Anticoagulant Peptides; Synthesis, Stability and Antithrombin Activity of Hirudin C-Terminal-Related Peptides and Their Disulfated Analog[1)]

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We designed a unique anticoagulant decapeptide, which possesses two *O*-sulfated tyrosine residues, based on the structure of hirudin's C-terminal functional domain. We first prepared a series of octa-, nona- and decapeptides with no sulfation, Suc–Phe–Glu–Pro–Ile–Pro–Glu–Tyr–Tyr–X–OH [X = bond, Leu or Leu–Gln], by a solution phase method and measured their thrombin times (TT) using human thrombin and rabbit plasma. The shortest octapeptide (3a) showed full antithrombin activity comparable to that of the lead compound hirudin (54—65), and the longest decapeptide (3c) prolonged TT most potently with an IC_{50} value of 5.8 μM. We consequently converted 3c to a disulfated decapeptide (NF-22) with SO_3·pyridine complex and compared its antithrombin activity with that of known hirudin-related peptides: hirugen, MDL28050 and hirulog-1. NF-22 showed potent antithrombin activity with an IC_{50} value of 0.3 μM, being more potent than hirugen and MDL28050 (IC_{50} values of 4.0 μM and 1.1 μM, respectively). NF-22 was as potent as hirulog-1. NF-22 showed no change in activity in aqueous solution for 10 d at 60 °C, and remained about 90% unchanged in rat plasma on incubation for 24 h at 37 °C, whereas the corresponding unsulfated peptide (3c) was completely digested under the same condition. NF-22 appears to be one of the most potent and stable peptide anticoagulants among the hirudin analogs.

Key words thrombin inhibitor; NF-22; hirudin; hirugen; MDL28050; hirulog-1

Thrombin inhibitors are considered as promising antithrombotics because of thrombin's pivotal role in the control of thrombosis and hemostasis: fibrinogen cleavage to form the fibrin polymer, activation of the blood coagulation factors, stimulation of platelet aggregation, and so on.[2)] Hirudin is the most potent thrombin inhibitor isolated from the blood-sucking leech *Hirudo medicinalis*. Hirudin contains 65 amino acid residues and the molecule can be divided into two regions based on the biological function: an N-terminal domain held together by three disulfide bridges and a C-terminal functional domain. The former covers thrombin's active site, and the latter interacts with its fibrinogen-specific binding site, termed the "anion-binding exosite."[3)] The C-terminal functional domain has a high content of acidic amino acid residues including a posttranslationally sulfated tyrosine residue at position 63. Hirudin and its analogs, including hirulogs, have proven to be effective *in vivo* models both for arterial and venous thrombosis.[4)] They react directly with thrombin in contrast to heparin, which acts as a cofactor of the plasma proteinase inhibitor antithrombin III.[5)] In addition, the bleeding tendency was lower than with heparin.[6)] Their advantages as anticoagulants superior to heparin has been clarified in clinical trials.[7)] On the other hand, shorter hirudin C-terminal peptides are of interest as thrombin inhibitors which interact with thrombin only at the anion-binding exosite, without blocking thrombin's active site.[8)] These short peptides are expected to be safer and cheaper than hirudin.

In this study, aiming to develop hirudin C-terminal peptides as improved anticoagulants, we have designed an anticoagulant decapeptide possessing two *O*-sulfated tyrosine residues, based on the structure of hirudin's C-terminal functional domain. We have conducted the study by reference to results published by others as well as to our own structure–activity studies (Chart 1). Namely, we recently reported the *O*-sulfation of Tyr-substituted hirudin C-terminal dodecapeptides (54—65) by both enzymic and chemical methods, and demonstrated that the peptide disulfated at positions 62 and 63 (**2**) inhibited thrombin most potently among the compounds tested.[9)] On the other hand, Merrell Dow's group reported that

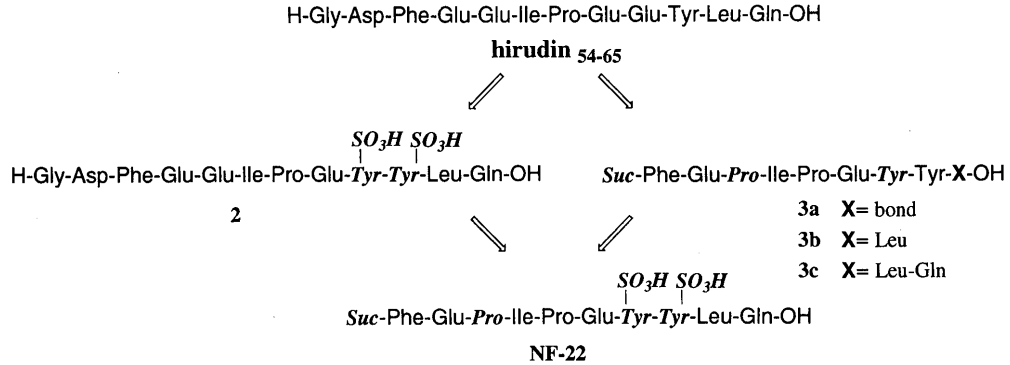

Chart 1. Design of the Disulfated Decapeptide as a Potent Thrombin Inhibitor Based on the Structure of Hirudin's C-Terminal Functional Domain

