 ($p < 0.001$)	52.6
5 Hma	(6)	0.40 ± 0.27 ($p < 0.001$)	73.7

*See Refs 19 and 24.

Table 6. Inhibitory effect of a single oral dose of 5 mg kg^{-1} of **2**, **3** and **5**, D-Hac-Pro-Arg-H, on thrombus formation in an arterio–venous shunt model and parallel changes in clotting times in rabbits*

Arterio–venous shunt in function for a 20 min period	Wet weight of thrombus (mg)		
	2, Dpl	**3**, Hpl	**5**, Hma
Before application†	77.1 ± 3.8	80.6 ± 8.7	93.7 ± 11.5
From 45 to 65 min after application†	24.9 ± 3.2‡	21.9 ± 2.45§	25.2 ± 9.3‡
Clotting times at 45 min relative to those at 0 min	TT, 8.65 APTT, 1.59 WBCT, 1.52	TT, 1.30‖ APTT, 1.36‖ WBCT, 1.11‖	TT, 16.84 APTT, 2.33 WBCT, 1.36

*See Refs 19 and 25. †$n = 5$. ‡$p < 0.001$. §$p < 0.01$. ‖Clotting times at 65 min relative to those at 0 min were 13.74 (TT), 2.16 (APTT) and 1.62 (WBCT).

By using the arterio–venous shunt model in rats[25] both the antithrombotic and anticoagulant effects of the peptides can be examined in self-controlled experiments. As it appears from Table 6 significant decrease in thrombus weight and delay in clotting times could be reached by each peptide.

Discussion

Table 1 includes three early structures as reference compounds: D-Phe-Pro-Arg-H (**R1**); the D-MePhe analog (**R2**), which has the similarly basic Me-NH as terminal group; and the Boc-D-Phe analog (**R3**), whose N-terminus is neutral, Boc-NH. **R1** and **R2** are about equipotent and more active than **R3** in each assay. Regarding the IC_{50} values measured in the TT and APTT, **R3** is only 2.2–1.2 times less potent than **R1** or **R2**. This finding suggests that it may be the size rather than the basicity of the N-terminus that controls the thrombin inhibiting potential of such tripeptide aldehydes. Thus D-Phe as a P_3 may favorably be replaced with its neutral hydroxy acid analog, D-Pla, or its suitable congeners. The α-OH of D-Pla is about as small as the NH_2 of D-Phe but cannot induce intramolecular condensation and is neutral like the bulky and hydrophobic Boc-NH.

Prompted by this speculation a series of D-Phe-Pro-Arg-H analogs having D-α-hydroxy acid residues (D-Hac) in P_3 has been prepared in order to further understand the role of the P_3 α-substituent as well as to develop new stable peptidyl arginals as oral anticoagulants.

The new D-Hac-Pro-Arg-H analogs could easily be prepared by using the THP group for protecting the α-OH of D-Hac as outlined in Scheme 1. Less convenient is this process when starting from DL-Hac because the introduction of the O-THP creates a new chiral center that makes the separation of diastereomeric intermediates more difficult. In the synthesis of D-Dpl-Pro-Arg-H (**2**) commenced from DL-Dpl, THP-DL-Dpl-Pro-Arg(Z)>, a three-residue intermediate (**g**), could only be resolved. For the synthesis of D-Nga(2)-Pro-Arg-H (**6**) the α-OH of DL-Nga(2) was protected with the *t*Bu. In this case, *t*Bu-DL-Nga(2)-Pro, a two-residue intermediate (**f**), could be separated. On the other hand, the *t*Bu had to be removed from the lactam, *t*Bu-D-Nga(2)-Pro-Arg(Z)>, because anhydrous acids, e.g. TFA, used as deblocking agents, would also decompose the Arg-H moiety.[12] Fortunately D-Nga(2)-Pro-Arg(Z)> could be converted into D-Nga(2)-Pro-Arg-H without complication.

Table 1 shows the anticoagulant activity of the new analogs **1–7** and reference arginals **R1–R3** as measured in the TT, WBCT, RCT and APTT assays (IC_{50}).[16] Regarding the antithrombin activities in the TT, **1–3** were similar to the more active **R1** and **R2** while **4–7** were similar to the less potent **R3**. Thrombin inhibition requires higher peptide concentration in the WBCT than in the TT (the WBCT:TT IC_{50} ratios indicate a factor of 7 on the average). This is because the amount of thrombin operating in the WBCT is much higher than that in the TT.[16] In the WBCT **1–3** were similarly or nearly as active as **R1** or **R2** while **4–7** showed about 2–3 times less potency than **R2**. On the other hand, **1–6** in the RCT and **1–4** in the APTT proved to be more active than **R1** or **R2**. As shown by the WBCT:RCT and WBCT:APTT IC_{50} ratios, **1–6** exerted higher activities

in the RCT (2.81–5.94 times) and APTT (1.60–4.35 times) than in the WBCT. It is of interest because **7**, the α-O-methyl derivative, as well as **R1** and **R2** were about equipotent in these three assays. Considering that the APTT and RCT operate at a 3–7 times higher Ca^{2+} concentration than the WBCT, one may suppose that anticoagulation by the α-hydroxy acyl compounds can be assisted by Ca^{2+}, at least if it is present in elevated concentration. The activity profile of **R3** was again different: higher potency in the APTT than in the RCT or WBCT. This result may be due to adsorption of **R3** on the blood cells because the potency order found seems to be inversely proportional to the cell content of the assay systems, i.e. it is low in platelet poor plasma (APTT) but rather high in platelet rich plasma (RCT) or whole blood (WBCT).

