Design of Weakly Basic Thrombin Inhibitors Incorporating Novel P1 Binding Functions: Molecular and X-ray Crystallographic Studies†

Giuseppina De Simone,*,‡ Valeria Menchise,‡ Salvatore Omaggio,‡ Carlo Pedone,‡ Andrea Scozzafava,§ and Claudiu T. Supuran*,§

Istituto di Biostrutture e Bioimmagini-CNR, via Mezzocannone 6, 80134 Napoli, Italy, and Laboratorio di Chimica Inorganica e Bioinorganica, Università degli Studi, via della Lastruccia 3, Rm 188, Polo Scientifico, 50019 Sesto Fiorentino (Firenze), Italy

Received August 5, 2002; Revised Manuscript Received April 21, 2003

ABSTRACT: To prepare weakly basic thrombin inhibitors with modified S1 anchoring groups, two series of compounds were synthesized by reaction of guanidine or aminoguanidine with acyl halides and N,N-disubstituted carbamoyl chlorides. pK_a measurements of these acylated guanidines/aminoguanidines showed a reduced basicity, with pK_a values in the range of 8.4−8.7. These molecules typically showed inhibition constants in the range of 150−425 nM against thrombin and 360−965 nM against trypsin, even though some bulky derivatives, such as *N,N*-diphenylcarbamoylguanidine/aminoguanidine and their congeners, showed much stronger thrombin inhibitory activity, with inhibition constants in the range of 24−42 nM. Unexpectedly, very long incubation times with both proteases revealed that aminoguanidine derivatives behaved as irreversible inhibitors. To assess the molecular basis responsible for the high affinity observed for these molecules toward thrombin, the crystal structure of the thrombin−hirugen−*N,N*-diphenylcarbamoylaminoguanidine complex has been solved at 1.90 Å resolution. The structural analysis of the complex revealed an unexpected interaction mode with the protease, resulting in an *N,N*-diphenylcarbamoyl intermediate covalently bound to the catalytic serine as a consequence of its hydrolysis together with the release of the aminoguanidine moiety. Surprisingly, in this covalent adduct a phenyl group was found in the S1 specificity pocket, which usually recognizes positively charged residues. These findings provide new insights in the design of low basicity serine protease inhibitors.

Thrombin[1] (EC 3.4.21.5) is a trypsin-like serine proteinase, which plays a key role in blood coagulation and hemostasis. In recent years this enzyme has become a highly investigated target for drug design, to develop orally active, low molecular weight inhibitors, with potential use as antithrombotic drugs (*1*−*5*).

Three-dimensional structure analysis of a large number of natural and synthetic inhibitors complexed with thrombin has provided important insights into the understanding of the structure−activity relationship for antithrombotic agents, allowing the rational design of new thrombin inhibitors with improved pharmacological properties (*5*, *6*). Thrombin shows a similar structure in all complexes examined so far (*7*, *8*−*12*). The catalytic triad is located in a cleft at the interface of two β-barrel domains and constitutes part of the active site region, together with the polar S1 specificity pocket and the hydrophobic S2 and aryl-binding sites (the proteinase binding sites are defined according to Schechter and Berger; see ref 13).

In recent years, much progress has been made in the design and synthesis of highly effective, active site directed inhibitors with affinity for the enzyme in the low nanomolar range, as well as specificity against other related serine proteases (such as trypsin) (*1*−*5*), but the "ideal" thrombin inhibitor has not yet been obtained (*2*, *3*). The majority of available potent inhibitors generally show very poor oral bioavailability, bind strongly to plasma proteins, and/or do not have an appropriate plasma half-life after oral dosing (*2*, *3*). Many of these pharmacokinetic problems are due to the high basicity of the first-generation thrombin inhibitors, which contain arginine or amidine-based S1 anchoring moieties. These problems seem to be circumvented to some degree by the recent report of a large number of weakly basic inhibitors, which do not contain the above-mentioned S1 anchoring group but incorporate isosters of these moieties, as well as other structural modifications that reduce the highly basic pK_a of the parent inhibitors (in the range of 11−13) (*3*). Among the most promising new S1 anchoring

† Supported by grants from the CNR (Italian National Research Council) Target Project Agenzia 2000 and from the MIUR.

* To whom correspondence should be addressed. Fax: +39-055-4573385 (C.T.S.) or (+39-081-5514305) (G.D.S.). E-mail: claudiu.supuran@unifi.it (C.T.S.) or gmg@chemistry.unina.it (G.D.S.).

‡ Università degli Studi.

§ Istituto di Biostrutture e Bioimmagini-CNR.

[1] Abbreviations: thrombin, human α-thrombin; MQPA, (2*R*,4*R*)-4-methyl-1-[N^{α}-((*RS*)-3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginyl]-2-piperidinecarboxylic acid; NAPAP, N^{α}-(2-naphthylsulfonylglycyl)-DL-*p*-amidinophenylalanylpiperidine; 4-TAPAP, N^{α}-(4-toluenesulfonyl)-DL-*p*-amidinophenylalanylpiperidine; 3-TAPAP, N^{α}-(4-toluenesulfonyl)-DL-*m*-amidinophenylalanylpiperidine; DAPA, 1-[N^{α}-dansyl-L-arginyl]-4-ethylpiperidine; hirugen, sulfated $Tyr^{63'}$ *N*-acetylhirudin 53′−64′; Dmc-azaLys-OH, N^{α}-(*N,N*-dimethylcarbamoyl)-α-azalysine; Hepes, (4-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Ts-Gly-Pro-Arg-Pna, *N*-tosylglycyl-L-prolyl-L-arginine *p*-nitroanilide. S1, S2, and S3 are the active site proteinase subsites, defined according to Schechter and Berger (*13*).

10.1021/bi020512l CCC: $25.00
Published on Web 07/09/2003

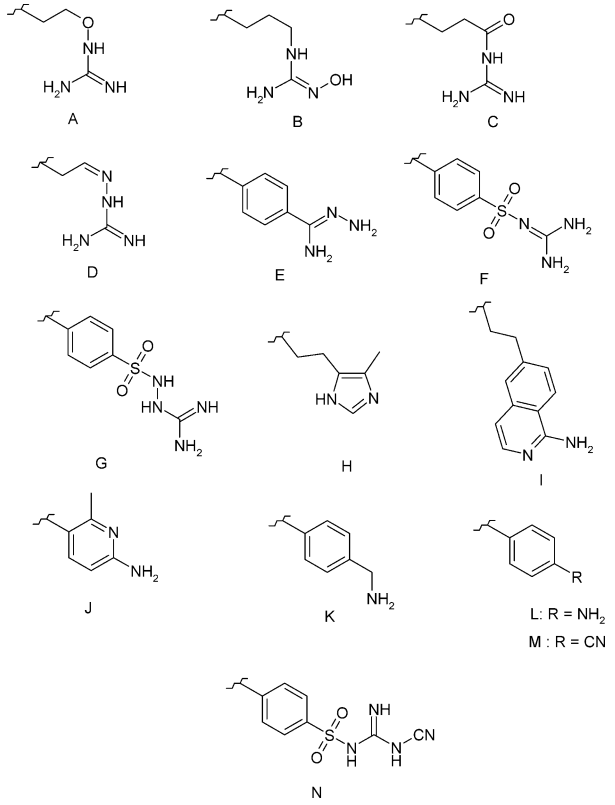


FIGURE 1: Examples of S1 anchoring groups with reduced basicity incorporated in thrombin inhibitors: A, oxoguanidine (*14*); B, hydroxyguanidine (*15*); C, acylguanidine (*16*); D, aminohydrazone (*17*); E, benzamidrazone (*18*); F, sulfonylguanidine (*19, 20*); G, sulfonylaminoguanidine (*21, 22*); H, imidazole (*23*); I, 1-aminoisoquinoline (*24*); J, 2-aminopyridine (*25*); K, benzylamine (*26*); L, aniline (*27, 28*), M, benzonitrile (*29*); N, arylsulfonyldicyandiamide (*30*).