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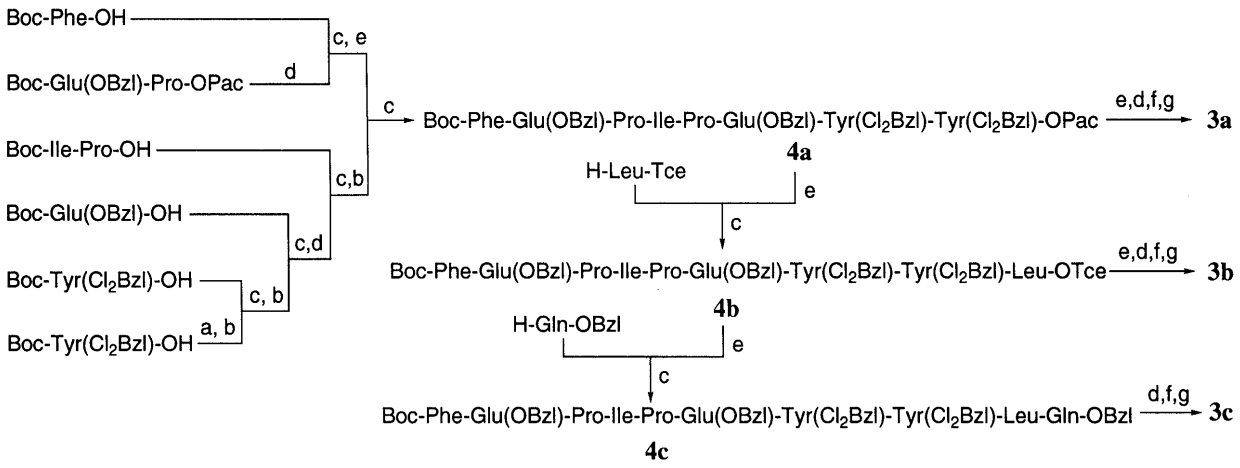

a) Pac-Br, Cs_2CO_3; b) TFA, then HCl/EtOAc; c) WSCI-HOBt; d) HCl/EtOAc; e) Zn/AcOH; f) succinic anhydride, Et_3N/DMF; g) HF/anisole

Chart 2. Scheme for the Synthesis of the Non-sulfated Hirudin C-Terminal-Related Peptides

the potency of hirudin (56—65) analogs increased with succinylation at the N-terminus[10] in addition to Pro substitution in place of Glu_{58}.[11] Based on these observations, we first prepared a series of *N*-succinyl octa-, nona-, and decapeptides (**3a**—**c**) which contained Pro_{58}, Tyr_{62} substituted hirudin's residues 56—63, and then among the peptides synthesized the most potent peptide was disulfated to afford the decapeptide NF-22. We tested these peptides for thrombin time (TT) to evaluate their anticoagulant activities and compared them with hirudin-related peptides known to be potent thrombin inhibitors: hirugen,[12] MDL28050[13] and hirulog-1.[14] In addition, we tested the stability of NF-22 both in an aqueous solution and a biological medium.

Chemistry

In the present study we chose the solution-phase method for synthesis of the novel peptides (**3a**—**c**). The synthetic routes are shown in Chart 2. Since the peptides designed here share a common sequence at the N-terminal octapeptidyl moiety, we first prepared an orthogonally protected octapeptide, Boc–Phe–Glu(OBzl)–Pro–Ile–Pro–Glu(OBzl)–Tyr(Cl_2Bzl)–Tyr(Cl_2Bzl)–OPac (**4a**), and then elongated the peptide chain toward the C-terminus if necessary. For the preparation of **4a**, three segments, Boc–Phe–Glu(OBzl)–Pro–OH, Boc–Ile–Pro–OH[15] and Boc–Glu(OBzl)–Tyr(Cl_2Bzl)–Tyr(Cl_2Bzl)–OPac, were obtained by stepwise elongation in 50, 76 and 61% yields, respectively, using a carbodiimide (DCC or WSCI·HCl) in the presence of HOBt as a coupling reagent. They were then coupled from the C-terminal segment by the WSCI/HOBt method to afford **4a** in 69% yield, and this product was treated with zinc powder in 90% AcOH to remove the carboxy-terminal Pac group. The partially deprotected octapeptide thus obtained was *N*-succinylated by treatment with 4 N HCl/EtOAc, followed by acylation with succinyl anhydride, and then treated with HF in the presence of anisole (9 : 1) at 0 °C for 1 h to obtain a crude non-sulfated octapeptide (**3a**), which was purified by gel filtration on a Sephadex LH-20 column. The nonapeptide (**3b**) was prepared by condensation of **4a** with Leu–OTce

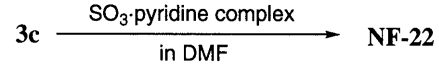

Chart 3. Preparation of Disulfated Hirudin C-Terminal-Related Decapeptide (NF-**22**)

to afford the protected peptide (**4b**). In this case, Tce ester was used instead of Pac ester to avoid racemization[16] of the amine component. On the other hand, we selected Pac ester for the coupling procedure to prepare the dipeptide, Boc–Tyr(Cl_2Bzl)–Tyr(Cl_2Bzl)–OPac, in spite of the occurrence of partial racemization at the C-terminal tyrosine residue, because most oligopeptide Pac-esters are easily purified by recrystallization to afford a diastereomerically pure product in fairly good yield, whereas the longer peptides are harder to purify by crystallization. The protected nonapeptide thus obtained was converted to the corresponding *N*-succinylated peptide (**3b**) according to the procedure described for the preparation of **3a**. The decapeptide (**3c**) was also prepared by condensation of **4b** with H–Gln–OBzl·HCl to afford **4c**, which was *N*-succinylated, followed by HF treatment. The overall yields of **3a**—**c** and NF-22 were 29, 22, 16 and 4.9% from Boc–Tyr(Cl_2Bzl)–OPac, respectively.

The known peptides hirudin (54—56),[11] hirugen, MDL28050 and hirulog-1, were synthesized by solid-phase methods using N^{α}-Fmoc chemistry.

