



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

United States Patent No.: 5,196,404  
Issued: 23 March 1993  
Filed: 6 July 1990  
Inventors: Maraganore *et al.*  
Title: INHIBITORS OF THROMBIN

RECEIVED  
FEB 20 2001

Commissioner for Patents  
Washington, D.C. 20231  
Box Patent Ext.

OFFICE OF PETITIONS

APPLICATION PURSUANT TO 35 U.S.C §156(d)(1) AND  
37 C.F.R. §1.740 FOR EXTENSION OF PATENT TERM

Hon. Commissioner

Applicants hereby request that the term of the above-identified United States Patent be extended pursuant to 35 U.S.C. 156. The information required by 37 C.F.R. §1.740(a) is provided below and in the Appendices.

- (1) The generic name of the approved product is bivalirudin. Its chemical name and physical characteristics are provided in Appendix A.
- (2) The regulatory review of the approved product occurred pursuant to section 505(b) of the Federal Food Drug and Cosmetic Act (See Appendix B).
- (3) The date on which the approved product received permission for commercial marketing was 15 December 2000.
- (4) The active ingredient in the approved product is bivalirudin. Bivalirudin has not been previously approved for commercial marketing or use under the Federal Food, Drug and Cosmetic Act, the Public Health Service Act, or the Virus-Serum-Toxin Act.
- (5) ~~This application is being submitted within the 60 day period permitted for submission pursuant to 37 C.F.R. §1.720(f). The last date upon which this application could be submitted is 15 February 2001.~~ *Sm 2/14/01*

OIE-0213

APP 1

(6) The patent for which an extension is being sought is in the name of the inventors John M. Maraganore, John W. Fenton, II and Toni Kline. The United States Patent No. is 5,196,404 ("The Patent"). The patent issued on 23 March 1993 and will expire on 23 March 2010. The patent is exclusively licensed by the applicant. (See Exhibit C)

(7) A copy of The Patent is provided herewith as Appendix D.

(8) A copy of a certificate of correction dated 18 October 1994 is provided herewith as Appendix E. A reexamination certificate issued 10 September 1996 is provided herewith as Appendix F. A receipt for a maintenance fee paid in August 1996 is provided herewith as Appendix G. A receipt for a maintenance fee paid in August 2000 is provided herewith as Appendix H. No disclaimer has been made for this patent.

RECEIVED

FEB 20 2001

OFFICE OF PETITIONS

(9) The approved product is covered by claims 1-6, 9, 13, 14, 16 and 21 of The Patent. This is illustrated for claim 1 below.

<u>Limitations of claim 1</u>	<u>Elements of the approved product</u>
A thrombin inhibitor consisting of:	Bivalirudin is a thrombin inhibitor (see Appendix I).
a catalytic site -directed moiety that binds to and inhibits the active site of thrombin;	The catalytic site -directed moiety of bivalirudin binds to and inhibits the active site of thrombin (see Appendix I).
wherein said catalytic site -directed moiety is selected from serine protease inhibitors .....	The catalytic site -directed moiety of bivalirudin is a serine protease inhibitor which inhibits the serine protease thrombin (See Appendix I).
a linker moiety characterized by a backbone chain having a calculated length of between about 18Å and about 42Å; and	The linker region of bivalirudin is the peptide Gly-Gly-Gly-Asp-Gly-Asp-Phe, a preferred linker within the 18Å to 42Å length range (See Appendix I and Appendix D at Col. 9, l. 25-47).
an anion binding exosite associating moiety;	Bivalirudin has a moiety that specifically binds the anion-binding exosite of thrombin (See Appendix I and Appendix D). Appendix D, at Col. 9, l. 50-67, defines the preferred anion-binding exosite associating moiety as W-B1-B2-B3-B4-B5-B6-B7-B8-Z, wherein W is a bond, B1 is Glu, B2 is Glu, B3 (see Appendix E, first page) is Ile, B4 is Pro, B5 is Glu, B6 is Glu, B7 is Tyr-Leu, B8 is a bond and Z is OH. As shown in Appendix I, this section of Appendix D describes the anion-binding exosite associating moiety of bivalirudin.
said catalytic site-directed moiety being bound to said linker moiety and said linker moiety being bound to said anion binding exosite associating moiety; (See Appendix D at Col. 9, l. 25-29)	In bivalirudin, the catalytic site-directed moiety is bound to the linker moiety by a peptide bond and the linker moiety is bound to the said anion binding exosite associating moiety by a peptide bond (See Appendix I).
wherein said inhibitor is capable of simultaneously binding to the catalytic site and the anion binding exosite of thrombin.	Bivalirudin is capable of simultaneously binding to the catalytic site and the anion binding exosite of thrombin (See Appendix I).

(10) The effective date of the investigational new drug (IND) application for the approved product was 2 November 1990. The IND number is 35,756.

The date on which a new drug application (NDA) was initially submitted was 23 December 1997. The NDA number is NDA 20-873.

The date on which the NDA was approved was 15 December 2000.

RECEIVED  
FEB 20 2001  
OFFICE OF PETITIONS

(11) Significant activities undertaken by the marketing applicant during the applicable regulatory review period with respect to the approved product and the significant dates applicable to such activities are set forth in Appendix J. Although significant activities were undertaken prior to 23 March 1993, these have been excluded as preceding the issue date of the patent, and therefor not being relevant to this analysis.

(12) In the opinion of the applicant, The Patent is eligible for the maximum statutory extension of 14 years from FDA approval (*i.e.* until 15 December 2014). (See Appendix K) This determination was made by

- (A) determining the number of days between the issue date of The Patent (23 March 1993) and the filing date of the NDA (23 December 1997) during such period of the regulatory review period = 1736 days;
- (B) calculating a period of days during which the applicant did not act with due diligence during such period of the regulatory review period (1996, when the program was being transferred from Biogen to applicant) = 366 days
- (C) taking the result of A-B and dividing the number of days by two = 685 days;
- (D) determining the number of days between the filing date of the NDA (23 December 1997) and the date of approval of the NDA (15 December 2000) during which applicant did act with due diligence during such period of the regulatory review period = 1088 days;
- (E) compiling the number of days from (C) + (D) = 1773 days;

In the opinion of the Applicant, therefore, the term of the patent should be extended to 15 December 2014. This determination is made by:

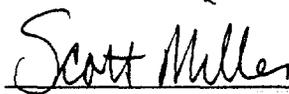
- (F) adding (E) to the date of expiration of The Patent (23 March 2010) to determine when the extended patent would expire = 29 January 2015;  
and
- (G) determining that this expiration date is greater than 14 years beyond the date of approval of the NDA (15 December 2014);
- (H) therefore the patent term should be extended to 15 December 2014).

(13) The applicant hereby acknowledges a duty to disclose to the Commissioner of Patents and Trademarks and the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension sought.

(14) A check in the amount of \$1,120.00 in payment of the fee pursuant to 37 C.F.R. §1.20(j) is enclosed herewith. The Commissioner is authorized to charge any additional fees to Deposit Account No. 06-1075.

(15) All inquiries and correspondence relating to this application should be directed to Paul Granger, Esq., The Medicines Company, One Cambridge Center, Cambridge, Massachusetts 02142, Telephone 617-225-9099.