groups recently reported are the following moieties: oxoguanidine (A) (pK_a 7) (*14*); hydroxyguanidine (B) (pK_a 9) (*15*); acylguanidine (C) (pK_a 7.6) (*16*); aminohydrazone (D) (pK_a 8.7) (*17*); benzamidrazone (E) (pK_a 8.9) (*18*); sulfonylguanidine (F) (pK_a 8.3) (*19, 20*); sulfonylaminoguanidine (G) (pK_a 8.4) (*21, 22*); imidazole (H) (pK_a around 7) (*23*); 1-aminoisoquinoline (I) (pK_a 7.5) (*24*); 2-aminopyridine (J) (pK_a around 7) (*25*); benzylamine (K) (pK_a 9.4) (*26*); aniline (L) (pK_a not provided but stated as "neutral") (*27, 28*); benzonitrile (M) (*29*); and arylsulfonyldicyandiamide (N) (pK_a of 7.9−8.2) (*30*) among others (Figure 1). Compounds incorporating some of these groups, such as LB-30057 (**1**), CGH 1668 (**2**), L-375378 (**3**), or the Organon derivative **4** (Figure 2), are currently under clinical investigation as antithrombotic drugs (*4, 5*).

In previous reports (*19*−*22*, *30*) we showed that derivatives incorporating benzenesulfonylguanidine/aminoguanidine or arylsulfonyldicyandiamide moieties as S1 anchoring groups (types **5** and **6**, Figure 2) not only possess good thrombin inhibitory properties (K_I values in the low nanomolar range) and specificity for thrombin over trypsin but also show pK_a values in the range of 8.0−8.4, making them interesting candidates for further investigations. In this paper, we extend our investigations for the search of new weakly basic serine protease inhibitors. Using sulfonylguanidine **7** and sulfonylaminoguanidine **8** derivatives (two compounds previously studied as candidates for the development of low basicity serine protease inhibitors) (*19, 21*) as lead molecules,

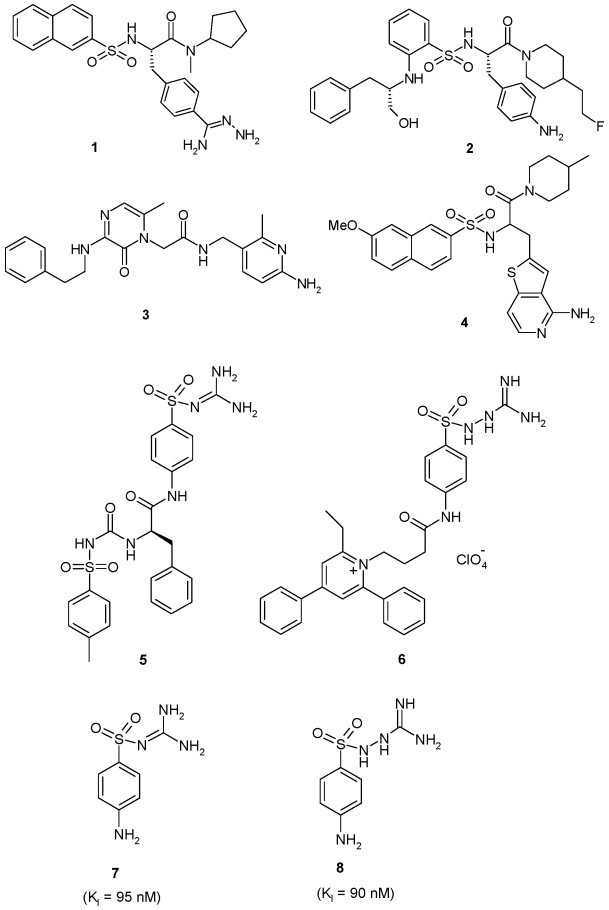


FIGURE 2: Examples of thrombin inhibitors: **1**, LB-30057; **2**, CGH 1668; **3**, L-375378; **4**, 2-(7-methoxynaphthalene-2-sulfonylamido)-3-(4-amino-5-azabenzothiophen-2-yl)propanoic acid 4-methylpiperidide; **5**, 4-[α-(4-toluenesulfonylureido)phenylalanylamido]-benzenesulfonylguanidine; **6**, 4-[(2-ethyl-4,6-diphenylpyridinium)-*N*-propylcarboxamido]benzenesulfonyl-*N*-1-aminoguanidine; **7**, 4-aminobenzenesulfonylguanidine; **8**, 4-aminobenzenesulfonyl-*N*-1-aminoguanidine.

we investigated the possibility of replacing the SO_2−NHC(═NH)NH_2 and SO_2−NHNHC(═NH)NH_2 binding functions of these two thrombin inhibitors with the corresponding acylated moieties, i.e., CO−NHC(═NH)NH_2 and CO−NHNHC(═NH)NH_2, respectively. These newly proposed series of compounds have been identified as a very simple class of low basicity thrombin inhibitors due to their K_I values. The molecular basis of their activity was derived by X-ray crystallographic studies on the complex between thrombin and one of these inhibitors. These findings could be useful for future drug design of serine protease inhibitors with low basicity and, thus, better bioavailability.

EXPERIMENTAL PROCEDURES

Materials. Human thrombin and human trypsin were purchased from Sigma-Aldrich (Milan, Italy); their concentrations were determined from their absorbance at 280 nm and the extinction coefficients furnished by the supplier. The activity of such preparations was in the range of 2500−3000 NIH units/mg. Acyl halides, N,N-disubstituted carbamoyl chlorides, guanidine hydrochloride, aminoguanidine carbonate, triethylamine, and other reagents used in the syntheses were commercially available compounds (from Sigma-Aldrich, Fluka, or Acros). Acetonitrile, acetone, dioxane,

ethyl acetate (E. Merck, Darmstadt, Germany), or other solvents used in the synthesis were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions. Inogatran was from Astra Hassle (Molndal, Sweden). Benzamidine, NAPAP, and Chromozym TH were from Sigma-Aldrich (Milan, Italy).

Synthesis. Melting points were determined on a heating plate microscope (not corrected), IR spectra as KBr pellets on a 400−4000 cm^{-1} Perkin-Elmer 16PC FTIR spectrometer, and ^1H NMR spectra on a Varian Gemini 300 apparatus (chemical shifts are expressed as δ values relative to Me_4Si as standard). Elemental analysis (±0.4% of the theoretical values, calculated for the proposed formulas; data not shown) was done on a Carlo Erba Instrument CHNS elemental analyzer, Model 1106. All reactions were monitored by thin-layer chromatography (TLC) using 0.25 mm precoated silica gel plates (E. Merck). Preparative HPLC was performed on a Dynamax-60A column (25 × 250 mm), with a Beckman EM-1760 instrument. The detection wavelength was 254 nm.