Sulfation of peptides was performed with SO_3·pyridine complex according to the modified procedure described by Fujii *et al.*[17] *O*-Sulfation of tyrosine was initially performed in DMF–pyridine mixture, but extensive racemization (>20%) of the C-terminal amino acid residue was observed. After exploring various reaction conditions, the problem was almost completely overcome by using a pyridine-free solvent system (Chart 3).[18] A small amount of trisulfate, being additionally sulfated at the carboxamide in the C-terminal Gln, was observed. However, it was so hydrophilic as to be easily separated from the disulfated product by RP-HPLC. The crude sulfated products were purified by salt formation with ammonia, sodium or calcium, followed by preparative

reverse-phase column chromatography under atmospheric pressure, eluted with aqueous MeOH, and they were isolated as powders after lyophilization. NF-22 was obtained in over 98% purity in 30% yield. The structures of the final products were confirmed by amino acid analysis after acidic hydrolysis with 6 N HCl (110 °C, 24 h). The sulfated peptide (NF-22) was also analyzed by enzymic digestion with V-8 protease and subsequently with aminopeptidase M for the determination of tyrosine *O*-sulfate.

Stability of NF-22 Tyrosine *O*-sulfate is a chemically labile residue that readily eliminates sulfonic acid.[19] We examined the stability of NF-22 under solution conditions. The solutions after incubation were analyzed by HPLC, and the concentrations of unchanged NF-22 and decomposed products were determined from the UV absorption at 230 nm.

In aqueous media, NF-22 is stable under weakly basic conditions (pH *ca.* 9). When a neutral solution of NF-22 in distilled water was heated at 60 °C for 10 d, no significant change was observed in the HPLC chromatogram. However, in 0.2 N HCl solution at room temperature, NF-22 gradually decomposed to afford a non-sulfate form (**3c**), although about 30% of the disulfate still remained after a 24-h incubation (data not shown). We then examined its resistance to proteolytic degradation in a biological medium. NF-22 was sufficiently stable in plasma. When NF-22 and **3c** were separately incubated in rat plasma for 24 h at 37 °C, over 80% of the former remained unchanged, while the latter was largely digested within 8 h and completely disappeared after a 24-h incubation (Fig. 1).

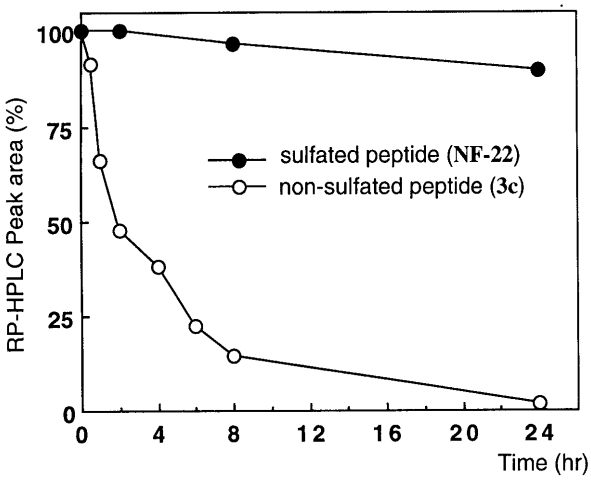

Fig. 1. Time Courses of the Residual Concentration of NF-22 (●) and **3c** (○) during Incubation in Rat Plasma

Biological Results

Anticoagulant activities were determined by measuring TT using human thrombin and rabbit plasma. IC_{50} were determined as the concentration (μM) of the inhibitor that doubled TT relative to a control containing no inhibitor (Table 1).

Native hirudin's C-terminal decapeptide (56—65) has been reported as the minimal structural unit for inhibitory action.[20] In this study, even the shortest octapeptide showed a strong antithrombin activity, comparable to that of the lead compound, hirudin (54—65). Addition of Leu at position 64 afforded the nonapeptide (**3b**), which showed no enhancement of activity relative to **3a**. The decapeptide (**3c**) showed the most potent activity, three times that of hirudin (54—56) or the octapeptide (**3a**).

Among the compounds synthesized herein, the decapeptide (**3c**) was selected, and sulfated at two tyrosine hydroxyl groups with SO_3·pyridine complex. The disulfated decapeptide (NF-22) showed a potent antithrombin activity with an IC_{50} value of 0.3 μM. NF-22 was found to be about 20-fold more effective than the corresponding non-sulfated peptide. We also measured the TT of known hirudin C-terminal-related peptides: hirugen, MDL28050 and hirulog-1, which showed IC_{50} values of 4.0, 1.1 and 0.2 μM, respectively.

Discussion

Here, we have described the synthesis, stabilities and antithrombin activities of anticoagulant peptides based on the structure of hirudin's C-terminal functional domain. We first investigated the minimal peptide length which possesses sufficient potency as a low-molecular-weight thrombin inhibitor. We prepared a series of *N*-succinyl octa-, nona-, and decapeptides which contain Pro_{58} and Tyr_{62}-substituted hirudin's residues 56—63. We evaluated their TT as a measure of their anticoagulant activities. Even the octapeptide exhibited some anticoagulant activity. Its potency was comparable to that of the lead peptide, hirudin (54—56). It is known that deletion of amino acid residues at positions 64 and/or 65,[13] as well

Table 1. Fibrin Clot Inhibition (Thrombin Time) of the Hirudin C-Terminal-Related Peptides

Compound	Structure (56 … 63)	IC_{50} (μM)
Hirudin (54—65)	H–Gly–Asp–Phe–Glu–Glu–Ile–Pro–Glu–Glu–Tyr–Leu–Gln–OH	18.8
3a	Suc–Phe–Glu–Pro–Ile–Pro–Glu–Tyr–Tyr–OH	17.3
3b	Suc–Phe–Glu–Pro–Ile–Pro–Glu–Tyr–Tyr–Leu–OH	14.0
3c	Suc–Phe–Glu–Pro–Ile–Pro–Glu–Tyr–Tyr–Leu–Gln–OH	5.8
NF-22	Suc–Phe–Glu–Pro–Ile–Pro–Glu–Tyr(SO_3H)–Tyr(SO_3H)–Leu–Gln–OH	0.3
Hirugen	Ac–Asn–Gly–Asp–Phe–Glu–Glu–Ile–Pro–Glu–Glu–Tyr(SO_3H)–Leu–OH	4.0
MDL28050	Suc–Tyr–Glu–Pro–Ile–Pro–Glu–Glu–Ala–Cha–D-Glu–OH	1.1
Hirulog-1	H–D-Phe–Pro–Arg–$(Gly)_4$–Asn–Gly–Asp–Phe–Glu–Glu–Ile–Pro–Glu–Glu–Tyr–Leu–OH	0.2

