67. Synthesis of a New Bivalent Hirudin Analog (Hirufos), which Includes a Stable 4′-Phosphono-L-phenylalanine Mimic of (L-Tyrosine O^4-Sulfate)-63

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The synthesis on solid phase of a new derivative of the anticoagulant protein hirudin is described (see *Scheme* and *Fig. 1*, **1**). The henicosapeptide is a bivalent conjugate of the C-terminus of hirudin and of the active-site-binding tetrapeptide D-Phe-Pro-Arg-Pro linked *via* a tetraglycine spacer. The peptide, for which the name hirufos was coined, incorporates a stable phosphono derivative of L-phenylalanine which, combined with the other structural modifications, leads to a potent anticoagulant agent. Synthesis was readily achieved by the (9*H*-fluoren-9-yl)-methoxycarbonyl (Fmoc) strategy followed by acidolytic cleavage from the resin and deprotection, including the liberation of the crucial phosphonic group on L-phenylalanine.

Introduction. – Hirudin is a *O*-sulfonated peptide of 65 amino acids firstly isolated from the salivary glands of the blood-sucking leech *Hirudo medicinalis* [1] and endowed with strong anticoagulant properties. The polypeptide binds tightly to the fibrinogen-recognizing site of α-thrombin, thus leading to an efficient inhibition of the protease [2]. X-Ray data analysis of the cocrystal convincingly demonstrated an interaction between the carboxy-terminal tail of hirudin (residues 48 to 65) and a long groove on the surface of the enzyme [3]. In addition, hirudin binds with its N-terminal part (Val-Val-Tyr) to the thrombin active sit cleft. The therapeutic potential of hirudin was soon realized, and the peptide (desulfohirudin) is currently produced on large scale by biotechnological means [4], and a number of advantages of recombinant hirudin over heparins are reported [5].

Extensive structure-activity relationship studies of the hirudin molecule led to the identification of a minimal C-terminal dodecapeptide for a measurable inhibition of clotting [6] [7]. Further chemical optimization of this sequence gave more potent and stable analogs such as MDL 28050, a N^{α}-succinylated, non-sulfonated peptide with nonnatural amino-acid substitutions [8]. The importance of sulfonation [9] [10] or phosphorylation [11] of L-tyrosine-63 was also investigated and found to consistently increase the anticoagulant activity. We previously developed an efficient strategy to replace these easily cleavable groups by stable phosphono-L-phenylalanine derivatives [11] [12] which, when incorporated into hirudin fragments, produced thrombin inhibitors with increased activity and duration of action. Further progress was achieved in the field by the design of bivalent peptide inhibitors in which the carboxy tail of hirudin is linked to a D-Phe-Pro-Arg-Pro sequence *via* a tetraglycine spacer [13]. The new class of inhibitors called hirulogs, and the similarly designed hirutonins [14] displayed nanomolar thrombin inhibitory activity. However, no attempt was made in these conjugates to incorporate the naturally occurring sulfate or its phosphate substitute in position 63, probably because of their instability toward hydrolysis.

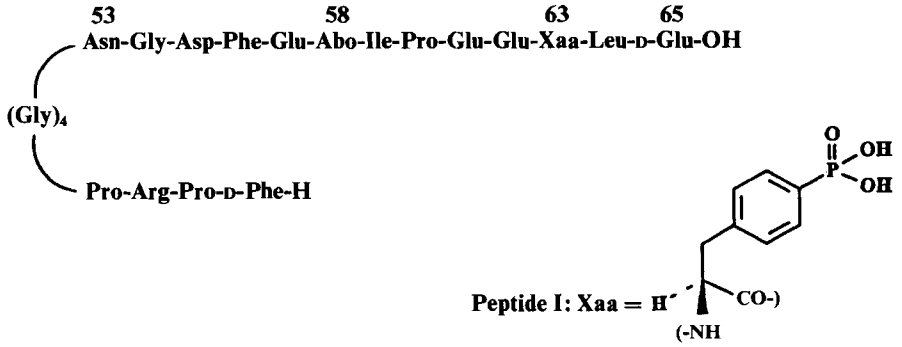


Fig. 1. *Structure of peptide* **I** (hirufos). Abo = (3*S*)-2-azabicyclo[2.2.2]octane-3-carboxylic acid.