General Procedure for the Preparation of Compounds. A predetermined amount of 10 mM acyl halide (or N,N-disubstituted carbamoyl chloride) was dissolved in 50 mL of acetone, and a 2-fold excess amount of guanidine hydrochloride or aminoguanidine carbonate dissolved in 15 mL of water was added dropwise, together with the stoichiometric amount of solid $NaHCO_3$ or NaOH needed for the neutralization of the acid formed in the reaction (*19*, *21*). The mixture was stirred magnetically at 25 °C for 5 h before being acidified with 0.1 N HCl to pH 7.0. The acetone was evaporated in vacuo, and the obtained acylated derivatives, which precipitated by leaving the above-mentioned reaction mixture at 4 °C overnight, were filtered before being abundantly washed in water (in order to remove the inorganic salts and excess guanidine/aminoguanidine) and recrystallized from ethanol or ethanol−water (2:1 v/v). Preparative HPLC [Dynamax-60A column (25 × 250 mm); 90% acetonitrile/6% methanol/4% water; flow rate of 30 mL/min] was sometimes done in order to obtain some of these pure compounds as colorless solids. All compounds were fully characterized by elemental analysis and IR, ^1H NMR, and ^{13}C NMR spectroscopy.

Measurements of Thermodynamic Inhibition Constants. The potency of standard and newly obtained inhibitors was determined by the inhibition of the enzymatic (amidolytic) activity of these serine proteases, at 21 °C, using Ts-Gly-Pro-Arg-pNA (Chromozym TH) from Sigma as substrate, using the method of Lottenberg et al. (*31*) (Table 1). The substrate was reconstituted as 4 mM stock in ultrapure water and adjusted to pH 4 with hydrochloric acid. Substrate concentrations were determined from absorbance at the isosbestic wavelength for the peptide−*p*-nitroanilide−*p*-nitroaniline mixtures. Extinction coefficients of 8270 $L{\cdot}mol^{-1}{\cdot}cm^{-1}$ in the buffer used [0.01 M Hepes−0.01 M Tris−0.1 M NaCl−0.1% poly(ethylene glycol) 6000] were employed. The rate of *p*-nitroanilide hydrolysis was determined from the change in absorbance at 405 nm using an extinction coefficient for *p*-nitroaniline of 9920 $L{\cdot}mol^{-1}{\cdot}cm^{-1}$ for the above-mentioned reaction buffer. Measurements were made using a Perkin-Elmer spectrophotometer interfaced with a PC. Initial velocities were estimated using the direct linear plot-based procedure as reported by Lottenberg et al. (*31*). K_I values were then determined according to Dixon, using a linear regression program (*32*). The K_I values determined are the means of at least three determinations.

Table 1: Acylated Guanidine/Aminoguanidine Derivatives **9**−**48** Prepared in the Present Study with Their Inhibition Data against Human Thrombin and Human Trypsin

R−CONHC(=NH)NH_2 (**9**−**28**)
R−CONHNHC(=NH)NH_2 (**29**−**48**)

		K_I (nM)[a]	
compound	R	thrombin	trypsin
9	Ph	425	521
10	*p*-F−C_6H_4−	410	515
11	*p*-Cl−C_6H_4−	402	504
12	*p*-Br−C_6H_4−	248	415
13	*p*-I−C_6H_4−	241	413
14	*p*-CH_3−C_6H_4−	409	513
15	*p*-Me_2N−C_6H_4−	370	493
16	C_6F_5−	165	430
17	*n*-C_8F_{17}	330	466
18	Ph_2CH−	42	110
19	2-thienyl	570	964
20	2-pyridyl	371	542
21	3-pyridyl	365	510
22	4-pyridyl	257	383
23	Me_2N−	124	256
24	Et_2N−	117	244
25	*i*-Pr_2N−	91	118
26	*n*-Bu_2N−	106	213
27	Ph(Me)N−	74	180
28	Ph_2N−	40	125
29	Ph−	412	489
30	*p*-F−C_6H_4−	381	437
31	*p*-Cl−C_6H_4−	375	429
32	*p*-Br−C_6H_4−	233	406
33	*p*-I−C_6H_4−	227	395
34	*p*-CH_3−C_6H_4−	403	476
35	*p*-Me_2N−C_6H_4−	354	481
36	C_6F_5−	150	413
37	*n*-C_8F_{17}	304	437
38	Ph_2CH−	34	98
39	2-thienyl	542	895
40	2-pyridyl	321	530
41	3-pyridyl	306	504
42	4-pyridyl	217	369
43	Me_2N−	98	243
44	Et_2N−	95	217
45	*i*-Pr_2N−	74	116
46	*n*-Bu_2N−	90	178
47	Ph(Me)N−	40	126
48	Ph_2N−	24	110
inogatran		15	nt
NAPAP		6	690

[a] K_I values were obtained from Dixon plots using a linear regression program from at least three different assays. Spreads around the mean (data not shown) were ±10−15% of the shown values.

pK_a Measurements. The half-neutralization point was measured by titrating the organic acids/bases with 0.05 N NaOH and 0.05 N HCl in EtOH−water (30% v/v), using a glass electrode, as described by Bell and Roblin (*33*) for some antibacterial sulfonamides and adapted by us for previously reported serine protease inhibitors (*19*, *21*).

Assessment of the Acylating Activity of Inhibitors. Thrombin and trypsin were incubated for various periods of time (typically 30 min to 6 h) with some of the most active inhibitors detected among the derivatives reported in this paper (for example, **28**, **38**, **48**, or NAPAP as standard). Enzyme concentrations were 5.4 nM for thrombin and 140 nM for trypsin, whereas the inhibitor concentration varied between 40 and 200 nM. Enzyme treated identically except for the presence of inhibitor was considered as the blank,

and its activity was considered as 100%. After the incubation period, the enzyme−inhibitor adduct was extensively dialyzed in the assay buffer [0.01 M Hepes−0.01 M Tris−0.1 M NaCl−0.1% poly(ethylene glycol) 6000] and the residual enzyme activity determined as described above, using the chromogenic substrate Ts-Gly-Pro-Arg-pNA. For the determination of the inactivation kinetic parameters by the prelevation method (*34*−*36*), a stock solution of 3.8 μM thrombin and 13.7 μM trypsin was incubated with variable concentrations of inhibitors (compounds **38** and **48**, respectively), working at ratios of enzyme:inhibitor between 1:1 and 1:20. Aliquots were taken from this stock at different periods (6, 12, 24, 48, 72, and 96 h, respectively) and diluted 1:100 with the assay buffer mentioned above, and the residual enzyme activity was assessed as described above. A linear regression program was employed for obtaining k_{inact}, as described in the literature (*34*−*36*).

Crystallization and X-ray Data Collection. Crystals of the α-thrombin−hirugen−*N*,*N*-diphenylcarbamoyl complex were obtained by soaking the enzyme−hirugen crystals (*37*) (monoclinic crystal form, space group *C*2) in solutions containing 5.0×10^{-4} M *N*,*N*-diphenylcarbamoylaminoguanidine (compound **48** in Table 1) at pH 7.0 for 4 days at 4.0 °C. In fact, crystals of human α-thrombin suitable for X-ray analysis can be grown properly in the presence of hirugen, which binds to the enzyme fibrinogen exosite and does not hinder small inhibitor accessibility to the active site.

Data collection was carried out on a Nonius DIP2030 imaging plate using Cu Kα radiation and one crystal of dimensions 0.3 mm × 0.3 mm × 0.8 mm. The crystal diffracted up to 1.90 Å resolution.

Diffracted intensities were processed using the HKL crystallographic data reduction package (Denzo/Scalepack) (*38*). A total of 125775 reflections were measured (unit cell parameters $a = 71.41$ Å, $b = 72.19$ Å, $c = 72.70$ Å, and $\beta = 100.57°$) and reduced to 28435 unique reflections (completeness 98.5% and 96.2% in the 20.0−1.90 Å and in the 1.97−1.90 Å resolution ranges, respectively; *R*-sym = 5.2%).