Table 2. Analytical Data of Synthetic Peptides

Compound	Method[a]	Phe	Glx[b]	Pro	Ile	Tyr	Leu	Tyr (SO_3H)	Gln	FAB-MS[c]
3a	A	1.00 (1)	2.08 (2)	2.06 (2)	0.96 (1)	1.92 (2)				1158
3b	A	1.00 (1)	2.10 (2)	2.08 (2)	0.95 (1)	2.06 (2)	1.03 (1)			1271
3c	A	1.00 (1)	3.09 (3)	1.99 (2)	0.95 (1)	1.96 (2)	0.98 (1)			1399
NF-22	A	1.00 (1)	3.13 (3)	1.91 (2)	0.98 (1)	2.05 (2)	0.95 (1)			—[d]
NF-22	B						1.00 (1)	1.95 (2)	1.06 (1)	

a) Method for hydrolysis: A, 6 M HCl containing 1% phenol at 110 °C for 24 h; B, treatment with V8 protease followed by digestion with aminopeptidase M dissolved in 0.1 M sodium phosphate buffer (pH 7.0) at 37 °C for 24 h. *b*) Glu and Gln. *c*) $[M+H]^+$; results were consistent with the calculated mass numbers. *d*) No significant peak was observed.

as removal of the acidic functionality at position 62,[20] causes a decrease in anticoagulant activity. That **3a** retains the activity despite such disadvantages of structural modification would reflect the positive contribution of *N*-succinylation and substitution of Glu with Pro at position 58. For residue 64, a hydrophobic side chain is favorable; it reaches into the same hydrophobic pocket of thrombin as Ile_{59}.[21] The nonapeptide (**3b**), with added Leu at this position, did not show any enhancement of the activity. Appropriate dipeptide units at both positions 64 and 65 seem to be required for the increment of the activity.

We next sulfated the decapeptide (**3c**), which was the most potent among the peptides synthesized, to afford a disulfated peptide (NF-22) and we compared its TT with those of known hirudin C-terminal-related peptides: hirugen, MDL28050 and hirulog-1. NF-22 showed a potent antithrombin activity with an IC_{50} value of 0.3 μM, being more potent than hirugen and MDL28050. Tyr_{63} is sulfated in native hirudin, of which the dissociation constant to thrombin is 10-fold smaller than that of desulfated hirudin.[22] Hirudin's C-terminal peptides also usually exhibit an increase of their anticoagulant activity by over 10-fold when the tyrosine residue at the position corresponding to native hirudin's Tyr_{63} is *O*-sulfated.[23] In our case, NF-22 prolonged TT 20-fold more potently than its precursor (**3c**). In contrast, hirugen showed only a 5-fold stronger activity than hirudin (56—65). This result suggests the importance of the role of residue 65 for the activity, as discussed above in connection with the comparison of the activities of **3b** and **3c**. MDL28050 is a relatively hydrophobic peptide, compared to native hirudin. It lacks a negatively charged residue at position 63. MDL28050 is a highly optimized hirudin analog selected from numerous candidates,[13] so such hydrophobic analogs seem to have a limitation on their activity and the negative charge at position 63 seems to be essential for the display of full activity as a functional domain in hirudin analogs. Hirulog-1 is a bifunctional icosapeptide which interacts with thrombin's active site and anion-binding exosite and thereby strongly inhibits thrombin. In this study, NF-22 was found to inhibit thrombin almost as potently as hirulog-1. These findings implied that adequate optimization of the functional domain of hirudin will enable even a small peptide to exert anticoagulant activity comparable to that of larger bifunctional inhibitors.

Much effort has been made to replace *O*-sulfated tyrosine in hirudin peptides with *p*-substituted phenylalanine derivatives which have a stable acidic function, such as carboxylic acid, sulfonic acid, methanesulfonic acid,[24] aminosulfonic acid,[23b] acetic acid, phosphoric acid,[19] *etc.* They might indeed be favorable for retaining the inhibitory potency. However, such modifications would not be convenient in large-scale synthesis. In contrast, NF-22 was easily prepared and surprisingly stable in both aqueous and biological media. Using our procedure, unsulfated peptide was prepared on a fairly large scale by a classical solution-phase method, and the sulfation was performable on a 10-gram scale even in a 300-ml flask. Therefore, this method should be usable on a larger scale, even for commercial production.

In conclusion, we have developed a unique disulfated hirudin C-terminal-related peptide (NF-22) endowed with potent antithrombin activity. NF-22 has some advantages over other known hirudin-related peptides, such as physicochemical and biological stabilities, as well as the simple and efficient synthetic procedure. NF-22 is a candidate for development as a new anticoagulant, and further pharmacological investigations *in vivo* in animal models are in progress.

Experimental

N^{α}-Boc–amino acids and N^{α}-Fmoc–amino acids were purchased from Peptide Institute, Inc., Osaka, Japan and Watanabe Chemical Industries, Ltd., Hiroshima, Japan. SO_3·pyridine complex was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI, U.S.A. Human α-thrombin and V8 protease were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Aminopeptidase M was from Pierce, Rockford, IL, U.S.A.