In the present study, we describe the synthesis of a new bivalent hirudin derivative (hirufos) which, due to the incorporation of a stable phosphono derivative of L-phenylalanine, includes all these structural features and displays improved pharmacological activity.

Results. – 1. *Synthesis.* The key synthon to be incorporated, Fmoc-Phe(4-PO_3Me_2)-OH (Fmoc = (9*H*-fluoren-9-yl)methoxycarbonyl), was obtained as described earlier by us [11]. Briefly, the $O^{4'}$-triflate of Boc-Tyr-OBzl (Boc = *tert*-butyloxycarbonyl) was treated with $(MeO)_2POH$ in the presence of *N*-methylmorpholine and of $[Pd^0(PPh_3)_4]$ and converted into the corresponding dimethyl phosphonate of L-phenylalanine, Boc-Phe(4′-PO_3Me_2)-OBzl. Hydrogenolytic removal of benzyl and exchange of Boc against Fmoc, using conventional methods, led to the desired synthon. The other nonclassical building block Fmoc-Abo-OH was prepared from (3*S*)-2-azabicyclo[2.2.2]octane-3-carboxylic acid (= Abo) [15] by a usual procedure [16].

The new hirudin analogue (peptide **I**, *Fig. 1*) was easily obtained by conventional peptide synthesis on solid phase [17] using the Fmoc strategy [18] for peptide elongation, except for the last amino acid D-Phe for which Boc was chosen as the protecting group (see *Scheme*). In this way, the protected peptide could be both deprotected and cleaved from the resin in one single step. The two methyl-ester groups of the 4′-(dimethoxyphosphoryl)-L-phenylalanine-63 unit were removed separately under optimized conditions by treatment of the crude product with Me_3SiBr/thioanisole/CF_3COOH 1:1:18 for 17 h at room temperature. Purification was obtained by prep. HPLC on reversed phase (H_2O/MeCN (0.1% CF_3COOH) gradient). The final compound, obtained as a bis-trifluoroacetate, had a single peak in HPLC (*Fig. 2*), the expected amino-acid content (±10%), and correct molecular weight as estimated by FAB-mass spectrometry.

2. Biological Activity. – The anticoagulant activity of peptide **I** (hirufos), when administered to the rat intravenously, is measured *ex vivo* in function of time over a period of 60 min (*Fig. 3*). It compares well with that of hirulog 1 (a bivalent 20-residue peptide containing Glu and Tyr instead of Abo and Phe(4-PO_3H_2), respectively, and lacking the C-terminal D-Glu residue [13]), and even with that of r-hirudin (= desulfohirudin(1–65)). It is also considerably more active in this test than its shorter monovalent analog (4-guanidinobenzoyl)-Gly-Asp-Phe-Glu-Abo-Ile-Pro-Glu-Glu-

