# New $N^{\alpha}$-Guanidinobenzoyl Derivatives of Hirudin-54–65 Containing Stabilized Carboxyl or Phosphoryl Groups on the Side Chain of Phenylalanine-63

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We report on the synthesis and pharmacological properties of a new series of thrombin inhibitors derived from hirudin carboxyl-terminal fragments. Two (arylphosphono)phenylalanines, *p*-$PO_3H_2$-L-Phe[1] and *m*-$PO_3H_2$-L-Tyr, and one (carboxymethyl)phenylalanine, *p*-$CH_2COOH$-L-Phe, were prepared and incorporated into position 63 of the modified hirudin's C-terminal dodecapeptide using the Fmoc solid-phase synthesis strategy. Substitution by any one of the residues led to very active analogs which doubled the thrombin time at low micromolar concentration ($C_{tt2}$) in vitro ($1\ \mu M < Ctt_2 < 3\ \mu M$) and potently increased the activated partial thromboplastin time (APTT) ex vivo. These compounds displayed a higher potency in vitro and a longer duration of action in vivo than both the corresponding sulfated or phosphorylated tyrosine counterparts.

## Introduction

Thrombin is one of the key enzymes of the blood coagulation system. Thrombin activities are diverse since they include the conversion of fibrinogen to fibrin monomer,[2] platelet activation by interacting with a specific platelet membrane receptor,[3] and control of its own zymogen activation.[4] The most potent thrombin inhibitor known is hirudin, a 65-amino acid polypeptide isolated from the blood sucking leech *Hirudo medicinalis*.[5] Only a portion of the carboxy terminus of the hirudin molecule is required for anticoagulant activity, the minimal peptide length being about 12 amino acid residues (Asn53 to Leu64).[6,7] Ionic interactions of the numerous negatively charged residues present in this COOH-terminal part of hirudin with a positively charged surface grove on thrombin have been shown to play a crucial role in hirudin–thrombin interactions and consequently in the anticoagulant activity of hirudin.[8] Additionally, tyrosine-63 is found sulfated in the natural purified protein, thus adding some ionic interactions to the overall binding energy of hirudin to thrombin[9,10] and increasing its specific activity. Structure–activity relationship (SAR) studies on this minimal sequence have been described, and the positional effects of sulfation have been extensively studied.[11,12] Since the sulfation of tyrosine-63 is known to affect the potency of hirudin and peptide derivatives of hirudin, we have prepared first an analog in which the sulfated tyrosine was replaced by a phosphorylated tyrosine. The phosphotyrosine substitution resulted in a molecule with essentially the same affinity for thrombin as the sulfated one. This initial modification had been suggested by the recent publication of an efficient synthesis of phosphorylated tyrosine residues that allows a relatively large scale synthesis of phosphorylated tyrosine-containing peptides.[13,14] To our knowledge, the synthesis of sulfated tyrosine has not yet been described, and the sulfation is performed on the entire peptide with relatively low yields and tedious purifications.[15] We then decided to incorporate more stable derivatives of phosphotyrosine and synthesized two (arylphosphono)phenylalanines, *p*-$PO_3H_2$-L-Phe and *m*-$PO_3H_2$-L-Tyr, and one (carboxymethyl)phenylalanine, *p*-$CH_2COOH$-L-Phe, which were successfully incorporated into peptides by conventional solid-phase peptide synthesis. These sulfated isosteric replacements and the incorporation of non-natural amino acids led to thrombin inhibitors with increased resistance to enzymatic degradation and improved anticoagulant potencies in vivo.

## Chemistry

Compounds **III** and **VI** (Table 1) were synthesized using the procedure described by Valerio et al.[16] for the synthesis of *O*-phosphonotyrosyl-containing peptides. Di-*tert*-butyl phosphate protecting groups were used for the coupling of the *O*-phosphonotyrosine and were removed concomitantly to the cleavage of the peptide from the resin. The preparation of the 4-(bis(alkyloxy)phosphinyl)phenylalanine residues **9** and **10** required for the synthesis of compound **VIII** is shown in Scheme 1. The synthesis of 4-(diethoxyphosphinyl)phenylalanine via trifluoromethane sulfonation of tyrosine and subsequent tetrakis(triphenylphosphine)palladium(0)-catalyzed coupling with diethyl phosphite was originally described by Petrakis and Nogabhushan.[17] We have developed the method to synthesize residues **9** and **10** with suitable protecting groups that have allowed their successful incorporation into peptides via standard solid-phase method using $N^{\alpha}$-Fmoc protection. The use of 45% $HBr/CH_3COOH$ for the deprotection of the precursor leading to peptide **VIII** prepared by incorporating derivative **9** gave rise to numerous side products involving the 4-guanidinobenzoyl part of the peptide. We then turned to the incorporation of residue **10**, for which the deprotection mixture (TFA/$Me_3SiBr$/thioanisole) used gave good yields and no side reactions.

