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Peptide-derived Transition State Analogue Inhibitors of Thrombin; Synthesis, Activity and Selectivity

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Abstract—In a study to combine the transition state analogue concept with the principle of catalytic site spanning, a series of peptide-derived transition state analogue (TSA) inhibitors of thrombin has been synthesized and tested. In the sequence H-D-Phe-Pro-Arg-Gly-OH (**2**) the Arg–Gly amide bond has been replaced by three classes of transition state analogues, being the ketomethylene, the hydroxyethylene and the hydroxymethylene amide bond replacements. Compound **12a**, in which the amide bond has been replaced by the ketomethylene group, was found to be the most potent thrombin inhibitor of the series studied. Subsequently, penta- and hexapeptide sequences with good affinity for thrombin were developed, i.e. H-D-Phe-Pro-Arg-Gly-Phe-OH (**16**) and H-D-Phe-Pro-Arg-Gly-Phe-Lys-OH (**26**). In these sequences the Arg–Gly amide bond was then replaced by the ketomethylene group. The resulting compounds **43a** and **47a**, respectively, were evaluated *in vitro* as inhibitors of thrombin and factor Xa. Compound **47a** was found to be the most potent thrombin inhibitor of the series studied (K_i = 29 nM). The combination of the transition state analogue concept and the principle of peptide elongation (tetrapeptide→hexapeptide) yields thrombin inhibitors of high potency and selectivity. The effects of these two alterations reinforce each other indicating a synergistic effect. This might be rationalized by entropy factors.

Introduction

Thrombin, also referred to as factor IIa, is a serine protease which occupies a central position in the blood coagulation cascade. Thrombin catalyzes the cleavage of four Arg–Gly bonds in the natural substrate fibrinogen in order to form fibrin.[1,2]

Inhibitors of the catalytic activity of thrombin have been considered as potential targets for drug design as anti-thrombotic and anti-coagulant agents. Recently, low molecular weight inhibitors of thrombin have been reported.[3–14] One type of such inhibitors is the class of the ketomethylene pseudo peptide analogues, in which the NH-group of the Arg–Gly amide bond has been replaced by a $-CH_2-$ group.[3,7] The resulting ketomethylene group has several advantageous features. It might form a tetrahedral hemi-ketal by reaction with the active site serine hydroxyl group, thus becoming a transition state analogue.[15,16] Moreover, the ketomethylene group is not prone to proteolytic degradation.

Recently, Bode and coworkers[17] reported that the occupation of thrombin's putative S' region[18a] with amino acid residues from fibrinogen might contribute to the binding of the latter. To us, this implies that peptide inhibitors spanning the S as well as the S' region of the active site (i.e. spanning the P as well as the P' region) might show improved activity and selectivity. In the course of our studies on peptide-derived transition state analogue inhibitors of thrombin, we wanted to combine the transition state analogue concept with the principle of catalytic site spanning. In particular we wanted to explore the effect of this combination of features on the affinity and selectivity of the inhibitor.

So far, the combination of spanning the P as well as the P' region[18a] with transition state analogues has hardly been studied. Szelke and Jones[19] prepared the ketomethylene transition state analogue D-Phe-Pro-Arg-ψ-[C(O)-CH_2]-Gly-Pip[18b] which showed appreciable inhibitory activity (K_i = 1.3 μM). This inhibitory activity could be further improved (K_i = 0.2 μM) by replacement of the N-terminal D-Phe residue by a D-diphenylalanine residue.[7] However, in both reports no further attention was paid to the optimization of the P' region. The hirutonines have been published recently by DiMaio *et al.*[20] These compounds are based on the hirulogs that bind to a region in thrombin called the anion binding exosite and in addition occupy the S_3–S_1 region of thrombin by a D-Phe-Pro-Arg-Pro moiety connected to a peptide sequence. This peptide sequence does not occupy the S' region adjacent to the S_1 pocket. In the hirutonines, the Arg–Pro dipeptide has been replaced by an Arg-ψ-[C(O)-$(CH_2)_n$]-moiety with n = 0–3. The anti-thrombin activity of the compound with n = 1 was reported to be 0.29 nM.

In the present study, we describe the synthesis and testing of several penta- and hexapeptides that span the P and P' regions. By incorporating various transition state analogues in the starting compound of our studies (**2**), we show that the ketomethylene group is the optimal replacement of the scissile peptide bond; this replacement enhances the inhibitory activity against thrombin. Remarkably, the effects of the amide bond

replacement and the region spanning reinforce each other. This systematic study of these two features yielded us a very potent and selective inhibitor of thrombin, having a K_i value of 29 nM.

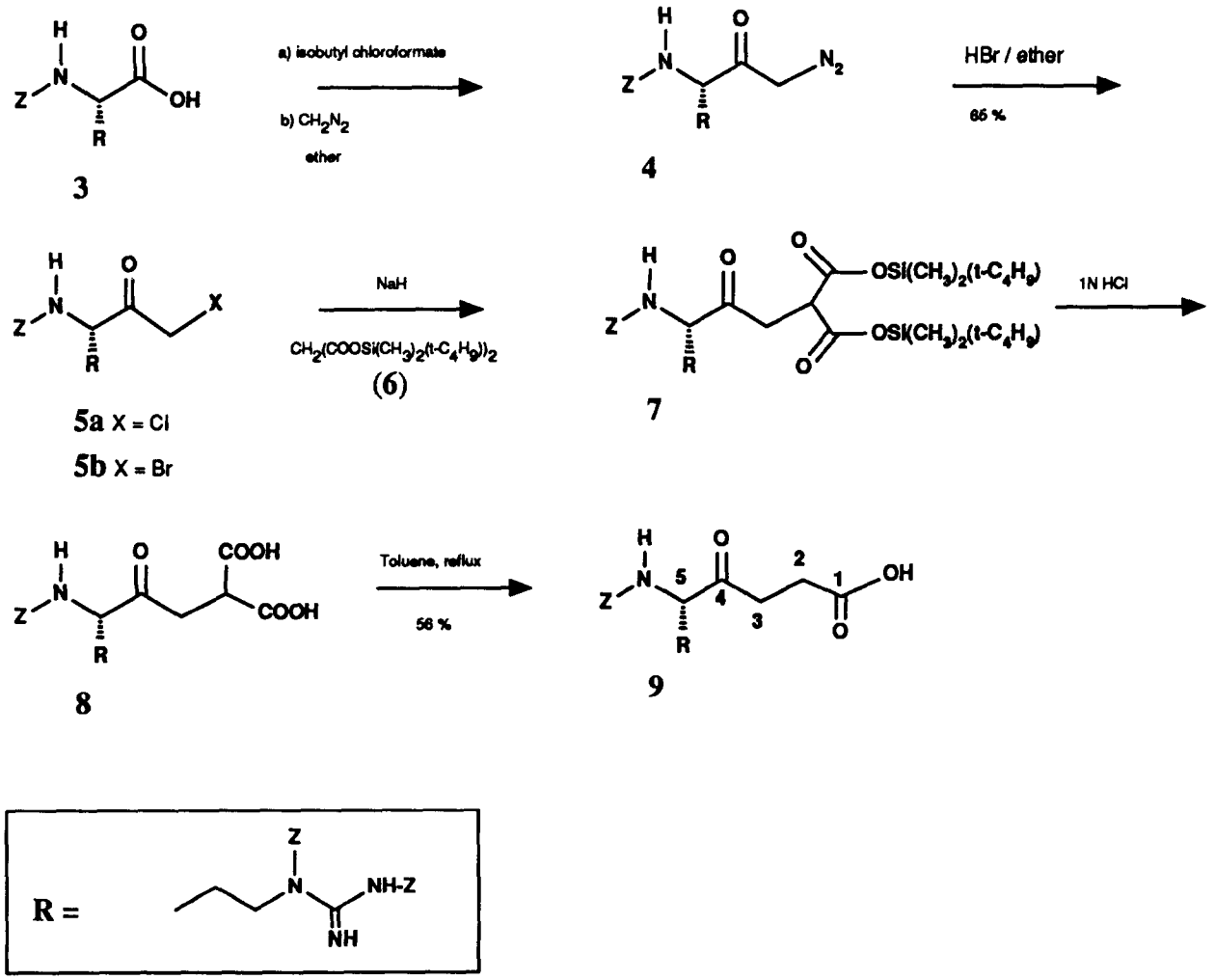
Results

Three regions can be distinguished in the structure of the inhibitors we aimed at, i.e. the P and P' regions[18] and the amide bond replacement. For the P region of the inhibitor we selected the peptide sequence D-Phe-Pro-Arg (**1**, Table 2) which has been widely used as a basis for thrombin inhibitors.[12a,13,21] This selection is also supported by X-ray data of thrombin inhibitors.[12b] Prior to the optimization of the P' region, the amide bond replacement was studied.

Optimization of the amide bond replacement

We planned to replace the Arg–Gly amide bond in the sequence D-Phe-Pro-Arg-Gly (**2**, Table 1) by three different amide bond replacements, i.e. the ketomethylene (C(O)-CH_2)-, the hydroxyethylene (CH(OH)-CH_2)- and the hydroxymethylene (CH(OH)) groups. In the first type, covalent bond formation between the ketomethylene carbonyl group and the active site serine residue[15a] might take place, thus mimicking the transition state of the amide bond hydrolysis. The latter two replacements can be considered as geometrical and stereo-electronic ground-state mimics.[15b]

For the synthesis of the compound with the ketomethylene amide bond replacement (Scheme 1, Z = benzyloxycarbonyl) we used the method described by García-López after some alterations.[22f,g] In order to avoid side reactions,[23,24] we used the, previously reported,[25] fully N-protected derivative N^{α},N^{δ},N^{ω}-tribenzyloxycarbonyl-L-arginine (**3**). The first step was elongation of the carbon chain at the C terminus in order to create the methylene moiety of the amide isostere. Accordingly, we prepared the bromomethylketone **5b**, since the chloromethylketone **5a** could not be reacted with the sodium salt of diethylmalonate. Reaction of **5b** with diethyl malonate yielded a product of which the malonate ester could not be saponified without loss of the benzyloxycarbonyl group at the δ-nitrogen. We therefore employed the di-(*t*-butyldimethylsilyl)malonate[26] (**6**) which was prepared from malonic acid, TEA and *t*-butyldimethylsilyl chloride in dry ether. The sodium salt of this compound was al-