Data processing statistics are given in Table 2.

Refinement. The structure of the thrombin−hirugen−*N*,*N*-diphenylcarbamoyl complex was analyzed by difference Fourier techniques using the PDB (*39*) file 1hah (*37*) as a starting model for refinement. Water molecules were removed from the starting model prior to structure factor and phase calculations. The crystallographic *R*-factor and *R*-free, calculated in the 20.0−1.90 Å resolution range on the basis of the starting model coordinates, were 0.323 and 0.311, respectively. Fourier maps calculated with $3F_o - 2F_c$ and $F_o - F_c$ coefficients (F_o are the observed structure factors for the complex and F_c those calculated on the basis of the model atomic coordinates) showed prominent electron density features in the active site region. After an initial refinement, limited to the enzyme−hirugen adduct structure (*R*-factor 0.236 and *R*-free 0.247), a model for the *N*,*N*-diphenylcarbamoyl moiety acylating Ser195 was easily built and introduced into the atomic coordinates set for further refinement, which proceeded to convergence with continuous map inspection and model updates. The refinement was carried out with the program CNS (*40*) while model building and map inspections were performed using the program O (*41*). The final crystallographic *R*-factor and *R*-free values calculated for the 28435 observed reflections (in the 20.00−1.90 Å resolution range) were 0.183 and 0.204, respectively. The refined model included 2473 complex atoms, 15 atoms belonging to the inhibitor, and 147 water molecules. The rms deviations from the ideal value of bond lengths and bond angles (*42*) were 0.010 Å and 1.53°, respectively. The average temperature factor (*B*) for all atoms was 32.19 Å^2. The stereochemical quality of the model was assessed by Procheck (*43*). The most favored and additionally allowed regions of the Ramachandran plot contained 100% of the non-glycine residues.

Table 2: Data Collection and Refinement Statistics

Data Collection Statistics (20.00−1.90 Å)	
temperature (K)	298
total reflections	125775
unique reflections	28435
completeness (%)	
overall	98.5
outermost data shell	96.2
R-sym[a]	
overall	0.052
outermost data shell	0.247
mean $I/\sigma(I)$	
overall	16.3
outermost data shell	3.4
Refinement Statistics (20.00−1.90 Å)	
R-factor[b] (%)	18.3
R-free[b] (%)	20.4
rmsd from ideal geometry	
bond lengths (Å)	0.010
bond angles (deg)	1.53
no. of protein atoms	2473
no. of water molecules	147
average *B* factor (Å^2)	32.19

[a] $R_{sym} = \sum|I_i - \langle I\rangle|/\sum I_i$ over all reflections. [b] *R*-factor $= \sum|F_o - F_c|/\sum F_o$; *R*-free was calculated with 10% of the data withheld from refinement.

The statistics for refinement are summarized in Table 2.

Accession Numbers. Coordinates and structure factors have been deposited with the Protein Data Bank (accession code 1NO9).

RESULTS AND DISCUSSION

Synthesis and K_I Measurements. The new compounds reported in this paper, of types **9**−**48** (Table 1), were obtained by reaction of acyl halides or N,N-disubstituted carbamoyl halides, with guanidine or aminoguanidine, similar to the procedure previously reported (*19*, *21*) for the alkyl/arylsulfonylation of guanidine/aminoguanidine. The reaction was performed in Schotten−Baumann conditions and led to the desired derivatives in high yields (65−90%; data not shown) (*19*, *21*). No protecting groups for the guanidino/aminoguanidino moieties were necessary, as we have shown previously that only one sulfonylation/acylation product was obtained in the reaction conditions of our experiments (*19*, *21*). The new compounds were fully characterized by standard procedures (elemental analysis, IR, ^{1}H NMR, and ^{13}C NMR spectroscopy), which confirmed their structure.

The following structure−activity correlations have been observed for the series of acylated guanidine/aminoguanidine derivatives **9**−**48** (Table 1): (i) Derivatives incorporating phenyl, 4-substituted phenyl, perfluoroaryl/alkyl, or pyridyl moieties showed moderate thrombin/trypsin inhibitory activity with inhibition constants in the range of 150−425 nM against thrombin and of 413−521 nM against trypsin. The

Table 3: pK_a Data of Several Serine Protease Inhibitors

compound	pK_a[a] for guanidino/amidino moiety
inogatran	12.3
NAPAP	12.6
sulfaguanidine	8.4
28	8.7
38	8.6
48	8.4

[a] pK_a values were determined in 30% EtOH−water (v/v) as described in Experimental Procedures.

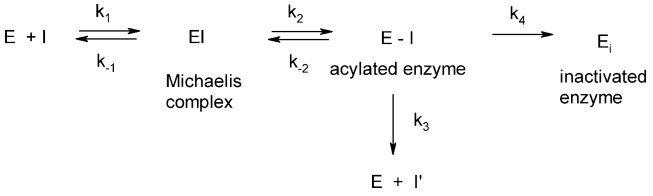
most potent inhibitors in this subseries were the perfluorophenyl-substituted derivatives **16** and **36** (K_I values of 165 and 150 nM, respectively, against thrombin). (ii) Stronger inhibition has been observed with compounds incorporating bulkier R groups, such as the diphenylacetic derivatives **18** and **38**, as well as the compounds obtained from N,N-disubstituted carbamoyl chlorides, **23**−**28** and **43**−**48**. These compounds showed inhibition constants in the range of 24−124 nM against thrombin and of 110−243 nM against trypsin. (iii) The least active compounds in this series were those containing 2-thienyl moieties in their molecule (**19** and **39**) with inhibition constants of 540−570 nM against thrombin and of 895−965 nM against trypsin. (iv) In the subseries of the compounds obtained from N,N-disubstituted carbamoyl chlorides, **23**−**28** and **43**−**48** (the best inhibitors reported here), clearly the enhanced thrombin inhibitory activity correlated with increasingly bulky groups substituting the urea moiety. Thus, the weakest inhibitors in this subseries were those containing two methyl groups (**23** and **43**), whereas the strongest ones were those with two phenyl moieties (**28** and **48**), which showed inhibitory power comparable to that of the very potent inhibitors NAPAP and inogatran (*44*). The other derivatives showed an intermediate activity between these two extremes. It is also interesting to note that the diphenylacetic derivatives **18** and **38** (isosteric with the ureas **28** and **48**, respectively) are the most active in the first subseries of derivatives, i.e., the one without an additional nitrogen, which has been mentioned in (i). (v) Without exception, acylaminoguanidines were more potent thrombin/trypsin inhibitors than the corresponding acylguanidines. (vi) All of these new inhibitors showed higher affinity for thrombin over the related protease trypsin, although an important discrimination is not achieved.

pK_a values have been measured for several simple derivatives investigated here to confirm their reduced basicity (Table 3). Indeed, such values were in the range of 8.4−8.7 pK_a units, proving that this anchoring group may constitute a valid alternative for obtaining serine protease inhibitors with weak basicity. Therefore, simply on the basis of their pK_a values, these compounds should present the expected increase in bioavailability.

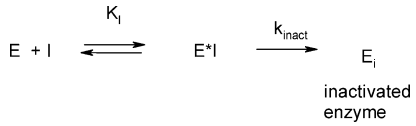
Kinetic Analysis of the Inhibitors. The inhibition data reported above have been obtained considering a reversible, noncovalent interaction between enzyme and inhibitor. More generally, the interaction of such a serine protease with an inhibitor can be described as shown in Scheme 1.