## Results

The hirudin-C-terminal related analogues were evaluated for their ability to inhibit the $\alpha$-thrombin-mediated hydrolysis of fibrinogen in vitro (thrombin time). The results are shown in Table 1. All the compounds were found to have micromolar activities as expressed by the concentration of the inhibitor tested that doubled thrombin time. The dessulfated or desphosphorylated analogs

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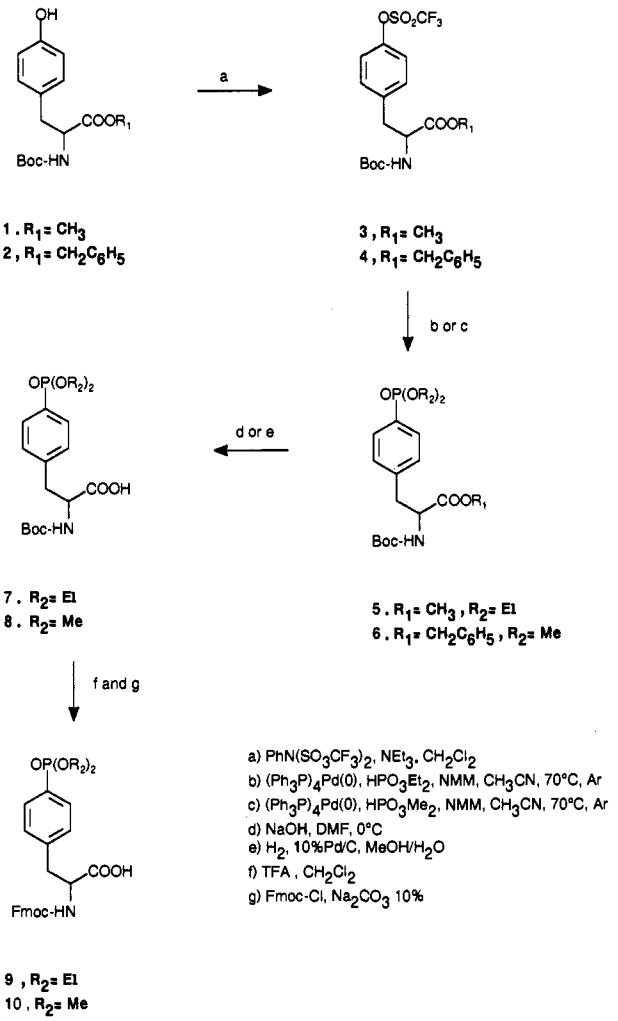
Table 1. Fibrin Clot Inhibition (Thrombin Time) of the Synthetic Analogs

| no. | | $C_{tt2}$[a] μM | n[f] |
|---|---|---|---|
| I[b] | Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH | 36.8 ± 1.9 | 8 |
| II[c] | Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr($SO_3H$)-Leu-Gln-OH | 2.5 ± 0.1 | 8 |
| III[d] | Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr($PO_3H_2$)-Leu-Gln-OH | 2.9 ± 0.2 | 4 |
| IV[e] | G-Gly-Asp-Phe-Glu-Abo-Ile-Pro-Glu-Glu-Tyr-Leu-OH | 16.5 ± 2.0 | 6 |
| V | G-Gly-Asp-Phe-Glu-Abo-Ile-Pro-Glu-Glu-Tyr-Leu-D-Glu-OH | 4.8 ± 0.7 | 6 |
| VI | G-Gly-Asp-Phe-Glu-Abo-Ile-Pro-Glu-Glu-Tyr($PO_3H_2$)-Leu-D-Glu-OH | 0.9 ± 0.1 | 6 |
| VII | G-Gly-Asp-Phe-Glu-Abo-Ile-Pro-Glu-Glu-Tyr(m$PO_3H_2$)-Leu-D-Glu-OH | 4.0 ± 0.3 | 3 |
| VIII | G-Gly-Asp-Phe-Glu-Abo-Ile-Pro-Glu-Glu-Phe(p$PO_3H_2$)-Leu-D-Glu-OH | 2.8 ± 0.1 | 3 |
| IX | G-Gly-Asp-Phe-Glu-Abo-Ile-Pro-Glu-Glu-L-Phe(p$CH_2COOH$)-Leu-D-Glu-OH | 1.0 ± 0.1 | 3 |
| X | G-Gly-Asp-Phe-Glu-Abo-Ile-Pro-Glu-Glu-D-Phe(p$CH_2COOH$)-Leu-D-Glu-OH | 50 ± 0.1 | 3 |

[a] $C_{tt2}$ = Concentration (μM) of the inhibitor that doubles thrombin time. [b] Hirudin-55–65. [c] Hirudin-55–65 sulfate. [d] Hirudin-55–65 phosphate. [e] G: 4-guanidinobenzoyl. [f] Number of independent experiments.