An anticoagulant applicable in therapy is obviously expected to inhibit the blood coagulation process while permitting the lysis of blood clot to proceed. The effects of compounds **1–3** and **5** on plasmin and plasminogen activation by tPA, UK and SK were examined by the fibrin plate assay.[18] The results obtained (Table 2) demonstrated that the new analogs were somewhat less inhibitory than **R2** (columns B). In comparison with **R3**, they exerted 6–16 times lower antiplasmin activity and 20–78 times lower effect on plasmin generation by tPA and UK.

Clotting time measurements (TT, APTT and WBCT) in conscious New Zealand white rabbits *ex vivo*[20] were used for the determination of the oral activity of **2**, **3** and **5** in a dose of 5 mg kg^{-1} (Table 3). Each assay indicated that the therapeutic effect[21] of each peptide appeared 30 min after application. As measured in the TT this activity persisted for 2.5 and 2.0 h with **2** and **5**, respectively, and for 1.5 h with **3**. Similar results were obtained in the APTT—the therapeutic effect of each peptide seemed to persist for 2 h. According to the WBCT the duration of peptide's effects were somewhat different, 1.0, 1.5 and 2.0 h for **3**, **5** and **2**, respectively. The more homogeneous results were obtained for **2**, which appeared to be the longest acting analog.

The antithrombotic effects of **2**, **3** and **5** in a single oral dose of 5 mg kg^{-1} were studied as described previously for the evaluation of D-MePhe-Pro-Arg-H.[22] The results obtained demonstrated that each analog induced a significant decrease in thrombus weight in a quantitative venous thrombosis model with stasis based on vascular lesion in rats[23] (Table 4) as well as in an extracorporeal arterio–venous shunt model in rabbits[25] (Table 6), and prevented the occlusion of the vessel in arterial thrombosis induced by mechanical damage in rats[24] (Table 5).

Conclusion

In conclusion, we have shown that substitution of the OH group for the NH_2 in D-Phe-Pro-Arg-H (**R1**) provides new analogs which have favorable chemical and biological properties similar to those of **R2** (GYKI-14766/LY294468). Apparently, the α-OH of the new P_3 residues cannot take part in the intramolecular condensation leading to an inactive heterocycle.[8] It is, however, surprising that the analogs of **R1** having neutral α-hydroxy acids in P_3, such as D-Dpl, D-Hpl and D-Hma, i.e. analogs GYKI-66104 (**2**), -66131 (**3**), and -66132 (**5**), could also be potent and selective anticoagulants/antithrombotics with significant oral activity. In view of these findings it is very likely that the less favorable biological properties of **R3** are due to the hydrophobicity and bulkiness of the N-terminus, Boc-NH, rather than its neutrality.

Experimental

Reactions were monitored, and the homogeneity of the products checked by TLC on Kieselgel 60 F_{254} plates (Merck, Darmstadt, BRG) with the following eluents (all v/v): (1) EtOAc:pyridine:AcOH:water (480:20:6:11); (2) EtOAc:pyridine:AcOH:water (240:20:6:11); (5) EtOAc:pyridine:AcOH:water (45:20:6:11); (6) EtOAc:pyridine:AcOH:water (30:20:6:11); (7) EtOAc:pyridine:HCOOH:water (480:20:6:5.5); (9) EtOAc:pyridine:AcOH:water (960:20:6:11); (10) EtOAc:diisopropyl ether (7:3); (12) chloroform:acetone (98:2); (13) hexane:EtOAc (2:1). Analytical HPLC was performed on a 'Pharmacia LKB analytical HPLC System Two' with UV/vis detector at 214 nm, utilizing a Vydac C-18 reversed phase 10 μm particle-size, 300 Å pore-size, 4 × 250-mm column, with eluent systems (A) 0.1% TFA in water and (B) 0.1% TFA in CH_3CN under gradient condition (flow rate) as follows: (1) 0–5 min: 0–15% B; 5–30 min: 15% B; (1 mL min^{-1}). (2) 0–30 min: 0–60% B; (1 mL min^{-1}). (3) 0–5 min: 0–6% B; 5–30 min: 6% B; 30–35 min: 6–18% B; (1.2 mL min^{-1}). The arginals are specified by *k'* values (capacity factor).[26] Mass spectra were taken on a Finnigan MAT 8430 instrument under the following operating conditions: (i) electron ionization (EI): electron energy, 70 eV; electron current, 100 μA; ion-source temperature, 250 °C; R = 1250; (ii) chemical ionization (CI): reagent gas, *i*-butane; electron energy, 250 eV; electron current, 200 μA; ion-source temperature, 250 °C; R = 1250. (iii) Fast atom bombardment (FAB): FAB-gas, xenon; gun voltage, 9 kV; matrix, *m*-nitro-benzylalcohol; source temperature, 25 °C; R = 1250.[27] ^1H NMR spectra were taken on a Bruker AC 250 instrument in D_2O or in DMSO-d_6.[28] Elemental analyses, indicated by symbols of elements, refer to data obtained ±0.4% of the theoretical values. All reagents used were from commercial sources and used without additional purification.

D-Hpl (3a)

A solution of D-Pla[29] (5 g, 0.03 mol; TLC R_f(2) 0.50) in glacial acetic acid (70 mL) was reacted with hydrogen over platinum oxide (0.25 g) at 40–50 °C. The reaction was monitored by TLC [final product: R_f (2) 0.65]. The reaction mixture was filtered, and the filtrate was concentrated *in vacuo*. Toluene (20 mL) was evaporated twice from the residue. The solid was crystallized

from hexane. The precipitate was filtered, washed with hexane, and dried *in vacuo* to afford pure **3a** (4.3 g, 83%): MS (EI) m/z 172 ($M^{+\cdot}$); ^1H NMR (DMSO-d_6) δ 0.75–1.05 (*m*, 2H), 1.1–1.3 (*m*, 3H), 1.35–1.6 (*m*, 3H), 1.6–1.85 (*m*, 5H), 3.97 (*m*, 1H), 5.1 (*b*, ~1H), 12.1 (*b*, ~1H); mp 90–93 °C; $[\alpha]_D^{20}$ +8.55° (*c* 1, MeOH). Anal. ($C_9H_{16}O_3$) C,H.