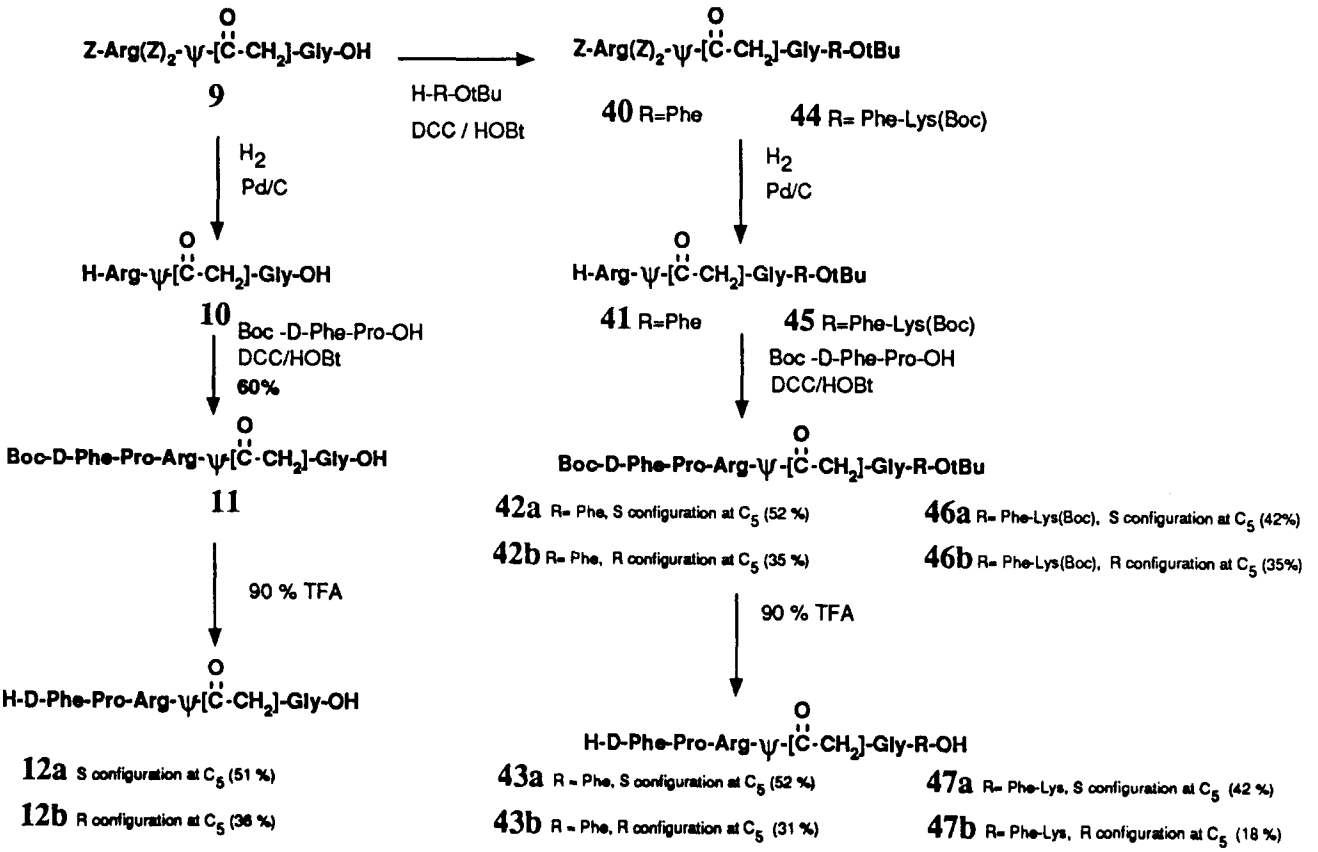


Scheme 1.

lowed to react with **5b** in THF to give **7**. Acidic workup of **7** gave the desired product **8**. The crude product **8** was decarboxylated by refluxing in toluene for 1 h to yield compound **9** in 56% yield based on **5b**. This compound was elongated by conventional peptide synthesis to yield compound **12a** (Scheme 2). For the assignment of the stereochemistry, see below.

Compounds **13** and **14** (Table 1) featuring the hydroxyethylene amide bond replacement were prepared from **9** by reduction with sodium borohydride followed by conventional peptide synthesis. The hydroxymethylene-containing compound **15** was prepared according to the method described by Castro *et al.*[27] For details of the synthesis of **13–15** see Ref. 28.

In Table 1 the results of the incorporation of the three replacements of the Arg–Gly amide bond in the sequence D-Phe-Pro-Arg-Gly are given. Table 1 shows that the ketomethylene group is the optimal replacement of the Arg–Gly amide bond in the tetrapeptide sequence D-Phe-Pro-Arg-Gly; this replacement (**2** → **12a**) enhances the inhibitory activity against thrombin appreciably. This ketomethylene replacement also causes an increase in selectivity as it hardly affects the anti-factor Xa activity.



Scheme 2.

Table 1. Inhibitory activities of the ketomethylene, the hydroxyethylene and the hydroxymethylene amide bond replacements in the sequence H-D-Phe-Arg-ψ-Gly-OH

		Thrombin		Factor Xa
	ψ	IC_{50} (10^{-3}M)	K_i (10^{-6}M)	IC_{50} (10^{-3}M)
2	C(O)-NH	**3.8**	**71.6**	**4.8**
12a	C(O)-CH_2	**0.99**	**25.1**	**4.7**
13	R-CH(OH)-CH_2	**>3.0**		**4.0**
14	S-CH(OH)-CH_2	**7.06**		**0.7**
15	S-CH(OH)	**>10**		**18.5**

On the basis of these findings it was decided to focus exclusively on the ketomethylene group.

Optimization of the P'-region

It was decided to elongate the peptide sequence D-Phe-Pro-Arg-Gly with one or two amino acid residues in the P' region and to search for the penta- and hexapeptide with the highest anti-thrombin activity. We wanted to pay attention to the nature as well as to the stereochemistry of the amino acid residues. For an optimal transition state stabilizing effect in further studies, it should also be ensured that the peptide selected is a substrate for thrombin and not merely an inhibitor.

The peptides **1**, **2** and **16–39** were synthesized by conventional peptide synthesis in solution and by solid phase peptide synthesis. Their analytical data are given in the Experimental (Table 6). The activities in the anti-thrombin and anti-factor Xa assay are given in Table 2.

Extension of **2** with a phenylalanine residue gave a pentapeptide (**16**) with considerably higher anti-thrombin activity. This pentapeptide and the Ala-analogue **19** were the starting points for the synthesis of a series of hexapeptides. Compounds **26** and **30** were obtained by elongation of **16** or **19**, respectively, with a lysine residue. This elongation leads to appreciable improvement of the inhibitory activity. Compounds **26** and **30** are better thrombin inhibitors than compound **39** which was used by Szelke *et al.*[3] to incorporate the ketomethylene group.

To obtain further insight into the role of the L-phenylalanine residue at the P_2' position we replaced this residue in compound **19** by a tyrosine residue (→ **20**) and its sulfated derivative (→ **21**), and in compound **30** by a cyclohexylalanine residue (→ **37**) or a tyrosine residue (→ **38**). These modifications hardly affect the anti-thrombin activity. Incorporation of a cyclohexylalanine residue (**37**) decreases only slightly the thrombin versus factor Xa selectivity. From these findings it can be concluded that small modifications of the phenylalanine residue are allowed.

Finally, the influence of the stereochemistry of the amino acid residues at the P_1–P_3' positions of the compounds **16**, **26** and **30** on the anti-thrombin activity was studied. Changing the chirality at either the arginine (→ **23**) or the alanine residue (→ **24**) of compound **19** is detrimental to the anti-thrombin activity. Changing the chirality at the P_2'-phenylalanine residue in compound **19** (→ **25**) decreases the

Table 2. Inhibitory activities of the peptides

							Thrombin		Factor Xa
	P_3 P_2	P_1	P_1'	P_2'	P_3'		IC_{50} (10^{-3}M)	K_i (10^{-6}M)	IC_{50} (10^{-3}M)
1	H-D-Phe-Pro-	Arg-	OH				**0.56**	**3.4**	**1.85**
2	H-D-Phe-Pro-	Arg-	Gly-	OH			**3.82**	**72**	**4.80**
16	H-D-Phe-Pro-	Arg-	Gly-	Phe-	OH		**0.33**	**7.6**	**1.13**
17	H-D-Phe-Pro-	Arg-	Gly-	His-	OH		**1.81**		**2.75**
18	H-D-Phe-Pro-	Arg-	Gly-	Glu-	OH		**>10**		**1.66**
19	H-D-Phe-Pro-	Arg-	Ala-	Phe-	OH		**0.47**		**6.20**
20	H-D-Phe-Pro-	Arg-	Ala-	Tyr-	OH		**0.32**		**3.47**
21	H-D-Phe-Pro-	Arg-	Ala-	Tyr(SO_3)-	OH		**2.41**		**3.54**
22	H-D-Phe-Pro-	Arg-	Ala-	Glu	OH		**6.19**		**2.83**
23	H-D-Phe-Pro-	D-Arg-	Ala-	Phe-	OH		**>10**		**2.34**
24	H-D-Phe-Pro-	Arg-	D-Ala-	Phe-	OH		**>10**		**4.29**
25	H-D-Phe-Pro-	Arg-	Ala-	D-Phe-	OH		**2.75**		**6.43**
26	H-D-Phe-Pro-	Arg-	Gly-	Phe-	Lys-	OH	**0.43**	**3.14**	**1.10**
27	H-D-Phe-Pro-	Arg-	Gly-	D-Phe-	Lys-	OH	**4.03**		**3.14**
28	H-D-Phe-Pro-	Arg-	Gly-	Phe-	D-Lys-	OH	**0.92**		**3.08**
29	H-D-Phe-Pro-	Arg-	Gly-	D-Phe-	D-Lys-	OH	**4.06**		**2.91**
30	H-D-Phe-Pro-	Arg-	Ala-	Phe-	Lys-	OH	**0.17**	**2.75**	**4.76**
31	H-D-Phe-Pro-	Arg-	Ala-	Phe-	Arg-	OH	**0.18**		**2.05**
32	H-D-Phe-Pro-	Arg-	Ala-	Phe-	Glu-	OH	**0.69**		**8.46**
33	H-D-Phe-Pro-	Arg-	Ala-	Phe-	Nle-	OH	**0.25**		**1.41**
34	H-D-Phe-Pro-	Arg-	Ala-	D-Phe-	Lys-	OH	**1.01**		**3.48**
35	H-D-Phe-Pro-	Arg-	Ala-	Phe-	D-Lys-	OH	**0.41**		**3.64**
36	H-D-Phe-Pro-	Arg-	Ala-	D-Phe-	D-Lys-	OH	**1.18**		**4.83**
37	H-D-Phe-Pro-	Arg-	Ala-	Cha-	Lys-	OH	**0.21**		**4.22**
38	H-D-Phe-Pro-	Arg-	Ala-	Tyr-	Lys-	OH	**0.15**		**5.27**
39	H-D-Phe-Pro-	Arg-	Gly-	Pro-	Arg-	OH	**1.33**		**2.16**

anti-thrombin activity, but not as dramatically as was seen for **23** and **24**. Changing the stereochemistry of the P_2' and P_3' residues in **26** (→ **27–29**) or **30** (→ **34–36**) does affect the activity. Comparison of the activities of these peptides indicates that for good anti-thrombin activity the L-stereochemistry of phenylalanine at P_2' is essential.

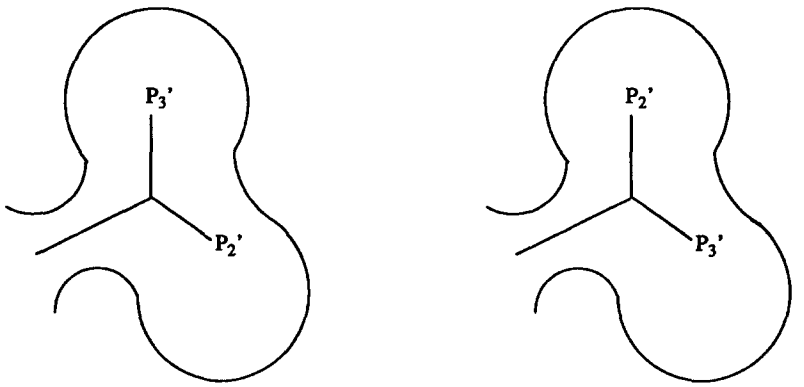
Molecular Modelling

In order to rationalize some of the structure–activity data from Table 2, we built structural models of several penta- and hexapeptides.[29–31] The X-ray structure of thrombin co-crystallized with its inhibitor H-D-Phe-Pro-Arg-CH_2Cl (PPACK)[31] was used as a starting point for our modelling studies because the D-Phe-Pro-Arg- sequence is identical to the P_3–P_1 sequence of the peptides studied by us.

Unfortunately, only scarce information is available concerning binding modes of inhibitors in the S' region of thrombin. Therefore, we decided to perform systematic conformational searches for the P' residues in the context of a rigid thrombin structure. In all calculations, the P_3–P_1 region remained close to its position observed in the X-ray structure of the thrombin–PPACK complex. From our modelling studies, it became clear that the P_2' residue and the C-terminus (P3' and the C-terminal carboxylate in the case of the hexapeptides and pentapeptides, respectively) of the inhibitor may adopt two main conformations, shown schematically in Figure 1. The conformation with the P_2' residue in the 'lower pocket' and the C-terminus in the 'upper pocket' is denoted orientation I, the corresponding one with the P_2' residue in the 'upper pocket' and the C-terminus in the 'lower pocket' is denoted orientation II.