Scheme 1. Synthesis of $N^{\alpha}$-Fmoc-L-phosphonophenylalanine Derivatives Suitable for Solid-Phase Synthesis




of hirudin-55–65 displayed a 15-fold decrease in activity as can be seen for compounds **II** and **III** compared to compound **I**. This result showed that phosphate is a good substitution for sulfate in the hirudin sequence. In order to develop a "targeted" antithrombin agent that specifically binds cells at a thrombus, we decided to incorporate an integrin-specific sequence. However, coupling a mimetic of the cell adhesive RGD sequence (guanidinobenzoyl-glycyl-aspartyl) to the N-terminus of hirudin-55–65 and substituting Glu58 by Abo, a generally well tolerated proline analog, did not affect the ability of hirudin-55–65 to inhibit fibrinogen hydrolysis by thrombin but led to a compound (**IV**) with a 2-fold increased activity. Addition of a D-Glu residue at the carboxyl part of compound **IV** roughly increased the activity 3-fold (**V**). Phosphorylation of Tyr63 in compound **V** gave compound **VI**, which appeared to be the most active derivative of the series in this in vitro test ($C_{tt2}$ = 0.9 μM).

Table 2. Anticoagulant Activity following Intravenous Administration of Hirudin C-Terminal Peptide Derivatives in Rats, at the Dose of 8 mg/kg

| | APTT[a] (% baseline) at time: | | | | | | |
|---|---|---|---|---|---|---|---|
| no. | 1.5 min | 3 min | 5 min | 15 min | 30 min | 60 min | n[b] |
| II | 179 ± 5 | 160 ± 6 | 147 ± 4 | 124 ± 13 | 120 ± 9 | 97 ± 10 | 6 |
| III | 139 ± 2 | 120 ± 3 | 108 ± 2 | 94 ± 1 | 83 ± 8 | 90 ± 3 | 6 |
| IV | 136 ± 8 | 123 ± 61 | 115 ± 5 | 108 ± 1 | 100 ± 7 | 98 ± 1 | 6 |
| V | 158 ± 4 | 145 ± 4 | 133 ± 4 | 115 ± 5 | 111 ± 9 | 96 ± 10 | 6 |
| VI | 201 ± 24 | 162 ± 16 | 147 ± 13 | 125 ± 11 | 110 ± 6 | 107 ± 3 | 4 |
| VII | 265 ± 28 | 244 ± 26 | 217 ± 16 | 192 ± 17 | 164 ± 14 | 163 ± 36 | 4 |
| VIII | 252 ± 5 | 225 ± 5 | 217 ± 2 | 200 ± 5 | 181 ± 3 | 187 ± 10 | 4 |
| IX | 213 ± 10 | 205 ± 8 | 188 ± 5 | 149 ± 5 | 141 ± 6 | 118 ± 10 | 4 |

[a] APTT: activated partial thromboplastin time, relative to untreated animals (100). [b] n = number of independent experiments.

Replacement of the *O*-phosphonotyrosine residue in compound **VI** by one of the two (arylphosphono)phenylalanines derivatives led to the active analogs **VII** and **VIII**. The stability of these peptides was assessed in proteolytic media in vitro and compared to reference peptides.[18] In rat kidney homogenates (10 mg of tissue/mL), which readily degrades hirudin-55–65, compounds **VII** and **VIII** were found to be stable for several hours (half-lives of 5 h), thus confirming the protection against peptidases activities provided by the presence of non-natural amino acids in the molecule and the property of phosphonic acid to be nonhydrolyzable phosphate mimetics. Introduction of racemic Fmoc-DL-Phe(*p*-$CH_2COOCH_3$)-OH into the sequence of hirudin-55–65 by solid-phase synthesis led to a mixture of the diastereoisomeric compounds **IX** and **X** which were easily separated by HPLC. As expected from previous SAR studies, compound **X** was 50 times less potent than compound **IX** in thrombin inhibition.[11] Compounds **VI–IX**, when compared to compounds **I–V**, demonstrated marked antithrombin activity in anesthetized rats after intravenous injection of 8 mg/kg (Table 2). These activities were dose related (data not shown). Maximum antithrombin activity for both compounds was observed 1.5 min after injection. Antithrombin activity of compound **VI**, the only arylphosphono derivative, declined rapidly and returned to initial values after 30 min, whereas it required more than 60 min for a similar loss to occur with compounds bearing the (arylphosphono)phenylalanines derivatives (**VII** and **VIII**) (Figure 1).