THP-D-Man·CHA (1d)

A solution of D-Man (**1a**) (3.05 g, 0.02 mol) in anhydrous methanol (30 mL) containing 4 drops of H_2SO_4 was refluxed for 3 h. The reaction mixture was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 (40 mL) and washed with water to neutrality. The organic solution was dried ($MgSO_4$), filtered, and concentrated *in vacuo* to give D-Man-OMe (**1b**) as an oil [3.3 g; TLC R_f (13) 0.45]. The ester **1b** was dissolved in CH_2Cl_2 (20 mL), and dihydropyran (2 mL, 0.022 mol) and 3 M HCl in EtOAc (0.3 mL) were added at constant stirring. After 16 h at room temperature the reaction mixture was diluted with CH_2Cl_2 (20 mL), washed with water to neutrality and evaporated *in vacuo*. The resultant oil [5.0 g of THP-D-Man-OMe, **1c**, TLC R_f (13) 0.70] was dissolved in MeOH (40 mL) and 1 M NaOH (20 mL). After 16 h at room temperature the reaction mixture was diluted with water (20 mL) and concentrated *in vacuo* up to the appearance of mild cloudiness. The solution obtained was washed with CH_2Cl_2 (10 mL). The aqueous layer was cooled to 5–10 °C, the pH was adjusted to 3 with 1 M $KHSO_4$ and extracted with CH_2Cl_2 (3 × 10-mL). The organic layers were pooled, washed with water to neutrality, dried ($MgSO_4$) and evaporated *in vacuo*. The THP-D-Man obtained was dissolved in diethyl ether and cyclohexylamine (2.3 mL, 0.02 mol) was added to the solution. After 5 h of standing at 5 °C, the crystals are filtered off, washed with diethyl ether and dried *in vacuo* to afford pure **1d** (4.2 g, 62%): MS (CI) m/z 237 (MH^+); TLC R_f (1) 0.77; mp 148–150 °C. Anal. ($C_{19}H_{29}NO_4$) C,H,N.

THP-DL-Dpl (2d)

(i) A stirred, cooled (0 °C) solution of 3,3-diphenyl-DL-lactic acid nitrile[30] (22.33 g, 0.1 mol) in diethyl ether (100 mL) containing methanol (4.04 mL, 0.1 mol) was saturated with dry HCl gas. After 1 h of standing without cooling, the solution was concentrated *in vacuo*. The residue was crystallized with diethyl ether (200 mL), filtered, washed with diethyl ether, and dried *in vacuo*. The resultant solid (mp 82–83 °C) was suspended in water (140 mL), stirred for 2 h at 50–60 °C, cooled to 5–10 °C, and stirred (1.5 h). The precipitate was filtered, washed with water, dried *in vacuo*, washed with hexane and dried *in vacuo* to afford pure DL-Dpl-OMe, **2b**, (12.8 g, 50%): TLC R_f (12) 0.40; mp 49 °C. Anal. ($C_{16}H_{16}O_3$) C,H. (ii) Compound **2b** (7.83 g, 0.0305 mol) was converted to THP-DL-Dpl-OMe (**2c**) and saponified in a similar manner to that for **1d** to give solid THP-DL-Dpl, **2d**, (7.9 g, 75%): MS (CI) m/z 327 (MH^+); TLC R_f (9) 0.70; mp 115–117 °C (sintering at 76 °C). Anal. ($C_{20}H_{22}O_4$) C,H.

THP-D-Hpl·CHA (3d)

THP-D-Hpl·CHA (**3d**) was prepared from **3a** in a similar manner to that for **1d** as crystalline salt (70%): MS (CI) m/z 257 (MH^+); TLC R_f (9) 0.67; mp 153–155 °C (sintering at 142 °C). Anal. ($C_{20}H_{37}NO_4$) C,H,N.

THP-D-Pla·CHA (4d)

THP-D-Pla·CHA (**4d**) was prepared from D-Pla[29] in a similar manner to that for **1d** as crystalline salt (60%): MS (CI) m/z 251 (MH^+); TLC R_f (9) 0.73; mp 148–150 °C. Anal. ($C_{20}H_{31}NO_4$) C,H,N.

THP-D-Hma·CHA (5d)

THP-D-Hma·CHA (**5d**) was prepared from D-Hma[31] (**5a**) in a similar manner to that for **1d** as crystalline salt (60%): MS (CI) m/z 243 (MH^+); TLC R_f (9) 0.62; mp 152–155 °C (sintering at 122 °C). Anal. ($C_{19}H_{35}NO_4$) C,H,N.

tBu-DL-Nga(2) (6d)

A solution of DL-Nga(2)[32] (20.2 g, 0.1 mol) in MeOH (150 mL) containing H_2SO_4 (0.3 mL) was refluxed for 3 h. The reaction mixture was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 (200 mL) and washed with water to neutrality. The organic solution was dried ($MgSO_4$), filtered, and concentrated *in vacuo* to give DL-Nga(2)-OMe (**6b**) as an oil (22 g, TLC R_f (2) 0.8). The oil was dissolved in CH_2Cl_2 (100 mL) and cooled to –25 °C, and CF_3SO_3H (0.88 mL, 0.01 mol) was added followed by isobutylene (120 mL). After 30 min of standing at –25 °C pyridine (1.0 mL) was added, and the reaction mixture was left to warm up to room temperature, and concentrated *in vacuo*. The oily residue was dissolved in benzene (100 mL), and washed sequentially with water, 5% $NaHCO_3$, water, 1 M $KHSO_4$, and water (3 × 20-mL each). The organic layer was dried ($MgSO_4$) and concentrated *in vacuo* to give an oil (**6c**, TLC R_f (12) 0.52). The oil was dissolved in 2 M NaOH (50 mL, 0.1 mol) and MeOH (100 mL). After stirring at room temperature overnight, the reaction mixture was saturated with carbon dioxide by adding dry-ice, washed with hexane (2 × 20-mL), acidified with solid $KHSO_4$ (13.6 g, 0.1 mol) and extracted with EtOAc (3 × 40-mL). The organic layers were combined, washed to neutrality with water, dried ($MgSO_4$) and concentrated *in vacuo*. The oily residue was mixed with hexane (100 mL). After 5 h of standing at 5 °C, the crystals formed were filtered, washed with hexane, and dried *in vacuo* to afford pure **6d** (13.2 g, 51%): MS (EI) m/z 258 ($M^{+\cdot}$); mp 114–116 °C; TLC R_f (9) 0.36. Anal. ($C_{16}H_{18}O_3$) C,H.