Respectfully submitted,



James F. Haley, Jr. (Reg. No. 27,794)

Attorney for Applicants

Scott D. Miller (Reg. No. 43,803)

Agent for Applicants

c/o FISH & NEAVE

1251 Avenue of the Americas

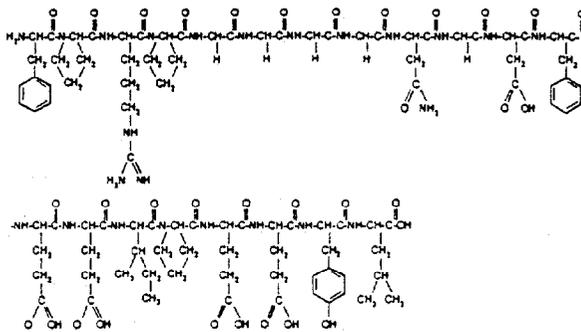
New York, New York 10020-1104

Tel: (212) 596-9000

**DESCRIPTION**

Angiomax™ (bivalirudin) is a specific and reversible direct thrombin inhibitor. The active substance is a synthetic, 20 amino acid peptide. The chemical name is D-phenylalanyl-L-prolyl-L-arginyl-L-prolyl-glycyl-glycyl-glycyl-L-asparagyl-glycyl-L-asparyl-L-phenylalanyl-L-glutamyl-L-glutamyl-L-isoleucyl-L-prolyl-L-glutamyl-L-glutamyl-L-tyrosyl-L-leucine trifluoroacetate (salt hydrate) (Figure 1). The molecular weight of Angiomax is 2180 daltons (anhydrous free base peptide). Angiomax is supplied in single-use vials as a white lyophilized cake, which is sterile. Each vial contains 250 mg bivalirudin, 125 mg mannitol, and sodium hydroxide to adjust the pH to 5 to 6 (equivalent of approximately 12.5 mg sodium). When reconstituted with Sterile Water for Injection the product yields a clear to opalescent, colorless to slightly yellow solution, pH 5-6.

Figure 1. Structural Formula for Bivalirudin



**CLINICAL PHARMACOLOGY**

**General:**

Angiomax directly inhibits thrombin by specifically binding both to the catalytic site and to the anion-binding exosite of circulating and clot-bound thrombin. Thrombin is a serine proteinase that plays a central role in the thrombotic process, acting to cleave fibrinogen into fibrin monomers and to activate Factor XIII to Factor XIIIa, allowing fibrin to develop a covalently cross-linked framework which stabilizes the thrombus; thrombin also activates Factors V and VIII, promoting further thrombin generation, and activates platelets, stimulating aggregation and granule release. The binding of Angiomax to thrombin is reversible as thrombin slowly cleaves the Angiomax-Arg<sub>1</sub>-Pro<sub>1</sub> bond, resulting in recovery of thrombin active site functions.

In *in vitro* studies, bivalirudin inhibited both soluble (free) and clot-bound thrombin, was not neutralized by products of the platelet release reaction, and prolonged the activated partial thromboplastin time (aPTT), thrombin time (TT), and prothrombin time (PT) of normal human plasma in a concentration-dependent manner. The clinical relevance of these findings is unknown.

**Pharmacokinetics:**

Bivalirudin exhibits linear pharmacokinetics following intravenous (IV) administration to patients undergoing percutaneous transluminal coronary angioplasty (PTCA). In these patients, a mean steady state bivalirudin concentration of 12.3 ± 1.7 mcg/mL is achieved following an IV bolus of 1 mg/kg and a 4-hour 2.5 mg/kg/h IV infusion. Bivalirudin is cleared from plasma by a combination of renal mechanisms and proteolytic cleavage, with a half-life in patients with normal renal function of 25 minutes. The disposition of bivalirudin was studied in PTCA patients with mild and moderate renal impairment and in patients with severe renal impairment. Drug elimination was related to glomerular filtration rate (GFR). Total body clearance was similar for patients with normal renal function and with mild renal impairment (60-89mL/min). Clearance was reduced approximately 20% in patients with moderate and severe renal impairment and was reduced approximately 80% in dialysis-dependent patients. See Table 1 for pharmacokinetic parameters and dose reduction recommendations. For patients with renal impairment the activated clotting time (ACT) should be monitored. Bivalirudin is hemodialyzable. Approximately 25% is cleared by hemodialysis.

Bivalirudin does not bind to plasma proteins (other than thrombin) or to red blood cells.

Table 1. PK parameters and dose adjustments in renal impairment

Renal Function (GFR, mL/min)	Clearance (mL/min/kg)	Half-life (minutes)	% reduction in infusion dose
Normal renal function (≥90 mL/min)	3.4	25	0
Mild renal impairment (60-90 mL/min)	3.4	22	0
Moderate renal impairment (30-59 mL/min)	2.7	34	20
Severe renal impairment (10-29 mL/min)	2.8	57	60
Dialysis-dependent patients (off dialysis)	1.0	3.5 hours	90

\* The ACT should be monitored in renally-impaired patients

**Pharmacodynamics:**

In healthy volunteers and patients (with ≥ 70% vessel occlusion undergoing routine angioplasty), bivalirudin exhibits linear dose- and concentration-dependent anticoagulant activity as evidenced by prolongation of the ACT, aPTT, PT, and TT. Intravenous administration of Angiomax produces an immediate anticoagulant effect. Coagulation times return to baseline approximately 1 hour following cessation of Angiomax administration.

In 291 patients with ≥ 70% vessel occlusion undergoing routine angioplasty, a positive correlation was observed between the dose of Angiomax and the proportion of patients achieving ACT values of 300 sec or 350 sec. At an Angiomax dose of 1.0 mg/kg IV bolus plus 2.5 mg/kg/h IV infusion for 4 hours, followed by 0.2 mg/kg/h, all patients reached maximal ACT values > 300 sec.

**Clinical Trials:**

Angiomax was evaluated in patients with unstable angina undergoing PTCA in 2 randomized, double-blind, multicenter studies with identical protocols. Patients must have had unstable angina defined as: (1) a new onset of severe or accelerated angina or rest pain within the month prior to study entry or (2) angina or ischemic rest pain which developed between four hours and two weeks after an acute myocardial infarction (MI). Overall, 4312 patients with unstable angina, including 741 (17%) patients with post-MI angina, were treated in a 1:1 randomized fashion with Angiomax or heparin. Patients ranged in age from 29-90 (median 63) years, their weight was a median of 80 kg (39-120kg), 68% were male, and 91% were Caucasian. Twenty-three percent of patients were treated with heparin within one hour prior to randomization. All patients were administered aspirin 300-325 mg prior to PTCA and daily thereafter. Patients randomized to Angiomax were started on an intravenous infusion of Angiomax (2.5 mg/kg/h). Within 5 minutes after starting the infusion, and prior to PTCA, a 1 mg/kg loading dose was administered as an intravenous bolus. The infusion was continued for 4 hours, then the infusion was changed under double-blinded conditions to Angiomax (0.2 mg/kg/h) for up to an additional 20 hours (patients received this infusion for an average of 14 hours). The ACT was checked at 5 minutes and at 45 minutes following commencement. If on either occasion the ACT was <350 seconds, an additional double-blinded bolus of placebo was administered. The Angiomax dose was not titrated to ACT. Median ACT values were: ACT in seconds (5<sup>th</sup> percentile-95<sup>th</sup> percentile): 345 sec (240-595 seconds) at 5 min and 348 sec (range 269-583 sec) at 45 min after initiation of dosing. Patients randomized to heparin were given a loading dose (175 IU/kg) as an intravenous bolus 5-minutes before the planned procedure, with immediate commencement of an infusion of heparin (15 IU/kg/h). The infusion was continued for 4 hours. After 4-hours of infusion, the heparin infusion

PN 1002



(A)

# Angiomax™

(bivalirudin)  
FOR INJECTION

the ACT was <350 seconds, an additional double-blind bolus of heparin (60 IU/kg) was administered. Once the target ACT was achieved for heparin patients, no further ACT measurements were performed. All ACTs were determined with the Hemochron® device. The protocol allowed use of open-label heparin at the discretion of the investigator after discontinuation of blinded study medication, whether or not an endpoint event (procedural failure) had occurred. The use of open-label heparin was similar between Angiomax and heparin treatment groups (about 20% in both groups).