Melting points were determined on a Yanagimoto melting point apparatus without correction. Column chromatography was performed on silica gel BW-200 and on ODS DM-1020T (Fuji Silisia Chemical Ltd., Aichi, Japan). The purity of protected peptides was monitored by analytical TLC on Merck Silica gel 60 F_{254} plates. Purity of the final products was monitored by RP-HPLC performed on a JASCO 800 system with a UV detector simultaneously monitored at 230 and 280 nm, utilizing a μ-Bondapak C18 column (3.9 × 150 mm). Samples were analyzed at 40 °C with gradient elution using a binary solvent system 0.1% TFA in water/0.1% TFA in acetonitrile at 1 ml/min. FAB-mass spectra were obtained using a JEOL JMS-DX303 spectrometer. Optical rotations were measured with a JASCO DIP-140 apparatus.

Amino acid compositions were determined according to the *o*-phthalaldehyde method by ion-exchange chromatography employing a Shimadzu LC-10A amino acid analyzer. Amino acid hydrolysates were prepared by treatment of samples in 6 M HCl (200 μl) containing 1% phenol at 110 °C for 24 h. The sulfated peptides were also treated with V8 protease followed by digestion with aminopeptidase M dissolved in 0.1 M sodium phosphate buffer, pH 7.0, at 37 °C for 24 h.

Hirudin (54—65), hirugen, MDL-28050 and hirulog-1 were synthesized by employing an Applied Biosystems (ABI) 430A peptide synthesizer using Fmoc chemistry. Fmoc-amino acid-*p*-alkoxybenzyl alcohol resin (0.25 mmol) was used as the starting resin for solid-phase synthesis. The synthesis was performed by single HOBt active ester couplings according

to the standard ABI-supplied protocol. After the coupling of the last amino acid, the growing peptide on the resin was acylated by using a mixture of the corresponding acid anhydride and DIEA. Subsequently, the peptide was cleaved from the resin with the concomitant removal of all protecting groups by treatment with TFA at room temperature for 1.5 h in the presence of 5% water and 5% anisole. The TFA was evaporated *in vacuo*, and the residue was triturated with ether. The crude peptide was removed from the resin by dissolution in aqueous acetic acid and filtration. The peptide was then purified by reverse-phase HPLC (>98% purity) on a Vydac C_{18} cartridge column (47 × 300 mm) using a linear gradient of 0—50% acetonitrile over 50 min at a flow rate of 80 ml/min on 0.1% TFA. Absorbance was monitored at 215 nm. Sulfation of Tyr_{63} in hirugen was carried out using SO_3·pyridine complex, which was applied to the synthesis of NF-22 as described below.

Boc-Tyr(Cl_2Bzl)-OPac (5) According to the procedure described by Wang *et al.*,[25] Boc-Tyr(Cl_2Bzl)-OH (41.8 g, 95.0 mmol) was dissolved in MeOH (270 ml), and this solution was treated with aqueous 1 N Cs_2CO_3 (95 ml) to maintain the pH at 7 (pH paper). The mixture was evaporated to dryness and the residue was reevaporated twice from 200 ml of DMF at below 40 °C. Pac-Br (18.9 g, 95.0 mmol) was added to the solution of cesium salt thus obtained in DMF (280 ml) at room temperature and the whole was stirred for 2 h. The precipitated cesium bromide was filtered off, and the filtrate was concentrated *in vacuo*. The residue was dissolved in EtOAc and this solution was washed with H_2O, saturated aqueous 10% Na_2CO_3 and brine. The solution was dried with $MgSO_4$, and concentrated *in vacuo*. The crude product was recrystallized from EtOAc–hexane to give the Pac ester **5** (50.2 g, 95%) as a white powder: mp 102—104 °C, $[\alpha]_D^{23}$ −21.0° (c=1.00, DMF). *Anal.* Calcd for $C_{29}H_{29}Cl_2NO_6$: C, 62.37; H, 5.23; N, 2.51. Found: C, 62.31; H, 5.31; N, 2.46.

Boc-Tyr(Cl_2Bzl)-Tyr(Cl_2Bzl)-OPac (6) A solution of **5** (50.1 g 89.7 mmol) in TFA was prepared at −20 °C, and stirred for 40 min at 4 °C, then concentrated *in vacuo*. The residue was taken up in 4 N HCl/EtOAc (50 ml) and treated with ether. The resulting precipitate was collected by filtration, and dried in a vacuum desiccator with NaOH. This amine component, Boc-Tyr(Cl_2Bzl)-OH (37.0 g, 84.0 mmol) and HOBt·H_2O (13.5 g, 88.2 mmol) were dissolved in DMF. Et_3N (12.3 ml, 88.2 mmol) and WSCI·HCl (17.7 g, 92.4 mmol) were added to the solution at −10 °C and the reaction mixture was stirred overnight at 5 °C. The resulting mixture was evaporated *in vacuo* at 30—40 °C. The product was extracted with EtOAc (600 ml) and washed with 1 N HCl, brine, saturated aqueous $NaHCO_3$ and finally brine, then dried over $MgSO_4$. The solution was evaporated *in vacuo*, and the crude product was recrystallized from CH_2Cl_2–hexane to afford **6** (51.4 g, 69%) as a white powder: mp 165—166 °C, $[\alpha]_D^{23}$ −7.6° (c=1.00, DMF). *Anal.* Calcd for $C_{45}H_{42}Cl_4N_2O_8$: C, 61.37; H, 4.81; N, 3.18. Found: C, 61.46; H, 4.59; N, 3.01.

Boc-Glu(OBzl)-Tyr(Cl_2Bzl)-Tyr(Cl_2Bzl)-OPac (7) This compound was prepared from Boc-Glu(OBzl)-OH (15.7 g, 63.5 mmol), Boc-Tyr(Cl_2Bzl)-Tyr(Cl_2Bzl)-OPac (48.7 g, 55.3 mmol), Et_3N (7.35 ml, 52.9 mmol), HOBt·H_2O (10.2 g, 66.7 mmol) and WSCI·HCl (11.7 g, 60.1 mmol) by the WSCI/HOBt method in DMF according to the procedure described for the preparation of **6**. It was obtained as a white powder (54.1 g, 93%) after purification by silica gel column chromatography using $CHCl_3$–MeOH (100 : 1) as the eluting solvent followed by recrystallization from $CHCl_3$–Et_2O: mp 155—157 °C, $[\alpha]_D^{23}$ −6.5° (c=1.0, $CHCl_3$). *Anal.* Calcd for $C_{57}H_{55}Cl_4N_3O_{11}$: C, 62.25; H, 5.04; N, 3.82. Found: C, 62.10; H, 4.98; N, 3.65.