D-MePla·CHA (7d)

D-Pla[29] (6.65 g, 0.04 mol) was methylated as described in Ref. 33. The product obtained as an oil was dissolved

in diethyl ether and cyclohexylamine (4.6 mL, 0.04 mol) was added to the solution. After 5 h of standing at 5 °C, the crystals are filtered off, washed with diethyl ether and dried *in vacuo* to afford pure **7d** (6.7 g, 60%): MS (EI) m/z 180 ($M^{+\cdot}$); ^{1}H NMR (DMSO-d_6) δ 2.87 (*dd*, 1H), 2.97 (*dd*, 1H), 3.2 (*s*, 3H), 3.92 (*dd*, 1H), 7.15–7.30 (*m*, 5H); $[\alpha]_D^{20}$ +21.3° (*c* 1; MeOH). TLC R_f (9) 0.70; mp 158–160 °C (sintering at 120 °C). Anal. ($C_{16}H_{25}NO_3$) C,H,N.

THP-D-Man-Pro·CHA (1f)

Compound **1d** (8.25 g, 0.025 mol) was dissolved in CH_2Cl_2 (50 mL) and 1 M $KHSO_4$ (30 mL). The organic layer was washed with water, dried ($MgSO_4$), and concentrated *in vacuo*. The oil was dissolved in DMF (15 mL) and cooled to 0 °C, and 2,4,5-trichlorophenol (5.12 g, 0.026 mol) was added followed by DCC (5.15 g, 0.025 mol). The reaction mixture was stirred at room temperature for 2 h at 0 °C. The resultant precipitate was removed by filtration, and proline (2.9 g, 0.025 mol) and triethylamine (3.5 mL, 0.025 mol) were added to the solution containing THP-D-Man-OTcp (**1e**). After the mixture stirred for 24 h at room temperature, the DMF was removed *in vacuo*. The residue was dissolved in 5% $NaHCO_3$ (30 mL). The cloudy solution obtained was washed with diethyl ether (3 × 20-mL), the pH is adjusted to 3 with solid $KHSO_4$, and extracted with EtOAc (3 × 40-mL). The combined EtOAc solutions were washed to neutrality with water, dried ($MgSO_4$), and concentrated *in vacuo*. The oil was dissolved in diethyl ether and cyclohexylamine (2.88 mL, 0.025 mol) was added to the solution. After 5 h of standing at 5 °C, the crystals are filtered off, washed with diethyl ether and dried *in vacuo* to afford pure **1f** (6.7 g, 62%): MS (FAB) m/z 334 (MH^+); ^{1}H NMR (DMSO-d_6) δ 1.6–2.2 (*m*, 10H), 3.25–3.9 (*m*, 4H), 4.15–4.3 (*m*, 1H), 4.55–4.75 (2*m*, 1H), 5.35–5.45 (2*s*, 1H), 7.25–7.55 (*m*, 5H), 12.5 (*b*, ~1H); TLC R_f (1) 0.35. Anal. ($C_{24}H_{36}N_2O_5$) C,H,N.

THP-DL-Dpl-Pro (2f)

THP-DL-Dpl-Pro (**2f**) was prepared from THP-DL-Dpl (**2d**) in a similar manner to that for **1f** except the product was triturated with diisopropyl ether to give the acid **2f** as an amorphous solid (54%): MS (FAB) m/z 424 (MH^+); TLC R_f (2) 0.50; mp 132–135 °C. Anal. ($C_{25}H_{29}NO_5$) C,H,N.

THP-D-Hpl-Pro·CHA (3f)

THP-D-Hpl-Pro·CHA (**3f**) was prepared from **3d** in a similar manner to that for **1f** as crystalline salt (56%): MS (FAB) m/z 354 (MH^+). TLC R_f (9) 0.47; mp 119–120 °C. Anal. ($C_{25}H_{44}N_2O_5$) C,H,N.

THP-D-Pla-Pro·CHA (4f)

THP-D-Pla-Pro·CHA (**4f**) was prepared from **4d** in a similar manner to that for **1f** as crystalline salt (60%): MS (FAB) m/z 348 (MH^+); TLC R_f (1) 0.35. Anal. ($C_{25}H_{38}N_2O_5$) C,H,N.