The studies were designed to demonstrate the safety and efficacy of Angiomax in patients undergoing PTCA as a treatment for unstable angina as compared with a control group of similar patients receiving heparin during and up to 24 hours after initiation of PTCA. The primary protocol endpoint was a composite endpoint called procedural failure, which included both clinical and angiographic elements measured during hospitalization. The clinical elements were: the occurrence of death, MI, or urgent revascularization, adjudicated under double-blind conditions. The angiographic elements were: impending or abrupt vessel closure. The protocol-specified safety endpoint was major hemorrhage.

The median duration of hospitalization was 4 days for both the Angiomax treatment group and the heparin treatment group. The rates of procedural failure were similar in the Angiomax and heparin treatment groups. Study outcomes are shown in Table 2.

Table 2. Incidences of In-hospital Clinical Endpoints in Randomized Clinical Trials Occurring within 7 Days

	ANGIOMAX™	HEPARIN
All Patients	n=2161	n=2151
<b>Efficacy Endpoints:</b>		
Procedural Failure <sup>1</sup>	7.9%	9.3%
Death, MI, Revascularization	6.2%	7.9%
Death	0.2%	0.2%
MI <sup>2</sup>	3.3%	4.2%
Revascularization <sup>3</sup>	4.2%	5.6%
<b>Safety Endpoint:</b>		
Major Hemorrhage <sup>4</sup>	3.5%	9.3%

<sup>1</sup> The protocol specified primary endpoint (a composite of death or MI or clinical deterioration of cardiac origin requiring revascularization or placement of an aortic balloon pump or angiographic evidence of abrupt vessel closure).

<sup>2</sup> Defined as: Q-wave MI; CK-MB elevation ≥ 2xULN, new ST- or T-wave abnormality, and chest pain ≥30 mins; OR new LBBB with chest pain ≥30 mins and/or elevated CK-MB enzymes; OR elevated CK-MB and new ST- or T-wave abnormality without chest pain; OR elevated CK-MB.

<sup>3</sup> Defined as: any revascularization procedure, including angioplasty, CABG, stenting, or placement of an intra-aortic balloon pump.

<sup>4</sup> Defined as the occurrence of any of the following: intracranial bleeding, retroperitoneal bleeding, clinically overt bleeding with a decrease in hemoglobin ≥3 g/dL or leading to a transfusion of ≥2 units of blood.

**INDICATIONS AND USAGE**

Angiomax is indicated for use as an anticoagulant in patients with unstable angina undergoing percutaneous transluminal coronary angioplasty (PTCA). Angiomax is intended for use with aspirin and has been studied only in patients receiving concomitant aspirin (see Clinical Trials and DOSAGE AND ADMINISTRATION).

The safety and effectiveness of Angiomax have not been established when used in conjunction with platelet inhibitors other than aspirin, such as glycoprotein IIb/IIIa inhibitors (see PRECAUTIONS, Drug Interactions).

The safety and effectiveness of Angiomax have not been established in patients with unstable angina who are not undergoing PTCA or in patients with other acute coronary syndromes.

**CONTRAINDICATIONS**

Angiomax is contraindicated in patients with:

- active major bleeding;
- hypersensitivity to Angiomax or its components.

**WARNINGS**

Angiomax is not intended for intramuscular administration. Although most bleeding associated with use of Angiomax in PTCA occurs at the site of arterial puncture, hemorrhage can occur at any site. An unexplained fall in blood pressure or hematocrit, or any unexplained symptom, should lead to serious consideration of a hemorrhagic event and cessation of Angiomax administration.

There is no known antidote to Angiomax. Angiomax is hemodialyzable (see CLINICAL PHARMACOLOGY, Pharmacokinetics).

**PRECAUTIONS**

**General:**

Clinical trials have provided limited information for use of Angiomax in patients with heparin-induced thrombocytopenia/heparin-induced thrombocytopenia-thrombosis syndrome (HIT/HITTS) undergoing PTCA. The number of HIT/HITTS patients treated is inadequate to reliably assess efficacy and safety in these patients undergoing PTCA. Angiomax was administered to a small number of patients with history of HIT/HITTS or active HIT/HITTS and undergoing PTCA in an uncontrolled, open-label study, and in an emergency treatment program and appeared to provide adequate anticoagulation in these patients. In *in-vitro* studies, bivalirudin exhibited no platelet aggregation response against sera from patients with a history of HIT/HITTS.

**Drug Interactions:**

Bivalirudin does not exhibit binding to plasma proteins (other than thrombin) or red blood cells. Drug-drug interaction studies have been conducted with the adenosine diphosphate (ADP) antagonist ticlopidine, and the glycoprotein IIb/IIIa inhibitor, abciximab, and with low molecular weight heparin. Although data are limited, precluding conclusions regarding efficacy and safety, a combination of



## DEPARTMENT OF HEALTH &amp; HUMAN SERVICES

Public Health Service

Food and Drug Administration  
Rockville MD 20857

NDA 20-873

DEC 15 2000

The Medicines Company  
Attention: Sonja Loar, Pharm. D.  
One Cambridge Center  
Cambridge, Massachusetts 02142

Dear Ms. Loar:

Please refer to your new drug application (NDA) dated December 23, 1997, received December 23, 1997, submitted under section 505(b) of the Federal Food, Drug, and Cosmetic Act for Angiomax™ (bivalirudin) Injection.

We acknowledge receipt of your submissions dated April 6, May 12 and 17, July 14, October 9, November 9, and December 1, 2000. Your submission of July 14, 2000, constituted a complete response to our May 11, 2000, action letter.

This new drug application provides for the use of Angiomax™ (bivalirudin) Injection as an anticoagulant in conjunction with aspirin in patients with unstable angina undergoing percutaneous transluminal coronary angioplasty (PTCA).

We have completed the review of this application, as amended, and have concluded that adequate information has been presented to demonstrate that the drug product is safe and effective for use as recommended in the agreed upon enclosed labeling text. Accordingly, the application is approved effective on the date of this letter.

The final printed labeling (FPL) must be identical to the enclosed labeling (text for the package insert) and submitted draft labeling (immediate container and carton labels submitted July 14, 2000). Marketing the product with FPL that is not identical to the approved labeling text may render the product misbranded and an unapproved new drug.

Please submit 20 paper copies of the FPL as soon as it is available, in no case more than 30 days after it is printed. Please individually mount ten of the copies on heavy-weight paper or similar material. Alternatively, you may submit the FPL electronically according to the guidance for industry titled *Providing Regulatory Submissions in Electronic Format - NDAs* (January 1999). For administrative purposes, this submission should be designated "FPL for approved NDA 20-873." Approval of this submission by FDA is not required before the labeling is used.

We remind you of your postmarketing commitment in your submission dated December 1, 2000. This commitment is listed below.