For the pentapeptides, the C_α–C_β dihedrals of the side chains of the P_2' residues were varied in increments of 60°, leading to a total of $2 \times 12 = 24$ conformations for the orientations I and II. For the hexapeptides, a larger increment of 120° was applied for the P_2' and P_3' side chain torsion angles. Thus a total of $2 \times 3 \times 3 = 18$ conformations were generated. Each conformation was optimized so that the energetically most favoured conformation of the peptide in thrombin's active site could be determined. Since solvent is not explicitly included in the calculations we applied a distance dependent dielectric function of $\epsilon = 4 \times r$ during the energy minimization and interaction energy analyses.[30] In Table 3 an interaction energy (E_{int}) analysis of a selection of the modelled peptides is given. The overall interaction energy (E_{int}) is the sum of the energy due to electrostatic interactions (E_{elec}) and Van der Waals interactions (E_{vdw}).

As can be seen from Table 3, the trend in the calculated interaction energies qualitatively agrees with the observed differences in binding for the thrombin inhibitors. Due to the significant down-scaling of the electrostatics, the differences in the van der Waals contribution to the interaction energy dominate



Orientation I **Orientation II**

Figure 1. The main orientations of the side chain at the P_2' position and the C-terminus.

Table 3. Calculated and experimental data of the binding between thrombin and a selection of the peptides prepared.

	I/II	E_{int}[a]	E_{elec}[a]	E_{vdw}[a]	K_i (μM)
2	-	-79.1	-27.8	-51.2	72
16	I	-94.8	-31.8	-63.1	7.6
26	I	-99.8	-29.3	-70.5	3.14
30	I	-102.6	-30.1	-72.5	2.75

[a](kcal mol^{-1})

the mutual differences. It turned out that orientation I is calculated significantly more favourably than orientation II for the compounds **16**, **26** and **30**. The active site area of the energy-minimized 'thrombin–**16**' and 'thrombin–**30**' complexes are depicted in Figures 2 and 3, respectively. Compound **26** binds in an analogous fashion to compound **30**.

In Figures 2 and 3, it can be seen that the aromatic side chain at the P_2' position is harboured in a rather hydrophobic pocket formed by Leu(40) and Leu(41), Glu(39), Gln(151), Asn(143) and Gly(193). This hydrophobic pocket is also able to harbour other aromatic moieties at the P_2' position such as the ones present in **17** (His), **20** (Tyr), **21** ($TyrSO_3$), **37** (Cha) and **39** (Pro). This observation is corroborated by the activities of these peptides (Table 2).

In orientation I, the C-terminal part of compounds **16** and **30** points toward the 'north' side of thrombin. In the 'thrombin–**16**' complex hydrogen bonding is observed between the ϵ-NH_3^+ of Lys(60F)[31] and the C-terminal carboxylate. In the corresponding complex of **30**, several hydrogen bonds are formed: between the C-terminal carboxylate and Arg(35), Lys(60F) and the carbonyl of P_2', and Asp(60E) and P_3' Lys residue. The latter interaction may explain the observed (slight) preference for arginine and lysine residues at the P_3' position. However, the P_3' position seems more promiscuous than the P_2' position since negatively charged (Glu, **32**) or hydrophobic (Nle, **33**) residues are also allowed at the P_3' position. Some of the inhibitors with inverted stereochemistry may adopt orientation II as modelling studies indicate.[28]

We note that our structural models generally agree with the model proposed by Stubbs and Bode[17] for the interaction between fibrinogen and thrombin, in particular in the S' region. In their model the P_1'–P_3' sequence, being Gly-Pro-Arg, adopts an orientation comparable to our orientation I, the P_3' arginine residue forming an interaction with Glu(192) or Glu(32).

Hydrolysis experiment

The peptides to be selected from our peptide optimization study should be substrates for thrombin. If not, a transition state intermediate is not reached and replacement of the Arg–Xaa amide bond by a TSA would be a futile exercise.

The peptides **26** and **30** were each treated under the conditions of the anti-thrombin assay, the only difference being the absence of the chromogenic substrate.

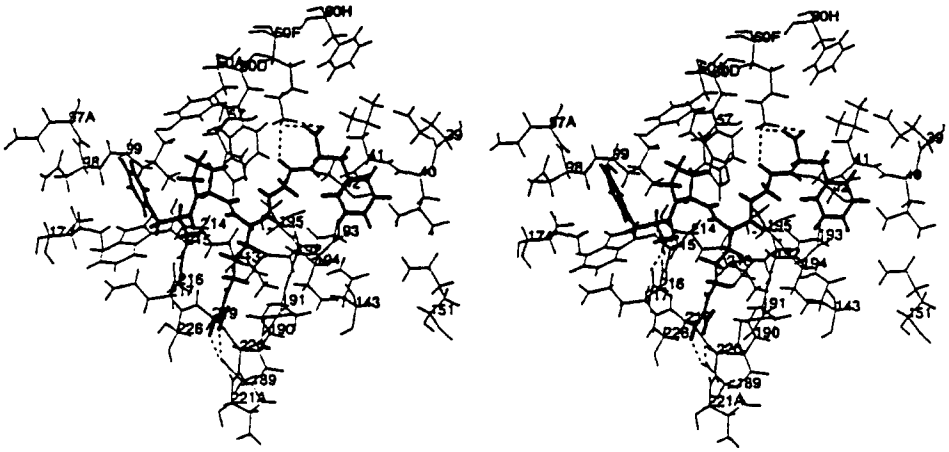


Figure 2. Interactions of compound **16** H-D-Phe-Pro-Arg-Gly-Phe-OH, within the active site of thrombin (orientation I).

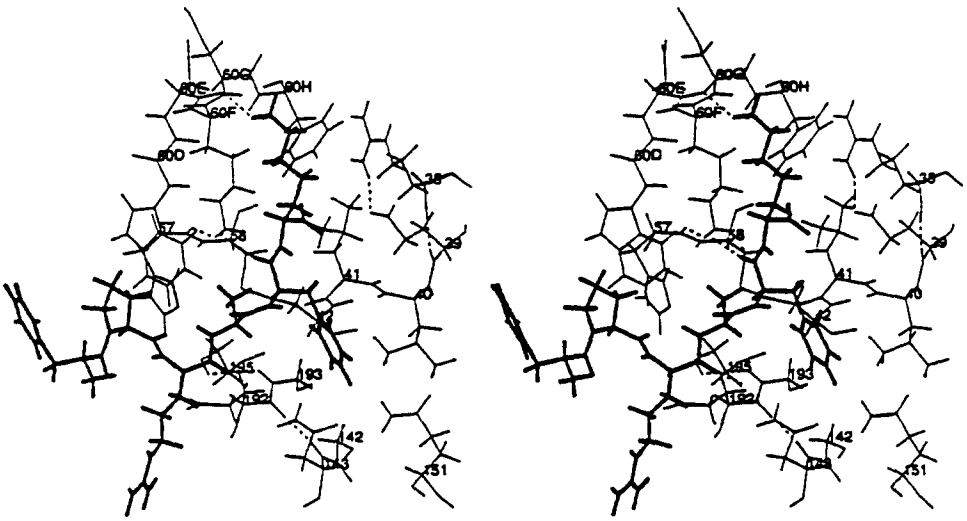


Figure 3. Interactions of compound **30** H-D-Phe-Pro-Arg-Ala-Phe-Lys-OH within the active site of thrombin (orientation I).

The release of H-D-Phe-Pro-Arg-OH (**1**) was measured by HPLC analysis as described in the Experimental. The results of these hydrolysis experiments are given in Table 4.

Table 4. Results of the hydrolysis experiments with **26** and **30**

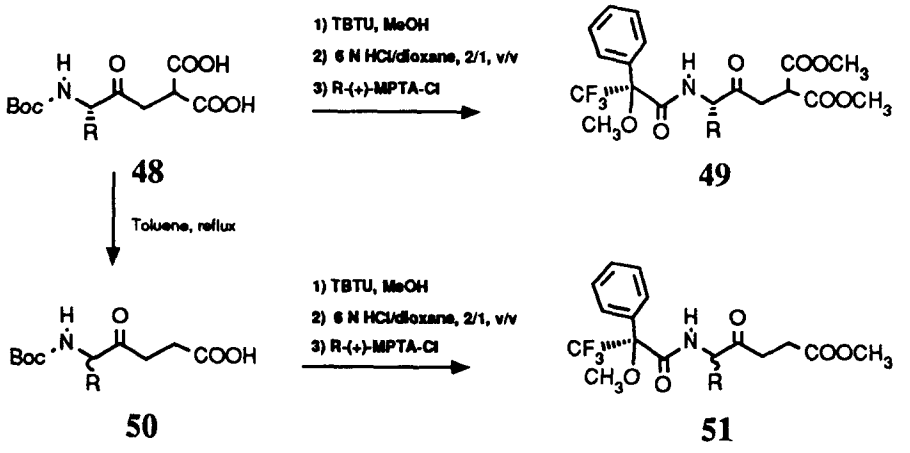
	Thrombin		% cleaved at:	
Compound	IC_{50} (10^{-3}M)	K_i (10^{-6}M)	90 min	16 h
26	**0.43**	**3.14**	**27**	**100**
30	**0.17**	**2.75**	**9**	**43.5**

Both compounds are hydrolyzed by thrombin; compound **26** is cleaved faster than **30**. This implies that of the former compound the transition state intermediate is better accommodated. From this we concluded that compound **26** is a better starting point than compound **30** for further studies. Another reason to focus further exclusively on **26** is that the synthesis of transition state analogues of the Arg–Xxx amide bond is less cumbersome when Xxx is a glycine residue lacking a centre of chirality.

Combination of two optimizations

On the basis of these findings the Arg–Gly amide bond in the peptides **16** and **26** was replaced by the ketomethylene group to yield **43** and **47**, respectively. The synthesis is depicted in Scheme 2. Compound **9**, which had been used previously for the synthesis of **12**, was now coupled to H-Phe-O*t*Bu using DCC/HOBt, to yield the protected tripeptide **40** in 43% yield. Subsequent hydrogenolysis gave **41**. This compound was coupled to Boc-D-Phe-Pro to give **42** in 87% yield as a mixture of two isomers (ratio 3:2) which could be separated by silica gel column chromatography. Removal of the acid-labile protecting groups of the separate isomers gave compounds **43a** and **43b**, re-spectively, whose ^{1}H NMR spectra were nearly identical except for the δ values of the β-CH_2 protons of the phenylalanine residue; both compounds had the same molecular weight. The same features were seen in the synthesis of compounds **12a/12b** and **47a/47b**, which have been synthesized using the same route as was used for the synthesis of **43a/43b**.

Supportive evidence for the assignment of the stereochemistry of compounds **12**, **43** and **47** was come by as follows. HPLC analysis of the derivatized amino acids obtained from each isomer by acid hydrolysis and subsequent derivatization with a chiral moiety,[32] indicated that epimerization had not occurred at the residues of phenylalanine, proline or lysine. On the basis of these findings it was concluded that epimerization had taken place at the α-carbon atom of the arginine residue. Subsequently, the stage of the synthetic scheme at which this epimerization had occurred was studied. Therefore, compound **48**, the N^{α}-Boc-protected analogue of compound **8** was prepared (Scheme 3). Part of this compound was converted into **49**, a Mosher's amide,[33] using reaction conditions that avoided racemization. The di-acid **48** was also subjected to the same decarboxylation conditions employed for the conversion of **8** to **9**. The resulting acid **50** was converted into the Mosher's amide **51**. Perusal of the ^{1}H NMR spectrum of **51** showed the presence of two signals at δ 3.29 and 3.42, corresponding to the methoxy groups of the methoxyphenyl trifluoroacetyl (MPTA) group, in a ratio of 3:1, respectively. This indicated the presence of two diastereomers, the major one having the *S*-configuration, the minor one the *R*-configuration at the C(5) carbon atom.[33] From this it was concluded that racemization must have occurred during the decarboxylation **48**→**50**, as the ^{1}H NMR spectrum of **49** showed only one signal at δ 3.29 corresponding to the methoxy of the MPTA group. A similar racemization must have occurred in the reaction sequence leading to **12a/12b**, **43a/43b** and **47a/47b**. So we concluded that the major isomers of these compounds, i.e. **12a**, **43a** and **47a**, have the *S*-chirality at C(5), corresponding to that of L-arginine. Consequently, the minor isomers **12b**, **43b** and **47b** must have the *R*-chirality at C(5). The anti-thrombin and anti-factor Xa activities of the resulting compounds are given in Table 5.