## Discussion

In this study, we have described the synthesis and pharmacological properties of a series of compounds

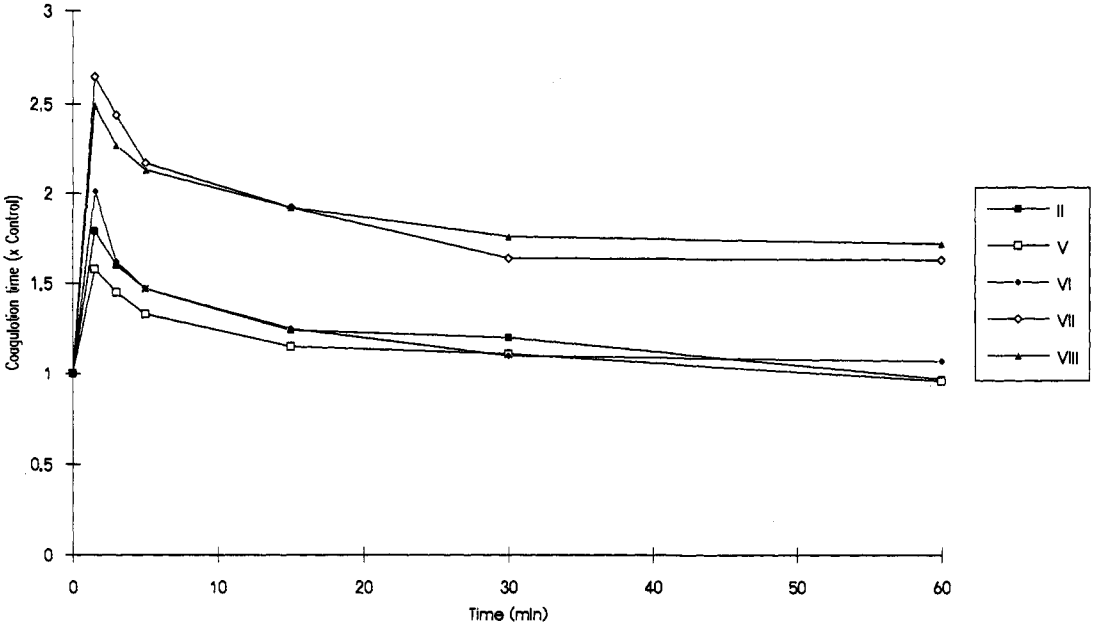



**Figure 1.** Anticoagulant effect of hirudin analogs after iv administration (8 mg/kg) in rats. Compound **VIII** increases maximally the coagulation time (ex vivo) from 18.1 ± 0.3 s (control) to 46.2 ± 0.8 s, after 1.5 min.

derived from hirudin carboxyl-terminal fragments. These hirudin fragments are known to exert their antithrombin activities by binding to the fibrinogen recognition site (anion binding exosite domain) of thrombin.[9] All analogs of the series contain a number of modifications as compared to the natural sequence, which are based on results published by others and on our own structure–activity studies.[11,19,20] For example, Glu58 is known to be one of the least important residues in the 55–65 sequence of hirudin, and introduction of Pro in this position resulted in increased potency.[11] Similarly, Abo (L-azabicyclooctanecarboxylic acid) proved to be a suitable proline analog when incorporated in position 58.[21] The most active analogs (**V–IX**) are all stabilized toward enzymatic degradation by the presence of an N-terminal guanidinoacyl group, of one or more non-natural amino acids, and of a COOH-terminal D-amino acid.

Another important goal was to find a stable nonhydrolyzable sulfate mimetic of tyrosine-63 which is sulfated in native hirudin and which increases the affinity constant of the hirudin–thrombin complex 10-fold.[22] On the basis of the assumption that the size of negatively charged sulfate and phosphate moieties are nearly the same, we decided to substitute phosphate for sulfate on Tyr63 of hirudin. The activity of phosphotyrosine-63–hirudin-55–65 appeared to be the same as its sulfated counterpart (compounds **II** and **III**). In order to stabilize the attachment of the phosphate group on the aryl moiety, the C–O bond was replaced by a C–P bond in a 4-phosphonophenylalanine (compound **VIII**), and in a new phosphonyl derivative of tyrosine[23] (compound **VII**). In the latter compound, the free phenolic function was maintained in position 4 and the phosponyl group attached to the aromatic ring in position 3. The in vitro anticoagulant activities of these analogs were relatively similar to the phosphotyrosine-containing compound (**VI**). On the contrary, in vivo studies showed a marked increase in their anticoagulant activities and duration of action confirming the stability of the phosphate mimetics in biological media (Figure 1). These results stress the importance of the simultaneous optimization of potency and stability to proteolysis of peptide-based drugs.[18]

In conclusion, a series of low molecular weight thrombin inhibitors derived from hirudin carboxyl-terminal fragments have been produced. A convenient synthesis of stable phosphotyrosine surrogates was developed, and these synthons were successfully incorporated into peptides by conventional methods. These modifications confer high activity and enhanced proteolytic resistance on the analogs. Compounds **VII** and **VIII**, which are strong anticoagulants and still active 60 min after their intravenous administration in rats, may be good antithrombotic candidates and may represent a valuable alternative to higher molecular weight thrombin inhibitors.