THP-D-Hma-Pro·CHA (5f)

THP-D-Hma-Pro·CHA (**5f**) was prepared from (**5d**) in a similar manner to that for **1f** as crystalline salt (60%): MS (FAB) m/z 340 (MH^+); TLC R_f (9) 0.23; mp 140–142 °C. Anal. ($C_{24}H_{42}N_2O_5$) C,H,N.

tBu-D-Nga(2)-Pro (6f)

(i) Compound **6d** (12.9 g, 0.05 mol) was dissolved in DMF (30 mL) and cooled to 0 °C, and 2,4,5-trichlorophenol (10.24 g, 0.052 mol) was added followed by DCC (10.3 g, 0.05 mol). The reaction mixture was stirred for 2 h at room temperature. The resultant precipitate was removed by filtration, and proline (5.8 g, 0.05 mol) and triethylamine (7.0 mL, 0.05 mol) were added to the solution containing *t*Bu-DL-Nga(2)-OTcp (**6e**). After the mixture was stirred for 24 h at room temperature, the DMF was removed *in vacuo*. The residue was dissolved in 5% $NaHCO_3$ (60 mL). The cloudy solution obtained was washed with diethyl ether (3 × 40-mL), the pH was adjusted to 3 with solid $KHSO_4$, and extracted with EtOAc (3 × 80-mL). The combined EtOAc solutions were washed to neutrality with water, dried ($MgSO_4$), and concentrated *in vacuo* to give a mixture of *t*Bu-L-Nga(2)-Pro and *t*Bu-D-Nga(2)-Pro (TLC R_f (1) 0.36 and 0.39, respectively) as an oil. (ii) The resultant oil was dissolved in a 2:1 mixture of MeOH and water (2.0 L), and applied to a 80 × 6-cm column prepared from Bio-Rad AG 1-X2 anion exchange resin 50–100 mesh in acetate form (2000 mL). The resin was washed with the above mixture of MeOH and water (4000 mL) at a flow rate of 15 mL min^{-1}. A 2:1 mixture of MeOH and 1 M AcOH was used as eluent, collecting 200 mL fractions. The fractions were monitored by TLC with system (1), the spots were visualized under UV light or by $KMnO_4$. The fractions containing the pure isomers were combined and concentrated to a third volume *in vacuo*. The resultant precipitate was redissolved by the addition of CH_3CN, and the solutions were freeze-dried. Yields: Isomer **I** (5.63 g, TLC R_f (1) 0.36), Isomer **II** (5.93 g, TLC R_f (1) 0.39) and mixture of **I** and **II** (1.0 g). On the basis of the chromatographic properties and enzyme inhibitory activities of the aldehyde derivatives prepared from them,[34] isomer **II** was identified as *t*Bu-D-Nga(2)-Pro, **6f** (0.0167 mol, 66.7%): MS (EI) m/z 355 ($M^{+\cdot}$); mp 90–93 °C; $[\alpha]_D^{20}$ –45.8° (*c* 1; MeOH). Anal. ($C_{21}H_{25}NO_4$) C,H,N.

D-MePla-Pro·CHA (7f)

Compound **7d** (7.0 g, 0.025 mol) was dissolved in EtOAc (50 mL) and 1 M $KHSO_4$ (30 mL). The organic layer was washed with water, dried ($MgSO_4$), and concentrated *in vacuo*. In flask 1, the resultant oil was dissolved in DMF (20 mL) and cooled to –15 °C, and *N*-methylmorpholine (2.75 mL, 0.025 mol) was added followed by isobutyl chloroformate (3.3 mL, 0.025 mol). The reaction mixture was stirred at –15 °C for 3 min. In flask 2, Pro-O*t*Bu·HCl (5.2 g, 0.025 mol) was dissolved in DMF (25 mL) and cooled to –15 °C, and tri-

ethylamine (3.5 mL, 0.025 mol) was added to the solution and stirred at −15 °C for 2 min. The content of flask 2 was added to flask 1, and the reaction mixture was stirred for 30 min (−15 °C) followed by 1.5 h at room temperature. The resultant precipitate was removed by filtration, and the mother liquor was concentrated *in vacuo*. The residue was dissolved in EtOAc (30 mL) and water (30 mL). The organic layer was separated and washed sequentially with 0.25 M H_2SO_4 (3 × 20-mL), water (2 × 20-mL), 5% $NaHCO_3$ (3 × 20-mL) and water (2 × 20-mL). The EtOAc layer was dried ($MgSO_4$), filtered, and concentrated *in vacuo* to give D-MePla-Pro-O*t*Bu as an oil. The oil was dissolved in TFA (25 mL). After 2 h of standing at room temperature, the solution was concentrated *in vacuo*. The residue was dissolved in EtOAc (50 mL) and extracted with 5% $NaHCO_3$ (3 × 30-mL). The aqueous layer was separated and acidified with 1 M $KHSO_4$ to pH 3 and extracted with EtOAc. The organic solution was dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The resultant oil was dissolved in diethyl ether and cyclohexylamine (2.88 mL, 0.025 mol) was added to the solution. After 5 h of standing at 5 °C, the crystals are filtered off, washed with diethyl ether and dried *in vacuo* to afford pure **7f** (6.6 g, 70%): MS (CI) m/z 278 (MH^+); ^1H NMR (DMSO-d_6) δ 1.6–2.3 (*m*, 4H), 2.85 (*d*, 2H), 3.15 (*s*, 3H), 3.3–3.8 (*m*, 2H), 4.18 (*m*, 2H), 7.15–7.30 (*m*, 5H); TLC R_f (1) 0.18; mp 139–140 °C. $[\alpha]_D^{20}$ −53.1° (*c* 1, MeOH). Anal. ($C_{21}H_{32}N_2O_4$) C,H,N.