Thus, the enzyme (E) and the inhibitor (I) form the Michaelis complex (E−I) by an equilibrium characterized by the kinetic (on/off) constants k_1 and k_{-1} (and the

Scheme 1



Scheme 2



thermodynamic inhibition constant $K_I = k_{-1}/k_1$). As stated earlier, the measurements of K_I reported above were made on the assumption that these new compounds behave as reversible inhibitors. We analyzed the possibility that some of them could act as inactivators of these serine proteases. In this case, the Michaelis complex in which the inhibitor is noncovalently bound to the enzyme reacts with chemical groups of the active site (generally the nucleophilic OH moiety of Ser195), acylating this moiety and giving rise to an acylated enzyme (E−I), by an equilibrium characterized by the kinetic constants k_2 and k_{-2}, respectively (Scheme 1). This acylated enzyme may be hydrolyzed, regenerating the active enzyme (step characterized by the kinetic constant k_3), or an inactivated enzyme may be formed due to further chemical reaction between certain moieties of the enzyme active site and the inhibitor molecule (step characterized by the kinetic constant k_4) (Scheme 1).

In the case of an irreversible inactivation, no distinction between the two enzyme−inhibitor species EI and E−I (Scheme 1) (which are now represented by the species E*I) can be made, and only the accumulation of E_i can be followed. Since generally also $k_3 \ll k_4$, the processes described in Scheme 1 can be simplified, leading to Scheme 2, in which only two thermodynamic/kinetic constants intervene: the inhibition constant K_I and a new parameter, the inactivation constant k_{inact}, which can be determined experimentally (*34*−*36*).

To assess whether the inhibitors reported here also act as acylating agents, in an initial group of experiments, the two serine proteases were incubated with different concentrations of potent inhibitors (such as compounds **28**, **38**, and **48**, with NAPAP also included in the experiments as standard) belonging to the two classes investigated here for variable periods of 30 min to 6 h (it should be stressed that the K_I presented in Table 1 were determined after the enzymes were incubated with the inhibitors for 30 min). After incubation, the enzyme−inhibitor adduct solutions were extensively dialyzed in buffer, and the recovered enzyme activity was assessed. The results of these experiments are shown in Table 4.

As seen from the data presented in Table 4, at 1−3 h of incubation, all of these compounds act as reversible inhibitors, similar to NAPAP, a well-known, standard serine protease inhibitor. Following incubation, close to 100% enzymatic activity (within the range of the experimental errors) was recovered, proving that the inhibition constants measured and shown in Table 1 indeed describe the behavior of this type of inhibitor. The only compounds that showed

Table 4: Recovered Enzymatic Activity after Incubation of Thrombin/Trypsin with Inhibitors **28**, **38**, **48**, and NAPAP for Variable Periods of Time, Followed by Dialysis[a]

	% enzyme activity[b]			
system	30 min	1 h	3 h	6 h
thrombin[c] + **28**[e]	97	96	98	97
thrombin[c] + **38**[e]	98	97	98	91
thrombin[c] + **48**[e]	97	99	96	83
thrombin[c] + NAPAP[e]	98	99	96	98
trypsin[d] + **28**[f]	99	96	95	97
trypsin[d] + **48**[f]	98	97	97	89
trypsin[d] + NAPAP[f]	97	96	98	97

[a] The activity of enzyme identically treated (but with no inhibitor added) was taken as 100%. [b] Errors in the range of 3−5% (from three different assays). [c] [Thrombin] = 5.4 nM. [d] [Trypsin] = 140 nM. [e] [Inhibitor] = 40 nM. [f] [Inhibitor] = 200 nM.

a deviation from this behavior were **38** and **48** at longer incubation times (6 h or longer; see Table 4; data not shown), cases in which a marked reduction in the enzyme activity recovered started to become obvious. These preliminary experiments allowed us to draw the conclusion that the acylaminoguanidines can act as enzyme inactivators using incubation times longer than 3 h, in contrast to acylguanidines and NAPAP which simply act as noncovalent, reversible serine protease inhibitors.

To determine the inactivation parameter k_{inact} for the acylaminoguanidine type of inhibitors, we used the prelevation method originally described by Kitz and Wilson (*34*) and subsequently adapted by Pochet et al. for following the inactivation of a large number of serine proteases, including thrombin and trypsin (*35*−*36*). In this method, the enzyme is incubated with variable concentrations of inhibitor, and the residual enzyme activity is assessed at different periods of time after a strong dilution of the enzyme−inhibitor adduct. This residual activity, ϵ, is defined by the equation (*34*−*36*):

$$[\epsilon] = [E]_T - [E_i] = [E] + [E^*I] \quad (1)$$

where $[E]_T$ = total enzyme concentration.

Considering the fact that K_I is defined by the equation $K_I = [I][E]/[E^*I]$, eqs 2−4 can be obtained:

$$[\epsilon] = [E]_T e^{-k_{obs}t} \quad (2)$$

$$k_{obs} = k_{inact}[I]/(K_I + [I]) \quad (3)$$

$$\text{for } [I] \ll K_I, \quad k_{obs} = (k_{inact}/K_I)[I] \quad (4)$$

By experimentally determining k_{obs} at different inhibitor concentrations, it was possible to assay the inactivation parameter k_{inact}. This was achieved for compounds **38** and **48**, against both thrombin and trypsin (Table 5).

As seen from data presented in Table 5, both of the acylaminoguanidine derivatives **38** and **48** act as irreversible inactivators against thrombin and trypsin. The kinetic parameter k_{inact} indicated the process as a rather slow reaction. Thrombin was acylated better than trypsin, although the differences between the two proteases were not relevant. The diphenylcarbamoyl derivative **48** was a slightly more efficient acylating agent with respect to the corresponding diphenylacetyl derivative **38**.

Table 5: Inactivation Parameters of Thrombin and Trypsin with Compounds **38** and **48**

	k_{inact} (s^{-1})[b]	
inhibitor[a]	thrombin	trypsin
38	$(1.4 \pm 0.3) \times 10^{-5}$	$(0.7 \pm 0.2) \times 10^{-6}$
48	$(4.8 \pm 0.6) \times 10^{-5}$	$(1.9 \pm 0.5) \times 10^{-6}$

[a] The ratio of enzyme/inhibitor was in the range of 1:1 to 1:20; [thrombin] = 3.8 μM; [trypsin] = 13.7 μM. [b] Mean from three different assays.

Crystallographic Studies. To assess the molecular basis responsible for the high affinity toward thrombin observed for these new classes of inhibitors, we solved the crystal structure of the hirugen-bound enzyme complexed with the most active compound (*N*,*N*-diphenylcarbamoylaminoguanidine) from these series (compound **48** in Table 1). Following extensive incubation between enzyme and inhibitor this molecule was shown to act as an irreversible inhibitor.

The three-dimensional structure was analyzed by difference Fourier techniques, the crystals being isomorphous to the native thrombin−hirugen complex (*37*). Inspection of the electron density within the enzyme active site, at various stages of the crystallographic refinement, demonstrated the occurrence of the bound aminoguanidine derivative. A careful analysis of this region clearly suggested the existence of a covalent bond between the thrombin Ser195OG atom and the inhibitor. The initial hypothesis of a specific binding to the carbonyl carbon atom of *N*,*N*-diphenylcarbamoylaminoguanidine (Figure 3, intermediate A), thus mimicking the first tetrahedral intermediate occurring during catalysis, was not supported by the electron density in the difference Fourier map. On the contrary, a species containing an *N*,*N*-diphenylcarbamoyl moiety bound to the enzyme (Figure 3, intermediate B), similar to a canonical acyl-enzyme form, was proposed as an intermediate with a chemical structure compatible with the observed data (Figure 4). This species is formed following the release of the aminoguanidine group from the tetrahedral *N*,*N*-diphenylcarbamoylaminoguanidine-bound enzyme mentioned above (Figure 3). The slow deacylation rate for this *N*,*N*-diphenylcarbamoyl−thrombin derivative during catalysis should be responsible for its recovery within the crystals. The nature of this intermediate was confirmed by electrospray ionization mass spectrometry measurements of the enzyme before and following crystallization.