Scheme 3.

Table 5. Inhibitory activities of the ketomethylene amide bond replacement in the sequences H-D-Phe-Pro- * -Arg-ψ-Gly-Q

				Thrombin		Factor Xa
	*[a]	ψ	Q	IC_{50} (10^{-3}M)	K_i (10^{-6}M)	IC_{50} (10^{-3}M)
2	S	C(O)-NH	OH	**3.8**	**71.6**	**4.8**
12a	S	$C(O)-CH_2$	OH	**0.99**	**25.1**	**4.7**
12b	R	$C(O)-CH_2$	OH	**1.6**		**48**
16	S	C(O)-NH	Phe-OH	**0.33**	**7.6**	**1.1**
43a	S	$C(O)-CH_2$	Phe-OH	**0.038**	**0.5**	**2.0**
43b	R	$C(O)-CH_2$	Phe-OH	**0.25**		**1.4**
26	S	C(O)-NH	Phe-Lys-OH	**0.43**	**3.14**	**1.1**
47a	S	$C(O)-CH_2$	Phe-Lys-OH	**0.0049**	**0.029**	**2.1**
47b	R	$C(O)-CH_2$	Phe-Lys-OH	**0.32**		**1.3**
52[b]	R/S	$C(O)-CH_2$	Pro-Arg-OH	-	**3.0**	-
53[c]	R	$C(O)-CH_2$	Pip	-	**1.3**	-

[a]Stereochemistry of the C^{α} arginine derivative; [b]see Ref. 19; [c]see Ref. 7.

Replacement of the Arg–Gly amide bond of **2**, **16** and **26** by the ketomethylene group (yielding **12a**, **43a** and **47a**, respectively) increases the anti-thrombin activity by a factor of 4, 9 and 88, respectively, based on the IC_{50} values and by a factor of 3, 15 and 108, respectively, based on the K_i values. The difference in anti-thrombin activity within the diastereomer pairs **12a/12b**, **43a/43b** and **47a/47b** is noteworthy. Compounds with the *S*-configuration at the α-carbon (C(5)) of the arginine residue have higher anti-thrombin activity than the corresponding C(5) epimers. This difference in activity increases by elongating the inhibitor (**12** → **43** → **47**). These results are depicted as a histogram in Figure 4.

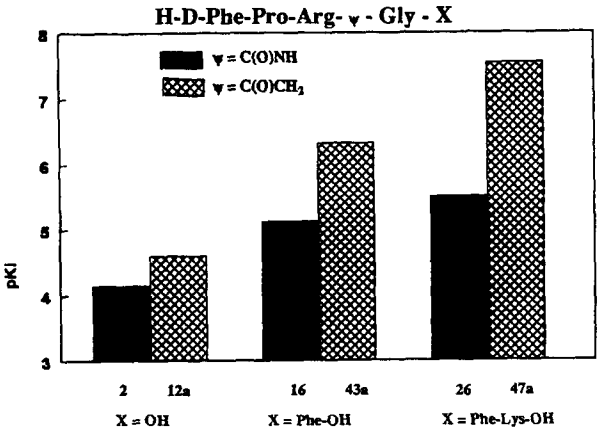


Figure 4. Anti-thrombin activities of the peptides (ψ = C(O)NH) and compounds having a ketomethylene amide bond replacement (ψ = $C(O)CH_2$).

Discussion

In the present study we have systematically investigated the interaction between thrombin and peptides with various transition state replacements of the Arg–Gly amide bond. We found that C-terminal elongation of the H-D-Phe-Pro-Arg sequence with one to three P' residues does not contribute dramatically to the overall binding. In fact, only the hexapeptide **30** shows appreciably higher thrombin inhibition than the compound H-D-Phe-Pro-Arg-OH (**1**) itself. However, when the scissile bond in the elongated peptides is replaced by a ketomethylene moiety, much more potent inhibitors result with K_i values up to 29 nM.

An interesting effect is noted with the latter class of compounds. The effect of replacing the scissile bond by the ketomethylene moiety becomes larger as the peptides become longer (Fig. 4). Several explanations can be given for this phenomenon. Firstly, one may argue that the price for loosing translational and rotational entropy has been paid for when the P' region binds to thrombin. As a result, the interaction between the transition state mimic and the oxyanion hole becomes more favourable due to the reduced loss of entropy. This effect will be larger as the binding becomes tighter. Another explanation is inspired by the work of Rutter *et al.*[34] who converted the specificity of trypsin into chymotrypsin-like specificity by site-directed mutagenesis. They found that extending binding sites contributes substantially to substrate specificity. It is possible that binding between the S' region of thrombin and the P' region of the peptides somehow stabilizes the transition state and with that increases

the ability of a transition state mimic to interact with the oxyanion hole.

Selectivity

The selectivity of the compounds prepared, i.e. their anti-thrombin versus anti-factor Xa activity deserves some further comments. The Ala derivatives **19** and **30** are more selective than the corresponding Gly-analogues **16** and **26**, respectively. It is also obvious that chain elongation (**19** → **30**) is accompanied by an increase in selectivity. A further increase in selectivity is observed upon replacement of the Arg–Xxx amide bond by a ketomethylene group; compare the activities of **26** and **47a**. This increase in selectivity is primarily due to an increase in anti-thrombin activity; the anti-factor Xa activity is hardly affected.

Introduction of the hydroxyethylene amide bond replacement in compound **2** has only an appreciable effect on the anti-factor Xa activity; compare the activities of **2** and **13** (Table 1). The increased anti-factor Xa selectivity of **15** as compared to the selectivity of **2**, suggests that the hydroxymethylene amide bond replacement should be studied in a peptide sequence optimized for its anti-factor Xa selectivity.

Combination of the optimal transition state analogue studied, i.e. the ketomethylene group, with the optimized P' sequence yields compound **47a** with high anti-thrombin activity (K_i = 0.029 μM) and selectivity. It compares favourably with an analogue reported by Szelke *et al.*[3] who replaced the Arg–Gly amide bond in the sequence derived from fibrinogen, the endogenous substrate of thrombin being H-D-Phe-Pro-Arg-Gly-Pro-Arg-OH (**39**, K_i = 1330 μM), by a ketomethylene group to give **52** having a K_i = 3 μM. Thus compound **47a** has a substantially higher affinity for thrombin than compound **52**.

Conclusion

In the present study we have investigated the effect of elongating the tetrapeptide H-D-Phe-Pro-Arg-Gly-OH (**2**) at the C-terminal side on its binding towards thrombin. It is concluded that additional binding can be obtained by elongation of this tetrapeptide with two amino acid residues. As of yet, we have no rationale for the loss of binding observed by elongation of the tripeptide H-D-Phe-Pro-Arg-OH (**1**) to the above-mentioned tetrapeptide. The most potently binding peptides are H-D-Phe-Pro-Arg-Ala-Phe-Lys-OH (**30**, K_i = 2.75 μM) and H-D-Phe-Pro-Arg-Gly-Phe-Lys-OH (**26**, K_i = 3.14 μM) which are only slightly better thrombin inhibitors than the tripeptide **1** (K_i = 3.4 μM), but appreciably better inhibitors than the tetrapeptide **2**. When the scissile amide bond in the hexapeptide **30** is replaced by a ketomethylene moiety, a 100-fold increase in binding towards thrombin is observed. The resulting compound **47a** is a potent inhibitor for thrombin with a K_i of 29 nM, close to the value of the well-known inhibitor argatroban (K_i = 19 nM).[10,11]

Combination of the transition state analogue concept with the principle of peptide elongation (tetrapeptide → hexapeptide) yields thrombin inhibitors of high potency and selectivity. The effects of these two alterations reinforce each other indicating a synergistic effect. This might be rationalized by entropy factors.

Experimental

Optical rotations were measured on a Perkin–Elmer Model 241 polarimeter. Analytical TLC was performed on silica gel plates (Merck, silica gel 60 F254), detection with Reindel–Hoppe's reagent (Cl_2/tolidine). Column chromatography was performed on silica gel 60, 230–400 mesh. Preparative high-performance liquid chromatography (HPLC) was carried out on a Waters Model 4000. Analytical HPLC was done on an HP 1090 M liquid chromatograph provided with a ternary solvent delivery system, being A: sodium phosphate/phosphoric acid buffer, pH 2.1; B: H_2O; C: CH_3CN:H_2O, 6:4. Several linear gradients were used. As supporting material a reversed-phase octadecylsilica (Supelcosil LC-18DB, 3-μm particles) column (150 × 4.6 mm I.D.) was used. Fast atom bombardment (FAB) mass spectra were recorded with a Finnigan MAT 90 mass spectrometer equipped with a WATV Cs ion gun, in a glycerol or nitrobenzyl alcohol matrix. ^{1}H NMR spectra were recorded on a Bruker AM 360. Chemical shifts were measured in δ units (ppm) relative to TMS in DMSO-d_6, $CDCl_3$ or CD_3OD and relative to DSS in D_2O. THF was distilled from $LiAlH_4$ and CH_2Cl_2 was distilled from CaH_2. Meldrum's acid was recrystallized from EtOH. Other chemicals were obtained from Janssen Chimica or Aldrich and were used without further purification.

Anti-thrombin and anti-factor Xa assays

The compounds were tested *in vitro* for their anti-thrombin and anti-factor Xa activities. The inhibition of human thrombin (obtained from the Centraal Laboratorium Bloedtransfusiedienst Amsterdam) was investigated by continuously monitoring the cleavage of the chromogenic substrate s-2238 (H-D-Phe-L-Pip-L-Arg-*p*-nitroanilide dihydrochloride, obtained from Kabi) by this enzyme at 37 °C, in the absence and presence of 3, 1, 0.3, 0.1, 0.03, 0.01 and 0.001 mM of the compound investigated (concentrations were calculated based on the peptide content). The amidolytic reaction was monitored by measuring the absorbance at 405 nm with a microtiter plate reader (Flow Laboratories). To a solution of s-2238 in 0. 1 M NaCl, 0.05 M Tris at pH 7.4, kept at 37 °C, a solution of the 'inhibitor' of the appropriate concentration was added to obtain the concentrations mentioned above. Subsequently a solution of thrombin (0.2 IU mL^{-1}) in 0.1 M NaCl, 0.3 % PEG (M_r 6000) and 0.05 M Tris pH 7.4 was added. The mixture was stirred thoroughly and then incubated at 37 °C. The increase in absorbance due to the release of the *p*-nitroaniline, which is cleaved off from s-2238 by

thrombin, was measured every 2 min for a period of 90 min.

The determination of the anti-factor Xa activities was similar to the measurement of anti-thrombin activity. In this case, the chromogenic substrate used was s-2222 (*N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide hydrochloride obtained from Kabi). Bovine factor Xa was used (2.13 nkat mL^{-1}, obtained from Kabi).

From the absorbances, measured at 90 min, we calculated the concentration at which 50% of the thrombin activity is reduced (the IC_{50}). The K_i values were determined according to Lineweaver and Burke by measuring initial velocities for a series of chromogenic substrate concentrations as functions of the inhibitor concentrations.

Molecular modelling procedures

All molecular modelling studies were carried out with Quanta/CHARMm version 3.3.1 (Molecular Simulations Inc., Burlington, MA). In the protocol used[29,30] the thrombin structure was kept rigid and only the peptide was allowed to move during the (constrained) energy minimization. Distance dependent dielectric functions of $\epsilon = 4R$ and $\epsilon = R$ were used for the energy minimization and interaction energy evaluations, respectively.[30] The nonbonded cutoff amounted to 15 Å; switching and shifting functions were used for the Van der Waals and dielectric interactions, respectively, between 11 and 14 Å. For the nonbonded list update frequency the default heuristic methodology was employed. The geometry optimizations were performed by initially applying 50 steps of steepest descents. Subsequently, an adopted basis Newton Raphson minimization was continued until the root-mean-square energy gradient was less than 0.10 kcal $Å^{-1}$. The analyses were done on the complexes with the most favourable interaction energy between protease and peptide.