## Experimental Section

**General Methods.** All peptides were synthesized by the solid-phase method of Merrifield[24] using standard procedures on a Milligen 9050 or a Labortec SP650 peptide synthesizer. Analytical data of the synthetic peptides are given in Table 3. The *p*-alkoxybenzyl alcohol resin, $N^{\alpha}$-protected (Fmoc) amino acids,[25] and reference peptide **II** were purchased from Bachem, except for Abo which was synthesized in-house by procedures previously described.[21] Single diisopropylcarbodiimide and *N*-hydroxybenzotriazole-mediated coupling reactions were run on the automatic synthesizer with the first amino acid routinely bound to the resin. Groups of the *tert*-butyl type were used for side-chain protection and $N^{\alpha}$-Fmoc for peptide elongation. Peptides were cleaved from the resin by TFA (10 mL/g of the resin) containing 30% of a dichloromethane/anisole/ethanedithiol (2/1/1, in mL/g of the resin) mixture. Reverse-phase HPLC was performed on a Waters 625 LC system equipped with a photodiode array UV detector, utilizing a DeltaPak C18 (spherical 5 μm) column (3.6 × 150 mm). Retention times are given for gradient elution at 1 mL/min in the binary solvent system 0.1% TFA in water/0.1% TFA in acetonitrile. Preparative HPLC was routinely performed on a Waters Prep LC 3000 system equipped with a Waters 490E multiwavelength detector on a PrePak cartridge (47 × 300 mm) filled with a C18-silica (300 Å, 15 μm) phase. The operating flow rate was 60 mL/min.

For amino acid analyses, peptides were hydrolyzed in 6 N HCl (0.3 mL) for 20 h at 110 °C in sealed tubes. Hydrolysates were analyzed with a Varian LC90 Star system. The whole procedure including liquid transfer, mixing, Fmoc-derivatization, pentane extraction, and separation on a Aminotag C18 (5-μm) column (4.6 × 150 mm) is completed within 40 min. Molecular weights of peptides were determined by FAB mass spectrometry on a Normag R10-10C apparatus. The samples were dissolved in a

Table 3. Analytical Data of Synthetic Peptides

| no. | Gly | Asp | Phe | Glx[a] | Leu | Abo | Ile | Pro | Tyr | % peptide content[b] | $[M+H]^+$ | HPLC $t_R$[d] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | | 1.04(1) | 1.02(1) | 4.95(5) | 1.02(1) | | 1.02(1) | 1.04(1) | 1.04(1) | 97 | c | c |
| II | | 1.03(1) | 1.02(1) | 5.0(5) | 1.04(1) | | 0.98(1) | 1.02(1) | 1.02(1) | 92 | c | c |
| III | | 0.96(1) | 0.97(1) | 4.78(5) | 1.07(1) | | 1.00(1) | 1.1(1) | 1.1(1) | 92 | 1491 | 10.2[e] |
| IV | 1.06(1) | 1.04(1) | 1.01(1) | 3.02(3) | 1.07(1) | 0.99(1) | 0.99(1) | 1.05(1) | 0.95(1) | 98 | 1526 | 19[f] |
| V | 1.02(1) | 1.08(1) | 0.99(1) | 4.21(4) | 0.85(1) | 0.8(1) | 0.90(1) | 1.1(1) | 1.04(1) | 93 | 1638 | 17.3[g] |
| VI | 1.05(1) | 1.03(1) | 1.05(1) | 4.01(4) | 0.91(1) | 0.92(1) | 0.94(1) | 1.1(1) | 1.05(1) | 93 | 1718 | 13.2[g] |
| VII | 1.10(1) | 0.97(1) | 1.05(1) | 3.78(4) | 1.01(1) | 0.93(1) | 1.0(1) | 1.03(1) | 1.1(1) | 90 | 1718 | 11.6[g] |
| VIII | 1.02(1) | 0.98(1) | 1.00(1) | 3.92(4) | 0.97(1) | 0.95(1) | 1.07(1) | 0.99(1) | | 86 | 1702 | 12[g] |
| IX | 0.99(1) | 0.99(1) | 1.04(1) | 4.03(4) | 0.96(1) | 0.90(1) | 0.97(1) | 1.04(1) | | 90 | 1680 | 11[h] |

[a] Glu and Gln. [b] One single peak in HPLC, difference to 100%: trifluoroacetate counter ion. [c] Reference peptides, nd. [d] Gradient $CH_3CN$ per 30 min in 0.1% TFA aqueous buffer at 1.0 mL/min, $t_0$ = 1.5 min. [e] 18–23% $CH_3CN$. [f] 25–40% $CH_3CN$. [g] 20–40% $CH_3CN$. [h] 25–30% $CH_3CN$.

glycerol–thioglycerol matrix (1/1), and ionization was effected by a beam of krypton atoms accelerated through 6–8 keV. $^1H$ NMR spectra were recorded on Bruker spectrophotometers at 200 or 400 MHz as indicated, with $Me_4Si$ as external standard.