THP-D-Man-Pro-Arg(Z)> (1g)

Compound **1f** (2.6 g, 0.006 mol) was dissolved in CH_2Cl_2 (20 mL) and 1 M $KHSO_4$ (7.0 mL). The organic layer was washed with water, dried ($MgSO_4$), and concentrated *in vacuo*. In flask 1, the resultant oil was dissolved in DMF (6.0 mL) and cooled to −15 °C, and *N*-methylmorpholine (0.66 mL, 0.006 mol) was added followed by isobutyl chloroformate (0.79 mL, 0.006 mol). The reaction mixture was stirred at −15 °C for 3 min. In flask 2, HCl·Arg(Z)>[8] (2.29 g, 0.007 mol) was dissolved in DMF (10 mL) and cooled to −15 °C. The content of flask 2 and triethylamine (2.1 mL, 0.015 mol) was added to flask 1 at −15 °C, and the reaction mixture was stirred for 2 h (−15 °C) followed by 1.5 h at room temperature. The resultant precipitate was removed by filtration, and the mother liquor was diluted with benzene (50 mL) and washed sequentially with water (4 × 20-mL), 0.1 M HCl (3 × 10-mL), water (3 × 20-mL). The organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was dissolved in a 240:20:6:5.5 mixture of EtOAc:pyridine:HCOOH:water (1.5 mL) and applied to a column prepared from silica gel (100 g) with EtOAc, and eluted with the same mixture of EtOAc:pyridine:HCOOH:water. Fractions were collected and pooled on the basis of TLC profile. The combined fractions were shaken with 1/3 volume of 1 M $KHCO_3$, the organic layer was washed to neutrality with water, dried ($MgSO_4$), and concentrated *in vacuo*. The resultant oil was dissolved in benzene, then repeatedly evaporated *in vacuo*. The residue was triturated with diisopropyl ether, and the solid was filtered and dried to give **1g** (2.5 g, 69%): MS (FAB) m/z 606 (MH^+); TLC R_f (EtOAc) 0.55.

THP-D-Dpl-Pro-Arg(Z)> (2g)

THP-DL-Dpl-Pro (**2f,** 2.60 g, 0.006 mol) was converted into a mixed anhydride and reacted with HCl·Arg(Z)>[8] (2.29 g, 0.007 mol) in a similar manner to that for **1g** to give the two diastereomeric peptide lactams, THP-L-Dpl-Pro-Arg(Z)> and THP-D-Dpl-Pro-Arg(Z)>, as a crude product [3.8 g, 90%; TLC R_f (10) 0.25 (**I**) and 0.30 (**II**) as two main components). This mixture was dissolved in CH_2Cl_2 (6 mL) and applied to a column prepared from silica gel (400 g) with a 1:1 mixture of EtOAc and cyclohexane, and was eluted with 7:3 mixture of EtOAc and cyclohexane. Fractions were monitored by TLC with system (10), the spots were visualized under UV light or by $KMnO_4$. The fractions containing the pure isomers were combined, concentrated *in vacuo*, and triturated with diisopropyl ether to afford isomer **I** [0.98 g, 47%; TLC R_f (10), 0.25] and **II** [1.0 g, 48%; TLC R_f (10), 0.30] as amorphous solid. On the basis of the enzyme inhibitory activities of the aldehyde derivatives prepared from the two products,[34] isomer **II** was identified as THP-D-Dpl-Pro-Arg(Z)> **2g**: MS (FAB) m/z 696 (MH^+). Anal. ($C_{39}H_{45}N_5O_7$) C,H,N.

THP-D-Hpl-Pro-Arg(Z)> (3g)

THP-D-Hpl-Pro-Arg(Z)> (**3g**) was prepared from **3f** in a similar manner to that for **1g** as an amorphous solid (65%): MS (FAB) m/z 626 (MH^+); TLC R_f (2) 0.77.

THP-D-Pla-Pro-Arg(Z)> (4g)

THP-D-Pla-Pro-Arg(Z)> (**4g**) was prepared from **4f** in a similar manner to that for **1g** as an amorphous solid (65%): MS (FAB) m/z 620 (MH^+); TLC R_f (EtOAc) 0.60.

THP-D-Hma-Pro-Arg(Z)> (5g)

THP-D-Hma-Pro-Arg(Z)> (**5g**) was prepared from **5f** in a similar manner to that for **1g** as an amorphous solid (69%): MS (FAB) m/z 612 (MH^+); TLC R_f (2) 0.74.

D-Nga(2)-Pro-Arg(Z)> (6g)

(i) Compound **6f** (2.8 g, 0.0078 mol) was converted into a mixed anhydride and coupled to HCl·Arg(Z)>[8] (2.61 g, 0.008 mol) in a similar manner to that for **1g** except the residue of the last evaporation was triturated with hexane. The solid was filtered, washed with hexane and dried to give *t*Bu-D-Nga(2)-Pro-Arg(Z)> (4.14 g, 85%): TLC R_f (9) 0.73. (ii) The O-*t*Bu-protected lactam thus obtained (4.08 g, 0.0065 mol) was dissolved in a 1:1 mixture of TFA and CH_2Cl_2 (20 mL). After 30 min of standing at room temperature, the mixture was diluted with CH_2Cl_2 (40 mL), washed to neutrality with water (3 × 20-mL) and 5% $NaHCO_3$ (20 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo* to give crude peptide **6g**. The peptide was applied to a column prepared from 100 g of silica gel with an eluent of

EtOAc:pyridine:AcOH:water (480:20:6:11). Fractions were collected and pooled on the basis of TLC profile (R_f (7) 0.45). The combined fractions were washed with 1 M $KHSO_4$ and water, dried ($MgSO_4$), and concentrated *in vacuo*. The residue was triturated with hexane, filtered, washed with hexane and dried *in vacuo* to yield pure **6g** (1.8 g, 48%): MS (FAB) m/z 572 (MH^+); TLC R_f (7) 0.45. Anal. ($C_{31}H_{33}N_5O_6$) C,H,N.

D-MePla-Pro-Arg(Z)> (7g)

Compound **7f** (2.73 g, 0.007 mol) was converted into a mixed anhydride and coupled to HCl·Arg(Z)>[8] (2.61 g, 0.008 mol) in a similar manner to that for **1g** except the pure oily product was triturated with hexane. The solid was filtered, washed with hexane and dried to give **7g** (2.5 g, 76%): MS (FAB) m/z 550 (MH^+); TLC R_f (9) 0.62.

