Synthesis of peptide p-nitroanilides mimicking fibrinogen- and hirudin-binding to thrombin

Design of slow reacting thrombin substrates

DIRK T.S. RIJKERS1,2, H. COENRAAD HEMKER2 and GODEFRIDUS I. TESSER1
1Catholic University of Nijmegen, Faculty of Natural Science, Department of Organic Chemistry, Toernooiveld, Nijmegen, The Netherlands and 2University of Limburg, Faculty of Medicine, Department of Biochemistry, Cardiovascular Research Institute, Maastricht, The Netherlands

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The design and synthesis of 20 peptide p-nitroanilides is described. The nitroanilides are used as thrombin substrates that share uncommon properties. These substrates are tested for their applicability to measure thrombin generation in activated plasma. This technique requires substrates that must slowly but selectively be hydrolyzed by thrombin. To ensure selectivity, thrombin's natural substrate and its most potent inhibitor were used as lead compounds. Eighteen peptide p-nitroanilides were synthesized using human fibrinogen Aα(7–16) decapetide as lead structure since this fragment constitutes the minimal sequence which binds to thrombin with high affinity. Two other chromogenic substrates were designed using 5-amino-2-nitrobenzoic acid as the chromophore. A peptide giving subsite interactions with the fibrinogen recognition exosite was coupled to the carboxyl function. As the peptide segment, the carboxyl terminal part of hirudin (residues 50–65) was chosen to ensure highly specific thrombin recognition. From the 20 nitroanilides synthesized, we indeed obtained a number of compounds which can be used as substrate in the thrombin generation assay. © Munksgaard 1996.

Key words: blood coagulation assay; chromogenic serine protease substrates; peptide p-nitroanilides; peptide synthesis; thrombin substrates

Thrombin is the pivotal enzyme in thrombosis and hemostasis. The thrombin generation curve, the course of appearance and disappearance of thrombin...
RATIONAL FOR DESIGN

Fibrinogen is the natural substrate of thrombin. The cleavage of fibrinogen by thrombin is highly specific since only four Arg-Xaa or Lys-Xaa bonds of the 376 present will be hydrolyzed (2). This remarkable specificity is confined to the N-terminal heptapeptide fragment of the Aα-chain (3, 4). In this region, fragment 1-23 (compound I, Fig. 1) interacts with the active site and determines the observed specificity (5).

By active-site mapping, insight was obtained into the contribution of each of the individual aminoacyl residues to the hydrolysis rate and demands of specificity. Van Nispen demonstrated (6) that the presence of residues Phe8 and Leu9 greatly enhances the rate of catalytic hydrolysis of the Arg16-Gly17 bond by thrombin. This has been confirmed by NMR measurements of complexes of the fibrinogen fragments 1-23 (7) and 7-16 (8) with thrombin. When bound to thrombin, the peptide chain folds back, enabling the side chain of Phe8 to interact with the argyl-binding site of thrombin. Similarly, the side chains of Leu9 and Val15 are bound into the apolar binding pocket, resulting in the occupation of both binding pockets as demonstrated with the inhibitor H-Phe-Pro-Arg-CH2Cl (9) and congeners. The chain-reversal demands a backbone conformation of the aminoacyl residue at position 12, which is incompatible with the presence of other residues than glycy. Recently, the crystal structure was determined of thrombin complexed with an analogue of fibrinopeptide A (10, 11). This investigation confirmed the chain-reversal, and the mentioned hydrophobic interactions. It was also found that Glu11 of the peptide forms a salt bridge with Arg173 of thrombin, thus stabilizing the peptide within the active-site cleft of the enzyme (11). The N-terminal hexapeptidyl part of the substrate points away from the enzyme and extends freely into solution. These experiments led to the conclusion that the sequence Phe8-Arg16 enables the highly specific binding interactions of compound I and congeners with thrombin (12).

We decided to synthesize small peptide p-nitroanilides derived from compound I in which Phe8 and Leu9 were omitted to arrive at substrates, which are attacked by thrombin with modified kinetic parameters fitting our continuous assay (1). The approach was based on a report (13) in which chloromethylketones of peptide fragments related to fibrinopeptide A were found to be recognized differently by thrombin when the positions 8, 9 and 12 were substituted with residues other than Phe, Leu and Gly, respectively. A general racemization-free synthesis was developed for compounds of the type Y-Val-X-pNA. The compounds synthesized in this study are given in Table 1. The substrates are defined herein as valyl-containing products, since this aminoacyl residue occurs in the P2 position fitting the S2 pocket of thrombin fairly well.

In the series studied, the amino group of the valyl residue is protonated or substituted either with a trim, polar aminoprotective function (Msc) (14), or with acyl(terminal) groups of variable bulkiness, length and polarity (Scheme 1). The presence of a valyl residue in these substrates results in a poor interaction with the S2 pocket of factor Xa which is so narrow that only glycy in position P2 will fit (15).

The nature of the basic aminoacyl group occupying the position of Arg16 was studied by varying the length of the side chain and its functional group (3a-c) (Scheme 1).

The importance of the conspicuous glycy spacer (positions 12-14) in the substrate which can attain a β-turn, was studied as the second approach to affect $K_m$ by modification of the linker length and its flexibility with compounds 3d-g. The peptide chain was changed by insertion of proline as a constraint either in position 13 (P4, 3h) or in position 12 (P5, 3i), cf. Kahn (16, 17). Variation of the length of the Gly spacer affects the ability to form the salt bridge between Glu11 and Arg173 of thrombin, and this would be revealed by the value of $K_m$. Proline is known to facilitate the formation of a β-turn and might thus replace the hydrophobic binding exerted by the residues Phe8 and Leu9, which causes the fold back of the peptide backbone and the formation of the salt bridge (Scheme 2).

The most potent thrombin inhibitor currently known is hirudin, a polypeptide isolated from the bloodsucking leech Hirudo medicinalis. Hirudin is a pentahexacontapeptide intramolecularly stabilized by three disulfide bridges, and containing a highly acidic carboxy-terminal segment including a sulfated tyrosyl residue (Fig. 2). It interacts with the active site of thrombin as well as with its important fibrinogen recognition exosite; factor Xa contrasts with thrombin by showing no interaction with hirudin.

