Articles

Conformationally Restricted Thrombin Inhibitors Resistant to Proteolytic Digestion†

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ABSTRACT: A new type of thrombin exo-site inhibitor has been designed with enhanced inhibitory potency and increased metabolic stability. With the aid of the model of the structure of the thrombin–hirudin fragment complex [Yue, S.-Y., DiMaio, J., Szewczuk, Z., Purisima, E. O., Ni, F., & Konishi, Y. (1992) *Protein Eng. 5*, 77–85], cyclic analogs of the hirudin fragment (hirudin$^{55-65}$) were designed and synthesized. In these analogs, the side chains of appropriately substituted residues, 58 and 61, were joined in order to restrict the conformation of the inhibitor. An analog with an 18-membered lactam ring showed higher antithrombin activity (IC_{50} = 0.57 μM) than the corresponding analogs with 17- or 16-membered rings and was 2-fold more potent than its linear counterpart. Even 4-fold greater enhancement was obtained when a shorter fragment, hirudin$^{55-62}$, was cyclized. This cyclization not only improved the potency but, more importantly, dramatically increased the resistance to proteolytic digestion. Remarkable enhancement of stability to proteolysis was observed for peptide bonds located in the exocyclic linear peptide segments. These results are discussed using molecular modeling.

The serine protease α-thrombin initiates blood coagulation by catalyzing proteolysis of fibrinogen to release fibrinopeptides A and B. The high affinity and catalytic specificity of α-thrombin toward fibrinogen are determined not only by the complicated surface topology around the active site of thrombin but also by interaction at the exo site distinct from the active site of α-thrombin (Fenton et al., 1988). Under normal conditions, there is a negligible amount of thrombin present in the plasma. An increase in the amount of enzyme could result in the formation of a clot. Thrombus formation inside the vessel may lead to thromboembolic disease, which is, besides cancer, one of the most common, serious contemporary disorders. The search for synthetic thrombin inhibitors has been intensified since such substances may be pharmacological tools to control blood coagulation.

The most potent, known thrombin inhibitor is hirudin, a 65 amino acid polypeptide isolated from the blood sucking leech *Hirudo medicinalis* (Badgy et al., 1976) (Figure 1). The high affinity and specificity of hirudin for α-thrombin are a result of its bivalent binding, in which the N-terminal domain of hirudin (residues 1–3) interacts with the thrombin active site and the carboxyl tail of hirudin (residues 48–65) wraps around the exo site of α-thrombin (Rydel et al., 1990; Grutter et al., 1990). Recently, potent thrombin inhibitors have been designed on the basis of the hirudin sequence (Maraganore et al., 1990; DiMaio et al., 1990). These inhibitors are constituted from an active site inhibitor, D-Phe-Pro-Arg, replacing the hirudin residues (1–47), an exo-site inhibitor, and a linker connecting these two inhibitors. Thus, they still mimic hirudin's distinctive mechanism by simultaneously occupying the active site and the exo site of thrombin. These

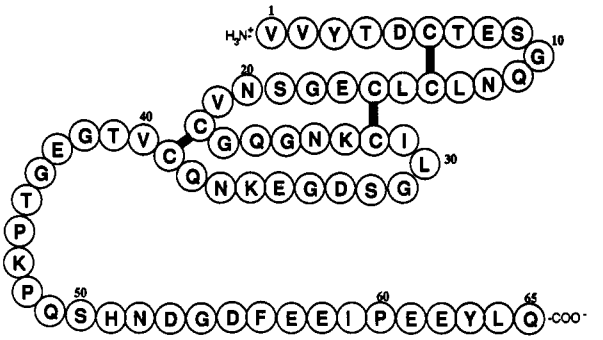

FIGURE 1: Primary structure of desulfo-hirudin.

peptide-based inhibitors are generally accessible to enzyme digestion and may have a short life in vivo. Further analogs have been designed to achieve higher potency and improve resistance to enzymatic degradation. This was achieved by substituting the scissile Arg–X peptide bond with ketomethylene pseudo-peptide bonds and by replacing the six-residue linker Gln-Ser-His-Asn-Asp-Gly with unnatural ω-amino acids (DiMaio et al., 1991; Kline et al., 1991; Szewczuk et al., 1992a). However, the C-terminal exo-site inhibitor may be accessible to enzyme digestion by both endo- and exopeptidases (Chang, 1983, 1990), because even in intact hirudin the C-terminal tail exhibits considerable flexibility in solution (Folkers et al., 1989; Haruyama & Wüthrich, 1989). Recent NMR experiments on the C-terminal fragment of hirudin$^{55-65}$ show that the free peptide is in the folded bound conformation less than 50% of the time (Ni et al., 1992).

A small hirudin C-terminal fragment, hirudin$^{55-65}$, binds specifically to the exo site of α-thrombin and inhibits the ability of α-thrombin to clot fibrinogen while having no effect on the activity of α-thrombin toward small substrates (Krstenansky

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& Mao, 1987; Dodt et al., 1990). The SAR[1] study of the undecapeptide suggested that residues 55–60[2] play a major role in binding to thrombin, where the side chains of Phe^{56}, Glu^{57}, and Ile^{59} showed critical contributions (Yue et al., 1992). On the other hand, residues Asp^{55}, Glu^{58}, Glu^{61}, Glu^{62}, Tyr^{63}, Leu^{64}, and Gln^{65} were found to be unessential for the activity and could be replaced by other amino acids, although only certain substitutions led to more active compounds (Krstenansky et al., 1990). Finally, removal of a positively charged N-terminal amino group from the peptide resulted in increased potency (e.g., desamino-hirudin$^{55-65}$ and desamino-hirudin$^{54-65}$ have a marked increase in potency over hirudin$^{55-65}$) (Krstenansky et al., 1987).

Cyclization of biologically active peptides with constrained conformations may enhance their activity (Hruby, 1982; Degrado, 1988; Hruby et al., 1990; Szewczuk et al., 1988) and proteolytic stability (Sham et al., 1988, 1990). In this paper, we describe how we used the SAR results above and the model of the structure of the C-terminal hirudin fragment bound to thrombin (Yue et al., 1992) for the design of side-chain to side-chain cyclized exo-site inhibitors with increased resistance to enzymatic degradation and improved potency. We used molecular modeling methods to analyze the conformational preference of the cyclic peptides.