THP-D-Man-Pro-Arg(Z)-H (1h)

To a stirred, cooled (–60 °C) solution of **1g** (2.5 g, 0.0041 mol) in dry THF (12 mL) lithium aluminum hydride 1 M in THF (3.1 mL, 0.0031 mol) was added. The reaction mixture was stirred for 30 min at –60 °C, then poured under cooling into 1 M $KHSO_4$ (30 mL). The solution was diluted with water (15 mL), and extracted with hexane (2 × 15-mL) and CH_2Cl_2 (3 × 15-mL). The combined CH_2Cl_2 solutions were washed sequentially with water, 5% $NaHCO_3$ and water (3 × 7-mL of each), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The resultant oil was triturated with hexane to give an amorphous solid **1h** (1.5 g, 60%): MS (FAB) m/z 608 (MH^+); TLC R_f (2) 0.38.

THP-D-Dpl-Pro-Arg(Z)-H (2h)

To a stirred, cooled (–60 °C) solution of **2g** (0.95 g, 0.00136 mol) in dry THF (8 mL) lithium aluminum hydride 1 M in THF (1.02 mL, 0.00102 mol) was added. The reaction mixture was stirred for 30 min at –60 °C, then acidified with 0.5 M H_2SO_4 to pH 3. The solution was extracted with 5 mL of diethyl ether. The bottom layer was neutralized with 5% $NaHCO_3$ (pH 6.5), and the solution was extracted with CH_2Cl_2 (3 × 5-mL). The combined CH_2Cl_2 solutions were dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The residue was triturated with diisopropyl ether to yield **2h** (0.86 g 90%): MS (FAB) m/z 698 (MH^+); TLC R_f (2) 0.40.

THP-D-Hpl-Pro-Arg(Z)-H (3h)

THP-D-Hpl-Pro-Arg(Z)-H (**3h**) was prepared from **3g** in a similar manner to that for **1h** as an amorphous solid (59%): MS (FAB) m/z 628 (MH^+); TLC R_f (2) 0.32.

THP-D-Pla-Pro-Arg(Z)-H (4h)

THP-D-Pla-Pro-Arg(Z)-H (**4h**) was prepared from **4g** in a similar manner to that for **1h** as an amorphous solid (60%): MS (FAB) m/z 622 (MH^+); TLC R_f (2) 0.45.

THP-D-Hma-Pro-Arg(Z)-H (5h)

THP-D-Hma-Pro-Arg(Z)-H (**5h**) was prepared from **5g** in a similar manner to that for **1h** as an amorphous solid (65%): MS (FAB) m/z 614 (MH^+); TLC R_f (2) 0.27.

D-Nga(2)-Pro-Arg(Z)-H (6h)

To a stirred, cooled (–60 °C) solution of **6g** (1.71 g, 0.003 mol) in dry THF (15 mL), lithium aluminum hydride 1 M in THF (2.25 mL, 0.00225 mol) was added. The reaction mixture was stirred for 30 min at –60 °C, then acidified with 0.5 M H_2SO_4 to pH 3–4. The solution was diluted with water (15 mL), washed with EtOAc (3 × 10-mL), and extracted with *n*-butanol saturated with water (3 × 10-mL). The combined *n*-butanol solutions were washed with 5% $NaHCO_3$ (10 mL) and water saturated with *n*-butanol (2 × 10-mL), and concentrated *in vacuo*. The residue was triturated with hexane to yield **6h** as an amorphous solid (1.13 g, 66%): MS (FAB) m/z 574 (MH^+); TLC R_f (6) 0.64.

D-MePla-Pro-Arg(Z)-H (7h)

D-MePla-Pro-Arg(Z)-H (**7h**) was prepared from **7g** in a similar manner to that for **1h** as an amorphous solid (70%): MS (FAB) m/z 552 (MH^+); TLC R_f (2) 0.50.

D-Man-Pro-Arg-H·0.5H_2SO_4 (1)

Compound **1h** (1.22 g, 0.002 mol) dissolved in EtOH (6 mL), water (4 mL) and 1 M H_2SO_4 (0.525 mL), was hydrogenated in the presence of 5% Pd/C catalyst (0.15 g) at about 10 °C and ambient pressure. After the removal of the Z-group was completed (1 h), the catalyst was filtered off and washed with 60% aqueous EtOH and water. The filtrate was concentrated to about half of its volume *in vacuo*, diluted with water to 10 mL, and the pH was adjusted to 2.8 with H_2SO_4 or Bio Rad AG1-X8 resin (hydroxide form). The solution was left to stand at room temperature until the complete cleavage of the THP (24 h). The aqueous solution was washed with CH_2Cl_2 (3 × 5-mL), the pH was adjusted to 3.6 with Bio Rad AG1-X8 resin (hydroxide form). The resin was removed by filtration and the filtrate lyophilized to give **1** as a white solid (0.6 g, 60%): MS (FAB) m/z 390 (MH^+); $[\alpha]_D^{20}$ –141.9° (*c* 1, H_2O); TLC R_f (5) 0.51; HPLC (3) k' = 8.33, 9.22, and 14.2.

D-Dpl-Pro-Arg-H·0.5H_2SO_4 (2)

Compound **2h** (0.8 g, 0.00115 mol) dissolved in DMF (6 mL) and 0.5 M H_2SO_4 (1.2 mL) was hydrogenated in the presence of 5% Pd/C catalyst (0.12 g) at about 10 °C and ambient pressure. After the removal of the Z-group was completed (1 h), the catalyst was filtered off and washed with 50% aqueous DMF (1 mL) and water (1 mL). The filtrate was concentrated *in vacuo*. The residue was dissolved in water (10 mL), and the pH was adjusted to 2.8 with H_2SO_4 or Bio Rad AG1-X8 resin (hydroxide form). The solution was left to stand at room temperature until the complete cleavage of the THP (24 h), and worked up in a similar manner to that

for **1** to give **2** as a lyophilized white solid (0.5 g, 70%): MS (FAB) m/z 480 (MH^+); $[\alpha]_D^{20}$ –170.04° (*c* 1, 0.05 M H_2SO_4:*t*BuOH, 1:1); TLC R_f (5) 0.50; HPLC (2) *k'* = 7.80 and 8.23.