Augmentation of cooperativity of the binding provided a new aim of research to arrive at bivalent thrombin inhibitors (18, 19) called hirulogs or hirulotins, respectively. Such inhibitors consist of the following structural elements: (1) an active-site directed part, which inhibits the active site; the sequence H-Phe-Pro-Arg-Pro being used most frequently; (2) a segment of hirudin, capable of interacting with the fibrinogen recognition exosite; usually the chain...
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### TABLE 1

**Substrates of the type Y-Val-X-pNA fitting the active site of thrombin like fibrinogen**

<table>
<thead>
<tr>
<th>Y</th>
<th>X</th>
<th>Code</th>
<th>Derived from</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>Lys</td>
<td>1a</td>
<td>H-Val-Arg-pNA (ref. 45)</td>
</tr>
<tr>
<td>Mac</td>
<td>Arg</td>
<td>2a</td>
<td>MeOMal-Val-Arg-pNA (ref. 45)</td>
</tr>
<tr>
<td>Mac</td>
<td>Lys</td>
<td>2b</td>
<td></td>
</tr>
<tr>
<td>Mac</td>
<td>Orn</td>
<td>2c</td>
<td></td>
</tr>
<tr>
<td>Mac</td>
<td>Nle</td>
<td>2d</td>
<td></td>
</tr>
</tbody>
</table>

| H-Gly  | Lys   | 3a   | Boc-Gly-Val-Arg-pNA                              |
| H-Gly  | Orn   | 3b   | **H-Gly-Val-pNA**                                |
| H-Gly  | Nle   | 3c   |                                                 |
| H-Glu  | Arg   | 3d   |                                                 |
| H-Glu-Gly | Arg | 3e   |                                                 |
| H-Glu-Gly-Gly | Arg | 3f   |                                                 |
| H-Glu-Gly-Pro-Gly | Arg | 3g   |                                                 |
| H-Glu-Pro-Gly | Arg | 3h   |                                                 |

Unprotected amino functions are suitably protonated (HCl) to give neutral solutions in plain water.

**SCHEME 1**

General synthesis of substrates of the type Y-Val-X-pNA, meant for investigation of the role of the aminoacyl group X in P$_1$ position in the recognition by thrombin; X represents position 16 in human fibrinogen Asf[1-23] tricosapeptide (I) cf. Fig. 1.

comprising residues 53-64 being used and (3) a peptide segment which spans the distance between the C-terminus of the active-site directed part and the N-terminus of the hirudin fragment.

The crystal structure of hirulog inhibited thrombin shows an important difference of the binding mode compared to hirudin (21). The active-site directed part of the inhibitor binds in a substrate-like manner. Consequently, these inhibitors will be cleaved by thrombin at the ~Arg-Pro~ bond albeit at a slow rate.

This type of thrombin inhibition prompted us to synthesize the chromogenic substrates 4 and 5 (Fig. 3), to bind S' subsites (post active-site interactions). Both consist of three parts: the acyl group ~Val-Arg~ occurring in the 'poor' thrombin sub-
Substrates for thrombin generation

![Chemical Structure 1](image1)

**FIGURE 3**
Amino acid sequence of a bifunctional substrate (compound 4), which interacts with the active site and the fibrinogen recognition exosite. The residues given in italics constitute the spacer segment between both interaction sites. Compound 5 is designed to interact with the primed subsites (S'→S'') of the active site of thrombin.

**SCHEME 3**
Synthesis of the pseudo-peptide acid Fmoc-Arg-ANBA-Gly-OH, to be used as building block in the solid phase synthesis of compounds 4 and 5 cf. Fig. 3. The guanidino function of arginine is protonated by HCl.

**METHODS OF SYNTHESIS**

Syntheses with nitroanilides are incompatible with conditions which attack or remove the nitroanilide function, these are: treatment with strong bases for hydrolysis or β-elimination, hydrazinolysis and catalytic hydrogenolysis. Acidolysis, however, is compatible with the conservation of the nitroanilide function.

Strict conservation of the chirality of aminoacyl residues excludes the activation of N-protected peptides with C-termini other than Gly or Pro. It is therefore risky to convert an existing peptide into a nitroanilide. The approaches which are compatible with this condition are stepwise elongation of the chain using activated N-protected amino acids and/or fragment condensation using azides to acylate an existing amino acid p-nitroanilide; azides are used to evade racemization during fragment condensations. Compounds of the types 1 and 2 (Scheme 1) were obtained by acylation of the α-amino function of the partially protected nitroanilide X with the active ester Boc-Val-ONSu, removal of the N-protection by acidolysis (1a), subsequent acylation with monomethyl malonate (2) or by acylation with Msc-Val-ONp (2a), respectively Msc-Val-ONSu(2b-il). Compounds of type 3 were obtained by acylation of the ω-amino group of the nitroanilide X with acylated valyl azides and subsequent treatment with acid (3, 3a-c). Compounds representing a modified glycyl-spacer were obtained by acylation of compound 1 and 3 respectively (Scheme 2), with Boc-Glu(OtBu)-ONSu and subsequent acidolysis (3d, e). Similarly, the introduction of Boc-Glu(OtBu)-Gly-ONSu into compound 3 afforded the substrate with the shortened glycyl-spacer (3f). The introduction of the complete glycyl spacer was performed by acylation of 3 with Boc-Gly-Gly-ONSu, removal of the Boc-group and acylation with Boc-Glu(OtBu)-ONSu (3g). The modified spacers were inserted using the same route, replacing Boc-Gly-Gly-ONSu by Boc-Gly-Pro-ONSu (3h) or Boc-Pro-Gly-ONSu (3i).

Deprotection of compounds containing the Boc-Glu(OtBu)→moiety (3d–1) was performed using Sieber’s method (26), to evade incomplete reactions as a consequence of the induced field effect by N⁺.

The ANBA-substrates 4 and 5 were synthesized by a combination of solid-phase and solution chemistry. Their syntheses required the pseudo-peptide Fmoc-
Arg-ANBA-Gly-OH·HCl, which was prepared first (Scheme 3).
The acid H-ANBA-OH was converted to its acid chloride. The amino function does not need protection, since it is a poor nucleophile due to the strong electron withdrawal by the nitro- and the carboxyl function. The acid chloride was coupled under Schotten-Baumann conditions to H-Gly-OAll in the presence of TEA in dry pyridine. The ensuing crude H-ANBA-Gly-OAll was purified by column chromatography on silica gel in a very low yield (13%), and was acylated with Fmoc-Arg-OH·HCl using POCl₃ in dry pyridine as described for the synthesis of protected amino acid p-nitroanilides (27). Despite the presence of a second electron-withdrawing function, the phosphorus oxychloride/pyridine activation appeared sufficiently strong to couple Fmoc-Arg-OH to H-ANBA-Gly-OAll in high yield (92%). The product was purified by counter-current distribution. The allyl function was removed by Pd-catalyzed nucleophilic transfer using Ph₃P as the catalyst as described by Albericio et al. (28). A catalytic amount of POCl₃ was used with regard to Fmoc-Arg-ANBA-Gly-OAll·HCl, which contrasts with the large excess used by the authors in the solid phase synthesis. Consequently, the deprotection lasted some 30 h at room temperature. The pseudo-peptide acid Fmoc-Arg-ANBA-Gly-OH·HCl was also purified by counter-current distribution. Although the side chain of arginine is only protected by protonation, Fmoc-Arg-ANBA-Gly-OH·HCl could be used as such in the solid-phase synthesis.