EXPERIMENTAL PROCEDURES

Materials. Human thrombin (3000 NIH units/mg), bovine fibrinogen (~75% protein, 90% clottable), normal human plasma, Tos-Gly-Pro-Arg-AMC HCl salt, and poly(ethylene glycol) 8000 were purchased from Sigma; chymotrypsin and pepsin were purchased from Boehringer Mannheim; trypsin was obtained from Worthington Biochemicals; 7-amino-4-methylcoumarin was from Aldrich, and the derivatives of amino acids for peptide synthesis were purchased from Aminotech and Advanced Chem Tech. The side-chain protecting groups for BOC-amino acids were benzyl for Glu, Asp, and Ser, Tos for His and Arg, and 2-bromobenzyloxycarbonyl for Tyr. The side chains of Fmoc-amino acids were protected by BOC for Lys and Orn and by tBu for Asp and Glu. BOC-Glu(OBzl)OCH_2-phenylacetylaminomethyl (Pam) resin (0.70 mmol/g), BOC-GlnOCH_2-phenylacetylaminomethyl resin (0.73 mmol/g), and *p*-methylbenzhydrylamine (BHA) resin HCl salt (0.62 mmol/g) were purchased from ABI. The solvents for peptide synthesis were obtained from B&J Chemicals and ABI. Tris and citric acid were purchased from Bio-Rad and Anachemia, respectively.

Peptide Synthesis. All linear peptides (Table I) were synthesized by the solid-phase method on an Applied Biosystem 430A peptide synthesizer using a standard ABI coupling protocol [*N*-methylpyrrolidone (NMP) cycle]. Only single coupling was performed followed by acetylation with acetic anhydride at the end of each cycle. For *N*-succinyl compounds P137, P141, P142, P143, P144, P173, P205, and P206, succinic anhydride (4-fold excess) was incorporated for 15 min in the presence of DIEA, after deprotection of the N-terminal amino acid residue.

The cyclic peptides listed in Table I were prepared by manual solid-phase techniques. The strategy of the synthesis was similar to the one described by Schiller et al. (1985b). The exocyclic C-terminal segment was assembled using standard BOC synthetic strategy (Steward & Young, 1984). Then, the segment to be cyclized, Fmoc[Glu or Asp](tBu)-Ile-Pro-[Lys or Orn](BOC), was assembled by the standard Fmoc synthetic procedure (Fields & Noble, 1990). The tBu and BOC protecting groups of the side chains to be linked were removed by 50% TFA in methylene chloride (1 × 1 min + 1 × 30 min). The peptide on the resin was cyclized by forming an amide bond between the side chains of Glu (or Asp) and Lys (or Orn) in DMF (15 mL/g of resin) at room temperature in the presence of 1-hydroxybenzotriazole (3 equiv), DIEA (6 equiv), and BOP (3 equiv), which is known as an efficient side-chain lactamizing reagent on the solid-phase resin (Felix et al., 1988). The cyclization was completed within 12 h on the basis of the ninhydrin test. After cyclization, the N-terminal Fmoc group was removed with a 50% piperidine/DMF solution (1 × 1 min + 1 × 10 min), and the resin was washed with DMF. The remaining N-terminal segments were assembled by a standard BOC synthetic strategy. Peptides were cleaved from the resin using HF in the presence of anisole (5% volume) and dimethyl sulfide (5% volume) at −5 °C for 60 min. After evaporation of the HF, the peptides were washed with ethyl and extracted with 50% acetic acid, followed by water prior to lyophilization.

The peptides were then purified by preparative HPLC on a Vydac C_4 column, 4.6 × 25 cm, using a linear gradient of 20–50% acetonitrile in 0.1% TFA (gradient 0.5%/min, flow rate 18 mL/min). If necessary, further purification to homogeneity was performed by semipreparative HPLC [20–40% acetonitrile (linear gradient 1%/min, flow rate 10 mL/min) in 0.1% TFA, Vydac C_{18}, 2 × 25 cm column]. Final products were obtained as lyophilysates with 98% or higher purity estimated by analytical HPLC [10–70% acetonitrile (linear gradient 1%/min, flow rate 1 mL/min) in 0.1% TFA, Vydac C_{18}, 0.46 × 25 cm column]. The peptides were identified with a Beckman Model 6300 amino acid analyzer and a SCIEX API III mass spectrometer. Amino acid analysis was used for peptide content determination. The purities of the crude products were estimated by their analytical HPLC profiles after HF cleavage. Analytical data are presented in Table II.

Fibrinogen Clotting and Amidolytic Assays. The fibrin clot formation induced by human thrombin was measured spectrophotometrically at 420 nm on a Varian DMS 90 spectrometer. Bovine fibrinogen (0.1%, 0.3 mL) in 0.05 M Tris-HCl buffer (pH 7.6 at 37 °C) containing 0.1 M NaCl and 0.1% poly(ethylene glycol) 8000 (called hereafter "running buffer") was premixed with various amounts of a thrombin inhibitor in the running buffer at 37 °C, and the volume of the premixed solution was adjusted to 0.98 mL with the running buffer. The time from the addition of human thrombin (20 μL, final concentration 0.2 NIH units/mL) to the inflection of the absorbance at 420 nm due to clot formation was recorded as a clotting time. The clotting time was plotted against inhibitor concentrations, and IC_{50} was estimated as the inhibitor concentration required for 50% inhibition of thrombin activity. The remaining thrombin activity was read from a standard curve relating the clotting time to the thrombin activity.

The inhibition of the amidolytic activity of human thrombin by the bifunctional inhibitor P175 was measured spectrophotometrically using Tos-Gly-Pro-Arg-AMC as the fluo-

[1] Abbreviations: AMC, 7-amino-4-methylcoumarin; BOC, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; Bzl, benzyl; DIEA, diisopropylethylamine; DMF, dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; HPLC, high-performance liquid chromatography; NMP, *N*-methylpyrrolidone; rms, root mean square; SAR, structure–activity relationships; Suc, succinyl; tBu, *tert*-butyl; TFA, trifluoroacetic acid; Tos, tosyl; Tris, tris(hydroxymethyl)aminomethane.