NDA 20-873

Page 2

Commit to completing Study TMC 98-10 entitled "Anticoagulant Therapy with Bivalirudin to Assist in the Performance of Percutaneous Coronary Intervention in Patients with Heparin-Induced Thrombocytopenia: An Open Label Study of Bivalirudin for Heparin-Induced Thrombocytopenia (HIT) or Heparin-Induced Thrombocytopenia and Thrombosis Syndrome (HITTS)" and submitting the full report for that study.

Final Report Submission: Within 36 months of the date of this letter.

Submit clinical protocols to your IND for this product. Submit nonclinical and chemistry, manufacturing, and controls protocols and all study final reports to this NDA. In addition, under 21 CFR 314.81(b)(2)(vii) and 314.81(b)(2)(viii), you should include a status summary of each commitment in your annual report to this NDA. The status summary should include expected summary completion and final report submission dates, any changes in plans since the last annual report, and, for clinical studies, number of patients entered into each study. All submissions, including supplements, relating to these postmarketing study commitments must be prominently labeled "Postmarketing Study Protocol", "Postmarketing Study Final Report", or "Postmarketing Study Correspondence."

Validation of the regulatory methods has not been completed. At the present time, it is the policy of the Center not to withhold approval because the methods are being validated. Nevertheless, we expect your continued cooperation to resolve any problems that may be identified.

Be advised that, as of April 1, 1999, all applications for new active ingredients, new dosage forms, new indications, new routes of administration, and new dosing regimens are required to contain an assessment of the safety and effectiveness of the product in pediatric patients unless this requirement is waived or deferred (63 FR 66632). We are waiving the pediatric study requirement for this action on this application.

In addition, please submit three copies of the introductory promotional materials that you propose to use for this product. All proposed materials should be submitted in draft or mock-up form, not final print. Please send one copy to the Division of Gastrointestinal and Coagulation Drug Products and two copies of both the promotional materials and the package insert directly to:

Division of Drug Marketing, Advertising, and Communications, HFD-42  
Food and Drug Administration  
5600 Fishers Lane  
Rockville, Maryland 20857

Please submit one market package of the drug product when it is available.

We remind you that you must comply with the requirements for an approved NDA set forth under 21 CFR 314.80 and 314.81.

NDA 20-873

Page 3

If you have any questions, call Julicann DuBeau, Regulatory Health Project Manager, at (301) 827-7310.

Sincerely,

*Vicki F.C. Rayhane* 12/15/00 FOR FH

Florence Houn, M.D., M.P.H., F.A.C.P.

Director

Office of Drug Evaluation III

Center for Drug Evaluation and Research

Enclosure

## LICENSE AGREEMENT

This LICENSE AGREEMENT is made and entered into this 21st day of March, 1997 by and between Biogen, Inc. ("Biogen"), a Massachusetts corporation, with principal offices located at 14 Cambridge Center, Cambridge, Massachusetts 02142, and The Medicines Company, a Delaware corporation, with principal offices located at One Cambridge Center, Cambridge, Massachusetts 02142 ("TMC").

RECEIVED

FEB 20 2001

### INTRODUCTION

OFFICE OF PETITIONS

1. Biogen is the owner of certain patents and other proprietary rights related to HIRULOG® bivalirudin and related hirudin-based peptide analogs, and has conducted clinical trials using HIRULOG® bivalirudin for the treatment of percutaneous transluminal coronary angioplasty, acute myocardial infarction and other diseases.
2. TMC is a biopharmaceutical company which is committed to the development and commercialization of prescription pharmaceutical products.
3. TMC desires to obtain an exclusive right and license in and to Biogen's technology, patent rights and proprietary know-how related to HIRULOG® bivalirudin and other hirudin-based peptides to develop and commercialize products based on such technology, patent rights and know-how worldwide.
4. Biogen is willing to grant a license to TMC on the terms and conditions set forth in this Agreement.

NOW, THEREFORE, in consideration of the mutual promises and other good and valuable consideration, the receipt and sufficiency of which are hereby acknowledged, the parties agree as follows:

SECTION 1 - DEFINITIONS.

As used in this Agreement, the following terms, whether used in the singular or plural, shall have the following meanings:

1.1 "Affiliate", as applied to either party, shall mean any corporation, partnership, joint venture or other legal entity which controls, is controlled by or is under common control with such party. For purposes of this definition, the term "control" shall mean (a) in the case of corporate entities, direct or indirect ownership or control of at least fifty percent (50%) of the outstanding equity entitled to vote for directors, and (b) in the case of non-corporate entities, direct or indirect ownership of at least fifty percent (50%) of the equity interest with the ability to otherwise control the management of the entity.

1.2 "AMI" shall mean acute myocardial infarction.

1.3 "AMITrial" shall have the meaning set forth in Section 4.2.

1.4 "Biogen Patent Rights" shall mean all patents and patent applications throughout the Territory, covering or relating to Biogen Technology, including any substitutions, extensions, reissues, reexaminations, renewals, continuations, continuations-in-part, divisionals and supplemental protection certificates, which Biogen owns or Controls. Biogen Patent Rights existing as of the Effective Date are set forth in Appendix A to this Agreement.

1.5 "Biogen Technology" shall mean all Technology which Biogen owns or Controls as of the Effective Date and which is reasonably useful in order to research, develop, make, use, sell or seek approval to market Product.

1.6 "Cardiology Indications" shall mean all therapeutic, prophylactic and diagnostic applications in humans related to the management of coronary vessel disease, including inter alia, PTCA, AMI and unstable angina. Cardiology Indications shall not include inter alia, arrhythmia not of ischemic etiology, embolic or hemorrhagic stroke or any medical condition resulting from an abnormality of venous circulation.

1.7 "Commercial Development Activities" shall mean pre and post Product-launch clinical studies that are not required for regulatory approval of Product in the Major Markets, pharmacoeconomic studies and sponsored educational programs for health care professionals at which clinical and/or pharmacoeconomic data related to Product are presented.

1.8 "Confidential Information" shall mean all information and materials, including without limitation, trade secrets and other proprietary information and materials (whether or not patentable) regarding a Party's Technology, products, business plans or objectives.

1.9 "Control" shall mean possession of the ability to grant a license or sublicense, as provided herein, without violating the terms of any agreement or other arrangement with any third party.

1.10 "CSL" shall mean CSL Limited (formerly Commonwealth Serum Laboratories Limited) or any successor in interest to the rights of CSL Limited under the CSL Agreement.

1.11 "CSL Agreement" shall mean a certain License and Supply Agreement between Biogen and CSL, dated as of September 30, 1991.

1.12 "Distributor" shall mean a person or entity in a country who (i) purchases Product or bulk Peptide from TMC or one of its Affiliates, and (ii) assumes responsibility for a portion of the promotion, marketing, sales and customer service effort related to Product in that country, and (iii) under an implied or express sublicense, sells Product in that country. The term Distributor shall not include a person or entity who provides a contract sales force to serve, in whole or in part, as TMC's sales force with respect to sales by TMC. For purposes of this Agreement, CSL shall be considered a Distributor of TMC based on the current CSL Agreement, and, to the extent that the CSL Agreement is modified, shall be considered a Distributor if it meets the criteria set forth in this definition. Without limiting the generality of the foregoing, Biogen acknowledges that TMC currently intends to contract with Innovex for sales support, and agrees that Innovex shall not be considered to constitute a Distributor by virtue of such sales support.

1.13 "Effective Date" shall mean the date of this Agreement.

1.14 "Existing Non-Cardiology Indications" shall mean those indications, set forth in Appendix E, that (a) are not Cardiology Indications and (b) as to which Biogen had data from human clinical trials as of the Effective Date.