Peptides 4 and 5 were synthesized using the Fmoc/Bu protocol (29). They were synthesized on a p-alkoxybenzyl alcohol resin (Wang-resin) (30). During the solid phase synthesis the side chains were protected with acid labile functions: Trt for Gin, Asn and His, Boc for Lys, 'Bu for Glu, Asp, Ser and Tyr. Fmoc-amino acids were coupled by DIPCDI/HOBt in DMF. Removal of the Fmoc-protection was performed with 25% piperidine in DMF. After each coupling step, the absence of free amino groups was checked with ninhydrin (Kaiser test, ref. 31), and remaining free amino functions were acylated by Ac₂O/DIPEA 2:1 v/v in DMF. The peptides were detached from the resin and deprotected by treatment with TFA/EDT/H₂O 95:2.5:2.5 v/v/v and precipitated with diethyl ether. The crude peptides were eluted over a weakly basic ion-exchange column in the acetate cycle to remove TFA. After purification by counter current distribution the peptides were obtained in a yield of 20 and 38%, respectively. The purity and identity of the peptides were determined by TLC, HPLC, AAA and NMR.

RESULTS AND DISCUSSION

The thrombin generation curve is the result of the activity of the prothrombin activating enzyme complex (prothrombinase complex) and the thrombin inactivation reactions, such as binding to the inhibitors antithrombin III and ζ₂-macroglobulin. The thrombin generation curve is an important tool in coagulation research, since it yields important information about the functioning of the coagulation cascade and of the anticoagulant properties of exogenous inhibitors.

We developed an assay in which the thrombin generation curve is obtained by measuring the velocity of product formation when thrombin splits an artificial (chromogenic) substrate (1). The chromogenic substrate is added to plasma and interferes competitively with the physiological steady state. To minimize competitive inhibition processes by the added substrate, the $K_m$ value of this synthetic substrate should be high. To prevent the substrate from being exhausted long before thrombin generation is over, a slowly reacting substrate has to be used, thus the turnover number ($k_{cat}$) has to be low. Furthermore, the chromogenic substrate must be free of any inhibitory activity on the coagulation factors which affects thrombin formation, especially, the substrate should have a very low affinity for and activity with factor Xa. The ideal substrate would be a highly specific thrombin substrate, which has no interaction with factor Xa, which does not disturb thrombin production and breakdown and is stable in plasma. The substrate currently used (MeOMal-Aib-Arg-pNA) has moderate sensitivity as well as moderate selectivity towards thrombin.

The aim of the present study was to develop a number of substrates which virtually eliminate these drawbacks and thus might be used in this thrombin assay. This contribution describes the design and synthesis of these substrates.

The kinetic parameters of hydrolysis served as a first basis for selection (Table 2). The values of the kinetic parameters of the best suited substrates should lie in the following ranges: for thrombin, $K_m =$ 800–1200 μM and $k_{cat} =$ 0.2–0.8 s⁻¹; for factor Xa, $K_m >$ 5000 μM and $k_{cat} >$ 0 s⁻¹. Based on these criteria we found substrates 1, 2a and 3f as the most suitable to be used in the continuous thrombin assay. The other substrates were found less suitable for different reasons, such as too high an activity with thrombin ($k_{cat}$ too high) (2, 3, 3h), too much interference with physiological substrates (thus $K_m$ too low) (3, 3g, 4), and too high an affinity for factor Xa (2, 3d, 3e) or too low a sensitivity for thrombin in plasma (3a, 3f). A detailed discussion of the biochemical properties will be given in a separate paper (32).

As expected, the ANBA-substrates 4 and 5 were not hydrolyzed by factor Xa (vide supra). Their hydrolysis by thrombin, however, showed some unexpected properties. The enormous decrease of the $K_m$ value of 4, while the turnover number is hardly unaffected with respect to 1 was surprising. The
### Substrates for thrombin generation

**TABLE 2**

Kinetic parameters of hydrolysis of the substrates by thrombin and factor Xa in buffer A at 37 °C

<table>
<thead>
<tr>
<th>No.</th>
<th>Substrate</th>
<th>Thrombin</th>
<th>Factor Xa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$k_m$</td>
<td>$k_{cat}$</td>
</tr>
<tr>
<td>1</td>
<td>2HCl-H-Val-Arg-pNA (1)</td>
<td>1239</td>
<td>0.23</td>
</tr>
<tr>
<td>2</td>
<td>2HCl-H-Val-Lys-pNA (1a)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>MeOMal-Val-Arg-pNA·HCl (2)</td>
<td>1007</td>
<td>14.4</td>
</tr>
<tr>
<td>4</td>
<td>MeOMal-Aib-Arg-pNA·HCl (2b)</td>
<td>830</td>
<td>0.46</td>
</tr>
<tr>
<td>5</td>
<td>Me-Val-Arg-pNA·HCl (2a)</td>
<td>882</td>
<td>0.79</td>
</tr>
<tr>
<td>6</td>
<td>Me-Val-Lys-pNA·HCl (2b)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Me-Val-Orn-pNA·HCl (2e)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Me-Val-Nle-pNA (2d)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Boc-Gly-Val-Arg-pNA·HCl (3')</td>
<td>244</td>
<td>11.2</td>
</tr>
<tr>
<td>10</td>
<td>2HCl-H-Gly-Val-Arg-pNA (3)</td>
<td>3234</td>
<td>25.4</td>
</tr>
<tr>
<td>11</td>
<td>HCl-H-Gly-Val-Lys-pNA (3a)</td>
<td>986</td>
<td>0.20</td>
</tr>
<tr>
<td>12</td>
<td>2HCl-H-Gly-Val-Orn-pNA (3b)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>HCl-H-Gly-Val-Nle-pNA (3e)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>2HCl-H-Glu-Val-Arg-pNA (3d)</td>
<td>932</td>
<td>1.44</td>
</tr>
<tr>
<td>15</td>
<td>2HCl-H-EGVR-pNA (3e)</td>
<td>3274</td>
<td>3.51</td>
</tr>
<tr>
<td>16</td>
<td>2HCl-H-EGGVR-pNA (3f)</td>
<td>1006</td>
<td>0.55</td>
</tr>
<tr>
<td>17</td>
<td>2HCl-H-EGGGVR-pNA (3g)</td>
<td>379</td>
<td>0.27</td>
</tr>
<tr>
<td>18</td>
<td>2HCl-H-EGPGVR-pNA (3h)</td>
<td>1074</td>
<td>9.67</td>
</tr>
<tr>
<td>19</td>
<td>2HCl-H-EGGGVR-pNA (3i)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>H-VR-ANBA-GH[His50-65]-OH (4)</td>
<td>4.6</td>
<td>0.43</td>
</tr>
<tr>
<td>21</td>
<td>H-EGGVR-ANBA-GFK-OH (5)</td>
<td>-</td>
<td>-</td>
</tr>
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</table>

*From ref. 45.