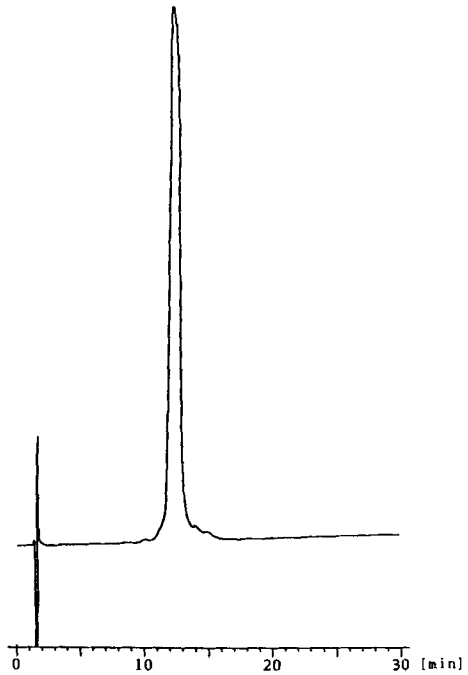


Fig. 2. *HPLC Analysis of peptide* **I**. Conditions: *Millipore Delta Pak* (5 μm, C_{18} 300 Å, 3.9 × 150 mm); 20–40% MeCN/H_2O (0.1% CF_3COOH) gradient within 30 min, flow rate 1 ml/min; detection 210 nm.

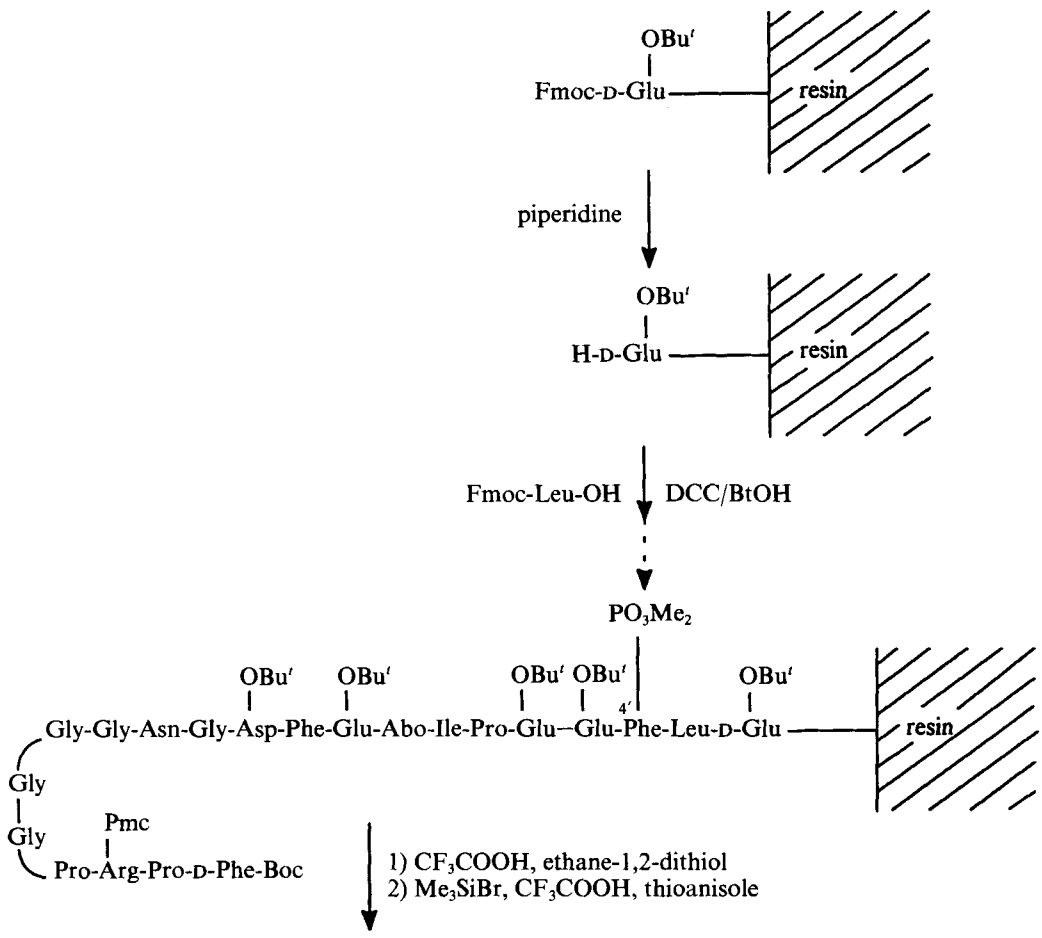
Phe(4′-PO_3H_2)-Leu-D-Glu-OH (= guanidinobenzoyl-hirufos(54–65)), which we described recently [11]. However, one should keep in mind that natural *O*-sulfonated hirudin was reported to be ten times more potent than its desulfonated counterpart (r-hirudin) [4] [13].