**Chemistry.** Compounds 1 and 2 (Scheme 1) were obtained from H-Tyr-OMe and H-Tyr-OBzl, respectively, by conventional method for Boc incorporation.[26]

**$N^{\alpha}$-Boc-4-[((trifluoromethyl)sulfonyl)oxy]-L-phenylalanine Methyl Ester (3).** A solution of 2 g (6.76 mmol) of 1 and 2.65 g (7.4 mmol) of phenylbis((trifluoromethyl)sulfonyl)amine (Aldrich) in 10 mL of dichloromethane was cooled in an ice bath, and 1.03 mL (7.4 mmol) of triethylamine was added dropwise. The resulting mixture was stirred for 1 h at 0 °C and allowed to warm up to room temperature over 2 h. The reaction was stopped by addition of 60 mL of diethyl ether, and the organic layer was washed with successive 20-mL portions of water, 1 M NaOH, water, and brine and dried. Filtration and concentration afforded an oil which was purified by flash chromatography on silica gel, eluting with pentane/ethyl acetate (80/20) to give 2.7 g (95%) of a yellow oil: $^1H$ NMR (300 MHz, $CDCl_3$): 1.40 (s, 9H), 5.05 (d, 1H), 3.7 (s, 3H), 4.5 (m, 1H), 3.05–3.45 (dd, 1H), 7.2 (s, 4H).

**$N^{\alpha}$-Boc-4-[((trifluoromethyl)sulfonyl)oxy]-L-phenylalanine Benzyl Ester (4).** This compound was prepared by using the procedure described for 3. From 4.3 g (11.6 mmol) of 2, was obtained 5.65 g (97%) of 4. $^1H$ NMR (300 MHz, $CDCl_3$): 1.4 (s, 9H), 5.05 (d, 1H), 7.1 (s, 4H), 3.05–3.15 (dd, 1H), 4.65 (m, 1H), 5.65 (dd, 2H), 7.35 (m, 5H).

**$N^{\alpha}$-Boc-4-(diethoxyphosphinyl)-L-phenylalanine Methyl Ester (5).** A suspension of 3 (0.470 g, 1.1 mmol), methylmorpholine (159 μL, 1.4 mmol), $(EtO)_2POH$ (174 μL, 1.32 mmol), and $Pd(0)(PPh_3)_4$ (38 mg, 0.032 mmol) in dry $CH_3CN$ (3 mL) was stirred at 70 °C in a sealed tube under $N_2$ for 15 h. The reaction was stopped by addition of 40 mL of ethyl acetate and the organic layer washed with 3 × 15 mL of 5% citric acid and 3 × 15 mL of aqueous 10% $NaHCO_3$ and dried ($Na_2SO_4$). The product was then purified by flash chromatography (silica gel, ethyl acetate/pentane, 3/7) affording 365 mg (80%) of the phosphonate 5. $^1H$ NMR (300 MHz, $CDCl_3$): 1.4 (s, 9H), 5.0 (d, 1H), 4.6 (m, 1H), 3.7 (s, 3H), 3.05–3.15 (dd, 1H), 7.25–7.5 (dd, 2H), 4.15 (m, 4H), 1.25–1.35 (t, 3H).

**$N^{\alpha}$-Boc-4-(dimethoxyphosphinyl)-L-phenylalanine Benzyl Ester (6).** This compound was synthesized analogous to 5 except that dimethyl phosphite ($(MeO)_2POH$) was used instead of diethyl phosphite ($(EtO)_2POH$). After usual workup procedures and chromatography, a yellow oil was obtained (45% yield). $^1H$ NMR (300 MHz, $CDCl_3$): 1.35 (s, 9H), 4.95 (d, 1H), 4.6 (m, 1H), 3.0–3.15 (dd, 1H), 7.1 (m, 2H), 7.6 (dd, 2H), 3.65–3.7 (s, 3H), 5.1 (dd, 2H), 7.25 (m, 5H).