**Experimental Procedures**

**General**

1H NMR spectra were recorded on Bruker AM 100 and AM 400 spectrometers. As an internal standard the residual solvent peak was used. Chemical shifts are given in parts per million (ppm). Optical rotations were measured on a Perkin Elmer 241 polarimeter in a 10 cm cuvette at room temperature. Melting points were determined with a Büchi melting point apparatus (Tottoli). Peptides were prepared by SPPS using a Laborteck SP 640 semi-automatic peptide synthesizer. Peptides (1 μmol) were hydrolyzed in 5.7 M HCl (Merck Suprapur 30% HCl) for 24 h at 120 °C and analyzed on a Varian Star amino acid analyzer. Analytical reversed-phase HPLC experiments were performed on a Varian LC equipped with a 9010 pump.
solvent delivery system, 9050 variable-wavelength UV-VIS detector and a C18 column (4.0 x 150 mm) using a gradient of 10--100% buffer B (0.08% TFA/10% H₂O v/v in CH₃CN) in buffer A (0.1% TFA in H₂O) as mobile phases at a flow rate of 0.2 mL/min. TLC was performed on Merck Silicagel 60F₂₅₄ plates, and column chromatography on Merck Kieselgel 60 70-230 Mesh ASTM (eluent: CH₂Cl₂/MeOH mixtures). Purification by counter current distribution (CCD) was performed on a Labor tec Craig counter current distribution apparatus using BuOH/AcOH/H₂O 4:1:5 v/v/v as solvent system. The partition coefficient K was calculated as follows:

\[ K = (r_{\text{max}} + 0.5)/(n - r_{\text{max}} + 0.5) \]

where \( r_{\text{max}} \) is the fraction with highest concentration of product, and \( n \) is the number of transfers. Spots were detected by UV-fluorescence quenching, ninhydrin (free amino functions), chlorine/TDM (NH groups) (36). Barton’s reagent (hydrazides) (37) and Sakaguchi’s reagent (arginine residues) (38). Pyridine was distilled over KOH and stored on 4 Å molecular sieves, dioxane was stored on 3 Å molecular sieves.

**Syntheses**

**Boc-Val-Arg-pNA·HCl.** Boc-Val-ONp (3.40 g, 10.0 mmol) was reacted with 2HCl·H-Arg-pNA (3.67 g, 10.0 mmol, 1.0 equiv.) in the presence of DIPEA (1.36 g, 10.6 mmol, 1.05 equiv.). Then DMF (75 mL) at room temperature for 16 h. After this period of stirring, DMF was evaporated in vacuo, and the residue was diluted with BuOH (100 mL). The organic solution was washed with H₂O, saturated NaHCO₃, H₂O and saturated NaCl (four times 30 mL each). The obtained organic solution was directly evaporated in vacuo. The residue was purified by CCD and lyophilized from AcOH. Yield: 4.71 g (89%), [\( \alpha \)] = -44.4° c = 0.59 MeOH, 1H NMR (CD₂OD):

\[ \delta = 0.94-0.98 \; [dd, \; 6H, \; \gamma-CH₃/\gamma'-CH₂-Val, \; J = 6.91 \; Hz]; \; 1.44 \; (s, \; 9H, \; Boc); \; 1.70 \; [m, \; 1H, \; \beta-CH-Val (J = 6.82 \; Hz)]; \; 1.82 \; (m, \; 2H, \; \gamma-CH₂-Arg); \; 2.03 \; (m, \; 2H, \; \beta-CH₂-Arg); \; 2.33 \; (m, \; 2H, \; \delta-CH₂-Arg); \; 3.88 \; [d, \; 1H, \; \alpha-CH-Val (J = 6.86 \; Hz)]; \; 4.56 \; (m, \; 1H, \; \alpha-CH-Arg); \; 7.84/7.86/8.20/8.22 \; (dd, \; 4H, \; aminopNA). \]

**Msc-Val-Arg-pNA·HCl (2a).** Msc-Val-ONp (116 mg, 0.30 mmol), 2HCl·H-Arg-pNA (120 mg, 0.33 mmol, 1.10 equiv.) were dissolved in DMF (3 mL) and TEA (51 μL, 0.37 mmol, 1.23 equiv.) was added. The suspension was stirred for 16 h at room temperature, and DMF was removed under reduced pressure. The oily residue was dissolved in H₂O (20 mL) and washed with diethyl ether (three times 5 mL). The aqueous phase was lyophilized. The product was purified by CCD and lyophilized from H₂O. Yield: 147 mg, (85%), [\( \alpha \)] = -37.6° c = 0.21 MeOH, 1H NMR (CD₂OD): 1.20 (s, 9H, Boc); 1.70-1.89 (m, 3H, B-CH₂-Val); 1.96-2.25 (dm, 2H, \( \delta-CH₂-Arg \)); 2.36 (m, 2H, \( \beta-CH₂-Arg \)); 3.26 (m, 2H, \( \beta-CH₂-Arg \)); 7.49 (m, 4H, aromatic pNA); 1.05-1.08 (m, 3H, B-CH₂-Arg); 1.62-1.94 (m, 3H, B-CH₂-Arg); 3.01 (m, 2H, \( \delta-CH₂-Arg \)); 3.82 (m, 2H, \( \delta-CH₂-Arg \)); 7.84/7.86/8.20/8.22 (dd, 4H, aminopNA).

**Msc-Arg-pNA·HCl (2a).** Msc-Arg-pNA (116 mg, 0.30 mmol) was dissolved in AcOH (10 mL), and 2.3 mL HCl in EtOAc (10 mL). The reaction mixture was stirred for 2.5 h at room temperature. The excess of hydrochloric acid was quenched with 1BuOH (50 mL) and the solution was evaporated in vacuo, and coevaporated with BuOH (twice 20 mL) and MeOH (three times 20 mL). The residue was dissolved in H₂O (50 mL) and lyophilized. Purification was done by CCD. Yield: 1.89 g (86%), [\( \alpha \)] = -5.1° c = 0.21 MeOH, 1H NMR (CD₂OD): 6:1.05/1.01-1.08/1.10 (dd, 6H, \( \gamma-CH₃/\gamma'-CH₂-Val \)); 1.70-1.89 (m, 3H, \( \gamma-CH₂-Arg/\beta-CH-Val \)); 2.03 (m, 2H, \( \beta-CH₂-Arg \)); 2.33 (m, 2H, \( \delta-CH₂-Arg \)); 3.88 (m, 2H, \( \beta-CH₂-Arg \)); 4.56 (m, 1H, \( \alpha-CH-Val \)); 5.48 (m, 1H, \( \alpha-CH-Arg \)); 7.85/7.87/8.20/8.22 (dd, 4H, aminopNA).