1.15 "FDA" shall mean the United States Food and Drug Administration.

1.16 "FTE" shall mean a full-time equivalent person year consisting of a total of one thousand eight hundred eighty (1,880) hours per year of work carried out by a Biogen employee.

1.17 "Field" shall mean all therapeutic, prophylactic and diagnostic applications in humans.

1.18 "First Commercial Sale" shall mean in each country of the Territory with respect to each Product, (i) the first sale of the Product by TMC or any of its Affiliates, Sublicensees or Distributors to a third party in such country in connection with the nationwide introduction of the Product by TMC, its Affiliates, Sublicensees or Distributors following marketing and/or pricing approval by the appropriate governmental agency for the country in which the sale is made, or (ii) when governmental approval is not required, the first sale in such country in connection with the nationwide introduction of the Product in that country.

1.19 "HRI" shall mean Health Research, Inc. or any successor in interest to the rights of Health Research Inc.'s under the HRI Agreement.

1.20 "HRI Agreement" shall mean a certain License Agreement between Biogen and HRI, dated as of June 6, 1990, as amended.

1.21 "IND" shall mean an Investigational New Drug application, as defined under the United States Federal Food, Drug and Cosmetic Act, as amended.

1.22 "MAA" shall mean an application for regulatory approval to sell Product in the European Union and similar in purpose to an NDA in the United States.

1.23 "Major Markets" shall mean the United States, the United Kingdom, Germany, France, Italy, Spain and the Benelux region.

1.24 "NDA" shall mean a New Drug Application or Product License Application or equivalent filing filed for Product with the FDA.

1.25 "Net Sales" shall mean the gross amount invoiced (not dependent on whether such invoices have been actually paid) on sales of Product by TMC and its Affiliates and Distributors to third parties, less the following items, as determined from the books and records of TMC or its Affiliates or Distributors, provided that such items do not exceed

reasonable and customary amounts in the country in which such sale or other disposition occurred: (i) freight, insurance and other transportation charges, if billed separately; (ii) amounts repaid or credited by reason of returns, rejections, defects, recalls or because of retroactive price reductions; (iii) sales taxes, excise taxes, value-added taxes and other taxes (other than income taxes) levied on the invoiced amount; (iv) import and export duties; (v) cash, trade and quantity discounts actually given or made; and (vi) rebates paid pursuant to government regulations. A sale of Product by TMC to an Affiliate or Distributor for resale of the Product by such Affiliate or Distributor shall not be considered a sale for the purpose of this provision, but the resale of such Product by the Affiliate or Distributor to a third party who is not an Affiliate or Distributor of TMC shall be a sale for purposes of this Agreement.

For purposes of this Agreement, "sale" shall mean any transfer or other distribution or disposition, but shall not include transfers or other distributions or dispositions of Product, at no charge, for pre-clinical, clinical or regulatory purposes or to physicians or hospitals for promotional purposes, provided such transfer, distribution or disposition is not made in exchange for lower prices on other TMC products or for other noncash consideration. In the event that consideration in addition to or in lieu of money is received for the sale of Product in an arms-length transaction, the fair market value of such consideration shall be included in the determination of Net Sales for such sale. To the extent that Product is sold in other than an arms-length transaction, Net Sales for such sale shall be the average sales price of Product if sold in an arms-length transaction during the applicable royalty reporting period in the country in which the non-arms-length transaction occurred.

In the event that Product is sold in the form of a combination Product containing one or more active ingredients or components in addition to Product, Net Sales for the combination Product shall be determined by multiplying actual Net Sales of the combination Product (determined by reference to the definition of Net Sales set forth above) during the royalty payment period by the fraction  $A/A+B$  where A is the average sale price of Product when sold separately in finished form and B is the average sale price of the other active ingredients or components when sold separately in finished form in each case during the applicable royalty reporting period in the country in which the sale of the combination Product was made, or if sales of both the Product and the other active ingredients or components did not occur in such period, then in the most recent royalty reporting period in which sales of both occurred. In the event that such average sale price cannot be determined for both Product and all other active ingredients or components included in the combination Product, Net Sales for purposes of determining payments under this Agreement shall be calculated by multiplying the Net Sales of the combination Product by the fraction  $C/C+D$  where C is the standard fully-absorbed cost of the Product portion of the combination and D is the sum of the standard fully-absorbed costs of all other active components or ingredients included in the combination Product, in each case, as determined by TMC using its standard accounting procedures consistently applied. In no event shall Net Sales of a combination Product be reduced to less than [REDACTED] of actual Net Sales of such combination Product (determined by reference to the definition of Net Sales set forth above) by reason of any adjustment provision set forth in this paragraph.

1.26 "New Non-Cardiology Indications" shall mean those indications that are neither Existing Non-Cardiology Indications nor Cardiology Indications.

1.27 "Peptide" shall mean one or more of the hirudin-based peptide analogs described in Appendix B to this Agreement.

1.28 "Product" shall mean the finished form of a product that comprises, contains or is Peptide and which or the manufacture, use or sale of which (i) is covered by a Valid Claim of any Biogen Patent Rights in the country where such Product is manufactured, used or sold and/or (ii) embodies any of the Biogen Technology.

1.29 "PTCA" shall mean percutaneous transluminal coronary angioplasty.

1.30 "Semilog Process" shall mean the joint biological/synthetic process for producing Peptide.

1.31 "Sublicensee" shall mean any third party expressly licensed by TMC to make, use and sell Product, but not including any Affiliate or Distributor of TMC.

1.32 "Technology" shall mean all information, data, concepts, formulas, methods, procedures, designs, compositions, plans, applications, specifications, techniques, processes, technical data, know-how, samples, biological materials, inventions, discoveries and the like which a party owns (in whole or in part) or Controls.

1.33 "Technology Transfer" shall mean the transfer of Biogen Technology to TMC, in accordance with Section 3.

1.34 "Technology Transfer Plan" shall have the meaning set forth in Section 3.1

1.35 "Territory" shall mean all countries of the world.

1.36 "TMC Patent Rights" shall mean all patents and patent applications throughout the Territory, covering or relating to TMC Technology, including any substitutions, extensions, reissues, reexaminations, renewals, continuations, continuations-in-part, divisionals and supplemental protection certificates, which TMC owns or Controls at any time.

1.37 "TMC Technology" shall mean all Technology which TMC owns or Controls as of the date of termination of this Agreement and which is reasonably useful in order to discover, research, develop, make, use, sell or seek approval to market Product.

1.38 "UCB Information" shall mean information related to the manufacturing of Peptide contained in the Chemistry, Manufacturing and Control (CMC) sections of Biogen's existing INDs for Peptide, and stability data generated by UCB on Peptide.

1.39 "Valid Claim" shall mean (i) a claim of a pending patent application which claim shall not have been canceled, withdrawn, abandoned or rejected by an administrative agency from which no appeal can be taken or which shall not have failed to issue as a patent within seven (7) years of the earliest claimed priority date or (ii) a claim of an issued and unexpired patent which has not lapsed or become abandoned or been declared invalid or unenforceable by a court of competent jurisdiction or an administrative agency from which no appeal can be or is taken.

## SECTION 2 - GRANT AND ASSIGNMENTS.

2.1 License Grant. Biogen hereby grants to TMC, and TMC hereby accepts from Biogen, a royalty-bearing right and license under Biogen Technology and Biogen Patent Rights to make, have made, import, use, offer to sell and sell Product in the Territory in the Field. The license granted to TMC under this Section 2.1 shall be exclusive subject only to the rights granted to CSL under the CSL Agreement and any rights retained by HRI under the HRI Agreement.