Boc-Ile-Pro-Glu(OBzl)-Tyr(Cl_2Bzl)-Tyr(Cl_2Bzl)-OPac (8) A solution of **7** (11.0 g, 10.0 mmol) in 4 N HCl/EtOAc (100 ml) was prepared at 4 °C, and stirred for 35 min at 4 °C, then EtOAc (100 ml) was added. The resulting precipitate was collected by filtration, and dried in a vacuum desiccator over NaOH. This amine component, without further purification, was dissolved in DMF and neutralized with Et_3N (1.40 ml, 10.0 mmol) at −5 °C. Boc-Ile-Pro-OH (3.28 g, 10.0 mmol) and HOBt (1.35 g, 10.0 mmol) were dissolved in the solution, then WSCI·HCl (2.10 g, 11.0 mmol) was added at −10 °C and the reaction mixture was stirred overnight at 5 °C. The resulting mixture was evaporated *in vacuo* at 30—40 °C. The product was solidified from EtOAc, washed with 1 N HCl, saturated aqueous $NaHCO_3$ and H_2O on a glass filter, and precipitated from DMF–Et_2O to afford **8** (10.2 g, 76%) as a white powder: mp 131—133 °C, $[\alpha]_D^{23}$ −28.9° (c=1.00, DMF). *Anal.* Calcd for $C_{68}H_{73}Cl_4N_5O_{13}$: C, 62.25; H, 5.04; N, 3.82. Found: C, 62.28; H, 5.26; N, 3.61.

Boc-Phe-Glu(OBzl)-Pro-OH (9) Boc-Glu(OBzl)-Pro-OPac[15] (120 g, 217 mmol) was dissolved in 3 N HCl/EtOAc (200 ml) at 4 °C. The mixture was stirred for 45 min at the same temperature, and then ether (1 l) was added. The resulting precipitate was collected by filtration, and dried in a vacuum desiccator over NaOH. This amine component was dissolved in 1-methyl-2-pyrrolidinone (100 ml) and diluted with DMF (300 ml) at room temperature, and then Et_3N (30.4 ml, 217 mmol) was added dropwise at −5 °C. Boc-Phe-OH (52.3 g, 197 mmol) and HOBt·H_2O (33.4 g, 217 mmol) were dissolved in the above solution, and then DCC (48.8 g, 236 mmol) in DMF (200 ml) was added dropwise at −10 °C. The reaction mixture was stirred for 1 h at the same temperature, then overnight at 5 °C. The resulting mixture was filtered to remove precipitated dicyclohexyl urea and evaporated *in vacuo* at 30—40 °C. The product was dissolved in EtOAc (1 l) and the solution was allowed to stand overnight. After filtration, the filtrate was washed with 1 N HCl, brine, saturated aqueous $NaHCO_3$ and finally brine, then dried over $MgSO_4$. The solution was evaporated *in vacuo*, and the crude product was purified by silica gel column chromatography using $CHCl_3$–MeOH (100 : 1) as the eluting solvent to afford Boc-Phe-Glu(OBzl)-Pro-OPac (124 g, 81%) as a white gum. The tripeptidyl Pac ester thus obtained (26.0 g, 37.1 mmol) was dissolved in AcOH (100 ml). To this solution, Zn powder (48.5 g, 742 mmol) was added, and the whole was vigorously stirred for 30 min at room temperature. Zinc was filtered off and the filtrate was concentrated *in vacuo*. Et_2O was added to the product, and the insoluble materials were filtered off. The filtrate was concentrated *in vacuo* and the residue was recrystallized from acetone–hexane to afford 13.7 g (63%) of **9** as a white powder: mp 148—150 °C, $[\alpha]_D^{23}$ −42.0° (c=1.00, DMF). *Anal.* Calcd for $C_{31}H_{39}N_3O_8$: C, 64.01; H, 6.76; N, 7.22. Found: C, 63.85; H, 6.99; N, 6.98.

Boc-Phe-Glu(OBzl)-Pro-Ile-Pro-Glu(OBzl)-Tyr(Cl_2Bzl)-Tyr(Cl_2Bzl)-OPac (4a) A solution of **8** (113 g, 86.0 mmol) in TFA (500 ml) was prepared at 4 °C, stirred for 30 min at the same temperature and concentrated *in vacuo*. The residue was taken up in 4 N HCl/EtOAc (100 ml), and Et_2O (1 l) was added. The resulting precipitate was collected by filtration, and dried in a vacuum desiccator over NaOH. This amine component, the carboxy component **9** (48.0 g, 83.0 mmol) and HOBt·H_2O (33.4 g, 217 mmol) were dissolved in DMF, and then Et_3N (12.8 ml, 91.3 mmol) and WSCI·HCl (19.1 g, 99.6 mmol) were added at −10 °C. The reaction mixture was stirred for 1 h at the same temperature, then overnight at 5 °C. The resulting mixture was poured slowly into ice-cold 10% aqueous citric acid solution (2 l). The solidified product was washed with saturated aqueous $NaHCO_3$ and H_2O on a glass filter, then dried over P_2O_5 to afford **4a** (134 g, 91%) as a white powder: mp 125—127 °C, $[\alpha]_D^{23}$ −41.5° (c=1.00, DMF). *Anal.* Calcd for $C_{94}H_{102}Cl_4N_8O_{18}$: C, 63.65; H, 5.80; N, 6.32. Found: C, 63.72; H, 5.82; N, 6.21.