**Boc-Gly-Val-NzHy·Boc-Gly-OH (1.80 g, 10.3 mmol).** Boc-Gly-ONp (1.70 g, 10.1 mmol) was dissolved in AcOH (10 mL), and HOBT (1.70 g, 11.1 mmol, 1.10 equiv.) were suspended in EtOAc (50 mL). This mixture was cooled on ice and the amino component was set free with 2.3 mL HCl in EtOAc (10 mL). The reaction mixture was stirred for 2.5 h at room temperature. Then, the excess of hydrochloric acid was quenched with tBuOH (50 mL) and the solution was evaporated in vacuo, and coevaporated with BuOH (twice 20 mL) and MeOH (three times 20 mL). The residue was dissolved in H₂O (50 mL) and lyophilized. Purification was done by CCD. Yield: 1.89 g (86%), [\( \alpha \)] = -5.1° c = 0.21 MeOH, 1H NMR (CD₂OD): 6:1.05/1.01-1.08/1.10 (dd, 6H, \( \gamma-CH₃/\gamma'-CH₂-Val \)); 1.70-1.89 (m, 3H, \( \gamma-CH₂-Arg/\beta-CH-Val \)); 2.03 (m, 2H, \( \beta-CH₂-Arg \)); 2.33 (m, 2H, \( \delta-CH₂-Arg \)); 3.88 (m, 2H, \( \beta-CH₂-Arg \)); 4.56 (m, 1H, \( \alpha-CH-Val \)); 5.48 (m, 1H, \( \alpha-CH-Arg \)); 7.85/7.87/8.20/8.22 (dd, 4H, aminopNA).

**Boc-Gly-Val-NzHy·HCl (1.80 g, 10.3 mmol).** Boc-Gly-ONp (1.70 g, 10.1 mmol) was dissolved in AcOH (10 mL), and HOBT (1.70 g, 11.1 mmol, 1.10 equiv.) were suspended in EtOAc (50 mL). This mixture was cooled on ice and the amino component was set free with DIPEA (1.85 mL, 10.6 mmol, 1.05 equiv.). Then DCC (2.16 g, 10.5 mmol, 1.04 equiv.) was added and the suspension was stirred overnight. After this period DCU was filtered off and the clear organic solution was subsequently washed with 2 n KHSO₄, H₂O, saturated NaHCO₃ and saturated NaCl (three times 30 mL each). The EtOAc layer was dried on Na₂SO₄, filtered and evaporated in vacuo, yielding an oil which
still contained residual DCU; it was therefore dissolved in diethyl ether and filtered, and appeared to be free of DCU (Rr(CHrClr/MeOH 9:1 vlv): 0.60, single spot). The ester was used as such in the hydrazinolysis.

The obtained oil was dissolved in MeOH (20 mL) and NaH2H2O (2.5 mL, 51.5 mmol, 5.1 equiv.) was added. This reaction mixture was stirred for 3 d at room temperature. After this period the reaction mixture was evaporated in vacuo and the residue was coevaporated with MeOH (three times 20 mL). The residue was recrystallized from diisopropyl ether. Yield: 2.16 g (75%), R1(CH2Cl2/MeOH 9:1 v/v): 0.37, m.p. 118-121 °C, [\(\alpha\)]D: -21.0, c: 0.70 MeOH, 1H NMR (cDCl3): 6: 0.91-0.96 (dd, 6H, \(\gamma\)-CH\((\text{CH}_3\)Val)); 1.45 (s, 9H, Boc); 2.11 (m, 1H, \(\beta\)-CH-Val); 3.64 (dd, 2H, \(\gamma\)-CH\((\text{CH}_3\)Val)); 4.30 (dd, 1H, \(\alpha\)-CH-Val'); 5.73 (m, 1H, NH); 7.17 (d, <2H, NH2); 8.46 (s, 1H, NH).

Boc-Gly-Val-Arg-pNA·HCl (3'). Boc-Gly-Val-NH3 (1.23 g, 4.26 mmol) was dissolved in DMF (40 mL). This solution was cooled to -20 °C. To this solution were added: 2.3 mL HCl in EtOAc (5.2 mL, 12.0 mmol, 2.81 equiv.) and tBuONO (0.60 mL, 5.04 mmol, 1.18 equiv.). This reaction mixture was stirred for 15 min at -20 °C (no Barton positive material is present: azide formation is complete). The reaction mixture was neutralized by adding DIPEA (0.25 mL, 1.17 mmol) and 2HClH-Arg-pNA (1.56 g, 4.26 mmol, 1.0 equiv.) was added. The reaction mixture was kept at 0°C for 16 h; at regular time intervals the pH was checked and when necessary DIPEA was added to maintain the pH at 7-8. When the reaction was complete, the solvent was evaporated in vacuo and the residue was diluted with BuOH. The solution was washed with H2O, saturated NaHCO3, H2O and saturated NaCl, respectively. After removal of the solvent under reduced pressure the residue was purified by column chromatography. Yield: 2.22 g (89%), R1(BuOH/AcOH/H2O 4:1:1 vlv): 0.66 (K:6.8 Hz), [\(\alpha\)]D: -28.7°, c: 0.57 MeOH, 1H NMR (CD2OD): 6: 0.99 (dd, 6H, \(\gamma\)-CH\((\text{CH}_3\)Val)); 1.40 (s, 9H, Boc); 1.51-2.10 (dm, 3H, \(\gamma\)-CH\((\text{CH}_3\)Arg)), 2.15 (dm, 1H, \(\beta\)-CH-Arg); 3.73 (m, 2H, \(\delta\)-CH-Arg); 3.26 (m, 2H, d-CH-Gly); 4.18 1.13 (d, 1H, \(\alpha\)-CH-Val); 4.28 (d, 1H, \(\alpha\)-CH-Arg); 4.53 (m, 1H, \(\alpha\)-CH-Arg); 7.88/7.90–8.20/8.23 (dd, 4H, arom pNA).

Substrates for thrombin generation

2HCl-H-Gly-Val-Lys-pNA (3a). Removal of the Boc-function as described for 1. Yield: 72%, Rr(BuOH/AcOH/H2O 4:1:1 vlv): 0.33 (K:0.32), [\(\alpha\)]D: -56.1°, c: 0.36 MeOH, 1H NMR (CD2OD): \(\delta\) = 0.98 (dd, 6H, \(\gamma\)-CH\((\text{CH}_3\)Val)); 1.33 (m, 2H, \(\gamma\)-CH-Lys); 1.73 (m, 2H, \(\delta\)-CH-Lys); 1.75 (m, 2H, \(\beta\)-CH-Lys(1H)); 1.92 (m, 1H, \(\beta\)-CH-Lys(1H)); 2.82 (m, 2H, \(\varepsilon\)-CH-Lys); 3.71 (dd, 2H, CH2-Gly); 4.05 (m, 1H, \(\alpha\)-CH-Lys); 7.39/8.05 (dd, 4H, arom pNA).