### 2.2 Sublicense Rights.

(a) TMC shall be entitled to extend the license granted to it under Section 2.1 to any of its Affiliates and to grant sublicenses to its rights for all indications in each country

of the Territory other than the United States and the countries of the European Union, provided that TMC shall obtain Biogen's consent prior to granting any sublicense in Canada or Japan which such consent Biogen agrees not to unreasonably withhold. TMC shall also be entitled to grant sublicenses to the rights granted to it under Section 2.1 in the United States and the countries of the European Union for New Non-Cardiology Indications, provided that TMC shall obtain Biogen's consent prior to granting any such sublicense which such consent Biogen agrees not to unreasonably withhold. All Affiliates and Sublicensees to whom TMC has extended or sublicensed its rights under Section 2.1 shall agree to be bound by all of the applicable terms and conditions of this Agreement. TMC shall advise Biogen of any extension of TMC's rights to its Affiliates and shall provide copies to Biogen of each sublicense promptly after such extension or sublicense becomes effective. TMC shall not have the right to grant sublicenses to its rights under Section 2.1 in the United States or any of the countries of the European Union with respect to Cardiology Indications or Existing Non-Cardiology Indications.

(b) TMC shall use commercially reasonable efforts to ensure that its Affiliates and Sublicensees to whom TMC has extended or sublicensed its rights under Section 2.1 shall comply with all applicable terms of this Agreement and shall make all payments of compensation due and make all reports due under this Agreement by reason of sales of Product by such Affiliates and/or Sublicensees.

(c) TMC shall use commercially reasonable efforts to ensure that all Sublicensees to whom TMC grants rights to make, use and sell Product in New Non-Cardiology Indications in the United States and the countries of the European Union market Product solely for use in New Non-Cardiology Indications.

2.3 Assignment of Agreements. Concurrently with execution of this Agreement, the parties shall execute (i) an Assignment of License in the form set forth in Appendix C hereto under which Biogen assigns to TMC, and TMC accepts assignment of, all of Biogen's rights and obligations under the HRI Agreement, and (ii) an Assignment of License and Supply Agreement in the form set forth as Appendix D hereto under which Biogen assigns to TMC, and TMC accepts assignment of, all of Biogen's rights and obligations under the CSL Agreement.

### SECTION 3 - TECHNOLOGY TRANSFER AND SUPPLY OF MATERIAL

3.1 Technology Transfer Plan. As soon as reasonably practical after the Effective Date, the parties shall meet to agree on a plan for Technology Transfer (the "Technology Transfer Plan"). The Technology Transfer Plan shall specify the Technology Transfer activities to be performed and the amount of time to be devoted to such activities. The parties shall review and update the Technology Transfer Plan, on a monthly basis, until the earlier to occur of (i) completion of Technology Transfer or (ii) the end of the Technology Transfer period, as set forth in Section 3.2. Biogen shall not be required to devote time or perform activities in connection with Technology Transfer beyond the time and activities shown on the Technology Transfer Plan unless both parties agree on an update to the Technology Transfer Plan.

3.2 Limitation on Technology Transfer. Notwithstanding anything in this Agreement to the contrary, Biogen shall not be obligated to devote more than the equivalent of 2.8 FTEs, in the aggregate, to Technology Transfer or, in the event that Biogen has devoted an equivalent of 2.8 FTEs to Technology Transfer, to perform Technology Transfer after the date which is four (4) months from the Effective Date. The

parties shall use their best efforts to complete Technology Transfer within four (4) months from the Effective Date. If Biogen has devoted an equivalent of 2.8 FTEs to Technology Transfer and Technology Transfer has not been completed by the end of such four (4) month period, the parties may extend the Technology Transfer period by mutual agreement.

3.3 Costs of Technology Transfer. TMC shall pay Biogen's fully-burdened costs associated with Technology Transfer, provided that the activities and the time spent performing the activities for which the costs are to be paid are reflected in the Technology Transfer Plan, as updated from time to time, or TMC has specifically requested the additional time or activities. Biogen shall bill TMC for Biogen's fully-burdened costs related to Technology Transfer on a monthly basis. TMC shall pay all Biogen invoices within thirty (30) days of receipt.

3.4 Assignment of Regulatory Filings and Other Product-Related Information. Biogen hereby assigns to TMC all of Biogen's right, title and interest in (a) its existing INDs and equivalent regulatory filings in the Territory related to Product and (b) subject to Section 5, any and all regulatory and clinical information related to Product that Biogen owns or Controls as of the Effective Date. Biogen and TMC shall jointly manage the transition of Biogen's INDs or equivalent regulatory filings to TMC under this Section in such a way as not to harm the existing relationship of either party with the relevant regulatory authorities. Biogen may elect, or TMC may request, to have one or more of Biogen's employees participate in meetings between TMC and regulatory authorities regarding assignment of Biogen's INDs or equivalent regulatory filings to TMC, provided that Biogen's right to elect to participate in any such meeting shall terminate on December 31, 1997. TMC shall pay all of Biogen's costs associated with the assignment of INDs or

equivalent regulatory filings to TMC, including the costs incurred by Biogen in sending Biogen representatives to meetings with regulatory authorities at the request of TMC.

### 3.5 Supply of Material.

(a) As soon as reasonably practical after the Effective Date but in any event within ninety (90) days after the Effective Date, Biogen shall deliver to TMC Biogen's existing inventory of Peptide as described in Appendix F (the "Biogen Inventory"). In addition, Biogen shall, at TMC's request, provided such request is made prior to September 18, 1997 (the "Completion Option Period"), initiate completion of processing by UCB Bioproducts S.A. (collectively "UCB") of approximately 30kg of Peptide intermediates (expressed in equivalent bulk drug substance quantities) stored at UCB as of the Effective Date under the terms of a Supply Agreement between Biogen and UCB, dated as of March 21, 1997 (the "Supply Agreement") (a copy of which has been provided to TMC), and shall deliver to TMC the resulting material (the "UCB Material"). TMC understands and agrees that "processing", as the term is used in this Section, of the 30kg of Peptide intermediates by UCB shall mean completion of the manufacturing of such portion of the 30kg of Peptide intermediates as UCB, in consultation with Biogen and TMC, determines is viable for further production (the "Unfinished Peptide") using the manufacturing process specified in Biogen's most recent IND for Peptide on file with the FDA as of the Effective Date. In the event that UCB reports to Biogen that any portion of the 30kg of Peptide intermediates is not viable, Biogen shall use reasonable efforts to confirm UCB's determination. "Processing" shall also include delivery by UCB with each batch of Peptide of a release certificate and access for Biogen and/or TMC to review the relevant batch records. "Processing" shall specifically not include (i) any analytical process-related or other validation work, (ii) qualification of plant, equipment or utilities, (iii) work towards a

supplemental IND, NDA or any other regulatory filing, (iv) any other work requested by regulatory authorities in connection with a regulatory filing, (v) any work associated with filing or inspection of the documentation or facilities by TMC or the regulatory authorities, or (vi) any supporting activities including further development work (process-related and analytical-related), stability standard or reference standard establishment or requalification (collectively "Ancillary Services"). At or prior to initiation of the completion of processing of the Unfinished Peptide under the terms of this Agreement, TMC shall meet with UCB to negotiate the terms, if any, under which UCB would be willing to provide, and TMC would be willing to accept, Ancillary Services in connection with the Peptide manufactured by UCB. Biogen represents that UCB is obligated to complete processing of the Unfinished Peptide if the request is made during the Completion Option Period whether or not TMC accepts Ancillary Services from UCB at the end of the negotiation described in the preceding sentence. Upon delivery to Biogen by TMC during the Completion Option Period of a request to have UCB complete processing of the Unfinished Peptide, TMC, UCB and Biogen shall meet to agree upon a delivery schedule for the resulting Peptide. Biogen represents that UCB has agreed to deliver the Peptide resulting from processing of the Unfinished Peptide within at least eighteen (18) months of receipt of the processing request. Biogen shall use commercially reasonable efforts to enforce the Supply Agreement after consultation with TMC.