D-Hpl-Pro-Arg-H·0.5H_2SO_4 (3)

D-Hpl-Pro-Arg-H·0.5H_2SO_4 (**3**) was prepared from **3h** in a similar manner to that for **1** as a lyophilized white solid (60%): MS (FAB) m/z 410 (MH^+); $[\alpha]_D^{20}$ –80.2° (*c* 1, H_2O); TLC R_f (5) 0.47; HPLC (2) *k'* = 35.25 and 37.40.

D-Pla-Pro-Arg-H·0.5H_2SO_4 (4)

D-Pla-Pro-Arg-H·0.5H_2SO_4 (**4**) was prepared from **4h** in a similar manner to that for **1** as a lyophilized white solid (60%): MS (FAB) m/z 404 (MH^+); $[\alpha]_D^{20}$ –114.23° (*c* 1, H_2O); TLC R_f (5) 0.55; HPLC (1) *k'* = 3.96 and 4.96.

D-Hma-Pro-Arg-H·0.5H_2SO_4 (5)

D-Hma-Pro-Arg-H·0.5H_2SO_4 (**5**) was prepared from **5h** in a similar manner to that for **1** as a lyophilized white solid (60%): MS (FAB) m/z 396 (MH^+); $[\alpha]_D^{20}$ –84.5° (*c* 1, H_2O); TLC R_f (5) 0.42; HPLC (2) *k'* = 5.70, 6.10 and 8.76.

D-Nga(2)-Pro-Arg-H·0.5H_2SO_4 (6)

Compound **6h** (1.0 g, 0.00175 mol) dissolved in EtOH (20 mL), water (2 mL) and 0.5 M H_2SO_4 (1.75 mL) was hydrogenated in the presence of 5% Pd/C catalyst (0.12 g) at about 10 °C and ambient pressure. After the reaction was completed (4 h), the catalyst was filtered off and washed with 60% aqueous EtOH (5 mL) and water (5 mL). The filtrate was concentrated to about half of its volume *in vacuo*, diluted with water to 15 mL, and the pH was adjusted to 3.3 with H_2SO_4 or Bio Rad AG1-X8 resin (hydroxide form). If the solution obtained was cloudy due to precipitated material, it was redissolved by adding *tert*-butanol (0.5–1.0 mL, max. 10%). The clear solution was lyophilized to give **6** as a white solid (0.78 g, 88%): MS (FAB) m/z 440 (MH^+); $[\alpha]_D^{20}$ –152.54° (*c* 1, 0.05 M H_2SO_4:*t*BuOH, 1:1); TLC R_f (5) 0.31; HPLC (1) *k'* = 8.25, 9.25 and 11.3.

D-MePla-Pro-Arg-H·0.5H_2SO_4 (7)

D-MePla-Pro-Arg-H·0.5H_2SO_4 (**7**) was prepared from **7h** in a similar manner to that for **6** as a lyophilized white solid (70%): MS (FAB) m/z 418 (MH^+); $[\alpha]_D^{20}$ –93.9° (*c* 1, H_2O); TLC R_f (5) 0.55; HPLC (2) *k'* = 5.50 and 7.55.

References and Notes

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13. According to a previous finding[9] and recent practice[14] the acidolytic removal of the *tert*-butyloxycarbonyl group from peptidyl arginals can safely be performed by 5 M H_2SO_4 at 50 °C; cleavage of the O-*tert*-butyl requires more vigorous conditions.

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genous thrombin (TT), or by recalcification (RCT, APTT). Clotting in fresh blood (WBCT) takes place spontaneously. Exogenous thrombin applied in the TT results in a final concentration of 2.5 NIH U mL^{-1}. Endogenous thrombin generated in the other assays could theoretically be present at final concentrations as high as about 50 NIH U mL^{-1} (APTT) or 100 NIH U mL^{-1} (RCT, WBCT). Thrombin inhibiting (anticlotting) activity is expressed in IC_{50}, which is the concentration (μM) required to double the clotting time.

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23. A quantitative venous thrombosis model with stasis based on vascular lesion in rats.[22] Thrombus formation was started 45 min after administration of the peptides (at the time of maximum effect) and lasted for 2 h.

24. Arterial thrombosis after mechanically-induced damage of the vessel wall in rats.[22] The time from the mechanical provocation to the final decrease in temperature (30 min) was by definition the occlusive thrombus time and was used for the estimation of the antithrombotic effect. The thrombotic occlusion was recorded by the decrease of vessel-surface temperature using a thermistor thermometer in contact with the arterial surface. Thrombus formation was started 45 min after administration of the peptides (at the time of maximum effect) and lasted for 2 h.

25. Extracorporeal arterio–venous shunt thrombosis model in rabbits.[22] A similar model in rats (Smith, J. R.; White, A. M. *Br. J. Pharmacol.* **1982**, *77*, 29) was adapted to rabbits whereby recording the thrombus growth and monitoring the changes in clotting times were possible in self-controlled experiments.

26. The acyl-arginals exist in equilibrium structures, i.e. in aldehyde, aldehyde hydrate and two aminocyclol forms. During HPLC analysis the aldehyde hydrate and the aminocyclol forms appear as separated peaks; these compounds can be specified by two or three *k'* values.

27. The mass spectra of cyclohexylamine (CHA) salts were taken from the discharged acids.

28. Due to the presence of tautomeric and rotameric forms the ^1H NMR spectra of **1** to **7** are extremely complex (*c.f.* Ref. 8), therefore they are not given here.

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34. It has been known from earlier experience[1] that peptidyl arginals, DL-Xaa-Pro-Arg-H, having a D-residue in P_3 possess much higher thrombin inhibiting activity than their L-residue containing congeners.

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