Boc-Glu(OtBu)-Gly-Val-pNA·HCl (3b). 'Obtained as described for (a). Yield 90%, R1(BuOH/AcOH/H2O 4:1:1 vlv): 0.78 (K:10.52), [\(\alpha\)]D: -40.7°, c: 0.28 MeOH, 1H NMR (CD2OD): 6: 0.78 (d, 6H, \(\gamma\)-CH\((\text{CH}_3\)Val)); 1.43 (m, 1H, Boc(18H)OBU(9H)); 1.51-2.12 (bm, 7H, \(\beta\)-CH2-Glu/\(\beta\)-CH2-Val/\(\beta\)-CH2-Arg/\(\gamma\)-CH2-Arg), 2.34 (m, 2H, \(\gamma\)-CH2-Glu); 3.23 (m, 2H, \(\delta\)-CH2-Arg); 4.08 (m, 1H, \(\alpha\)-CH-Glu); 4.16 (m, 1H, \(\alpha\)-CH-Val); 4.54 (m, 1H, \(\alpha\)-CH-Arg); 7.86/8.88–8.21/8.23 (dd, 4H, arom pNA).
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\[ \delta = 0.99 \pm 0.01 (d, 6H, \gamma-CH_3/-CH_3-VaI); 1.43 \pm 0.03 (s, 18H, Boc(9H)/O'Bu(9H)); 1.69 \pm 0.16 (bm, 7H, \beta-CH_2-Glu/-\beta-CH-VaI/\gamma-CH_2-Glu); 2.31 (m, 2H, \gamma-CH_2-Glu); 3.24 (m, 2H, \delta-CH_2-Arg); 3.90 (dd, 2H, CH_2-Gly); 4.04 (m, 1H, \alpha-CH-Glu); 4.16 (d, 1H, \alpha-CH-VaI); 4.52 (m, 1H, \alpha-CH-Arg); 7.97/9.82 \pm 0.20/0.22 (dd, 4H, arom pNA).

(c) \( n = 2 \): Obtained as described for (a). Yield 79%, \( R_f (BuOH/ACOH/H_2O \ 4:1:1 \ v/v/v) = 0.67 \) \((K = 5.91)\), \( [\alpha]_D = -40.0^\circ \), \( e = 0.14 \) MeOH, \( 1^H \) NMR (CD_2OD): \( \delta = 0.99/1.01 \) (dd, 6H, \( \gamma-CH_2/-CH_2-VaI \)); 1.46 [s, 18H, Boc(9H)/O'Bu(9H)]; 1.73 (m, 2H, \( \gamma-CH_2-VaI \)); 1.87 \pm 0.07 (m, 1H, \( \beta-CH_2-Arg(1H) \)); 2.00 (m, 3H, \( \beta-CH_2-VaI/\beta-CH-VaI(1H) \)); 2.25 (m, 1H, \( \beta-CH_2-Glu(1H) \)); 2.34 (m, 2H, \( \gamma-CH_2-Glu \)); 3.23 (m, 2H, \( \delta-CH_2-Arg \)); 3.76 (m, 2H, N-CH_2-Pro); 3.89/3.93/3.97/4.01 (dd, 4H, CH_2-Gly(2 \times 2H)); 4.12 (d, 1H, \( \alpha-CH-VaI \)); 4.37 (m, 1H, \( \alpha-CH-Glu \)); 4.43 (m, 1H, \( \alpha-CH-Pro \)); 4.55 (m, 1H, \( \alpha-CH-Arg \)); 7.88/9.70 \pm 8.21/8.23 (dd, 4H, arom pNA).

Depletion of the N-terminal Boc-Glu(O'Bu) ~ residue. The Boc- and BtBu functions were removed by the method of Sieber et al. (26). In general: the protected peptide was dissolved in pre-cooled (\(-5^\circ C\)) concentrated HCl (37%). After stirring for 5 min at 0°C, the reaction mixture was diluted with ACOH (10 volumes) and lyophilized, dissolved in H_2O and lyophilized again. The crude deprotected product was purified by CDD and lyophilized from H_2O. The fluffy, off-white products were further dried in a vacuum desiccator on NaOH-pellets to reduce the slightly hygroscopic character of the fresh lyophilisates.

2HCl-H-Glu-Val-Arg-pNA (3d). Yield 81%, \( R_f (BuOH/ACOH/H_2O \ 4:1:1 \ v/v/v) = 0.36 \) \((K = 0.37)\), \( [\alpha]_D = -43.0^\circ \), \( e = 0.10 \) MeOH, \( 1^H \) NMR (D_2O): \( \delta = 0.78 \pm 0.07 (dd, 6H, \gamma-CH_2/-CH_2-VaI \)); 1.52 (m, 2H, \( \gamma-CH_2-VaI \)); 1.72 [m, 2H, \( \beta-CH_2-VaI(1H)/\beta-CH-VaI \]); 1.94 [m, 3H, \( \beta-CH_2-VaI(1H)/\beta-CH_2-Glu \)]; 2.25 (m, 2H, \( \gamma-CH_2-Glu \)); 3.04 (m, 2H, \( \delta-CH_2-Arg \)); 3.93 (m, 1H, \( \alpha-CH-Glu \)); 4.00 (d, 1H, \( \alpha-CH-VaI \)); 4.28 (m, 1H, \( \alpha-CH-Arg \)); 7.51/8.07 (dd, 4H, arom pNA).

2HCl-H-Glu-Gly-Val-Arg-pNA (3e). Yield 84%, \( R_f (BuOH/ACOH/H_2O \ 4:1:1 \ v/v/v) = 0.34 \) \((K = 0.36)\), \( [\alpha]_D = -40.8^\circ \), \( e = 0.26 \) MeOH, \( 1^H \) NMR (CD_2OD): \( \delta = 0.98/1.01 \pm 0.00 (dd, 6H, \gamma-CH_2/-CH_2-VaI \)); 1.70 (m, 2H, \( \gamma-CH_2-VaI \)); 1.84 (m, 1H, \( \beta-CH-VaI \)); 1.97/2.13 (dm, 2H, \( \beta-CH_2-VaI \)); 2.13 (m, 2H, \( \gamma-CH_2-Glu \)); 2.56 (m, 2H, \( \gamma-CH_2-Glu \)); 3.24 (m, 2H, \( \delta-CH_2-Arg \)); 3.97/4.00/4.05/0.09 (dd, 2H, CH_2-Gly); 4.02 (m, 1H, \( \alpha-CH-Glu \)); 4.23 (d, 1H, \( \alpha-CH-VaI \)); 4.53 (m, 1H, \( \alpha-CH-Arg \)); 7.86/7.89 \pm 8.21/8.23 (dd, 4H, arom pNA).