(b) As part of Technology Transfer, Biogen shall provide to TMC copies of quality control release test results existing as of the Effective Date related to the Biogen Inventory. Biogen shall perform additional HPLC tests on the Biogen Inventory only at TMC's request and expense in accordance with the Technology Transfer Plan.

(c) Biogen represents that Peptide delivered to TMC as part of the Biogen Inventory was stored under the conditions set forth in Appendix E.

(d) TMC shall reimburse Biogen for the amount due to UCB for delivery of the UCB Material, up to [REDACTED]. TMC shall also reimburse Biogen for any storage costs for the Biogen Inventory and the UCB Material incurred by Biogen after the Effective Date. In addition, TMC shall reimburse Biogen for all freight, storage, duties, taxes and insurance costs incurred in connection with delivery of the Biogen Inventory and the UCB Material to TMC, including but not limited to those costs incurred in shipping the Biogen Inventory to and from Europe and storing the Biogen Inventory in Europe. All payments to be made by TMC to Biogen under this paragraph shall be made within thirty (30) days of receipt of each invoice therefor from Biogen.

#### SECTION 4 - DUE DILIGENCE.

4.1 Investment. TMC shall use commercially reasonable efforts to expend at least \$20 million (not including amounts spent on or as part of the AMI Trial) in connection with pre-launch and post-launch commercialization activities related to Product for the PTCA and AMI indications within two (2) years of the later of the date of approval of a NDA for Product in the PTCA indication and the date of approval of a NDA for Product in the AMI indication. Commercialization activities may include Commercial Development Activities.

4.2 Diligence. TMC shall use commercially reasonable efforts (defined, for purposes of this Agreement, as those efforts consistent with the efforts that would be exerted by a mid-size biopharmaceutical company in the development and sale of its own products) to develop and commercialize Product in each of the Major Markets. TMC shall

develop Product for use in the treatment of PTCA and AMI. Without limiting the generality of the foregoing, TMC shall use commercially reasonable efforts to meet the following diligence milestones:

- (a) Commence a phase III clinical trial of Product in the AMI indication (the "AMI Trial") by December 31, 1998.
- (b) File an NDA for Product in the PTCA indication by December 31, 1998.
- (c) File an NDA for Product in AMI indication by December 31, 2001.
- (d) File an MAA for Product in AMI indication by December 31, 2001.
- (e) Commence marketing and sales of Product in the United States in each indication (i) within six (6) months of receipt of a license from the FDA to market and sell Product in such indication, if no approvable letter is issued with respect to such indication or (ii) within four (4) months of receipt of the applicable FDA license, if an approvable letter is issued with respect to such indication.

4.3 AMI Trial. TMC shall use a lead investigator for the AMI Trial who is a nationally recognized expert in cardiology. TMC shall provide to Biogen a draft of the protocol for the AMI Trial, and Biogen shall have the right to review and comment on such protocol. The parties acknowledge and agree that the phase III study design for the AMI Trial will be a mortality trial substantially based on Biogen's phase II results with Peptide and streptokinase. TMC shall be the sponsor of the AMI Trial for purposes of 21 C.F.R. section 312 et. seq. TMC shall review the protocol for the AMI Trial with the FDA, and shall use reasonable efforts to obtain the FDA's advice that the protocol is reasonable for obtaining marketing approval of Product in the AMI indication.

#### 4.4 Consequences of Failure to Satisfy Diligence Obligation

(a) If at any time Biogen believes that TMC has not satisfied its diligence obligations under Section 4.1 and 4.2, then Biogen shall so notify TMC. Within fifteen (15) days of the date of such notice, the parties shall meet to discuss TMC's performance. If TMC is able to demonstrate to Biogen's satisfaction that TMC used commercially reasonable efforts to meet its diligence obligations, the parties shall negotiate in good faith to set new milestones which are reasonable in light of any difficulties or any unforeseen events which TMC may have encountered. If TMC is unable to demonstrate to Biogen's satisfaction that TMC used commercially reasonable efforts to meet its diligence obligations and if TMC does not agree with Biogen's assessment, the parties shall enter into binding arbitration, under the terms of Section 14.7, within ten (10) days of the meeting between the parties held under this Section, provided, that the arbitrators selected by the parties pursuant to Section 14.7 to arbitrate any issue that arises under this Section 4.4(a) shall each be an expert in the field of drug development in the United States.

(b) In the event that TMC agrees with Biogen's determination that TMC failed to satisfy its diligence obligations under Section 4.1 or 4.2 or an arbitration panel convened under paragraph (a) of this Section 4.4 determines that TMC failed to satisfy its diligence obligations under Section 4.1 or 4.2, Biogen shall have the right and option to terminate this Agreement for material breach by TMC under Section 10.2.

4.5 Japan Diligence In the event that TMC informs Biogen in writing that it does not intend to develop, register, manufacture, market or sell, or sublicense a third party to develop, register, manufacture or sell, Product in Japan, and TMC's reasons for choosing not to enter Japan are not related to potential parallel import or pricing issues or regulatory or patent obstacles outside of TMC's control, Biogen shall have the right and

option to (i) terminate the license set forth in Section 2.1 as to Japan only on sixty (60) days prior written notice to TMC and (ii) to exercise its rights under Section 10.5.

4.6 Transdermal Product. No later than December 31, 1998, TMC shall submit to Biogen a development plan for the transdermal application of Product (the "Transdermal Plan"). The Transdermal Plan shall include commercially reasonable milestones for development and commercialization of a transdermal Product. In the event TMC does not use commercially reasonable efforts to meet the milestones set forth in the Transdermal Plan, Biogen shall have the right to terminate this Agreement as to the transdermal application of Product.

4.7 Status Reports. Within forty-five (45) days of the end of each calendar quarter, TMC shall provide to Biogen a written report describing in reasonable detail the status of development and commercialization activities related to Product, including the nature of the development and commercialization activities undertaken by TMC and its Sublicensees and Distributors, if any, during the preceding quarter, the results obtained and the goals and plans for the next quarter. After Product launch, the status report provided to Biogen under this Section shall include rolling four-quarter sales forecasts for Product. TMC shall furnish to Biogen copies of final study reports from clinical trials related to Product as soon as such reports are available. At Biogen's request from time to time, TMC shall provide to Biogen verbal updates on the status of development and commercialization efforts.

## SECTION 5 - CONFIDENTIALITY

5.1 Treatment of Confidential Information. Each party agrees that it shall maintain the Confidential Information of the other party in strict confidence and shall not

disclose any such Confidential Information to a third party or use such Confidential Information for any purpose other than as contemplated under this Agreement. Each party agrees to exercise reasonable precautions to prevent and restrain the unauthorized disclosure or use of the Confidential Information of the other party by any of the receiving party's directors, officers, agents or employees. TMC acknowledges and agrees that all regulatory and clinical information assigned to TMC under Section 3.4 (b) shall, except as provided in Section 5.2, continue to be Confidential Information of Biogen for purposes of this Section 5.