[2] The numbering of the inhibitor residues is based on the hirudin sequence.

Table I: Formulas of Hirudin C-Terminal Fragments and Their Analogs

No.	Structure
	45[a] 50 55 60 65
P53	Ac-D-Phe-Pro-Arg-Pro-Gln-Ser-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH
P175	Ac-D-Phe-Pro-Arg-Pro-Gln-Ser-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Lys-Glu-Tyr-Leu-Gln-OH[b]
P24	Ac-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH
P177	Ac-Asp-Phe-Glu-Glu-Ile-Pro-Lys-Glu-Tyr-Leu-Gln-OH
P178	Ac-Asp-Phe-Glu-Asp-Ile-Pro-Lys-Glu-Tyr-Leu-Gln-OH
P179	Ac-Asp-Phe-Glu-Asp-Ile-Pro-Orn-Glu-Tyr-Leu-Gln-OH
P137	Suc-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH
P141	Suc-Phe-Glu-Gln-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH
P142	Suc-Phe-Glu-Glu-Ile-Pro-Lys(Ac)-Glu-Tyr-Leu-Gln-OH
P143	Suc-Phe-Glu-Gln-Ile-Pro-Lys(Ac)-Glu-Tyr-Leu-Gln-OH
P144	Suc-Phe-Glu-Glu-Ile-Pro-Lys-Glu-Tyr-Leu-Gln-OH
P206	Suc-Phe-Glu-Glu-Ile-Pro-Glu-Glu-OH
P173	Suc-Phe-Glu-Glu-Ile-Pro-Lys-Glu-OH
P205	Suc-Phe-Glu-Glu-Ile-Pro-Lys-NH_2

[a] The numbering of the residues is based on the hirudin sequence. [b] The substituted amino acid residues are underlined.

rogenic substrate. The reaction was monitored on a Varian-Cary 2000 spectrophotometer in the fluorescence mode (λ_{ex} = 383 nm, λ_{em} = 455 nm), and the fluorescent intensity was calibrated using 7-amino-4-methylcoumarin. The substrate was dissolved in 21% (v/v) dimethyl sulfoxide in the running buffer (pH 7.8 at 25 °C) to obtain a 0.3 mM stock solution. The inhibitor was dissolved in the running buffer, and the pH was adjusted to 7.8. The substrate (final concentration ranged from 1 to 8 μM) and the inhibitor were premixed, and the running buffer was added to adjust the volume to 2.99 mL. The reaction was initiated by adding 0.01 mL of human thrombin (final concentration 0.033 NIH unit/mL). The initial velocity of the fluorescence change was recorded at various substrate and inhibitor concentrations. The data were analyzed using a nonlinear regression program, RNLIN in the IMSL library (IMSL, 1978), to estimate the kinetic parameters (K_M, V_{max}, and K_i).

Proteolytic Assays. Proteolytic stability of the inhibitors against pepsin, trypsin, chymotrypsin, and human plasma [that may contain every enzyme associated with living tissue (Fishman & Doellgast, 1975)] was measured using HPLC (Hewlett-Packard, Model 1090). An inhibitor (100 μg) and the enzyme(s) in the ratio of 100 to 1–2 (w/w) (for the plasma experiment the ratio was based on lyophilized human plasma) were incubated at 37 °C in 0.2 mL of the appropriate buffer [100 mM ammonium bicarbonate (pH = 7.8) for trypsin and chymotrypsin, 5% acetic acid for pepsin, and 50 mM Tris-HCl, pH 7.8, for plasma proteases). Aliquots of the reaction solution were removed at intervals, and the reaction was arrested by changing the pH. These aliquots, in most cases, were injected directly onto a Vydac C_{18} column and eluted with a linear gradient of acetonitrile containing 0.1% TFA from 10 to 70% over 60 min at a flow rate of 1 mL/min. The column eluent was monitored by the absorbencies at 206 and 260 nm. The fractions were collected and identified by amino acid analysis on a Beckman Model 6300 analyzer and mass spectrometry (SCIEX API III).

Molecular Modeling. Molecular modeling of the cyclic inhibitors was carried out using Quanta/CHARMm (version 3.2.1) (Polygen Corp.; Brooks et al., 1983), for constrained energy minimization, and SYBYL (version 5.4) (Tripos Associates Inc.) and MacroModel/BatchMin (version 3.1) (Mohamadi et al., 1990), for conformational search, on an Iris 4D/280 (Silicon Graphics Inc.). The thrombin–hiru-

Table II: Analytical Data of Synthetic Peptides

peptide	purity[a] (%)	amino acid composition (calcd values)[b] Asx	Ser	Glx	Gly	Ile	Leu	Tyr	Phe[c]	His	Lys	Orn	Arg	Pro	MW_{obsd} (MW_{calc})[b]
P175	20	3.15 (3)	0.91 (1)	5.46 (5)	1.02 (1)	0.94 (1)	1.05 (1)	0.97 (1)	2.00 (2)	0.95 (1)	0.90 (1)		1.01 (1)	3.03 (3)	2570.8 (2570.8)
P24	87	1.18 (1)		6.01 (5)		1.01 (1)	1.10 (1)	1.01 (1)	1.00 (1)					1.16 (1)	1452.7 (1452.6)
P177	14	1.04 (1)		4.38 (4)		0.97 (1)	1.01 (1)	0.97 (1)	1.00 (1)		0.98 (1)			1.00 (1)	1434.0 (1433.7)
P178	16	1.92 (2)		3.18 (3)		0.92 (1)	1.02 (1)	0.94 (1)	1.00 (1)		0.87 (1)			1.04 (1)	1419.7 (1419.7)
P179	15	2.02 (2)		3.28 (3)		0.93 (1)	1.02 (1)	0.96 (1)	1.00 (1)			0.97 (1)		1.03 (1)	1406.0 (1405.6)
P137	80			5.53 (5)		0.98 (1)	1.02 (1)	1.00 (1)	1.00 (1)					0.94 (1)	1395.4 (1395.6)
P141	69			5.36 (5)		0.92 (1)	1.07 (1)	1.01 (1)	1.00 (1)					1.01 (1)	1394.8 (1394.6)
P142	80			4.47 (4)		0.95 (1)	1.07 (1)	0.99 (1)	1.00 (1)		0.91 (1)			1.06 (1)	1436.8 (1436.7)
P143	80			4.45 (4)		0.96 (1)	1.07 (1)	1.01 (1)	1.00 (1)		0.92 (1)			1.03 (1)	1435.7 (1435.7)
P144	30			4.44 (4)		0.96 (1)	1.06 (1)	0.99 (1)	1.00 (1)		0.90 (1)			1.04 (1)	1376.9 (1376.7)
P206	90			4.33 (4)		0.96 (1)			1.00 (1)					0.93 (1)	991.5 (991.4)
P173	41			3.26 (3)		0.98 (1)			1.00 (1)		1.00 (1)			0.97 (1)	972.5 (972.5)
P205	70			2.20 (2)		0.96 (1)			1.00 (1)		1.00 (1)			0.96 (1)	842.5 (842.4)