The main protein−inhibitor interactions are schematically depicted in Figure 5. According to this figure, the bound molecule presents a peculiar spatial arrangement with respect to the structure of the other thrombin−inhibitor complexes described so far (*5*, *6*). In particular, the *N*,*N*-diphenylcarbamoyl moiety covalently bound to Ser195OG presents one of the two phenyl groups accommodated in the S1 specificity pocket, stabilized by several van der Waals interactions (<5.0 Å) with residues Asp189, Ala190, Cys191, Ser214, Trp215, Gly216, Gly219, and Cys220. This finding is very uncommon since the S1 subsite is usually involved in polar-dependent interactions with basic amino acids. Moreover, the inhibitor molecule does not occupy the S2 and the aryl-binding subsites. In fact, the second phenyl group, occurring at van der Waals distances (<5.0 Å) from thrombin residues Glu192, Trp60D, Trp215, and Gly216,

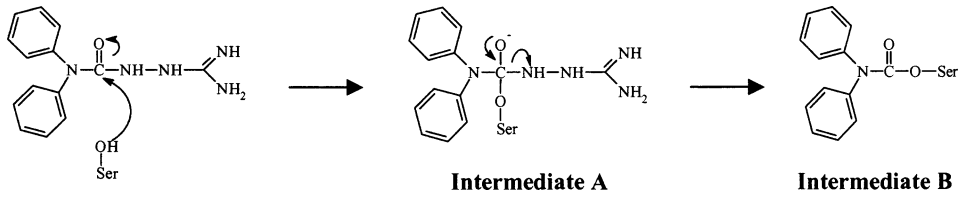


FIGURE 3: Reaction of thrombin with *N*,*N*-diphenylcarbamoylaminoguanidine.

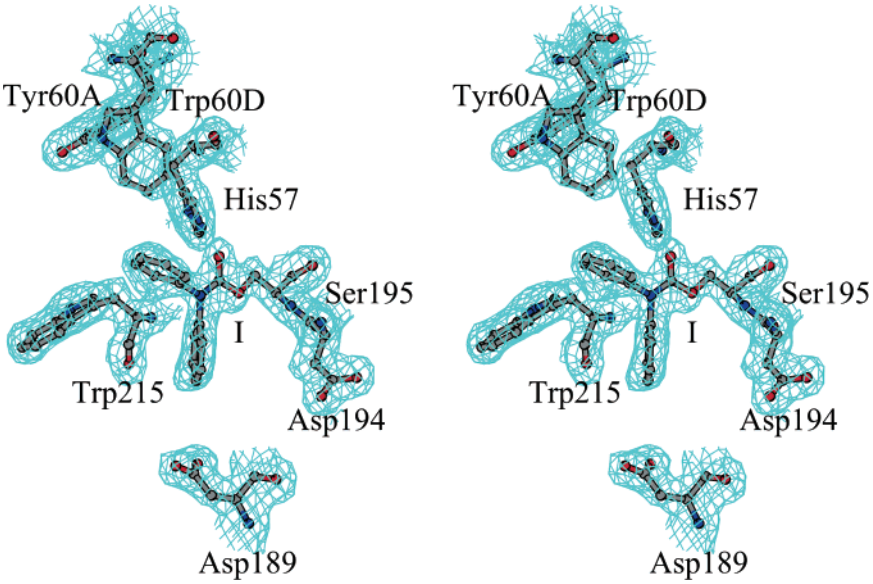


FIGURE 4: Simulated annealing (*29*) omit $|2F_o - F_c|$ electron density map, computed at 1.90 Å and contoured at 1.0 σ. The catalytic site region is shown. The *N*,*N*-diphenylcarbamoyl molecule covalently bound to the catalytic serine is identified with the I label.

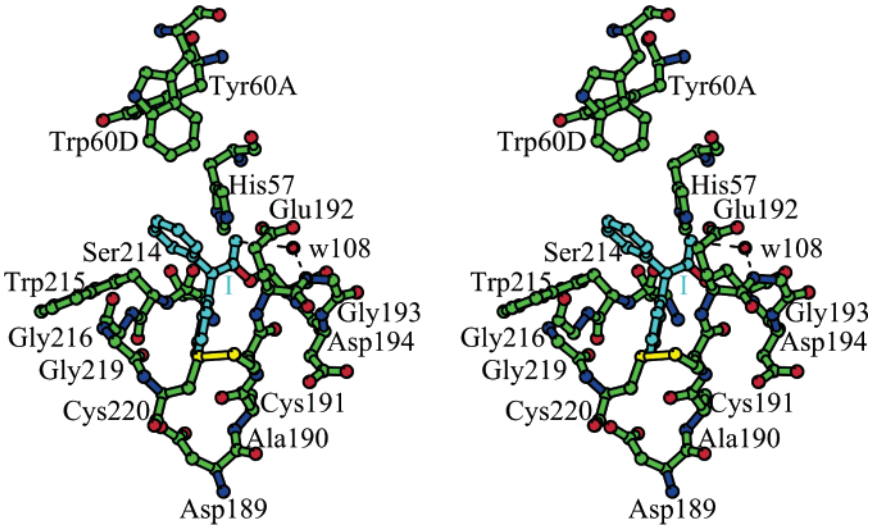


FIGURE 5: Stereoview of the active site region in the thrombin−*N*,*N*-diphenylcarbamoyl complex showing the residues participating in recognition of the acylating *N*,*N*-diphenylcarbamoyl molecule (labeled I). Hydrogen bonds are shown.

points toward S2 but is too small in structure to be hosted by this subsite.

Finally, a careful analysis of the diphenylcarbamoyl−thrombin intermediate geometry within the active site revealed that the carbonyl group of the scissile *N*,*N*-diphenylcarbamoyl moiety does not properly fill the oxyanion binding hole. In fact, the C═O bond is rotated by about 90° with respect to the canonical orientation, producing the functionally effective H-bond interactions with Gly193N and Ser195N. In this case a water molecule (W108) that, together with the His57NE2 atom, is hydrogen bonded to the carbonyl oxygen of the inhibitor occupies the oxyanion hole (Figure 5).

The three-dimensional structure of the thrombin molecule within the complex was clearly very similar to that observed in other thrombin−inhibitor complexes reported in the literature to date (*5*, *37*). When superimposing all of the thrombin Cα atoms with those of the enzyme in the thrombin−hirugen complex (PDB code 1hah) used as a reference (*37*), an rms deviation of 0.32 Å was calculated. Weak or missing electron densities, as already noted in other thrombin complexes crystallized in the same monoclinic

form, were observed in the N-terminal (residues 1H-1D) and C-terminal (residues 14M−15) regions of the A-chain, in the γ autolysis loop (residues 148−149E), and in the C-terminal (residues 246−247) segment of the B-chain.

A careful analysis of the catalytic site region revealed that the structure of the enzyme has undergone some minor changes following inhibitor binding. In particular, residues His57 and Asp102, within the catalytic triad, were entirely superimposable to their corresponding residues in the thrombin−hirugen complex, whereas the side chain conformation of Ser195 was different. As a matter of fact, a variation in the Ser195 χ^1 angle from −87° (thrombin−hirugen complex) to −58° (structure reported here) was observed as a consequence of the inhibitor covalent binding.