Discussion. – Compared to the 3′-phosphono-L-tyrosine, which we introduced recently into a C-terminal fragment of hirudin [12], the amino acid 4′-phosphono-L-phenylalanine used in this study was considerably easier to synthesize, showed the same stability towards hydrolytic splitting, and was not detrimental to biological activity [11]. It appears, therefore, to be a more convenient substitute for tyrosine sulfate or -phosphate in these peptide analogs.

The synthesis on solid phase was carried out without major difficulties, apart from a longer reaction time (14 h) required for the introduction of Abo in the peptide chain. Final deprotection could probably be achieved in one single step. However, since this would expose the peptide to more drastic conditions for a longer period of time, a two-step procedure was preferred, in which the saponification of the dimethyl phosphonate was performed separately. The overall yield (4.2%) for more than forty steps indicates that a synthesis on a 100-g scale could be envisaged by the same procedure in the research laboratory.

The high anticoagulant potency of peptide **I** compared to hirulog 1 and to recombinant desulfohirudin seems to be due to the apparent additivity of at least three factors

Scheme



Peptide I

Abo = (3*S*)-2-Azabicyclo[2.2.2]octane-3-carboxylic acid; Pmc = 2,2,5,7,8-pentamethylchromane-6-sulfonyl; Fmoc = (9*H*-fluoren-9-yl)methoxycarbonyl; Boc = *tert*-butyloxycarbonyl.

observed to individually enhance activity: *1*) the optimization of the affinity and stability of the C-terminal dodecapeptide of hirudin, *2*) the coupling in a conjugate of an active-site-recognizing tetrapeptide with the C-terminal fragment of hirudin, and *3*) the introduction and stabilization of a phosphonic group on the side chain of residue 63, thus mimicking the naturally occurring O^4-sulfo-L-tyrosine-63. An extensive pharmacological evaluation of peptide **I** (hirufos) will be published elsewhere.

Thanks are due to Dr. *J. P. Volland* and collaborators for expert analytical characterization of the products.