[a] The purity of the crude product was estimated from the HPLC profile. [b] Theoretical values are in parentheses. [c] Phe was used to normalize the amino acid composition of the peptides.

din$^{55-65}$ model (Yue et al., 1992) was used as the starting structure of the exo-site inhibitor with minor modifications of the ϕ and ψ dihedral angles of residues 56–62 to those of hirugen bound to thrombin (Skrzypczak-Jankun et al., 1991). The three C-terminal residues (Tyr^{63}–Gln^{65}) of the inhibitors P142, P177, P178, and P179 were neglected to increase the speed of the computation, and the corresponding inhibitors in computation are designated as P142t, P177t, P178t, and P179t, respectively. The energy minimization of P177t constrained to the same backbone conformation as the corresponding residues in the thrombin–hirudin complex was carried out as follows. The native sequence was mutated accordingly while maintaining the same side-chain orientation as in the native hirudin peptide. The side chains of Glu^{58} and Lys^{61} were linked to form an amide bond through the removal of an O atom from the 58th residue and the conversion of $-NH_3^+$ of the 61st residue into –NH–. A harmonic energy minimization was first carried out in which the ϕ and ψ dihedral angles were constrained to be the same as that of the native peptide with a force constant of 400 kcal/mol. The CHARMm and polar hydrogen force field (Brooks et al., 1983) was used in the calculation with a 10-Å cutoff and a distance-dependent dielectric function ($\epsilon = 2r$). The energy minimization was done using the adopted Basis Newton–Raphson algorithm until an energy change <0.0001 kcal/mol between two consecutive steps was achieved.

An extensive search of low-energy conformations for P177t, P178t, and P179t was carried out using the BatchMin program (Mohamadi et al., 1990) and Monte Carlo multiple minimum (MCMM) method (Chang et al., 1989) with the AMBER united atoms (Weiner et al., 1984) and a distance-dependent dielectric function ($\epsilon = 4r$). The energy window in the search was set to 4.8 kcal/mol, and all rotatable bonds with the exception of the C_β–C_γ bond of the 56th residue and all the C–COO^- and peptide bonds were subject to rotation in the computation. Nine, seven, and five runs were calculated for P177t, P178t, and P179t, respectively. The starting conformations for each of these runs were randomly generated using SYBYL and subjected to 500 steps of energy minimization. At least three long conformational searches with 10 000 generated conformations and several shorter runs with 5000–10 000 conformers were carried for each inhibitor. For each conformation generated, 40 steps of truncated Newton conjugate gradient minimization (Ponder & Richards, 1987), implemented in the BatchMin program, was calculated. The low-energy conformers (1547, 1042, and 654 conformers for P177t, P178t, and P179t, respectively) were compared with the bound conformer of P142t, based on the relative position in space of 10 main-chain atoms involved in the cyclizations, using X-PLOR (version 2.1) (Brünger et al., 1987).

RESULTS AND DISCUSSION

Synthesis of the Cyclic Inhibitors. Peptides were cyclized on the solid support to minimize dimerization or polymerization which are common in solution synthesis even at low concentrations of peptides (Bodanszky, 1984). A small amount of dimers formed on the resin was separated from the corresponding monomer by reverse-phase HPLC, as was shown in the example of P173 (Figure 2A). The purified monomer and the dimer were identified by their molecular weights using an ion-spray mass spectrometer. Panels B and C of Figure 2 show the mass spectra of peaks I and II eluted at 38 and 52 min (Figure 2A), respectively. Since the isotope peaks are separated by 1.0 and 0.5 Da consecutively for singly charged and doubly charged species, the peaks in panels B and C of Figure 2 correspond to the singly and doubly charged peptides with molecular weights of 972.5 (monomer) and 1945.0 (dimer), respectively. Similarly, the monomer and the dimer of other cyclic peptides were also well separated by HPLC (Schiller et al., 1985a). Thus, the monomers listed in Table I are not contaminated by the corresponding dimers. All linear and cyclic peptides synthesized were identified by mass spectrometry and amino acid analysis and are listed in Table II.