Boc-Phe-Glu(OBzl)-Pro-Ile-Pro-Glu(OBzl)-Tyr(Cl_2Bzl)-Tyr(Cl_2Bzl)-OH (10) **4a** (2.50 g, 1.34 mmol) was dissolved in 90% AcOH (8 ml), then Zn powder (2.63 g, 40 mmol) was added, and the whole was vigorously stirred for 2 h at room temperature. Zinc was filtered off and the filtrate was poured into ice-cold 0.1 N HCl (100 ml). The solidified material was washed with H_2O and Et_2O. The crude product was reprecipitated from $CHCl_3$–Et_2O to afford 2.08 g (89%) of **10** as a white powder: mp 109—112 °C, $[\alpha]_D^{23}$ −39.5° (c=1.00, DMF). *Anal.* Calcd for $C_{86}H_{96}Cl_4N_8O_{17}$: C, 62.39; H, 5.84; N, 6.77. Found: C, 62.41; H, 5.76; N, 6.56.

Suc-Phe-Glu-Pro-Ile-Pro-Glu-Tyr-Tyr-OH (3a) A solution of **10** (1.66 g, 1.00 mmol) in 4 N HCl/EtOAc (20 ml) was prepared at 4 °C and stirred for 1 h at the same temperature, then Et_2O (100 ml) was added. The resulting precipitate was collected by filtration, and dried in a vacuum desiccator over NaOH. Succinyl anhydride (110 mg, 1.10 mmol) was added to a mixture of the amino component hydrochloride thus obtained and NMM (0.22 ml, 2 mmol) in DMF at 4 °C. The reaction mixture was stirred at the same temperature overnight, then poured into ice-cold 10% citric acid (100 ml). The resulting precipitate was collected by filtration, then washed on a glass filter with H_2O, and dried in a vacuum desiccator over P_2O_5. This succinylated peptide was dissolved in anhydrous HF (10 ml) and anisole (1 ml) at 0 °C. After 30 min, HF was evaporated *in vacuo* at below 5 °C. The residue was washed with dry Et_2O (2 × 30 ml) by successive centrifugation and decantation. The crude product was passed through a column of anion-exchange resin (Diaion PA-308, AcO^- form) in aqueous AcOH and then purified by Sephadex LH-20 column chromatography with 50% aqueous MeOH. The product was lyophilized to give a white powder (12.3 g, 80%): mp 165—170 °C, $[\alpha]_D^{23}$

$-50.6°$ ($c=1.00$, DMF). *Anal.* Calcd for $C_{57}H_{72}N_8O_{18}\cdot 3H_2O$: C, 56.52; H, 6.49; N, 9.25. Found: C, 56.58; H, 6.22; N, 9.18. The results of amino acid analysis and FAB-mass spectroscopy are given in Table 2.

Boc-Phe-Glu(OBzl)-Pro-Ile-Pro-Glu(OBzl)-Tyr(Cl_2Bzl)-Tyr(Cl_2Bzl)-Leu-OTce (4b) Compound **10** (14.0 g, 8.45 mmol), H-Leu-OTce·*p*-TsOH[26] (5.50 g, 12.7 mmol) and HOBt·H_2O (1.43 g, 9.30 mmol) were dissolved in DMF, and then Et_3N (1.2 ml, 8.5 mmol) and WSCI·HCl (1.94 g, 10.1 mmol) were added at -8 °C. The mixture was stirred for 1 h at the same temperature, then overnight at 5 °C. The resulting mixture was poured slowly into ice-cold 10% aqueous citric acid solution (500 ml). The precipitated product was washed with saturated aqueous $NaHCO_3$ and H_2O on a glass filter, then dried over P_2O_5 to afford **4b** (16.1 g, 100%) as a white powder: mp 124—129 °C, $[\alpha]_D^{23}$ $-38.0°$ ($c=1.00$, DMF). *Anal.* Calcd for $C_{94}H_{108}Cl_7N_9O_{18}$: C, 59.42; H, 5.73; N, 6.63. Found: C, 59.56; H, 5.80; N, 6.51.

Boc-Phe-Glu(OBzl)-Pro-Ile-Pro-Glu(OBzl)-Tyr(Cl_2Bzl)-Tyr(Cl_2Bzl)-Leu-OH (11) Prepared from **4b** (14.0 g, 7.37 mmol) and Zn powder (9.6 g, 147 mmol) in 90% AcOH (200 ml) according to the procedure described for the preparation of **10**. White powder (12.8 g, 98%): mp 150—155 °C, $[\alpha]_D^{23}$ $-38.1°$ ($c=1.00$, DMF). *Anal.* Calcd for $C_{92}H_{107}Cl_4N_9O_{18}$: C, 62.48; H, 6.10; N, 7.13. Found: C, 62.59; H, 6.01; N, 7.05.

Suc-Phe-Glu-Pro-Ile-Pro-Glu-Tyr-Tyr-Leu-OH (3b) According to the procedure described for the preparation of **3a**, **11** (800 mg, 0.45 mmol) was succinylated with succinyl anhydride (48 mg, 0.48 mmol) and NMM (53 ml, 0.48 mmol) in DMF (5 ml) to afford 730 mg (94%) of the succinylated product. This product (400 mg) was deprotected by HF treatment to give a white powder (186 mg, 66%): mp 157—162 °C, $[\alpha]_D^{23}$ $-53.8°$ ($c=1.00$, DMF). *Anal.* Calcd for $C_{63}H_{83}N_9O_{19}\cdot 3H_2O$: C, 57.13; H, 6.77; N, 9.52. Found: C, 57.38; H, 6.85; N, 9.68. The results of amino acid analysis and FAB-mass spectroscopy are given in Table 2.