Analysis of the solvent molecule distribution around the active site showed an overall agreement with the solvent structure for other thrombin complexes previously reported (*5*, *37*). However, it is worth noting that the hydrophobic nature of the inhibitor causes a release of several water molecules from the complex to the bulk solvent. In particular, a comparison between the present structure and several thrombin−inhibitor complexes (*5*, *37*) reveals the lack in our structure of four water molecules: three of these (W519, W429, and W462 in the thrombin−hirugen structure) are displaced from the S1 pocket by one of the phenyl groups, while the fourth (W580 in the thrombin−hirugen structure) is displaced from the S2 pocket by the other phenyl group.

Several structural studies on serine proteinase acyl-enzymes have allowed the identification of different factors responsible for the acyl-enzyme stabilization (*45*−*47*). According to these investigations a structural explanation of the low rate of hydrolysis of the acyl-enzyme in the structure here reported could be tentatively provided. In fact, the incorrect positioning of the carbonyl function of the diphenylcarbamoyl moiety within the oxyanion hole and the absence of specific interactions with Gly193 and Ser195 could affect the electrophilic character of this group, significantly reducing its reactivity toward water. This peculiar orientation could be due to the rigidity of the carbamylic bond with respect to that observed for natural substrates, as well as the aforementioned absence of interactions with the S2 and aryl-binding subsites.

These findings are in agreement with studies previously reported for the thrombin−Dmc-azaLys acyl-enzyme complex (*47*). In fact, also in this case the absence of a significant hydrolysis of the acyl-enzyme species results from an improper occupation of the proteinase oxyanion binding hole by the acyl bond carbonyl group. Unproductive orientation of the acyl bond was associated with the planar and rigid nature of the inhibitor as well as the absence of interactions with the S2 and aryl-binding subsites of the proteinase. According to this hypothesis, the introduction in α-azalysine derivatives of substituents able to interact with the S2 and aryl-binding subsites allows an appropriate orientation of the carbonyl group within the oxyanion hole and a consequent increase in the deacylation rate of the complex (*48*).

Concluding Remarks. A large number of active site directed thrombin inhibitors have been synthesized and structurally characterized so far (*5*). The most potent synthetic inhibitors often incorporate into their structure a guanidino or amidino group as in the case of the arginine-based MQPA and DAPA and benzamidine-based NAPAP, 4-TAPAP, and 3-TAPAP derivatives (*49*−*57*). Some of these compounds have been extensively tested for clinical applications and even registered in some countries for use in deep vein thrombosis. The X-ray structure of some of these inhibitors complexed with thrombin revealed that they are able to bind the enzyme active site in a "U-shaped" conformation (*56*, *57*). The N- and C-terminal hydrophobic moieties fill the aryl-binding site and S2 cavity, respectively. The basic arginine or benzamidine group is located in the specificity S1 pocket and interacts with Asp189.

In this paper, we report the synthesis and biochemical characterization of two new classes of thrombin inhibitors containing the aforementioned guanidine functionality. These new compounds present good thrombin inhibitory properties with K_I values in the low nanomolar range. All of these compounds initially act as noncovalent, reversible inhibitors, but only the acylaminoguanidines are able to acylate the active site Ser195 when incubated for long periods with the enzyme. The inactivation parameters k_{inact} show these processes to be rather slow.

The crystallographic analysis of the complex between thrombin and the most active molecule from these series revealed a distinctive inhibitor binding mode. As a matter of fact, this species forms an intermediate covalently bound to the catalytic serine as a consequence of its hydrolysis and the release of the aminoguanidine moiety. Moreover, no canonical interactions usually reported for classical inhibitors were observed. The S1 subsite was occupied by a phenyl group instead of a charged one, and the S2 or the aryl-binding sites, conventionally involved in binding to substrates and inhibitors, were totally empty.

On the basis of these results, we hypothesize that other members of the acylaminoguanidine class of compounds act as suicide inhibitors, leading to a covalent intermediate in which the specificity S1 pocket of the enzyme is occupied by a hydrophobic moiety. These findings shed more light on the design of low basicity thrombin inhibitors, which may have important clinical applications as antithrombotic drugs. Moreover, it is envisaged that this approach may also be adapted to other therapeutically important serine proteases, which are targets for drug design (*58*), such as factor Xa, tryptase, u-PA, or the viral serine proteases (isolated in herpes viruses or in the hepatitis C virus).

ACKNOWLEDGMENT

We thank Giuseppe Perretta and Maurizio Amendola for technical support.

REFERENCES

1. Vacca, J. P. (2000) *Curr. Opin. Chem. Biol. 4*, 394−400.
2. Leung, D., Abbenante, G., and Fairlie, D. P. (2000) *J. Med. Chem. 43*, 305−341.
3. Hauptmann, J., and Stürzebecher, J. (1999) *Thromb. Res. 93*, 203−241.
4. Rewinkel, J. B. M., and Adang, A. E. P. (1999) *Curr. Pharm. Des. 5*, 1043−1075.
5. Pavone, V., De Simone, G., Nastri, F., Galdiero, S., Staiano, N., Lombardi, A., and Pedone, C. (1998) *Biol. Chem. 379*, 987−1006.
6. Lombardi, A., De Simone, G., Galdiero, S., Staiano, N., Nastri, F., and Pavone, V. (1999) *Biopolymers 51*, 19−39.
7. Bode, W., Huber, R., Rydel, T. J., and Tulinsky, A. (1992) in *Thrombin Structure and Function* (Berliner, L. J., Ed.) Plenum Press, New York.

8. Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) *EMBO J. 8*, 3467−3475.
9. Bode, W., Turk, D., and Karshikov, A. (1992) *Protein Sci. 1*, 426−471.
10. Stubbs, M. T., and Bode, W. (1993) *Thromb. Res. 69*, 1−58.
11. Tulisky, A., and Qiu, X. (1993) *Blood Coagulation Fibrinolysis 4*, 305−312.
12. Di Cera, E., Dand, Q. D., and Ayala, Y. M. (1997) *Cell. Mol. Life Sci. 53*, 701−730.
13. Schechter, I., and Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162.
14. Misra, R. N., Kelly, Y. F., Brown, B. R., Roberts, D. G. M., Cong, S., and Seiler, S. M. (1994) *Bioorg. Med. Chem. Lett. 4*, 2165−2171.
15. Kim, S., Hwang, S. Y., Kim, Y. K., and Oh, Y. S. (1997) *Bioorg. Med. Chem. Lett. 7*, 769−774.
16. Adang, A. E. P., Lucas, H., de Man, A. P. A., Engh, R. A., and Grootenhuis, P. D. J. (1998) *Bioorg. Med. Chem. Lett. 8*, 3603−3608.
17. Soll, R. M., Lu, T., Tomczuk, B., Illig, C. R., Fedde, C., Eisenagel, S., Bone, R., Murphy, L., Spurlino, J., and Salemme, F. R. (2000) *Bioorg. Med. Chem. Lett. 10*, 1−4.
18. Oh, Y. S., Yun, M., Hwang, S. Y., Hong, S., Shin, Y., Lee, K., Yoon, K. H., Yoo, Y. J., Kim, D. S., Lee, S. H., Lee, Y. H., Park, H. D., Lee, C. H., Lee, S. K., and Kim, S. (1998) *Bioorg. Med. Chem. Lett. 8*, 631−634.
19. Supuran, C. T., Scozzafava, A., Briganti, F., and Clare, B. W. (2000) *J. Med. Chem. 43*, 1793−1806.
20. Scozzafava, A., Briganti, F., and Supuran, C. T. (1999) *Eur. J. Med. Chem. 34*, 939−952.
21. Clare, B. W., Scozzafava, A., Briganti, F., Iorga, B., and Supuran, C. T. (2000) *J. Enzyme Inhib. 15*, 235−264.
22. Supuran, C. T., Briganti, F., Scozzafava, A., and Ilies, M. A. (2000) *J. Enzyme Inhib. 15*, 335−356.
23. Wiley, M. R., Weir, L. C., Briggs, S. L., Chirgadze, N. Y., Clawson, D., Gilford-Moore, D. S., Schacht, A. L., Smith, G. F., Vasudevan, V., Zornes, L. L., and Klimkowski, V. J. (1999) *Bioorg. Med. Chem. Lett. 9*, 2767−2772.
24. Rewinkel, J. B. M., Lucas, H., van Galen, P. J. M., Noach, A. B. J., van Dinther, T. G., Rood, A. M. M., Jenneboer, A. J. S. M., and van Boeckel, C. A. A. (1999) *Bioorg. Med. Chem. Lett. 9*, 685−690.
25. Issacs, R. C. A., Cutrona, K. J., Newton, C. L., Sanderson, P. E. J., Solinsky, M. G., Baskin, E. P., Chen, I. W., Cooper, C. M., Cook, J. J., Gardell, S. J., Lewis, S. D., Lucas, R. J., Lyle, E. A., Lynch, J. L., Naylor-Olsen, A. M., Stranieri, M. T., Vastag, K., and Vacca, J. P. (1998) *Bioorg. Med. Chem. Lett. 8*, 1719−1724.
26. Hanessian, S., Balaux, E., Musil, D., Olsson, L. L., and Nilsson, I. (2000) *Bioorg. Med. Chem. Lett. 10*, 243−247.
27. Ambler, J., Baker, E., Brown, L., Butler, P., Farr, D., Dunnet, K., Le Grand, D., Janus, D., Jones, D., Menear, K., Mercer, M., Smith, G., Talbot, M., and Tweed, M. (1998) *Bioorg. Med. Chem. Lett. 8*, 3583−3588.
28. Ambler, J., Baker, E., Bentley, D., Brown, L., Butler, K., Butler, P., Farr, D., Dunnet, K., Le Grand, D., Hayler, J., Janus, D., Jones, D., Menear, K., Mercer, M., Smith, G., Talbot, M., and Tweed, M. (1999) *Bioorg. Med. Chem. Lett. 9*, 737−742.
29. Lee, S. L., Alexander, R. S., Smallwood, A., Trievel, R., Mersinger, L., Weber, P. C., and Kettner, C. (1997) *Biochemistry 36*, 13180−13186.
30. Clare, B. W., Scozzafava, A., and Supuran, C. T. (2001) *J. Enzyme Inhib. 16*, 1−13.
31. Lottenberg, R., Christensen, U., Jackson, C. M., and Coleman, P. L. (1981) *Methods Enzymol. 80*, 341−361.
32. Hemker, H. C., Willems, G. M., and Beguin, S. (1986) *Thromb. Haemostas*is 56, 9−17.
33. Bell, P. H., and Roblin, R. O. (1942) *J. Am. Chem. Soc. 64*, 2905−2917.
34. Kitz, R., and Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245−3249.
35. Doucet, C., Pochet, L., Thierry, N., Pirotte, B., Delarge, J., and Reboud-Ravaux, M. (1999) *J. Med. Chem. 42*, 4161−4171.
36. Pochet, L., Doucet, C., Dive, G., Wouters, J., Masereel, B., Reboud-Ravaux, M., and Pirotte, B. (2000) *Bioorg. Med. Chem. 8*, 1489−1501.
37. Skrzypczak-Jankun, E., Carperos, V. E., Ravichandran, K. G., Tulinsky, A., Westbrook, M., and Maraganore, J. M. (1991) *J. Mol. Biol. 221*, 1379−1393.
38. Otwinowski, Z., and Minor, W. (1997) *Methods Enzymol. 276*, 307−326.
39. Abola, E. E., Sussman, J. L., Prilusky, J., and Manning, N. O. (1997) *Methods Enzymol. 277*, 556−571.
40. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr., Sect. D 54*, 905−921.
41. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr., Sect. A 47*, 110−119.
42. Engh, R. A., and Huber, R. (1991) *Acta Crystallogr., Sect. A 47*, 392−400.
43. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) *J. Appl. Crystallogr. 26*, 283−291.
44. Steinmetzer, T., Batdorsdhjin, M., Kleinwachter, P., Seyfarth, L., Greiner, G., Reissmann, S., and Stürzebecher, J. (1999) *J. Enzyme Inhib. 14*, 203−216.
45. Ringe, D., and Mottonen, J. M. (1986) *Biochemistry 25*, 5633−5638.
46. Stoddard, B. L., Bruhnke, J., Porter, N., Ringe, D., and Petsko, G. A. (1990) *Biochemistry 29*, 4871−4879.
47. Nardini, M., Pesce, A., Rizzi, M., Casale, E., Ferraccioli, R., Balliano, G., Milla, P., Ascenzi, P., and Bolognesi, M. (1996) *J. Mol. Biol. 258*, 851−859.
48. De Simone, G., Balliano, G., Milla, P., Gallina, C., Giordano, C., Tarricone, C., Rizzi, M., Bolognesi, M., and Ascenzi, P. (1997) *J. Mol. Biol. 269*, 558−569.
49. Okamoto, S., Kinjo, K., Hijikata, A., Kikumoto, R., Tamao, Y., Ohkubo, K., and Tonomura, S. (1980) *J. Med. Chem. 23*, 827−830.
50. Okamoto, S., Hijikata, A., Kikumoto, R., Tonomura, S., Hara, N., Ninomiya, K., Maruyama, A., Sugano, M., and Tamao, Y. (1981) *Biochem. Biophys. Res Commun. 101*, 440−446.
51. Kikumoto, R., Tamao, Y., Ohkubo, K., Tezuka, T., Tonomura, S., Okamoto, S., Funahara, Y., and Hijikata, A. (1980) *J. Med. Chem. 23*, 830−836.
52. Kikumoto, R., Tamao, Y., Ohkubo, K., Tezuka, T., Tonomura, S., Okamoto, S., and Hijikata, A. (1980) *J. Med. Chem. 23*, 1293−1299.
53. Kikumoto, R., Tamao, Y., Tezuka, T., Tonomura, S., Hara, N., Ninomiya, K., Hijikata, A., and Okamoto, S. (1984) *Biochemistry 23*, 85−89.
54. Stürzebecher, J., Markwardt, F., Voigt, B., Wagner, G., and Walsmann, P. (1983) *Thromb. Res. 29*, 635−642.
55. Stürzebecher, J., Markwardt, F., Voigt, B., and Wagner, G. (1984) *Thromb. Res. 36*, 457−467.
56. Banner, D. W., and Hadvary, P. (1991) *J. Biol. Chem. 266*, 20085−20093.
57. Brandstetter, H., Turk, D., Hoeffken, H. W., Grosse, D., Stürzebecher, J., Martin, P. D., Ewards, B. F. P., and Bode, W. (1992) *J. Mol. Biol. 226*, 1085−1099.
58. Ilies, M. A., Scozzafava, A., and Supuran, C. T. (2002) *Expert Opin. Ther. Pat. 12*, 1181−1214.

BI020512L