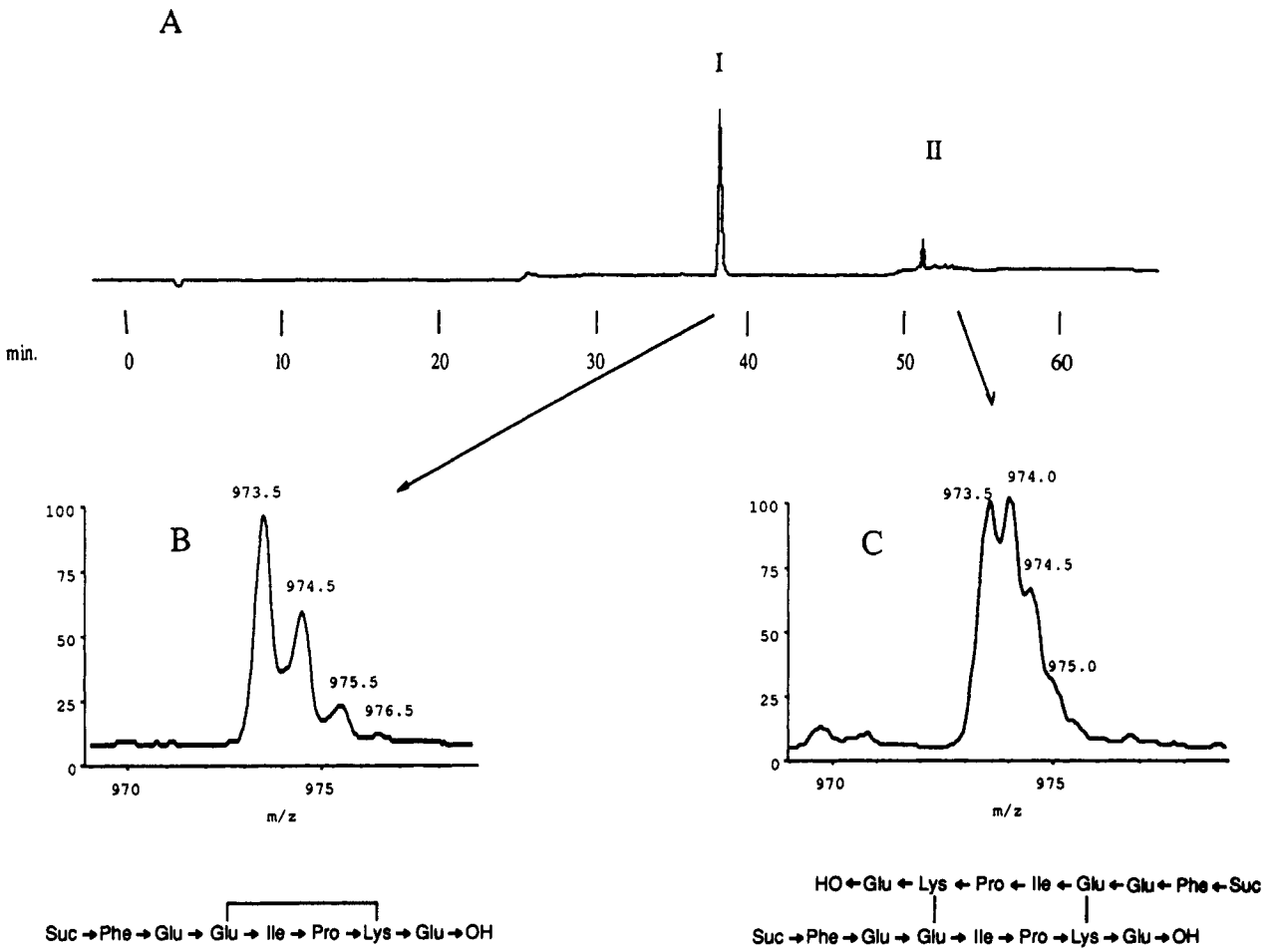

FIGURE 2: (A) HPLC elution profile of the crude product obtained in the solid-phase synthesis of P173. Peaks: I, cyclic monomer; II, side-chain-linked cyclic dimer. The mass spectra and structures of peaks I and II are shown in (B) and (C), respectively.

Residues To Be Cyclized. The cyclic structures have been generated by joining the side chains of two amino acid residues by means of a lactam bridge. We chose residues 58 and 61 for cyclization since the side chains of Glu^{58} and Glu^{61} in the hirudin C-terminal fragment are not critical for the activity and can be replaced by Gly (Yue et al., 1992) or Ala (Krstenansky et al., 1990) with a minimal reduction of the activity. These side chains are facing the same side in the bound conformation (Rydel et al., 1990; Ni et al., 1990; Yue et al., 1992) and are oriented favorably for cyclization. Furthermore, two amino acid residues that are important for the inhibitory actiivty, Ile^{59} and Pro^{60}, are located between the amino acids to be linked. There are two effects which have to be considered in the cyclization. First, the modification may induce a conformational change of the segment involved in cyclization, affecting the binding potency. Second, the lactam bridge formation requires the loss of two charges of Glu^{58} and Glu^{61} and alters the chemical profile of the C-terminal peptide. To dissect out the contributions of these two effects, we carried out the modification of the peptide in several stages. First, the change in chemical property was studied by replacing Glu^{58} and Glu^{61} with Gln and N^{ϵ}-acetyl-Lys, respectively, which contain the side-chain moieties present in the cyclic peptide. The substitution of Glu^{58} in desamino-hirudin$^{55-65}$ with Gln (P141) affected the potency by only 1.1-fold with an IC_{50} of 1.22 μM (Table III). Similarly, the substitution of Glu^{61} in desamino-hirudin$^{55-65}$ with N^{ϵ}-acetyl-Lys (P142) affected the potency by only 1.2-fold with an IC_{50} of 1.30 μM. The double mutations of Glu^{58} and Glu^{61} in desamino-hirudin$^{55-65}$ with Gln and N^{ϵ}-acetyl-Lys, respectively (P143), affected the potency by 1.3-fold with an IC_{50} of 1.42 μM. Thus, although Glu^{58} has a close contact with Arg^{77A} of thrombin in the thrombin–hirudin complex (Rydel et al., 1991), the side chains of Glu^{58} and Glu^{61} are not contributing to the inhibitory activity and can be replaced by other side chains for cyclization with a minimal loss of activity.