2HCl-H-Glu-Gly-Val-Arg-pNA (3f). Yield 94%, \( R_f (BuOH/ACOH/H_2O \ 4:1:1 \ v/v/v) = 0.28 \) \((K = 0.34)\), \( [\alpha]_D = -26.9^\circ \), \( e = 0.13 \) MeOH, AAA: Glu(1.00) 1.11, Gly(2.00) 1.97; Val(1.00) 0.99; Arg(1.00) 0.93, \( 1^H \) NMR (D_2O): \( \delta = 0.74 \pm 0.04 (dd, 6H, \gamma-CH_2/-CH_2-VaI \)); 1.52 (m, 2H, \( \gamma-CH_2-Glu \)); 1.68 (m, 1H, \( \beta-CH-VaI \)); 1.77/1.89 (dm, 2H, \( \beta-CH_2-VaI \)); 1.99 (m, 2H, \( \beta-CH_2-Glu \)); 2.37 (m, 2H, \( \gamma-CH_2-Glu \)); 3.05 (m, 2H, \( \delta-CH_2-Arg \)); 3.84 [m, 4H, CH_2-Gly(2 \times 2H)]; 3.94 (m,
2H, z-CH-Val/ z-CH-Glu); 4.29 (m, 1H, z-CH-Arg); 7.52/8.06 (dd, 4H, arom pNA).

2HCl-H-Glu-Gly-Gly-Gly-Val-Arg-pNA (3g). Yield 91%, Rf (BuOH/ AcOH/H2O 4:1:1 v/v/v): 0.20 (K = 0.28), [z]D = -16.4° c = 0.14 MeOH, AAA: Glu (1.00) 1.18; Gly (3.00) 2.92; Val (1.00) 0.99; Arg (1.00) 0.91, 1H NMR (D2O): δ = 0.78 (dd, 6H, γ-CH3/γ-CH3-Val); 1.54 (m, 2H, γ-CH2-Arg); 1.73/1.82 (dm, 2H, β-CH2-Arg); 1.93 (m, 1H, β-CH-Val); 2.04 (m, 2H, β-CH2-Glu); 2.42 (dd, 2H, γ-CH2-Glu); 3.08 (dd, 2H, δ-CH2-Arg); 3.81/3.89 (m, 6H, CH2-Gly); 4.23 (m, 1H, z-CH-Arg); 7.55/8.09 (dd, 4H, arom pNA).

2HCl-H-Glu-Gly-Pro-Gly-Val-Arg-pNA (3h). Yield 90%, Rf (BuOH/ AcOH/H2O 4:1:1 v/v/v): 0.17 (K = 0.33), [z]D = -50.8° c = 0.12 MeOH, AAA: Glu (1.00) 1.18; Gly (2.00) 1.97; Pro (1.00) 1.04; Val (1.00) 0.94; Arg (1.00) 0.87, 1H NMR (D2O): δ = 0.78 (dd, 6H, γ-CH3/γ-CH3-Val); 1.55 (m, 2H, γ-CH2-Arg); 1.64-2.14 (bm, 9H, β-CH2-Arg); 3.08 (m, 4H, δ-CH2-Arg); 3.46 (m, 2H, N-CH2-Pro); 3.80 (s, 2H, CH2-Gly); 3.98 (m, 4H, α-CH-Arg/CH-Fmoc/CHr-Fmoc); 1.31/1.74 (dm, 4H, γ-CH2l B-CHr-Arg); 3.09 (m, 2H, d-CH2-Arg); 4.04 (d, 2H, CH2-Gly); 4.16 (t, 2H, CH2-Fmoc); 4.23 (m, 1H, z-CH-Arg/CHr-Pro); 7.55/8.11 (dd, 4H, arom pNA).

H-ANBA-Gly-OAll. H-ANBA-OH (2.0 g, 11 mmol) was refluxed in SOCl2 (20 mL) with one drop of DMF, the suspension became clear after 10 min of refluxing. After 2 h, excess SOCl2 was removed under reduced pressure and the residue was diluted with dry dioxane (10 mL). The acid chloride was added dropwise to a solution of Tos-OH-H-Gly-OAll (3.16 g, 11 mmol, 1.0 equiv.) in dry pyridine (40 mL) in the presence of TEA (3.43 mL, 24.8 mmol, 2.25 equiv.). After 1 h of stirring at room temperature, the reaction mixture was evaporated in vacuo. The residue was dissolved in EtOAc (100 mL) and subsequently washed with H2O, saturated NaHCO3, H2O, 2N HSO4 and saturated NaCl (three times 30 mL each). After drying on Na2SO4, the solvent was removed under reduced pressure [Yield 1.18 g (38%), Rf (BuOH/ AcOH/H2O 4:1:1 v/v/v): 0.82]. The residue was purified by column chromatography. Yield 0.40 g (13%) dark yellow foam, Rf (CH3Cl/MeOH 95:5 v/v): 0.24, Rf (CHCl3/MeOH/ AcOH 95:20:3 v/v/v): 0.62, 1H NMR (CDCl3): δ = 4.26 (d, 2H, CH2); 4.66 (m, 2H, H-CH2-CH2-O- CO-) allyl); 5.34 (m, 2H, CH2-CH3 = C-CH3); 5.85 (m, 1H, CH-CH3); 6.32 (m, <1H, NH); 6.59-6.68 (m, 2H, arom ANBA H4/H6); 7.95/8.05 (d, 1H, arom ANBA H3).

Fmoc-Arg-ANBA-Gly-OAll HCl. Fmoc-Arg-OH-HCl (0.62 g, 1.43 mmol) and H-ANBA-Gly-OAll (0.40 g, 1.43 mmol) were coupled by POCl 3 in pyridine as described by us (27). After purification by CCD (K = 5.12), the product was lyophilized from AcOH and obtained as a fluffy pale yellow powder. Yield 0.91 g (92%), Rf (CHCl3/MeOH/ AcOH 95:20:3 v/v/v): 0.20, Rf (BuOH/ AcOH/H2O 4:1:1 v/v/v): 0.74, [z]D = -22.6°, c = 0.24 MeOH, 1H NMR (DMSO-d6): δ = 1.50-1.75 (dm, 4H, γ-CH2l β-CH2-Arg); 3.08 (m, 2H, δ-CH2-Arg); 4.04 (d, 2H, CH2-Gly); 4.16 (t, 1H, CH-Fmoc); 4.21 (m, 1H, z-CH-Arg); 4.28 (d, 2H, CH2-Fmoc); 4.63 (d, 2H, H-CH2-CH2-O-CO- allyl); 5.22/5.37 (2 × dd, 2H, CH2=CH-C=CH3); 5.93 (m, 1H, CH=CH- allyl); 7.32/7.41 (m, 4H, arom Fmoc); 7.73 (m, 2H, arom ANBA H4/H6); 7.89 (m, 4H, arom Fmoc); 8.11 (d, 1H, arom ANBA H3).