For all calculations the coordinates of the human thrombin–PPACK complex[12b] were used. The generation of the various peptide conformations was performed by combining the coordinates of D-Phe-Pro-Arg from PPACK with two different backbone conformations for the P' residues, denoted I and II. In addition, a limited grid scan was carried out for the P' side chains. Starting geometries for the P' backbones were derived from the bovine pancreatic trypsin inhibitor (BPTI) backbone as observed in its complex with trypsin[31] (pdb-code 2ptc; P1: $\psi = 39$; $\varpi = 164$; P_1': $\phi = 87$; $\psi = 164$; $\varpi = 167$; P_2': $\phi = -112$) and a manually modelled peptide backbone in which the P_2' φ dihedral was set on 101 degrees. The P_2' and P_3' side chains were generated with standard geometries for amino acids using increments of 60 and 120 degrees for $\chi 1$ dihedrals of the penta- and hexapeptides, respectively. Thus in total 24 pentapeptide and 18 hexapeptide conformations were generated and minimized in the context of thrombin.

Abbreviations

Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DiPEA, di-isopropylethylamine; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; HONSu, *N*-hydroxysuccinimide; Mtr, 2,3,6-trimethyl-4-methoxybenzenesulphonyl; NEM, *N*-ethylmorpholine; TBTU, N,N,N',N'-tetramethyl-O-(benzyltriazol-1-yl)-uronium tetrafluoroborate; TEA, triethylamine; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.

Hydrolysis experiments

In these experiments the Arg–Xaa containing peptides were incubated with thrombin in a buffer of pH 7.4, for 90 min. The reaction was stopped by filtration of the mixture and subsequent injection of the filtrate on an HPLC column, which was preceeded by a precolumn. Human thrombin was used in a solution having a concentration of 50 U mL^{-1} which was diluted with water to obtain a final concentration of 0.2 U mL^{-1}. The concentration of the stock solution of the peptide investigated was 0.01 M.

To 700 µL of TNP-buffer (tromethamine/NaCl/polyethylene glycol 6000) was added 200 µL of the thrombin solution and 100 µL of the peptide solution. In this mixture the final concentration of the peptide was 0.001 M, the thrombin activity was 0.04 U mL^{-1}. This mixture was incubated at 37 °C for 90 min. The mixture was filtrated and injected on a C-18 HPLC column (Supelcosil LC-18-DB * 88400 AB), using a ternary solvent system: A: 0.5 M NaH_2PO_4/H_3PO_4 pH 2.1; B: H_2O; C: $CH_3CN:H_2O$, 6:4, v/v. Gradient: A: 20%; B: 65 → 20%; C: 15 → 60% in 37 min. The gradient curve was linear, with slope 1. Injection volume: 200 µL of the filtered mixture. Flow: 1 mL min^{-1}, detection: 210 nm.

The product of hydrolysis in all cases should be H-D-Phe-Pro-Arg-OH (being compound **1** in Table 2). This tripeptide was prepared and incubated with thrombin, using the conditions described above; after 90 min the HPLC spectrum showed one peak. The retention time of the tripeptide was 34 min (± 1 min). The final concentration of the tripeptide was 0.0003 M, the area of the peak in the HPLC spectrum served as a reference for determination of the concentration of H-D-Phe-Pro-Arg-OH released in the hydrolysis experiments of the peptides.

Peptide synthesis

The peptides **1**, **2** and **16–39** were synthesized by conventional peptide synthesis in solution and by solid phase peptide synthesis. Analytical data are given in Table 6.

(3S)-3-(Benzyloxycarbonylamino)-1-diazo-6-[N-(N,N'-dibenzyloxycarbonylguanidino)]-2-oxohexane (4)

Compound **3** (20.75 g, 36 mmol) was dissolved in THF (200 mL). The solution was cooled to −15 °C and *N*-

Table 6. Analytical data of the peptides

						TLC R_f[a]	HPLC purity (%)	$[\alpha]_D$[e]	peptide content (%)
1	Phe 0.99	Pro 0.87	Arg 1.02			0.39[b]	84.2	-113.6	69.7
2	Phe 1.05	Pro 0.89	Arg 1.04	Gly 1.03		0.47	92.5	-117.3	73.3
16	Phe 1.88	Pro 0.88	Arg 1.06	Gly 1.06		0.44[b]	79.9	-71.6	63.8
17	Phe 1.03	Pro 0.83	Arg 1.05	Gly 1.14	His 0.93	0.36	77.8	-57.2	48.5
18	Phe 0.98	Pro 1.01	Arg 1.00	Gly 0.99	Glu 1.03	0.44[b]	89.9	-84.2[f]	83.6
19	Phe 2.00	Pro 0.99	Arg 1.00	Ala 1.01		0.45[c]	95.5	-73.5	65.6
20	Phe 1.01	Pro 1.00	Arg 1.01	Ala 1.01	Tyr 0.96	0.44[a]	94.5	-90.6	79.1
21	Phe 1.00	Pro 0.99	Arg 0.99	Ala 1.01	Tyr 0.70	0.29[d]	78.3	-42.6	35.9
22	Phe 0.99	Pro 1.00	Arg 1.00	Ala 1.02	Glu 0.99	0.68[c]	97.5	-117.8	73.3
23	Phe 2.03	Pro 0.99	Arg 0.98	Ala 1.00		0.45[c]	98.3	-44.4	73.7
24	Phe 2.01	Pro 1.01	Arg 0.99	Ala 0.99		0.46[c]	96.7	-70.4	75.9
25	Phe 1.99	Pro 1.03	Arg 0.99	Ala 0.99		0.46[c]	97.5	-147.0	95.8
26	Phe 1.98	Pro 1.02	Arg 1.00	Gly 1.00	Lys 1.01	0.35[c]	98.5	-74.1	69.3
27	Phe 2.03	Pro 0.96	Arg 0.99	Gly 1.01	Lys 1.00	0.35[b]	94.9	-81.0	78.6
28	Phe 1.98	Pro 1.01	Arg 0.98	Gly 1.01	Lys 1.02	0.35[b]	93.2	-56.3	72.3
29	Phe 2.02	Pro 0.98	Arg 1.00	Gly 0.99	Lys 1.01	0.35[b]	97.7	-79.8	79.1
30	Phe 1.98	Pro 1.03	Arg 0.99	Ala 1.00	Lys 1.01	0.29[b]	99.8	-98.9	86.4
31	Phe 1.96	Pro 1.00	Arg 2.02	Ala 1.01		0.43[a]	92.9	-79.9	68.0
32	Phe 1.98	Pro 1.01	Arg 1.01	Ala 1.00	Glu 1.00	0.34[c]	97.2	-116.2	85.1
33	Phe 2.00	Pro 0.98	Arg 1.00	Ala 1.01	Nle 1.01	0.24[d]	94.3	-99.6	81.5
34	Phe 2.02	Pro 1.03	Arg 1.01	Ala 0.92	Lys 1.02	0.24[c]	98.8	-119.2	76.7
35	Phe 1.99	Pro 0.99	Arg 1.01	Ala 1.00	Lys 1.01	0.24[c]	98.8	-91.5	71.0
36	Phe 2.01	Pro 0.96	Arg 1.01	Ala 1.03	Lys 0.99	0.24[c]	93.3	-89.3	70.7
37	Phe 1.01 Lys 1.01	Pro 1.00	Arg 1.01	Ala 1.00	Cha 0.99	0.40[b]	96.9	-81.4	78.8
38	Phe 0.99 Lys 1.09	Pro 1.01	Arg 1.00	Ala 1.02	Tyr 0.89	0.25[d]	87.7	-89.4	78.8
39	Phe 0.96	Pro 2.00	Arg 1.98	Gly 1.06		0.46[b]	85.9	-73.4	37.2

[a]TLC system B:P:A:W (*n*-butanol:pyridine:HOAc:H_2O) 4:1:1:2; [b]EtOAc:P:A:W 6:2:2:1; [c]B:P:A:W 8:3:1:4; [d]B:P:A:W 16:3:1:4; [e]determined at 20 °C, c = l, H_2O; [f]c = 0.5, 5% HOAc in H_2O; [g]c = l, MeOH.

methylmorpholine (4.59 mL, 36 mmol) was added, followed by isobutyl chloroformate (5.62 mL, 43 mmol). The mixture was stirred at −15 °C for 45 min. An ethereal solution of diazomethane (hazardous!, generated from *N*-nitrosomethylurea, carcinogen) was added until the solution was yellow. The mixture was kept overnight at 4 °C and after 20 h all starting material was gone. This crude reaction mixture was used as such for the synthesis of **5b**. R_f 0.67 (toluene:EtOH, 8:2, v/v).

*(3S)-3-(Benzyloxycarbonylamino)-1-bromo-6-[*N-(N,N'-*dibenzyloxycarbonylguanidino)]-2-oxohexane (5b)*

At 0 °C an anhydrous solution of HBr in ether was added slowly to the crude reaction mixture of **4** until the pH of the mixture was approximately 2. Stirring was continued for 1 h. The reaction mixture was diluted with ether and washed with H_2O until the organic layer was neutral. The organic layer was dried ($NaSO_4$), filtered and concentrated *in vacuo*. The residue was recrystallized from *i*-Pr_2O/CH_2Cl_2 (12:1, v/v) to give **5b** as white crystals (15.29 g, 65%, based on **3**): R_f 0.81 (toluene:EtOH, 8:2, v/v); ^{1}H NMR ($CDCl_3$, 360 mHz) δ 1.55–1.88 (4H, *m*, 2 × H_4 and 2 × H_5), 3.82–3.94 (4H, *m*, 2 × H_6, 2 × H_1), 4.46–4.57 (1H, *m*, H_3), 5.06 (4H, *s*, 2 × CH_2Z), 5.23 (2H, *s*, CH_2 Z), 6.19 (1H, *d*, NH), 7.26–7.39 (15H, *m*, ArH), 9.33 (2H, *d*, 2 × NH); MS (FAB) m/z 653 $(M + H)^+$; $[\alpha]^{25}_D$ −5.0° (*c* 1.0, $CHCl_3$).

*Di-(*tert*-butyldimethylsilyl)malonate (6)*

Malonic acid (7.5 g, 74.9 mmol) was suspended in 1,2-dichloroethane (35 mL). To this suspension *tert*-butyldimethylsilyl chloride was added (22.6 g, 150 mmol) followed by TEA (21.0 mL, 150 mmol). The suspension was refluxed for 1.5 h and cooled to room temperature. Dry ether was added (250 mL) and Et_3N·HCl was removed by filtration. The filtrate was immediately concentrated and purified by distillation under reduced pressure (1 mm Hg) to give **6** as an oil (15.75 g, 63.2%): bp 115–117 °C (1 mm Hg); ^{1}H NMR ($CDCl_3$, 200 mHz) δ 0.30 (12H, *s*, 4 × CH_3), 0.93 (18H, *s*, 2 × $(CH_3)_3C$), 3.36 (2H, *s*, CH_2).

*(5S)-5-(Benzyloxycarbonylamino)-2-carboxy-8-[*N-(N,N'-*dibenzyloxycarbonylguanidino)]-4-oxo-octanoic acid (8)*

To a solution of compound **5b** (11.44 g, 17.54 mmol) in dry THF (75 mL), cooled to 0 °C in an ice bath, a solution of the sodium salt of **6** [prepared by reaction of sodium hydride (0.84 g, 35.08 mmol) with **6** (11.66 g, 35.08 mmol)] in THF was added dropwise. After the addition was complete, stirring was continued for 3 h at room temperature and **7** was formed. EtOH was added and the mixture was cooled to 0 °C. To the mixture of **7** 1 M HCl was added until pH 2 and stirring was continued for 30 min. The organic layer was washed with 5% $NaHCO_3$, H_2O, dried ($NaSO_4$) and concen-

trated *in vacuo*. A small portion of the crude product was purified by silica gel column chromatography (CH_2Cl_2:MeOH, 95:5) to give **8** as an oil. Compound **8** was used without purification for the synthesis of **9**. R_f 0.51 (CH_2Cl_2/MeOH, 8:2, v/v); ^{1}H NMR (DMSO-d_6, 200 mHz) δ 1.43–1.88 (4H, *m*, 2 × H_6 and 2 × H_7), 2.88 (1H, *br s*, H_2), 3.78–4.16 (5H, *m*, H_5 and 2 × H_8 and 2 × H_3), 4.98–5.03 (4H, *m*, 2 × CH_2 Z), 5.23 (2H, *s*, CH_2 Z), 7.27–7.47 (15H, *m*, ArH); MS (FAB) m/z 676 (M + H)$^+$.