Table III: Inhibition of Fibrin Clot Formation

peptide	compound	IC_{50}[a,b] (μM)
P24	desamino-hirudin$^{54-65}$	1.21 ± 0.17[c]
P177	desamino-cyclo[Glu^{58},Lys^{61}]hirudin$^{54-65}$	0.568 ± 0.007
P178	desamino-cyclo[Asp^{58},Lys^{61}]hirudin$^{54-65}$	1.56 ± 0.08
P179	desamino-cyclo[Asp^{58},Orn^{61}]hirudin$^{54-65}$	9.67 ± 0.65
P137	desamino-hirudin$^{55-65}$	1.12 ± 0.04[c]
P141	desamino-[Gln^{58}]hirudin$^{55-65}$	1.22 ± 0.13
P142	desamino-[$Lys(Ac)^{61}$]hirudin$^{55-65}$	1.30 ± 0.18
P143	desamino-[Gln^{58},$Lys(Ac)^{61}$]hirudin$^{55-65}$	1.42 ■ 0.05
P144	desamino-cyclo[Glu^{58},Lys^{61}]hirudin$^{55-65}$	0.361 ± 0.019
P206	desamino-hirudin$^{55-62}$	51.0 ± 7.3
P173	desamino-cyclo[Glu^{58},Lys^{61}]hirudin$^{55-62}$	12.2 ± 0.9
P205	desamino-cyclo[Glu^{58},Lys^{61}]hirudin$^{55-61}$-amide	145 ± 12

[a] IC_{50} = molar dose of peptide that inhibited 50% of the thrombin activity. [b] Mean of three determinations ± SEM. [c] IC_{50} values of desamino-irudin$^{54-65}$ and desamino-hirudin$^{55-65}$ reported by Owen et al. (1988) were 5.2 and 1.7 μM, respectively.

Effect of Ring Size. To determine the dependence of the antithrombin activity on the ring size, a series of cyclic analogs (P177, P178, and P179) with 16-, 17-, and 18-atom rings, respectively, were synthesized. Among these inhibitors, desamino-cyclo[Glu^{58},Lys^{61}]hirudin$^{55-65}$, P177 (analog with an 18-membered lactam ring), showed the highest inhibitory activity with IC_{50} = 0.568 μM, while desamino-cyclo-[Asp^{58},Lys^{61}]hirudin$^{55-65}$, P178 (analog with a 17-membered

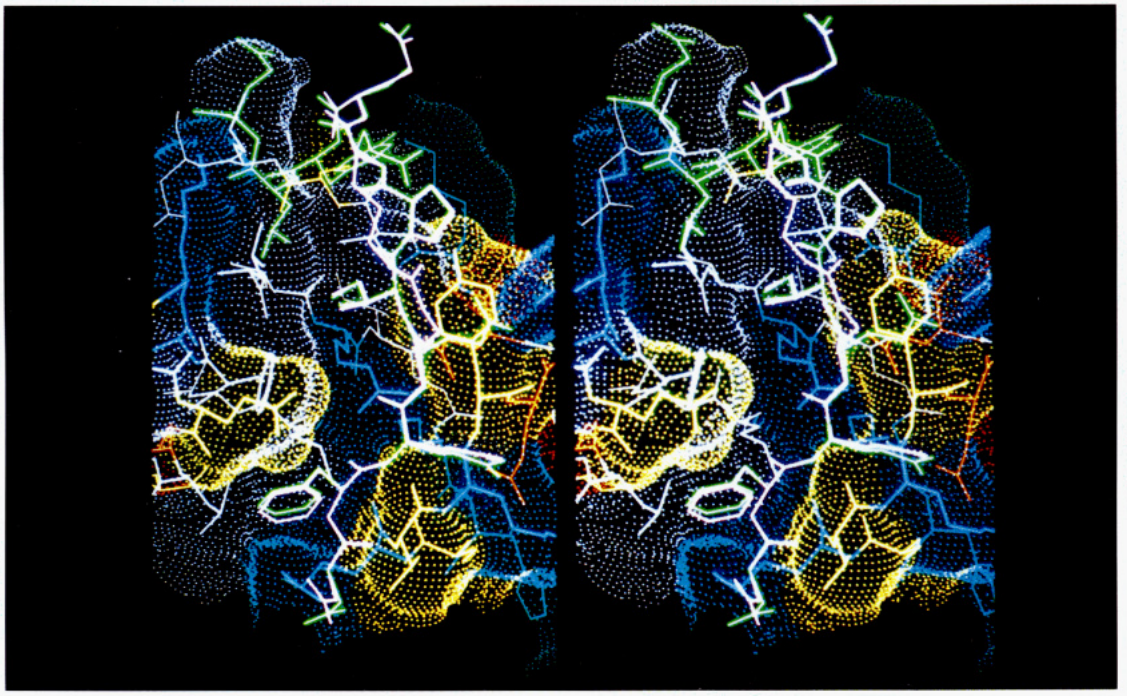

FIGURE 3: Stereoview of P177t (pink) and P24 (green) superposition on the thrombin exo site (loops 32–42 and 65–85). The molecular surface of the thrombin exo site is multicolored: red, acidic residues; blue, basic residues; white, hydrophobic residues; yellow, other residues.

lactam ring), and desamino-cyclo[Asp^{58},Orn^{61}]hirudin$^{55-65}$, P179 (analog with a 16-membered lactam ring), showed lower activity (IC_{50} = 1.56 and 9.67 μM, respectively) as listed in Table III. Since all the cyclic compounds, P177, P178, and P179, showed comparable potencies to that of the linear peptide P24, they may adopt the conformation similar to the desired one (Yue et al., 1992; Skrzypczak-Jankun et al., 1991) without the introduction of strain in the covalent geometry.

Steric Overlap between Cyclic Inhibitors and Thrombin in the Complex. In designing cyclic peptides, the bridges that connect amino acid side chains should not have any steric overlap with an enzyme or receptor upon its binding. Although there are some examples of tight-binding cyclic peptides formed by connecting the side chains of amino acids known to project toward the interior of an enzyme binding pocket (Szewczuk et al., 1992b) or even interact with the receptor through aromatic groups (Szewczuk et al., 1988), the bridges of the cyclic inhibitors were designed to orient away from the protein. Energy-minimized structures of the 18-membered ring inhibitor, with its harmonic constraints on the main-chain dihedral angles to maintain the bound conformation, have side-chain conformations at the 58th and 61st residues quite different from those of hirudin (Figure 3). When the main chain of the low-energy conformation was superimposed with the hirudin C-terminal fragment in the thrombin–hirudin$^{55-65}$ complex (Yue et al., 1992), the modified side chains projected outward toward the solvent and had no steric overlap with thrombin. Furthermore, since the cyclization of P177 did not deteriorate the activity but rather improved the IC_{50} 2-fold, the steric overlap between P177 and thrombin in the complex is very unlikely.