**$N^{\alpha}$-Boc-4-(diethoxyphosphinyl)-L-phenylalanine (7).** To a solution of compound 5 (187.5 mg, 0.45 mmol) in DMF (2.5 mL) cooled to 0 °C was slowly added 1 M NaOH (0.5 mL, 1.1 equiv). After 15 min at 0 °C and 2 h at room temperature, the reaction was stopped by acidification with 1 N HCl (0.65 mL, 1.1 equiv). After evaporation, the residue was dissolved in 20 mL of ethyl acetate and washed with 3 × 10 mL of $H_2O$ and 2 × 10 mL of 5% NaCl. The organic layer was dried over $Na_2SO_4$ and evaporated in vacuo. A 130-mg (72%) yield of a white powder was obtained. $^1H$ NMR (300 MHz, DMSO): 7.45–7.6 (m, 2H), 4.15 (m, 1H), 4.0 (q, 4H), 2.15–3.15 (2 dd, 2H), 1.3 (s, 9H), 1.2 (t, 6H), 7.1 (d, NH).

**$N^{\alpha}$-Boc-4-(dimethoxyphosphinyl)-L-phenylalanine (8).** A solution of 6 (6.67 g, 14.4 mmol) in methanol/water (60 mL/40 mL, v/v) was reduced under hydrogen atmosphere (50 psi) using 10% palladium carbon as catalyst. After filtration and evaporation of the solvent, a clear oil was obtained (5.53 g, 81% yield). $^1H$ NMR (300 MHz, $CD_3CN$): 7.2 (m, 2H), 4.4 (m, 1H), 4.0 (q, 4H), 3.0–3.25 (2 dd, 1H), 1.35 (s, 9H), 7.65 (dd, 2H), 5.53 (d, 1H).

**$N^{\alpha}$-Fmoc-4-(diethoxyphosphinyl)-L-phenylalanine (9).** $N^{\alpha}$-Boc deprotection of 7 (1 g, 2.5 mmol) was performed using standard procedures.[27] The Fmoc group was introduced according to Carpino.[25] The overall yield for the two steps was 82.7%. $^1H$ NMR (200 MHz, DMSO): 12.9 (s, 1H), 7.3–8.0 (m, 12H), 4.2 (m, 4H), 4.0 (m, 4H), 2.95–3.2 (dd, 2H), 1.2 (t, 6H).

**$N^{\alpha}$-Fmoc-4-(dimethoxyphosphinyl)-L-phenylalanine (10).** The same procedure as for 9 was used starting with 8 (1.6 g, 4.2 mmol). The overall yield of the two steps was 75%. $^1H$ NMR (300 MHz, $CD_3CN$): 7.85 (d, 2H), 7.60 (d, 2H), 7.4 (m, 2H), 4.15 (dd, 1H), 4.3 (m, 2H), 6.05 (d, 1H), 4.45 (m, 1H), 3.0–3.25 (dd, 1H), 7.35 (dd, 2H), 7.65 (m, 2H), 3.65–3.7 (s, 3H).

**$N^{\alpha}$-Fmoc-*O*-(*O'*,*O''*-di-*tert*-butylphosphono)-L-tyrosine (11).** This compound was synthesized according to previously described procedures,[13] starting with $N^{\alpha}$-Fmoc-L-Tyr-OH (0.605 g, 1.5 mmol); 0.640 mg (70% yield) of 11 was obtained. MS (FAB): *m/e* 596 ($MH^+$).

**$N^{\alpha}$-Fmoc-*O*-benzyl-*o*-(dimethylphosphono)-L-tyrosine (12).** The synthesis of this compound is described elsewhere.[23]

**$N^{\alpha}$-Fmoc-4-((methoxycarbonyl)methoxy)-D,L-phenylalanine (13).** Compound 13 was synthesized as previously described.[28] Total yield from 4-(bromomethyl)phenylacetic acid, 32%. $^1H$ NMR (500 MHz, DMSO): 7.90 (d, 2H), 7.3–7.4 (t, 2H), 4.2 (m, 3H), 7.65 (d, 1H), 12.8 (s, 1H), 2.85 (t, 1H), 3.05 (d, 1H), 7.15 (m, 4H), 3.75 (s, 2H), 3.70 (s, 3H).

**[(4-Guanidinobenzoyl)Gly54,Abo58,Tyr(*m*-$PO_3H_2$)63,D-Glu65]hirudin-54–65 (VII).** This compound was synthesized analogous to the procedure described by Paladino et al.[23] The protected peptide was assembled starting with Fmoc-D-Glu-($OBu^t$)-*p*-alkoxybenzyl alcohol-resin (0.45 g, 0.18 mmol), and building block 12 was incorporated in position 63. After cleavage of the peptide from the resin, the benzyl and methyl groups of building block 12 were removed as follow: 250 mg of the peptide was subjected to a catalytic hydrogenation (10% palladium charcoal) in methanol/water (3:1) for 24 h at room temperature. After filtration and evaporation of the solvent, the peptide was treated with a mixture of 0.8 mL of $Me_3SiBr$, 0.7 mL of thioanisole, and 6 mL of TFA for 5 h at 0 °C. After evaporation, the residue was purified by preparative HPLC on reverse phase and lyophilized; 60 mg of pure VII was obtained. MS: *m/e* $MH^+$ 1718.