Boc-Phe-Glu(OBzl)-Pro-Ile-Pro-Glu(OBzl)-Tyr(Cl_2Bzl)-Tyr(Cl_2Bzl)-Leu-Gln-OBzl (4c) Prepared from **11** (1.00 g, 0.57 mmol), H-Gln-OBzl·HCl (295 mg, 0.57 mmol) [prepared from Boc-Gln-OBzl[25] by 3 N HCl/EtOAc treatment], Et_3N (119 ml, 0.85 mmol), HOBt·H_2O (131 mg, 0.85 mmol) and WSCI·HCl (130 mg, 0.68 mmol) by the WSCI/HOBt method in DMF according to the procedure described for the preparation of **4b**. White powder (1.08 g, 96%): mp 133—136 °C, $[\alpha]_D^{23}$ $-35.5°$ ($c=1.00$, DMF). *Anal.* Calcd for $C_{104}H_{121}Cl_4N_{11}O_{20}$: C, 62.87; H, 6.14; N, 7.75. Found: C, 62.83; H, 6.09; N, 7.68.

Suc-Phe-Glu-Pro-Ile-Pro-Glu-Tyr-Tyr-Leu-Gln-OH (3c) According to the procedure described for the preparation of **3a**, **4c** (18.5 g, 9.31 mmol) was succinylated with succinyl anhydride (1.00 g, 10.0 mmol) and Et_3N (1.4 ml, 10 mmol) in DMF (36 ml). The product was chromatographed on a silica gel column with $CHCl_3$–MeOH (50 : 1) to afford 12.9 g (71%) of the succinylated product, and then 2.00 g of the product was deprotected by HF treatment using 18 ml of anhydrous HF and 2 ml of anisole. The crude product was precipitated from MeOH–Et_2O and chromatographed on a Diaion HP-20 column with aqueous MeOH. The purified product was lyophilized to give a white powder (0.94 g, 67%): mp 164—166 °C, $[\alpha]_D^{23}$ $-47.0°$ ($c=1.00$, DMF). *Anal.* Calcd for $C_{68}H_{91}N_{11}O_{21}\cdot 4H_2O$: C, 55.54; H, 6.79; N, 10.48. Found: C, 55.28; H, 6.17; N, 10.33. The results of amino acid analysis and FAB-mass spectroscopy are given in Table 2.

Suc-Phe-Glu-Pro-Ile-Pro-Glu-Tyr(SO_3H)-Tyr(SO_3H)-Leu-Gln-OH Calcium Salt (NF-22) SO_3·pyridine complex (3.12 g, 20 mmol) was added to a solution of **3c** (2.80 g, 2.00 mmol) in DMF (20 ml) and the mixture was stirred for 1 h at room temperature. Diisopropyl ether (100 ml) was added to the reaction mixture. The resulting oily insoluble material was separated by decantation and washed with diisopropyl ether (100 ml), and the solvent was removed again by decantation. $Ca(OH)_2$ (2.5 g, 32 mmol) suspended in ice-cold water (50 ml) was added to the residue, and the mixture was stirred for 1 h at 4 °C. Insoluble materials were removed by filtration. The filtrate was concentrated *in vacuo*. The crude product was purified by ODS column chromatography with MeOH–0.1 N AcOH (0 : 100—1 : 9) as the eluting solvent and then lyophilized to give a white powder (1.02 g, 30%): mp >250 °C, $[\alpha]_D^{23}$ $-82.6°$ ($c=0.50$, H_2O). The results of amino acid analysis are given in Table 2.

Measurement of Thrombin Time (TT) TT was measured according to the modified procedure of Maraganore *et al.*[23a] Rabbit plasma was separated from the blood collected from the ear artery of male Japanese white rabbits weighing about 3.0 kg with a plastic syringe containing 1/10 volume of sodium citrate (3.8%). A mixture of the plasma thus obtained (90 μl) and 10 μl of a peptide solution or 33% ethanol (control) was added to 200 μl of the thrombin solution (2.5 U/ml), and TT was measured in an Amelung-Coagulometer (KC4, Baxter). TT of the control plasma was 17.8 ± 0.3 s (mean $\pm$S.E., $n=3$).

Determination of Peptides in Rat Plasma A peptide (NF-22 or **3c**) was dissolved at a concentration of 0.1 mg/ml in rat plasma, and the solution was incubated at 37 °C. A 100 μl aliquot of the mixture was taken at 0 (control), 0.5, 1, 2, 4, 6, 8 and 24 h after the addition, and mixed with 100 μl of 20% trichloroacetic acid. After separation of precipitated plasma proteins by centrifugation, 50 μl of the soluble fraction thus obtained was analyzed by HPLC on a μ-Bondapak C_{18} (3.9×300 mm) using a linear gradient of 10—40% acetonitrile in 0.1% TFA over 30 min at a flow rate of 1.5 ml/min. Absorbance was monitored at 230 nm. NF-22 and **3c** were detected at 20.1 and 27.2 min, respectively, after the injection. The concentration of remaining peptide was expressed as percent of the concentration at 0 h.

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References and Notes

1) All amino acids are the L enantiomer unless otherwise noted. Standard abbreviations for amino acids, protecting groups and peptides are used [*Eur. J. Biochem.*, **138**, 9—37 (1984)]. Other abbreviations include: Boc = *tert*-butyloxycarbonyl, Cha = cyclohexylalanine, Cl_2Bzl = 2,6-dichlorobenzyl, DCC = dicyclohexylcarbodiimide, DIEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, Fmoc = 9-fluorenylmethoxycarobnyl, HOBt = 1-hydroxybenzotriazole, NMM = *N*-methylmorpholine, ODS = octadecylated silica gel, Pac = phenacyl, RP-HPLC = reverse-phase high-performance liquid chromatography, suc = succinyl, Tce = 2,2,2-trichloroethyl, TFA = trifluoroacetic acid, WSCI = water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride]. Numbering of hirudin analogs is based on that for hirudin variant-1: Bagdy D., Barabas E., Graf L., Petersen T. E., Magnussen S., *Methods Enzymol.*, **45**, 669—678 (1976).
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