Fmoc-Arg-ANBA-Gly-OH-HCl. Fmoc-Arg-ANBA-Gly-OAll.HCl (398 mg, 0.574 mmol) was dissolved in 25 mL DMF/ AcOH/NMM (5:1:0.1 v/v/v), argon was bubbled through the solution during 10 min and Pd(PPh3)4 (30 mg, 0.026 mmol, 0.045 equiv.) was added. The obtained reaction mixture was stirred for 30 h at room temperature. After this period of stirring, solvent was removed under reduced pressure and the oily residue was purified by CCD (K = 4.18). Pure product was lyophilized from AcOH and obtained as a fluffy off-white material. Yield 306 mg (82%), Rf (BuOH/ AcOH/H2O 4:1:1 v/v/v): 0.53, [z]D = -33.3°, c = 0.11 DMF, 1H NMR (DMSO-d6): δ = 1.23-1.75 (dm, 4H, γ-CH2l β-CH2-Arg); 3.09 (m, 2H, δ-CH2-Arg); 3.64 (m, 2H, CH2-Gly); 4.21 (m, 1H, z-CH-Arg); 4.28 (d, 2H, CH2-Fmoc); 4.63 (d, 2H, H-CH2-CH2-O-CO- allyl); 5.22/5.37 (2 × dd, 2H, CH2=CH-C=CH3); 5.93 (m, 1H, CH=CH- allyl); 7.32/7.41 (m, 4H, arom Fmoc); 7.61 (m, 2H, arom ANBA H4/H6); 7.89 (m, 4H, arom Fmoc); 8.11 (d, 1H, arom ANBA H3).

Substrates for thrombin generation

Solid-phase-peptide-synthesis

The peptides were synthesized by solid-phase techniques following the Fmoc/ Bu protocol on a Wang resin. The Fmoc-amino acids (3 equiv. over substitution level of the resin) were coupled with DIPCDI (3 equiv.) and HOBr (3 equiv.) at room temperature for 1 h using DMF as the solvent. The side-chain protection was chosen as follows: Glu(Trt), Asn(Trt), His(Trt), Lys(Boc), Glu(O"Bu), Boc-Leu(Amt), Asp(O"Bu), Arg(Boc), Thr(O"Bu), Ser(O"Bu), Val(O"Bu), Ile(O"Bu), Leu(O"Bu), Phe(O"Bu), Thr(O"Bu), Gly(Boc), Trp(Boc), and Tyr(O"Bu). The peptides were purified by reverse phase HPLC. The UV absorption spectrum of these peptides was measured at the primary amino acids at 214 nm and the side-chain groups at 290 nm.
Asp(O'Bu), Ser(O'Bu) and Tyr(O'Bu). Fmoc protection was removed by treatment with 25% piperidine in DMF (three times for 6 min each). The peptides were detached from the resin and deprotected by treatment with TFA/H\textsubscript{2}O/EDT 95:2.5:2.5 v/v/v at room temperature for 4 h. The peptides were precipitated with diethyl ether. The precipitate was decanted and subsequently washed with diethyl ether and finally dried over NaOH-pellets. The crude deprotection product was eluted over a Merck II (weakly basic ion-exchanger, acetate form) ion-exchange column with H\textsubscript{2}O/AcOH 95:5 v/v as eluent. After lyophilization of the crude, the peptides were purified by C18. The purity and identity of the peptides were assessed by TLC, analytical HPLC, amino acid analysis and NMR.

H-VR-ANBA-G[His50-65]-OH; H-Val-Arg-ANBA-Gly-Ser-His-Asp-Aryl-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH (4). Quantities used: Fmoc-Gln(Trt)-O-Resin: 0.50 g (0.25 mmol), crude yield: 370.9 mg (59%), yield after purification: 128.4 mg (72%), yield after purification: 126.1 mg (20%), R\textsubscript{f}(BuOH/AcOH/H\textsubscript{2}O 4:1:1 v/v/v): 0.02, R\textsubscript{f}(BuOH/pyr/AcOH/H\textsubscript{2}O 4:1:1:2 v/v/v/v): 0.02, K = 0.11, HPLC: R\textsubscript{f} = 26.7 min, [\delta] = -38.2°, c = 0.11 AcOH; -42.1°, c: 0.19 DMF/H\textsubscript{2}O 1:1 v/v, AAA: Glu(1.00) 4.66; Leu(1.00) 0.86; Tyr(1.00); Pro(1.00) 0.86; Ile(1.00) 0.77; Phe(1.00) 1.22; Asp/Asn(3.00) 3.17; Gly(2.00) 2.47; His(1.00) 0.87; Ser(1.00); Arg(1.00) 1.04; Val(1.00) 1.06.

H-EGGV-ANBA-GFK-OH; H-Glu-Gly-Gly-Val-Tyr(1.00); Pro(1.00) 0.86; Leu(1.00) 0.86; Arg(1.00) 1.04; Val(1.00) 1.06, 1H NMR (aromatic part; DMSO-\textsubscript{d}6): \[ \delta = 6.60; 6.62-6.99; 7.01 (dd, 4H, arom Tyr); 7.13-7.30 (m, 5H, arom Phe); 7.30 [s, 1H, arom His (H5)]; 7.69 (m, 2H, arom ANBA); 8.08 (d, 1H, arom ANBA); 8.24 [bs, 1H, arom His (H2)].

**Kinetic parameters**

The hydrolysis experiments were run in buffer A (0.05 M Tris·HCl, 0.1 M NaCl, pH 7.35) containing 0.5 g/L bovine serum albumin (Sigma, Bornem, Belgium) at 37°C. The liberation of p-nitroaniline was monitored at 405 nm in a dual wavelength spectrophotometer made in our laboratory, using a personal computer for data recording. In a polystyrene micro-cuvette (total volume 500 μL), buffer A and substrate were added to obtain a final substrate concentration between 1 and 2000 μM. After 5 min of incubation at 37°C, enzyme solution was added to achieve a final concentration between 0.5 and 100 nM. The measurement was carried out in a thermostatted cuvette-holder at 37°C. The Michaelis constant \( K_m \) and catalytic constant \( k_{cat} \) were obtained by measuring initial reaction velocities at seven different substrate concentrations. The data given are mean values of three experiments.

**REFERENCES**