**[(4-Guanidinobenzoyl)Gly54,Abo58,Phe(*p*-$PO_3H_2$)63,D-Glu65]hirudin-54–65 (VIII).** This compound was synthesized analogous to the procedure described for general methods. The deprotection was carried out depending on which building block was used. Building block 9: 80 mg of the peptide was treated with 16 mL of 40% $HBr/H_2O$ and 4 mL of $CH_3COOH$ for 20 h at room temperature. The mixture was then evaporated, and the residue was taken up in $H_2O$ and purified by preparative HPLC. Lyophilization of the eluate gave 10 mg of the desired pure peptide VIII. Building block 10: 300 mg of the peptide prepared by incorporating building block 10 was treated with a mixture of 45 mL of TFA, 2.7 mL of $Me_3SiBr$, and 2.4 mL of

thioanisole for 17 h at room temperature. The mixture was evaporated and the product purified by preparative HPLC. After lyophilization 100 mg of the desired peptide **VIII** was obtained. MS: *m/e* $MH^+$ 1702.

**[(4-Guanidinobenzoyl)Gly54,Abo58,Phe(*p*-$CH_2COOH$)-63,D-Glu65]hirudin-54–65 (IX).** This compound was synthesized as described in the general methods: 88 mg of the peptide prepared by incorporating building block **13** was treated with 0.878 mL of 0.5 N NaOH and 2.7 mL of MeOH for 14 h at rt. After neutralization with 0.44 mL of 1 N HCl the mixture was evaporated and lyophilized. Preparative HPLC afforded separation of the two diastereoisomeric peptides: 12.4 mg of pure **IX** was obtained. MS: *m/e* $MH^+$ 1680.

**Plasma Anticoagulant Activity.** Sprague–Dawley rats (300–400 g, Charles River, France) were anesthetized with pentobarbital sodium (60 mg/kg ip), and a jugular vein and carotid artery were cannulated for drug administration and blood sampling, respectively. Catheters were purged with citrated physiological serum (1/40). For in vitro assays, 20 mL of blood was drawn into a siliconated tube containing 1/10 final volume of 3.8% trisodium citrate. For in vivo assays, blood samples (1.5 mL) were collected at 30 min before and 1.5, 3, 5, 15, 30, and 60 min after administration of the peptides (1 mL). After each sampling, 1.5 mL of physiological serum was injected into the carotid artery. The collected samples were then centrifuged for 15 min at 3000*g* in a way to obtain platelet poor plasma. Plasma was conserved during 4 h at 20 °C. TT and APTT were measured using a ST4 coagulometer (Diagnostica Stago, France) and were assayed with a Thrombin Prest (Diagnostica Stago) kit containing 1.5 NIH/mL of thrombin, and a CK Prest (Diagnostica Stago) kit, respectively. For the thrombin time (TT) assay, 150 μL of thrombin (1.5 NIH/mL) was added to plasma (45 μL) and incubated 2 min in the presence of the vehicule (5 μL) at 37 °C. The clotting time was immediately measured. The control thrombin time (obtained in the presence of vehicule alone) was 23.4 ± 0.4 s (mean ± SEM, $n$ = 28) which provided a time corresponding to TT assay obtained in human plasma. In the presence of the inhibitor (5 μL), the thrombin time was prolonged sufficiently to enable the determination of $C_{tt2}$ as the concentration (μM) of the inhibitor that doubles thrombin time relative to a control containing no inhibitor. For the activated partial thromboplastin time (APTT), 100 μL of plasma were mixed with 100 μL of a thromboplastin solution at 37 °C. The clotting time was obtained 3 min after recalcification with 100 μL of a $CaCl_2$ solution. The average clotting time was 18.1 ± 0.3 s, $n$ = 30.

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## References

(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC–IUB Joint Commission on Biomedical Nomenclature: Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem. J.* 1984, *219*, 345–373. The following further abbreviations were used: AAA, amino acid analysis; Abo, 2-azabicyclo[2.2.2]-octane-3(*S*)-carboxylic acid; APTT, activated partial thromboplastin time; Boc, *tert*-butyloxycarbonyl; $C_{tt2}$, concentration of the inhibitor that doubles thrombin time; DCC, *N,N'*-dicyclohexylcarbodiimide; DMF, dimethylformamide; FAB, fast atom bombardment; Fmoc, 9-fluorenylmethoxycarbonyl; NMM, *N*-methylmorpholine; TFA, trifluoroacetic acid; TT, thrombin time.

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