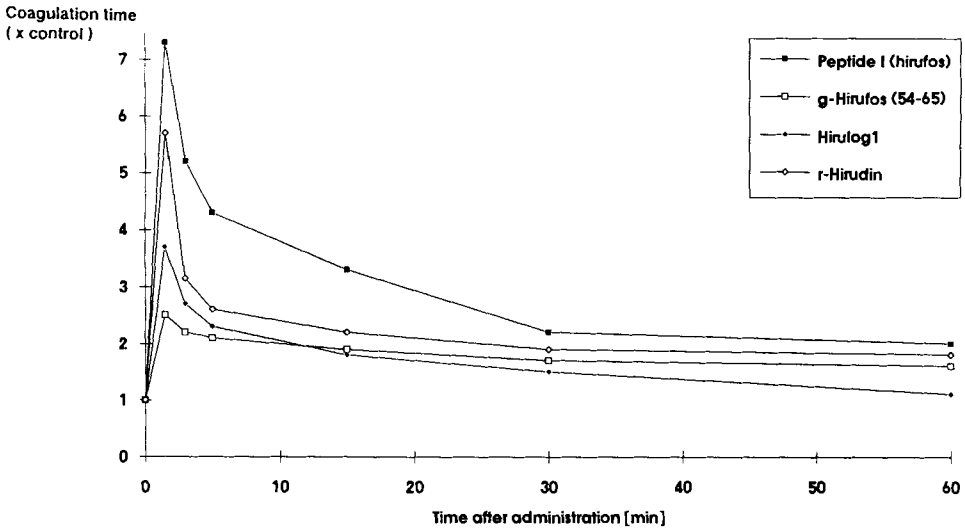


Fig. 3. *Anticoagulant activity* (measured *ex vivo*) *of peptide* **I** *compared to g-hirufos(54–65)* (g = 4′-guanidinobenzoyl) [11], *to hirulog 1, and to desulfohirudin* (= r-hirudin). The activity is expressed as the factor by which the control time is multiplied after intravenous administration to the rat at the dose of 0.8 mg/kg (8 mg/kg for g-hirufos(54–65)).

Experimental Part

General. The commercially available protected amino acids and the resin-linked Fmoc-D-Glu(OBu^t) starting material were from *Bachem AG.* The reagents were from *Fluka AG*, except for trifluoromethanesulfonamide, dimethyl phosphite and Me_3SiBr which were from *Aldrich.* The synthesis was performed on the semi-automatic synthesizer *Labortec SP650* on which deprotection and washings (3 × DMF, 3 × i-PrOH, 3 × CH_2Cl_2, alternatively) were carried out automatically, while addition of the next amino acid and of the coupling reagents occurred manually. Peptide **I** was purified by HPLC on *Waters-Prep-LC-3000* system on a *PrePak*® cartridge (47 × 300 mm) filled with a C_{18}-silica (300 Å, 15 μ) phase. Specific rotations (*c* in g/100 ml): *Perkin-Elmer-241* polarimeter. ^{1}H-NMR Spectra: *Bruker* spectrometer at 200 MHz with $SiMe_4$ as external standard. FAB-MS: *Nermag-R10-10C* apparatus; glycerol/thioglycerol matrix; ionization with Kr-atoms. Amino-acid analysis: the peptide was hydrolyzed in 6N HCl for 20 h at 100° and the hydrolyzate automatically analyzed in a *Varian-LC-90-Star* system, after transformation to Fmoc derivatives and separation on *Aminotag-C_{18}* (5 μm) phase.

Fmoc-Abo-OH. The (3*S*)-2-azabicyclo[2.2.2]octane-3-carboxylic acid (= H-Abo-OH; 3 g, 19.3 mmol) was dissolved in dioxane (15 ml) and 10% aq. Na_2CO_3 soln. (50 ml) and treated dropwise with a soln. of Fmoc-Cl (5.5 g, 21.2 mmol) dioxane (20 ml) under cooling to 0°. Precipitation slowly occurred, while the reaction was allowed to proceed for 4 h at 0°, then for 14 h at r.t. The mixture was taken up in H_2O (200 ml) and washed 3 times with Et_2O. After acidification of the H_2O phase to pH 1 with 6N HCl, the product was extracted into AcOEt (3 × 150 ml). The org. phase was dried and evaporated and the resulting gel reprecipitated from Et_2O/pentane. The white solid (6.2 g, 85%) was gathered by filtration and washed with pentane. R_f ($CHCl_3$/MeOH/AcOH/H_2O 70:25:0.5:4.5) 0.69. HPLC (*Millipore Delta Pak* 5 μm, *C_{18}* 300 Å, 3.9 × 150 mm; 40–100% MeCN/H_2O (0.1% CF_3COOH) gradient within 30 min, flow rate 1 ml/min): t_R 16 min. $[\alpha]_D^{22} = -43.6$ (*c* = 1, MeOH). ^{1}H-NMR (200 MHz, (D_6)DMSO): 1.4–2.0 (*m*, 8 H); 2.15 (*m*, 1 H); 4.05 (*m*, 2 H); 4.15–4.40 (*m*, 3 H); 7.35 (*m*, 4 H); 7.62 (*m*, 2 H); 7.90 (*d*, 2 H). FAB-MS: 378 (*M*H$^+$). Anal. calc. for $C_{23}H_{23}NO_4$ (377.4): C 73.19, H 6.14, N 3.71; found: C 72.93, H 6.19, N 3.95.