Conformational Preferences of the Cyclic Peptides. The conformational preferences of the cyclic peptides were investigated through a conformational search of peptides P177t, P178t, and P179t. The details of the calculation are described under Experimental Procedures, and the results are summarized in Figure 4. Although the conformations of exocyclic segments varied, the low-energy conformers of the lactam rings might be classified into two groups. One group of the

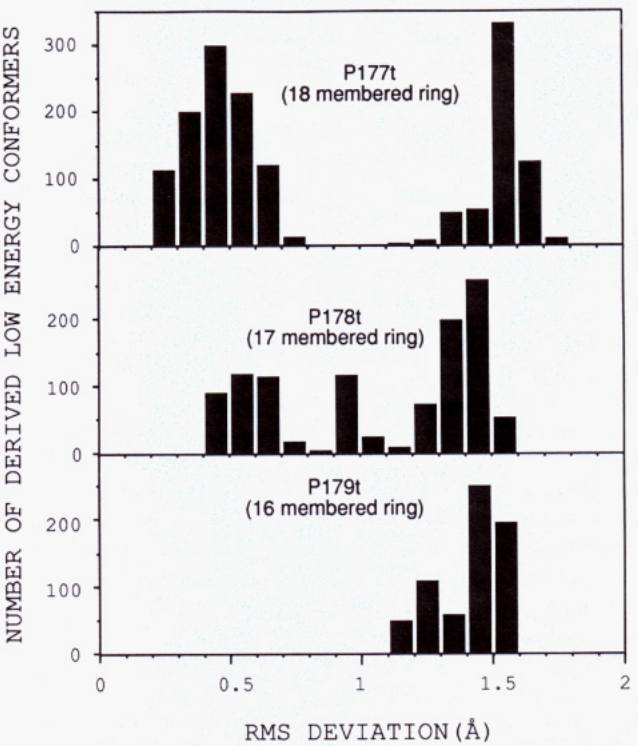

FIGURE 4: Graphical representation of numbers of low-energy conformers of 18- (P177t), 17- (P178t), and 16-membered (P179t) ring cyclopeptides found in the conformational search as a function of rms deviation from the exo-site inhibitor-bound conformation. The comparison was made on the basis of the position of the 10 main-chain atoms involved in the lactam ring. The details of the search are described under Experimental Procedures.

conformers has a significant similarly to the bound conformation of exo-site inhibitors with small rms deviations around 0.4–0.5 Å. The other group of the conformers was different from the active conformation; typically ψ of Ile^{59} was around –60°, whereas the corresponding ψ was around –150° in the active conformation. As summarized in Figure 4, P177t with an 18-atom ring prefers the one close to the bound confor-

Table IV: Proteolytic Stability of the Exo-Site Inhibitors

	incubation time (h)														
	chymotrypsin				trypsin				plasma proteases			pepsin			
inhibitor	0.1	1	2	3	0.1	1	2	4	0.1	1	2	0.1	1	2	4
P143	4[a]	80	100		2	30	45	50	0	10	30	10	100		
P144	0	0	0	0	0	0	0	0	0	0	3[b]	10	100		
P173	0	0	0	0	0	0	0	0	0	0	0	0	10	20	30

[a] The percentage of the inhibitors hydrolyzed by proteases was estimated from the peak areas in the HPLC profiles. [b] The digestion products were not determined.

mation. The preference is shifted to the inactive conformation in P178t with a 17-atom ring. This preference is shifted further in P179t with a 16-atom ring such that no conformation close to the bound one was observed in its 654 low-energy conformers. These conformational preferences correlated with their potencies, i.e., IC_{50} of 0.568 μM for P177, 1.56 μM for P178, and 9.67 μM for P179. Thus, the IC_{50} of these peptides and the molecular modeling suggest that the effect of cyclization of the peptides is not only to restrict the conformational flexibility of the peptide but also to change the conformational preference of the peptide. Further design of cyclization to stabilize the active conformation may improve the potency of the exo-site inhibitor.

Effect of Exocyclic Segments. Conformationally constrained cyclic peptides are expected, for entropic reasons, to bind to the enzyme more tightly than the corresponding linear analog. It was therefore somewhat puzzling that the cyclic inhibitors were only 2–4 times more potent than the corresponding linear analogs. A possible explanation may be the observation that the C-terminal fragment of the linear inhibitor is not completely disordered in solution but already has a relatively stable conformation in solution, similar to the bound one (Ni et al., 1992), in which Tyr^{63} and Leu^{64} form a hydrophobic cluster with Ile^{59}. The further effect of this hydrophobic cluster on conformational stabilization induced by cyclization was examined by synthesizing truncated analogs of the linear (P206) and cyclic inhibitors (P173). Removal of the three C-terminal residues (Tyr^{63}–Leu^{64}-Gln^{65}) in the linear peptide (P206) affected IC_{50} 46-fold to 51 μM (Table III). The cyclization of this truncated inhibitor (P173) improved IC_{50} 4-fold to 12.2 μM. Since the improvement is very similar to that of the untruncated exo-site inhibitor, the C-terminal tetrapeptide may not contribute to further enhancement of the conformational stabilization induced by cyclization. On the other hand, the attachment of the active site inhibitor suppressed the effect of the cyclization; i.e., the linear bifunctional inhibitor, P53, was only slightly less potent with IC_{50} = 20.4 nM and K_i = 5.6 nM (Yue et al., 1992) than those of the corresponding cyclic inhibitor (P175) with IC_{50} = 17.5 nM and K_i = 4.3 nM (K_M = 2.5 μM; V_{max} = 0.58 μM/min for the fluorogenic substrate Tos-Gly-Pro-Arg-AMC). Thus, although the active site inhibitor and the exo-site inhibitor are separated by a flexible hexapeptide (-Gln-Ser-His-Asn-Asp-Gly-), they may not be independent either in solution or in the state bound to thrombin.