(5S)-5-(Benzyloxycarbonylamino)-8-[N-(N,N'-dibenzyloxycarbonylguanidino)]-4-oxo-octanoic acid (9)

Decarboxylation of the crude compound **8** was carried out by refluxing in toluene for 1 h. After column chromatography (CH_2Cl_2:MeOH, 85:15), the isosteric dipeptide **9** was obtained as a white powder (6.21 g, 56%); R_f 0.17 (CH_2Cl_2:MeOH, 9:1); ^{1}H NMR ($CDCl_3$, 200 mHz) δ 1.50–1.85 (4H, *m*, 2 × H_6 and 2 × H_7), 2.51 (2H, *m*, 2 × H_2), 2.69 (2H, m, 2 x H_3), 3.91 (2H, *m*, 2 × H_8), 4.28 (1H, *m*, H_5), 5.06 (2H, *s*, CH_2Z), 5.08 (1H, *d*, CH Z, J = 13.5 Hz), 5.12 (1H, *d*, CH Z, J = 13.5 Hz), 5.22 (2H, *s*, CH_2 Z), 7.28–7.45 (15H, *m*, ArH); MS (FAB) m/z 633 (M + H)$^+$.

(5S)-5-Amino-8-guanidino-4-oxo-octanoic acid dihydrochloride (10)

Compound **9** (0.303 g, 0.48 mmol) was dissolved in MeOH:HOAc:H_2O, 5:1:1, a solution of 1 M aqueous HCl was added (1.44 mL). Pd/C (10%) was added (30 mg) and H_2 was bubbled through the solution. When the reaction was complete according to TLC the catalyst was removed by filtration and the solvent was evaporated. Compound **10** was used without further purification. A small amount was purified by column chromatography (EtOAc:pyridine:HOAc:H_2O, 6:2:2:1) to obtain an analytically pure sample. R_f 0.10 (EtOAc: pyridine:HOAc:H_2O, 6:2:2:1); ^{1}H NMR (D_2O, 360 mHz) δ 1.59–2.21 (4H, *m*, 2 × H_6 and 2 × H_7), 2.07 (*s*, HOAc), 2.56–2.59 (2H, *m*, 2 × H_2), 2.82–2.96 (2H, *m*, 2 × H_3), 3.18–3.29 (2H, *m*, 2 × H_8), 4.35–4.38 (1H, *m*, H_5).

(5S)-5-[tert-Butyloxycarbonyl-D-phenylalanyl-prolylamino]-8-guanidino-4-oxo-octanoic acid (11)

A solution of Boc-D-Phe-Pro-OH (273 mg, 0.75 mmol) in DMF (8 mL) was cooled to 0 °C. DCC (155 mg, 0.75 mmol) and HONSu (86.3 mg, 0.82 mmol) were added. After stirring for 1 h a solution of **10** (0.48 mmol) in H_2O (5 mL) which was brought to pH 7.5 with NEM, was added to the solution. Stirring was continued at room temperature for 16 h. DCU was removed by filtration followed by evaporation of the filtrate to dryness. The residue was dissolved in EtOAc, washed with 5% $NaHCO_3$, 5% $KHSO_4$ and brine. The organic layer was dried over Na_2SO_4. After evaporation of the solvent the crude reaction product was obtained, which was purified by silica gel column chromatography (EtOAc:pyridine:HOAc:H_2O, 6:2:2:1) to give **11** in a 60% yield. R_f 0.61 (EtOAc:pyridine:HOAc:H_2O, 6:2:2:1); ^{1}H NMR ($CDCl_3$, 360 mHz) δ 1.39 (9H, *s*, Boc), 1.56–2.05 (8H, *m*, 2 × H_6, 2 × H_7 and γ-CH_2 Pro), 2.36–2.53 (2H, *m*, 2 × H_2), 2.65–2.74 (2H, *m*, 2 × H_3), 2.90–3.19 (5H, *m*, β-CH_2 Phe, δ-CH Pro and 2 × H_8), 3.62 (1H, m, δ-CH Pro), 4.24–4.58 (3H, *m*, α-H Pro, α-H Phe and H_5), 7.20–7.30 (5H, *m*, ArH); HPLC-purity 97.6%; MS (FAB) m/z 575 (M + H)$^+$.

*(5S)- and (5R)-5-[D-Phenylalanyl-prolylamino]-8-guanidino-4-oxo-octanoic acid acetate (**12a** and **12b**, respectively)*

Compound **11** (162 mg, 0.28 mmol) was treated with 90% TFA (7.5 mL) and anisol (28 μL). The mixture was stirred for 1 h at room temperature and then poured into an excess of ether, which causes the deprotected compound to precipitate. The ether layer was decanted and fresh ether was added. This procedure was repeated twice and then the resulting white solid was filtered off. The residue was dissolved in H_2O:*t*-BuOH (1:1, v/v) and Dowex-2 (X-8, acetate form) was added. After stirring for 1 h, the resin was removed by filtration. The filtrate was lyophilized to give the product in its acetate form. This product was purified by silica gel column chromatography (EtOAc:pyridine:HOAc:H_2O, 6:2:2:1). HPLC showed that the compound is a mixture of two compounds in a ratio of 1:1.4. These two compounds were separated by preparative HPLC.

12a: Yield 51%; ^{1}H NMR (D_2O, 360 mHz) δ 1.50–2.14 (8H, *m*, 2 × H_6 and 2 × H_7, β-CH_2 and γ-CH_2 Pro), 1.93 (*s*, HOAc), 2.39–2.46 (2H, *m*, 2 × H_2), 2.74–2.89 (3H, *m*, 2 × H_3 and δ-CH Pro), 3.10–3.27 (4H, *m*, β-CH_2 Phe and 2 × H_8), 3.52 (1H, *m*, δ-CH Pro), 4.39 (1H, *dd*, α-H Pro, J_1 = 4 Hz, J_2 = 8.5 Hz), 4.45 (1H, *dd*, H_5, J_1 = 5 Hz, J_2 = 9 Hz), 4.49 (1H, *dd*, α-H Phe, J_1 = 6 Hz, J_2 = 9 Hz), 7.29–7.45 (5H, *m*, ArH). HPLC-purity: 97.8%; $[\alpha]^{25}_D$ –75.8° (*c* 1, H_2O); MS (FAB) m/z 475 (M + H)$^+$; amino acid analysis: Phe:Pro = 1.01:0.99; peptide content: 76.2%.

12b: Yield: 36%; ^{1}H NMR (D_2O, 360 mHz) δ 1.53–2.17 (8H, *m*, 2 × H_6 and 2 × H_7, β-CH_2 and γ-CH_2 Pro), 1.94 (*s*, HOAc), 2.39–2.45 (2H, *m*, H_2), 2.72–2.86 (3H, *m*, 2 × H_3 and δ-CH Pro), 3.10–3.27 (4H, *m*, β-CH_2 Phe and 2 × H_8), 3.52 (1H, *m*, δ-CH Pro), 4.38 (1H, *dd*, α-H Pro, J_1 = 2.5 Hz, J_2 = 8.5 Hz), 4.46 (1H, *dd*, H_5), 4.55 (1H, *dd*, α-H Phe, J_1 = 7 Hz, J_2 = 9 Hz), 7.31–7.48 (5H, *m*, ArH). HPLC-purity: 95.9%; $[\alpha]_D$ –115.9° (*c* 1, H_2O); MS (FAB) m/z 475 (M + H)$^+$; amino acid analysis: Phe:Pro = 1.01:0.99; peptide content: 78.3%.

N-[(5S)-5-[(Benzyloxycarbonylamino)-8-[N-(N,N'-dibenzyloxycarbonylguanidino)]-4-oxo-octanoyl]-phenylalanine tert-*butyl ester (40)*

To a mixture of **9** (250 mg, 0.39 mmol), which had been cooled to 0 °C, HOBt (79.1 mg, 0.58 mmol) and DCC (88.7 mg, 0.43 mmol) in DMF (2.5 mL) were added. A solution of H-Phe-O*t*Bu·HCl (0.21 g, 0.59 mmol) in DMF (2 mL), adjusted to pH 7.2 with NEM, was added.

After a similar workup procedure as described for **11**, the crude product was purified by silica gel column chromatography (CH_2Cl_2:EtOAc, 8:2) to give **40** (139 mg, 43%): R_f 0.51 (CH_2Cl_2:EtOAc, 95:5); ^{1}H NMR ($CDCl_3$, 200 mHz) δ 1.39 (9H, *s*, $(CH_3)_3C$), 1.46–1.72 (4H, *m*, 2 × H_6 and 2 × H_7), 2.29–2.37 (2H, *m*, 2 × H_2), 2.65–2.74 (2H, *m*, 2 × H_3), 3.01–3.06 (2H, *m*, β-CH_2 Phe), 3.89–3.98 (2H, *m*, 2 × H_8), 4.34 (1H, *m*, α-H Arg), 4.68 (1H, *m*, α-H Phe), 5.06 (2H, *s*, CH_2Z), 5.11 (2H, *s*, CH_2Z), 5.22 (2H, *s*, CH_2Z), 5.86–5.99 (2H, *m*, 2 × NH), 7.15–7.42 (20H, *m*, ArH), 9.24–9.49 (1H, *br d*, NH); $[\alpha]^{25}_D$ +24.2° (*c* 0.5, $CDCl_3$); MS (FAB) m/z 835 $(M + H)^+$.

N-*[(5S)-5-amino-8-guanidino-4-oxo-octanoyl]-phenylalanine* tert-*butyl ester (41)*

Compound **40** (119 mg, 0.14 mmol) was deprotected to give **28**, following the procedure as described for compound **10**. After removal of the catalyst the filtrate was concentrated to a smaller volume. This solution was used in the reaction to give **41**. R_f 0.33 (EtOAc:pyridine:HOAc:H_2O, 6:2:2:1).

N-*[(5S)-* *and* N-*[(5R)-5-[*tert-*Butyloxycarbonyl-*D*-phenylalanyl-prolylamino]-8-guanidino-4-oxo-octanoyl]-phenylalanine* tert-*butyl ester (42a and 42b, respectively)*

Boc-D-Phe-Pro-OH (72.7 mg, 0.20 mmol), HOBt (40 mg, 0.30 mmol) and DCC (41.4 mg, 0.22 mmol) were dissolved in DMF (5 mL). The solution of compound **41** (89.6 mg, 0.14 mmol) in DMF was brought to pH 7.5 with NEM and added to the activated carboxylic acid. After workup as described for **40**, a mixture of **42a** and **42b** was obtained which was separated by column chromatography (EtOAc:pyridine:HOAc:H_2O, 80:20:6:5). The spectra of both compounds were recorded.

42a: Yield 52%; R_f 0.27, (EtOAc:pyridine:HOAc:H_2O, 80:20:6:5); ^{1}H NMR ($CDCl_3$:CD_3OD, 9:1, 360 mHz) δ 1.39 (9H, *s*, $OC(CH_3)_3$), 1.44 (9H, *s*, Boc), 1.53–1.88 (8H, *m*, 2 × H_6 and 2 × H_7, β-CH_2 and β-CH_2 Pro), 2.39–2.55 (2H, *m*, 2 × H_2), 2.68–2.83 (3H, *m*, 2 × H_3 and δ CH Pro), 2.88–3.16 (6H, *m*, 2 × H_8 and 2 × β-CH_2 Phe), 3.59 (1H, m, δ-CH_2 Pro), 4.27–4.57 (3H, *m*, H_5, α-H Pro and α-H Phe), 4.62 (1H, *t*, α-H L-Phe, J_1 = 6 Hz), 7.18–7.32 (10H, *m*, ArH); MS(FAB) m/z 778 $(M + H)^+$; $[\alpha]^{25}_D$ −34.8° (*c* 0.4, MeOH).