H-D-Phe-Pro-Arg-Pro-Gly-Gly-Gly-Gly-Asn-Gly-Asp-Phe-Glu-Abo-Ile-Pro-Glu-Glu-Phe(4′-PO_3H_2)-Leu-D-Glu-OH · 2 CF_3COOH (**I**). The protected henicosapeptide was assembled on the resin starting with Fmoc-D-Glu-(OBu^t)-*p*-alkoxybenzyl-alcohol-resin (3 g, 1.8 mmol) in the semi-automatic synthesizer and using 20% piperidine in dimethylformamide (DMF; 2 × 15 min) for deprotection. Fmoc Splitting and washings were carried out

automatically. The protected amino acids were introduced in the following order: Fmoc-Leu-OH, Fmoc-Phe(4′-PO_3Me_2), Fmoc-Glu(OBu^t)-OH, Fmoc-Glu(OBu^t)-OH, Fmoc-Pro-OH, Fmoc-Ile-OH, Fmoc-Abo-OH, Fmoc-Glu(OBu^t)-OH, Fmoc-Phe-OH, Fmoc-Asp(OBu^t)-OH, Fmoc-Gly-OH, Fmoc-Asn-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-Arg(Pmc)-OH (Pmc = 2,2,5,7,8-pentamethylchromane-6-sulfonyl), Fmoc-Pro-OH, Boc-D-Phe-OH. The following activating agents were used for coupling: BtOH (i-Pr)$_2$EtN/TBTU (BtOH = 1*H*-benzotriazol-1-ol; TBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) for Fmoc-Phe(4′-PO_3Me_2), for Fmoc-Abo-OH and for Fmoc-Glu(OBu^t)-OH (when coupled to Abo) or BtOH/DCC (DCC = *N*,*N*′-dicyclohexylcarbodiimide) in all other cases. Coupling time (under shaking at r.t.) was 2 h, except for the introduction of Fmoc-Abo-OH where it was 14 h. Cleavage from the resin and simultaneous removal of all protecting groups, except the methyl-ester functions on phosphono-L-phenylalanine, was achieved by treatment of the resin-bound peptide (5.8 g) with mixture of CF_3COOH (37.5 ml), ethane-1,2-dithiol (2.5 ml), and anisole (2.5 ml) for 90 min at r.t. Filtration, evaporation, and washing with Et_2O followed. The crude product (1.8 g) was then stirred in CF_3COOH (200 ml), Me_3SiBr (12 ml), and thioanisole (10.8 ml) for 5 h at 0°. The solid residue obtained from the filtrate after evaporation (414 mg) was submitted to prep. reversed-phase HPLC (gradient 20–40% *B* in *A* in 30 min, *A* = 0.1% CF_3COOH/H_2O, *B* = 0.1% CF_3COOH/MeCN): t_R 12 min. Yield 180 mg (4.2%). $[\alpha]_D^{23} = -54.5$ (*c* = 1, AcOH). Amino-acid analysis: Arg 0.90 (1), Asp + Asn 2.03 (2), Abo + Ile 1.98 (2), Glu 4.26 (4), Gly 5.06 (5), Leu 1.04 (1), Phe 2.1 (2), Pro 2.94 (3), Phe(4′-PO_3H_2) n.d. FAB-MS: 2381 (*M*H$^+$, calc. for *M* ($C_{106}H_{150}N_{25}O_{36}P$) 2381.4).

Pharmacological Methods. The biological activity of hirufos (peptide **I**) was assessed *ex vivo* according to *Broersma et al.* [19] after *in vivo* administration to the rat by the intravenous (*i.v.*) route. It was expressed as the factor by which the prothrombin time was increased at the given dose and in function of time. The reference peptides were synthesized in house, and r-hirudin was purchased from *Serva*.

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