Proteolytic Stability. Proteolytic stability is an important factor in designing peptide-based inhibitors. The cyclization of the exo-site inhibitors improved their proteolytic stability dramatically as summarized in Table IV. The cleavage sites of the inhibitors by chymotrypsin, trypsin, human plasma proteases in the coagulation cascade, and pepsin were investigated using the cyclic inhibitor P144 and its linear analog with similar chemical moieties, P143 (Figure 6). The corresponding hirudin fragment (P137) was cleaved by the

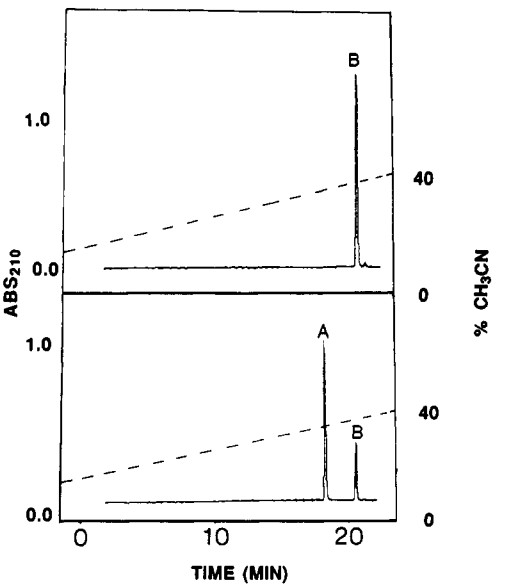

FIGURE 5: HPLC profile of P143 (1) before and (2) after 1 h of incubation with chymotrypsin. Peak B corresponds to the intact peptide while peak A is its chymotryptic product. Suc-Phe-Gln-Ile-Pro-Lys(Ac)-Glu-Tyr-OH. The details of the digestion are described under Experimental Procedures.

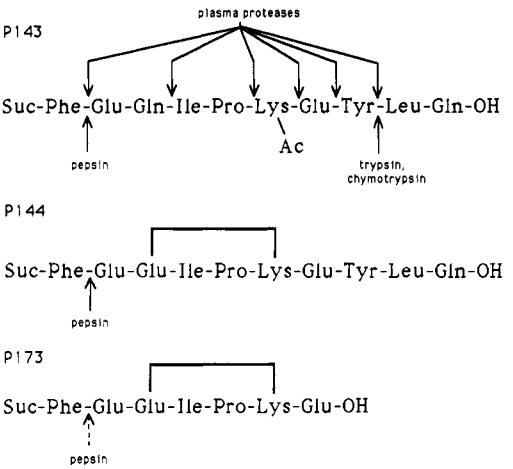

FIGURE 6: Peptide bonds of the inhibitors P143, P144, and P173 hydrolyzed by chymotrypsin, trypsin, pepsin, and human plasma proteases.

enzymes tested similar to the analog P143 but slightly slower than P143 (results not shown). The cleavage of the linear peptide P143 by chymotrypsin is shown in Figure 5. After 1-h incubation, 80% of P143 (Peak B) was digested by chymotrypsin and the product Suc-Phe-Glu-Gln-Ile-Pro-Lys-(Ac)-Glu-Tyr-OH was eluted at 18 min on the HPLC chromatogram (peak A in Figure 5). Amino acid analysis and ion-spray mass spectrometry identified the product as desamino-hirudin[55–63]. Thus, chymotrypsin, which preferentially cleaves the carboxyl end of Tyr, Trp, Phe, and Leu,

cleaved the peptide bond at the carboxyl end of Tyr^{63} as expected. This relatively rapid cleavage of P143 (80% in 1 h) was not observed in the cyclized P144. No hydrolysis of P144 was observed even after 4 h of incubation. Trypsin preferentially cleaves peptide bonds at the carboxyl end of basic residues. Although trypsin contained *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK–chymotrypsin inhibitor), it still possessed some chymotryptic-like activity, which may be due to the presence of a small amount of ψ-trypsin, an autolysis product of trypsin (Keil-Dlouha et al., 1971), resulting in 30% cleavage at the Tyr^{63}–Leu^{64} peptide bond after 1 h of incubation of P143 with trypsin. Again the cyclization completely protected the peptide from digestion by trypsin. Pepsin is a nonspecific protease but prefers to cleave the carboxyl end of Phe and Leu. Pepsin completely cleaved the Phe^{56}–Glu^{57} peptide bond of P143 within 1 h. Another slower cleavage at the carboxyl end of Glu^{62} was observed, although it was not clear whether these cleavages were independent or sequential. The same cleavage sites (at the carboxyl ends of Phe^{56} and Glu^{62}) by pepsin were also observed in the recombinant hirudin (Chang, 1990). The cyclization showed no effect on the protection of the peptide from peptic digestion. Interestingly, a shorter cyclic peptide, P173, which ends at Glu^{62} and has a reasonable antithrombin potency of IC_{50} = 12 μM, was fairly stable against peptic digestion, and only 30% of P173 was digested at the carboxyl end of Phe^{56} after 4 h of incubation with pepsin. Human plasma is an important in vitro system to investigate the stability of the inhibitor in the bloodstream. It is reasonable to suggest that the plasma may contain every enzyme associated with living tissue (Fishman & Doellgast, 1975). The linear inhibitor P143 was digested in human plasma at the carboxyl end of Phe^{56}, Gln^{58}, Pro^{60}, N^{ϵ}-acetyl-Lys^{61}, Glu^{62}, and Tyr^{63} after 2 h of incubation. Whereas the specific enzymes involved in the digestion of P143 are unknown, numerous endopeptidases in the plasma have been shown to hydrolyze small peptides (Ishida et al., 1989). The effect of the cyclization was obvious since P144, which is the cyclic form of P143, was digested only 3% in 2 h of incubation with human plasma as compared to 30% for P143.

Thus, the cyclization of the exo-site inhibitor improved the proteolytic stability in most cases. The peptide bonds within a ring of cyclic peptides have been reported to be resistant to proteolytic digestions (Nishino & Izumiya, 1982; Sham et al., 1990). Our results demonstrated that the ring effect extends to the exocyclic segment so that it is also resistant to some of the proteolytic digestions.

CONCLUSION

This study showed that a proper cyclization of thrombin exo-site inhibitors based on the hirudin C-terminal sequence not only improved the potency of the inhibitors but also dramatically increased the proteolytic stability of the inhibitors against various proteases. It is noteworthy that the cyclization protected the exocyclic peptide bonds of the inhibitors.

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