42b: Yield 35%; R_f 0.20 (EtOAc:pyridine:HOAc:H_2O, 80:20:6:5); ^{1}H NMR ($CDCl_3$:CD_3OD, 9:1, 360 mHz) δ 1.37 (9H, *s*, O*t*Bu), 1.39 (9H, *s*, Boc), 1.50–1.86 (8H, *m*, 2 × H_6 and 2 × H_7, β-CH_2 and γ-CH_2 Pro), 2.38–2.61 (3H, *m*, 2 × H_2 and δ-CH Pro), 2.75–2.81 (2H, *m*, 2 × H_3), 2.88–3.15 (6H, *m*, 2 × H_8 and 2 × β-CH_2Phe), 3.64 (1H, *m*, δ-CH Pro), 4.31 (1H, *dd*, H_5, J_1 = 3 Hz, J_2 = 8 Hz), 4.43 (1H, *dd*, α-H Phe, J_1 = 6.5 Hz, J_2 = 10 Hz), 4.47 (1H, *dd*, α-H Pro, J_1 = 3 Hz, J_2 = 9 Hz), 4.62 (1H, *t*, α-H L-Phe, J_1 = J_2 = 6.5 Hz), 7.18–7.35 (10H, *m*, ArH); MS (FAB) m/z 778 $(M + H)^+$; $[\alpha]^{25}_D$ −21.9° (*c* 0.4, MeOH).

N-*[(5S)-5-[D-Phenylalanyl-prolylamino]-8-guanidino-4-oxo-octanoyl]-phenylalanine acetate (43a)*

To **42a** (135 mg, 0.14 mmol) 90% TFA (5 mL) with anisole (28 μL) was added. After workup (as described for **12**), the freeze-dried product was purified by silica gel column chromatography (*n*-BuOH:pyridine:HOAc:H_2O, 4:0.75:0.25:1) to obtain **43a**: R_f 0.19 (*n*-BuOH:pyridine:HOAc:H_2O, 4:0.75:0.25:1); ^{1}H NMR (D_2O, 360 mHz) δ 1.38–1.52 (4H, *m*, 1 × H_6 and 2 × H_7 Arg, γ-CH Pro), 1.75–1.90 (3H, *m*, 1 × H_6, β-CH and γ-CH Pro), 2.10 (1H, *m*, β-CH Pro), 2.44–2.49 (2H, *m*, 2 × H_2), 2.72–2.83 (3H, *m*, 2 × H_3 and δ-CH Pro), 2.92 (1H, dd, β-CH Phe, J_1 = 9 Hz, J_2 = 14 Hz), 3.15–3.22 (4H, *m*, 2 × β-CH Phe), 3.21 (2H, *t*, 2 × H_8), 3.51 (1H, *m*, δ-CH Pro), 4.34 (1H, *dd*, α-H Pro, J_1 = 4.5 Hz, J_2 = 8.5 Hz), 4.37–4.41 (2H, *m*, H_5, α-H Phe), 4.42 (1H, *dd*, α-H L-Phe), 7.27–7.45 (10H, *m*, ArH); MS (FAB) m/z 622 $(M + H)^+$; $[\alpha]^{25}_D$ −31.9° (*c* 0.1, H_2O); amino acid analysis: Phe:Pro = 1.98:1.02; peptide content: 91.9%. HPLC-purity: 96.4%

N-*[(5R)-5-[D-Phenylalanyl-prolylamino]-8-guanidino-4-oxo-octanoyl]-phenylalanine acetate (43b)*

Compound **43b** was obtained by reaction of **42b** in an analogous manner to **42a**: R_f 0.16 (*n*-BuOH:pyridine:HOAc:H_2O, 4:0.75:0.25:1); ^{1}H NMR **43b** (D_2O, 360 mHz) δ 1.46–1.69 (4H, *m*, 1 × H_6, 2 × H_7, γ-CH Pro), 1.57–1.74 (3H, *m*, 1 × H_6, 1 × β-CH and 1 × γ-CH Pro), 2.05 (1H, *m*, β-CH Pro), 2.41–2.53 (2H, *m*, 2 × H_2), 2.75–2.82 (3H, *m*, 2 × H_3 and δ-CH Pro), 2.92 (1H, *dd*, β-CH Phe, J_1 = 9 Hz, J_2 = 14 Hz), 3.00 (1H, *m*, β-CH Phe), 3.09–3.21 (4H, *m*, 2 × H_8 and 2 × β-CH Phe), 3.51 (1H, *m*, δ-CH Pro), 4.27 (1H, *dd*, α-H Phe), 4.30–4.37 (2H, *dd*, α-H Pro and H_5 Arg), 4.43 (1H, *dd*, α-H Phe, J_1 = 4.5 Hz, J_2 = 8.5 Hz), 7.24–7.43 (10H, *m*, ArH); MS (FAB) m/z 622 $(M + H)^+$; $[\alpha]^{25}_D$ +26.3° (*c* 0.04, H_2O); amino acid analysis: Phe:Pro = 1.99:1.01; peptide content: 82.6%; HPLC-purity: 82.1 % and 9.7% of **43a**.

N-*[5(S)-5-[(Benzyloxycarbonylamino)-8-[*N-(N,N'-*dibenzyloxycarbonylguanidino)]-4-oxo-octanoyl]-phenylalanyl-*N^e*(*tert-*butyloxycarbonyl)-lysyl-*tert-*butylester (44)*

Compound **9** (1.00 g, 1.58 mmol) and HOBt (2.39 mmol, 0.32 g) were dissolved in DMF (10 mL); after cooling the mixture to 0 °C, DCC (1.74 mmol, 0.36 g) was added. A solution of H-Phe-Lys(Boc)-O*t*Bu (2.49 mmol) in DMF (10 mL) was adjusted to pH 7.5 with NEM and added to the activated **9**. After workup (as described for **11**) the crude product was purified by silicagel column chromatography (CH_2Cl_2:EtOAc, 8:2) to obtain **44** as a white solid (1.02 g, 61%): R_f (**44**) 0.29 (CH_2Cl_2:EtOAc, 8:2); ^{1}H NMR ($CDCl_3$:CD_3OD, 9:1, v/v, 360 mHz) δ 1.42 (9H, *s*, O*t*Bu), 1.45 (9H, *s*, Boc), 1.32–1.82 (10H, *m*, 2 × H_6 and 2 × H_7 Arg, β-CH_2, γ-CH_2 and δ-CH_2 Lys), 2.25–2.42 (2H, *m*, 2 × H_2), 2.60–2.77 (2H, *m*, 2 × H_3), 2.85–2.92 (2H, *m*, β-CH_2 Phe), 3.00–3.06 (2H, *m*, ε-CH_2 Lys), 3.89–4.08 (2H, *m*, 2 × H_8 Arg), 4.14–4.18 (1H, *dd*, α-H Lys, J_1 = 4 Hz, J_2 = 9 Hz), 4.25–4.29 (1H, *m*, H_5 Arg, J_1 = 5.5 Hz, J_2 = 8.5 Hz),

4.61–4.66 (1H, *m*, α-H Phe, J_1 = 5.5 Hz, J_2 = 8.5 Hz), 5.05 (2H, *m*, CH_2-Z), 5.09 (2H, *m*, CH_2-Z), 5.24 (2H, *m*, CH_2-Z), 7.15–7.32 (20H, *m*, ArH). MS (FAB) m/z 1063 $(M + H)^+$; HPLC-purity: 85.4 %; $[\alpha]^{25}_D$ –9.2° (*c* 1, $CHCl_3$).

N-*[5(S)-5-[Amino-8-guanidino-4-oxo-octanoyl]-phenylalanyl*-N^{ε}-*(*tert*-butyloxycarbonyl)-lysyl*-tert-*butyl ester (45)*

Compound **44** (0.47 mmol, 0.5 g) was dissolved in DMF (5 mL) and 1 M aqueous HCl was added (0.47 mL). Next, Pd/C (10%) was added (50 mg) and H_2 was bubbled through the solution. When the reaction was complete according to TLC the catalyst was removed by filtration. The filtrate was used for the synthesis of compound **46**. R_f **45** 0.43 (EtOAc:pyridine:HOAc:H_2O, 6:2:2:1).

N-*[5(S)-5-[*tert*-Butyloxycarbonyl-D-phenylalanyl-prolylamino]-8-guanidino-4-oxo-octanoyl]-phenylalanyl*-N^{ε}-*(*tert*-butyloxycarbonyl)-lysyl*-tert-*butyl ester (46)*

To a mixture of Boc-D-Phe-Pro-OH (0.66 mmol, 0.24 g), HOBt (0.99 mmol, 0.13 g) in DMF (7.5 mL) cooled to 0 °C, DCC (0.73 mmol, 0.15 g) was added. To a solution of compound **45** (0.47 mmol) in DMF (5 mL) NEM was added until pH 7.5. This was added to the activated carboxylic acid. After workup (as described for **11**) a mixture of two products was obtained which were separated by silica gel column chromatography (EtOAc:pyridine:HOAc:H_2O, 80:20:6:5) to give **46a** (0.28 g, 42% yield) and **46b** (0.23 g, 35% yield). The spectra of both products were recorded.

46a: R_f 0.27 (EtOAc:pyridine:HOAc:H_2O, 80:20:6:5); ^{1}H NMR ($CDCl_3$, 360 mHz) δ 1.39 (9H, *s*, $OC(C\underline{H}_3)_3$), 1.42 (9H, *s*, $OC(C\underline{H}_3)_3$), 1.45 (9H, *s*, $OC(C\underline{H}_3)_3$), 1.34–1.98 (14H, *m*, 2 × H_6 and 2 × H_7, β-CH_2, γ-CH_2 and δ-CH_2 Lys, β-CH_2 and γ-CH_2 Pro), 2.38–2.45 (2H, *m*, 2 × H_2), 2.67–2.71 (2H, *m*, 2 × H_3), 2.84–2.95 (4H, *m*, 3 × β-CH Phe and δ-CH Pro), 3.02 (2H, *t*, ε-CH_2 Lys, *J* = 7 Hz), 3.14–3.20 (3H, *m*, 2 × H_8 Arg and β-CH Phe), 3.63 (1H, *m*, δ-CH Pro), 4.22 (1H, *dd*, α-H Lys, J_1 = 5.5 Hz, J_2 = 9 Hz), 4.24–4.34 (2H, *m*, α-H Arg and α-H Pro), 4.52 (1H, *t*, α-H Phe, *J* = 7.5 Hz), 4.61 (1H, *dd*, α-H L-Phe, J_1 = 5 Hz, J_2 = 9 Hz), 7.18–7.32 (10H, *m*, ArH); HPLC-purity: 84.5% and 13.8% of **46b**; MS (FAB) m/z 1006 $(M + H)^+$.

46b: R_f 0.23 (EtOAc:pyridine:HOAc:H_2O, 80:20:6:5); ^{1}H NMR ($CDCl_3$, 360 mHz) δ 1.38 (9H, *s*, $OC(C\underline{H}_3)_3$), 1.41 (9H, *s*, $OC(C\underline{H}_3)_3$), 1.44 (9H, *s*, $OC(C\underline{H}_3)_3$), 1.28–1.98 (14H, *m*, H_6 and H_7 Arg, β-CH_2, γ-CH_2 and δ-CH_2 Lys, β-CH_2 and γ-CH_2 Pro), 2.32–2.52 (2H, *m*, 2 × H_2), 2.64–2.79 (4H, *m*, 2 × H3), 2.77–2.99 (4H, *m*, 3 × β-CH_2 Phe, δ-CH Pro), 3.03 (2H, *t*, ε-CH_2 Lys, $J_1 = J_2$ = 7 Hz), 3.12–3.21 (3H, *m*, β-CH Phe and H_8 Arg), 3.67 (1H, m, δ-CH Pro), 4.23 (1H, *dd*, α-H Lys, J_1 = 5.5 Hz, J_2 = 8.5 Hz), 4.28 (1H, *dd*, α-H Pro, J_1 = 3.5 Hz, J_2 = 10 Hz), 4.38–4.43 (1H, *m*, α-H Arg), 4.49 (1H, *t*, α-H Phe, $J_1 = J_2$ = 7.5 Hz), 4.62 (1H, *dd*, α-H Phe, J_1 = 5 Hz, J_2 = 9 Hz), 7.18–7.35 (10H, *m*, ArH); HPLC-purity: 88.9% and 10.3% of **23a**; MS (FAB) m/z 1006 $(M + H)^+$.

N-*[5(S)-5-[D-Phenylalanyl-prolylamino]-8-guanidino-4-oxo-octanoyl]-phenylalanyl-lysine acetate (47a)*

Compound **46a** (0.28 g, 0.28 mmol) was dissolved in 90% TFA (5 mL) with anisole (23 μL). After workup (as described for **12**) the freeze-dried product (0.18 g, 0.24 mmol) was purified by preparative HPLC using a ternary solvent system (A: 0.1% TFA, 20%; B: H_2O 10 min 55%, then in 30 min to 35%; C: CH_3CN:H_2O, 6:4, v/v, for 10 min 25%, then in 30 min to 45%). The fractions with purified product were evaporated to remove the acetonitrile; the aqueous layer was freeze-dried. Compound **47a** was obtained as a white solid (88 mg, 42% yield): R_f 0.12 (EtOAc:pyridine:HOAc:H_2O, 6:2:2:1); ^{1}H NMR (D_2O, 360 mHz) δ 1.32–1.40 (2H, *m*, γ-CH_2 Lys), 1.54–1.91 (11H, *m*, 2 × H_6 and 2 × H_7, β-CH_2 and δ-CH_2 Lys and β-CH and γ-CH_2 Pro), 1.93 (*s*, HOAc), 2.10 (1H, *m*, β-CH Pro), 2.47 (2H, *m*, 2 × H_2, *J* = 7 Hz), 2.74–2.81 (3H, *m*, H_3 and δ-CH Pro), 3.00 (2H, *t*, ε-CH_2 Lys, *J* = 7 Hz), 2.99 (1H, *dd*, β-CH Phe, J_1 = 9 Hz, J_2 = 14 Hz), 3.12 (1H, *dd*, β-CH Phe, J_1 = 8.5 Hz, J_2 = 13 Hz), 3.19–3.26 (4H, *m*, 2 × β-CH_2 Phe and H_8), 3.52 (1H, *m*, δ-CH Pro), 4.15 (1H, *dd*, α-H Lys, J_1 = 5.5 Hz, J_2 = 8 Hz), 4.36 (1H, *dd*, α-H Pro, J_1 = 5 Hz, J_2 = 8.5 Hz), 4.39 (1H, *dd*, H_5, J_1 = 4.5 Hz, J_2 = 9 Hz), 4.49 (1H, *dd*, α-H Phe(-Lys), J_1 = 6 Hz, J_2 = 9 Hz), 4.62 (1H, *m*, α-H Phe, J_1 = 5.5 Hz, J_2 = 8.5 Hz), 7.28–7.48 (10H, *m*, ArH); HPLC-purity: 97.4%; peptide content: 70.5%; amino acid analysis: Phe:Pro:Lys = 2.01:0.98:1.00; MS (FAB) m/z 750 $(M + H)^+$; $[\alpha]^{25}_D$ –73.2° (*c* 0.33, H_2O).

N-*[5(R)-5-[D-Phenylalanyl-prolylamino]-8-guanidino-4-oxo-octanoyl]-phenylalanyl-lysine acetate (47b)*

Compound **46b** (0.23 g, 0.23 mmol) was treated as described for **47a** (see above) to obtain **47b** after purification by preparative HPLC (for conditions see **47a**). Compound **47b** was obtained as a white solid (14.1 mg, 18%): R_f 0.11 (EtOAc:pyridine:HOAc:H_2O, 6:2:2:1); ^{1}H NMR (D_2O, 360 mHz) δ 1.31–1.40 (2H, *m*, γ-CH_2 Lys), 1.48–1.92 (11H, *m*, H_6 and H_7, β-CH_2 and δ-CH_2 Lys and β-CH and γ-CH_2 Pro), 2.03 (*s*, HOAc), 2.09 (1H, *m*, β-CH Pro), 2.39–2.56 (2H, *m*, 2 × H_2), 2.72–2.86 (3H, *m*, 2 × H_3 and δ-CH Pro), 2.98 (2H, *t*, ε-CH_2 Lys, *J* = 7.5 Hz), 2.97 (1H, *dd*, β-CH Phe, J_1 = 9.5 Hz, J_2 = 14 Hz), 3.10–3.28 (2H, *m*, 3 × β-CH Phe and H_8 Arg), 3.49 (1H, *m*, δ-CH Pro), 4.15 (lH, *dd*, α-H Lys, J_1 = 5.5 Hz, J_2 = 8 Hz), 4.36 (1H, *dd*, α-H Pro, J_1 = 4 Hz, J_2 = 9 Hz), 4.37 (1H, *dd*, H_5, J_1 = 5.5 Hz, J_2 = 9 Hz), 4.53 (1H, *m*, α-H Phe), J_1 = 6 Hz, J_2 = 9 Hz), 4.62 (1H, *dd*, α-H D-Phe, J_1 = 5.5 Hz, J_2 = 9 Hz), 7.28–7.46 (10H, *m*, ArH); HPLC-purity: 97.7%; peptide content 64.8 %; amino acid analysis: Phe:Pro:Lys = 2.01:1.00:1.00; MS (FAB) m/z 750 $(M + H)^+$; $[\alpha]^{25}_D$ –30.7° (*c* 0.6, H_2O).

*Methyl-(5S)-5-[(R)-α-methoxy-α-(trifluoromethyl)phenyl-acetyl]amino-2-carboxymethyl-8-[*N-*(*N,N'-*dibenzyloxycarbonylguanidino)]-4-oxo-octanoate (49)*

Compound **48** (0.23 g, 0.36 mmol, synthesized using the same procedure as was used for **8**) was dissolved in

CH_2Cl_2:MeOH, 9:1 (10 mL); TBTU was added (0.23 g, 0.72 mmol) and TEA (0.1 mL, 0.72 mmol). The mixture was stirred at room temperature; every 10 min the pH was adjusted to 7.5 with TEA. After 1 h no starting compound could be detected; 0.1 M HCl was added (10 mL) and stirring was continued for 15 min. The mixture was diluted with CH_2Cl_2 and washed with water, 5% $NaHCO_3$ and water. After drying on Na_2SO_4, the solvent was evaporated. The crude product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc, 9:1) to obtain a white solid (0.13 g, 55% yield). This compound (59 mg, 0.09 mmol) was dissolved in dioxane (1.3 mL), 6 M HCl (2.6 mL) was added and the mixture was stirred for 30 min. After 30 min the volume of the reaction mixture was reduced to approximately 20%, CH_2Cl_2 was added (0.5 mL) and to this solution (0.07 mmol in 0.5 mL) a solution of *R*-(+)-MPTA-Cl was added dropwise under argon. After stirring for 16 h at room temperature no starting compound could be detected with TLC analysis. Water was added (40 μL) and the solvent was evaporated. The crude product was purified by silica gel column chromatography to give **49** as a yellow oil (22 mg, 31% yield): R_f 0.72 (CH_2Cl_2:EtOAc, 9:1); ^{1}H NMR ($CDCl_3$, 200 mHz) δ 1.63–2.04 (4H, *m*, 2 × H_6 and 2 × H_7), 3.03 (2H, *dd*, 2 × H_3, J_1 = 8 Hz, J_2 = 14 Hz), 3.29 (narrow *q*, α-OCH_3), 3.69 (3H, *s*, OCH_3), 3.71 (3H, *s*, OCH_3), 3.83 (1H, *m*, H_2), 3.98–4.08 (2H, *m*, 2 × H_8), 4.64 (1H, *m*, H_5), 5.13 (2H, *m*, CH_2 Z), 5.25 (2H, *m*, CH_2 Z), 7.29–7.60 (15H, *m*, ArH); ^{19}F NMR ($CDCl_3$, 188 mHz) δ −70.2 (*s*); MS (FAB) 786 $(M + H)^+$.

(5S)-5-(t-Butyloxycarbonylamino)-8-[N-(N,N'-dibenzyloxycarbonylguanidino)]-4-oxo-octanoic acid (50)

Decarboxylation of the crude compound **48** was carried out by refluxing in toluene for 1 h. After evaporation of the solvent, **50** was obtained as an oil: R_f 0.17 (CH_2Cl_2:MeOH, 9:1); ^{1}H NMR (360 mHz, $CDCl_3$) δ 1.40 (9H, *s*, Boc), 1.40–1.85 (4H, *m*, 2 × H_6 and 2 × H_7), 2.37–2.78 (4H, *m*, 2 × H_2 and 2 × H_3), 3.83–3.94 (2H, *m*, 2 × H_8), 4.27 (1H, *m*, H_5), 5.16 (1H, *s*, CH_2 Z), 5.22 (2H, *s*, CH_2 Z), 7.28–7.42 (10H, *m*, ArH); MS (FAB) m/z 598 $(M + H)^+$. HPLC-purity: 93.7%.

Methyl-(5S)-5-[(R)-α-methoxy-α-(trifluoromethyl)phenylacetyl]amino-8-[N-(N,N'-dibenzyloxycarbonylguanidino)]-4-oxo-octanoate (51)

Compound **50** was dissolved in CH_2Cl_2:MeOH, 9:1 (10 mL); TBTU was added (0.1 g, 0.31 mmol) and TEA (45 μL, 0.31 mmol). The mixture was stirred at room temperature; every 10 min the pH was adjusted to 7.5 with TEA. After 1 h no starting compound could be detected; 0.1 M HCl was added (10 mL) and stirring was continued for 15 min. The mixture was diluted with CH_2Cl_2 and washed with water, 5% $NaHCO_3$ and water. After drying on Na_2SO_4, the solvent was evaporated. The crude product was purified by silica gel column chromatography (CH_2Cl_2:EtOAc, 9:1) to obtain the methyl ester as a white solid (39 mg, 20% yield). This compound (40 mg, 0.07 mmol) was dissolved in dioxane (1 mL), 6 M HCl (2 mL) was added and the mixture was stirred for 30 min. After 30 min the volume of the reaction mixture reduced to approximately 20%, CH_2Cl_2 was added and to this solution (0.07 mmol in 0.5 mL) a solution of *R*-(+)-MPTA-Cl was added dropwise under Ar. After stirring for 16 h at room temperature no starting compound could be detected. Water was added (40 μL) and the solvent was evaporated. The crude product was purified by silica gel column chromatography (CH_2Cl_2:EtOAc, 97:3) to give **51** as a yellow oil (5 mg, 10% yield). R_f 0.74 (CH_2Cl_2:EtOAc, 9:1). ^{1}H NMR ($CDCl_3$, 200 mHz) δ 1.55–1.96 (4H, *m*, 2 × H_6 and 2 × H_7), 2.42–2.56 (2H, *m*, 2 × H_2), 2.61–2.79 (2H, *m*, 2 × H_3), 3.29 (narrow *q*, α-OCH_3),3.42 (narrow *q*, α-OCH_3), 3.62 (s, OCH_3), 3.64 (*s*, OCH_3), 3.89–4.06 (2H, *m*, 2 × H_8), 4.66 (1H, *m*, H_5), 5.11–5.18 (2H, *m*, CH_2 Z), 5.24 (1H, *s*, CH_2 Z), 7.28–7.52 (15H, *m*, ArH); ^{19}F NMR ($CDCl_3$, 188 mHz) δ −70.2 (*s*); MS (FAB) 728 $(M + H)^+$.

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