5.2 Exceptions.

The provisions of Section 5.1 shall not apply to Confidential Information which:

(i) was known to the receiving party prior to its disclosure by the disclosing party;

(ii) either before or after the date of disclosure to the receiving party becomes generally known to the public by some means other than a breach of this Agreement;

(iii) is subsequently disclosed to the receiving party by a third party having a lawful right to make such disclosure and who is not under an obligation of confidentiality to the disclosing party;

(iv) is independently developed by or for the receiving party without reference to or reliance upon the Confidential Information received from the disclosing party;

(v) is required by law, rule, regulation or bona fide legal process to be disclosed, provided that the receiving party takes all reasonable steps to restrict and

maintain the confidentiality of such disclosure and provides reasonable notice to the disclosing party; or

(vi) is approved for release by the parties.

The non-disclosure and non-use obligations under Section 5.1 shall terminate as to any Confidential Information twelve (12) years after receipt of such Confidential Information by the receiving party.

5.3 Permitted Disclosures. Notwithstanding anything to the contrary contained in Section 5.1, TMC may disclose the Confidential Information of Biogen licensed to TMC under Section 2.1 or assigned to TMC under Section 3.4, other than the UCB Information, to third parties who (i) need to know the same in order for TMC to secure regulatory approval for the sale of Product or (ii) need to know the same in order to work towards the commercial development of Product or to manufacture Product or (iii) need to know the same in order to determine whether to enter into a sublicense agreement with TMC with respect to the manufacture, use and/or sale of Product provided that such parties, other than regulatory authorities, are bound by obligations of confidentiality and non-use at least as stringent as those set forth in this Section 5. In addition, TMC may disclose Confidential Information of Biogen (other than UCB Information or any other Confidential Information of Biogen as to which Biogen would be required to obtain the consent of a third party with respect to further disclosure) to potential investors who have a need to know the same in order to assess the status of their investment in TMC or to determine whether to invest in TMC, provided that (i) the information to be disclosed is of a type customarily disclosed to investors and (ii) the investors to whom the information is disclosed are bound by obligations of confidentiality and non-use with respect to such information at least as stringent as those set forth in this Section 5.

5.4 UCB Information. Notwithstanding anything herein to the contrary, TMC shall not use the UCB Information for any purpose other than supporting the regulatory filings for Peptide assigned to TMC by Biogen under Section 3.4 ("Existing Regulatory Filings"), and shall not disclose the UCB Information to any third party other than regulatory authorities. TMC shall return to UCB all documents containing UCB Information in TMC's possession in the event that maintaining UCB Information is no longer required for purposes of supporting the Existing Regulatory Filings, and shall take all reasonable steps to return promptly to UCB any UCB Information in the possession of the FDA which might be returned to TMC (except as otherwise required by the FDA) and to inform the FDA that communication of such UCB Information to any third party requires UCB's express written consent.

SECTION 6 - PAYMENT OBLIGATIONS.

6.1 License Fee. In consideration of the rights granted by Biogen, TMC shall pay to Biogen a nonrefundable, noncreditable license fee of [REDACTED] on the Effective Date.

6.2 Milestone Payments. TMC shall make each of the following nonrefundable, noncreditable payments to Biogen within thirty (30) days of the first achievement of each of the following milestones:

	<u>Event</u>	<u>Payment</u>
(a)	First Commercial Sale of Product in the United States for treatment in AMI	[REDACTED]
(b)	First Commercial Sale of Product in Europe for treatment in AMI	[REDACTED]

6.3 Royalties.

(a) TMC shall pay to Biogen earned royalties on Net Sales of Product sold by TMC and/or its Affiliates and/or its Distributors at the following rates:

<u>Annualized Net Sales in a Calendar Year in Territory</u>	<u>Royalty Rate on Net Sales of Product</u>
Less than or equal to [REDACTED]	[REDACTED]
Greater than [REDACTED] but less than or equal to [REDACTED]	[REDACTED]
Greater than [REDACTED] but less than or equal to [REDACTED]	[REDACTED]
Greater than [REDACTED] but less than or equal to [REDACTED]	[REDACTED]
Greater than \$ [REDACTED]	[REDACTED]

(b) Notwithstanding anything in this Agreement to the contrary, sales by Sublicensees shall be included as TMC sales solely for purposes of determining the royalty rate applicable to sales by TMC and/or its Affiliates and/or its Distributors.

(c) The applicable royalty rate for a given calendar year shall be based on the rate determined by reference to total Net Sales during the year, and shall be applied retroactively to the first dollar of such Net Sales in such calendar year. Adjustment payments shall be made as necessary in accordance with Section 6.7.

(d) The obligation to pay royalties and a percentage of Sublicense Royalty Income (as defined in Section 6.5) shall continue, on a country-by-country basis, from the date of the First Commercial Sale of Product in a country until the later of (i) twelve (12) years after the date of the First Commercial Sale of such Product in such country or (ii) the date on which the Product or its manufacture, use or sale is no longer covered by a Valid Claim of any Biogen Patent Rights in such country.

6.4 Royalty Offset.

(a) Subject to paragraph (d) of this Section 6.4, the royalty rates set forth in Section 6.3 shall be reduced, on a country-by-country basis, by [REDACTED] with respect to Net Sales of any Product in any calendar year if (i) neither such Product nor its use or sale is covered during any part of such year by a Valid Claim of a Biogen Patent Right in such country and (ii) third parties selling Comparable Products, as defined below, have, in the aggregate, during such year [REDACTED] or more of the volume-based market share in such country. For purposes of this Section, "Comparable Product" shall mean a product which, if sold on the Effective Date by a third party in the United States without a license from Biogen, would infringe a Valid Claim of Biogen Patent Rights related to Product existing as of the Effective Date.

(b) Subject to paragraph (c) and (d) of this Section 6.4, in the event that TMC, in order to manufacture, use or sell Product in a country in the Territory, reasonably determines that it must make a royalty payment to one or more third parties (a "TMC Third Party Payment") to obtain a license or similar right to manufacture, use or sell Product in such country, TMC may reduce the royalty payment due Biogen under Section 6.3 on sales of Product, on a country-by-country basis, by the amount of such TMC Third Party Payments paid on such sales up to a [REDACTED] percentage points reduction in the applicable royalty rate set forth in Section 6.3. The offset available under this paragraph (b) shall not apply to royalty payments made or due under the HRI Agreement.

(c) Subject to paragraph (d) of this Section 6.4, with respect to any sales as to which TMC is paying royalties at the [REDACTED] royalty rates under Section 6.3, TMC may, in addition to other offsets available under paragraphs (a) and (b) above, reduce the royalty payment due Biogen under Section 6.3 by the amount of any payments

made by TMC to HRI under the HRI Agreement on such sales, but not more than the amounts that would be payable to HRI at the rates in effect under the HRI Agreement on the Effective Date.

(d) Until the later of (i) the date of receipt of marketing approval for Product from the FDA for the AMI indication or (ii) the fourth anniversary of the date of the First Commercial Sale of Product in any country in the PTCA indication, TMC may offset against the royalty payment due to Biogen (A) any costs incurred by TMC after the First Commercial Sale of Product for PTCA in development or commercialization of Product for the AMI indication, provided that the costs are incurred as part of a development or commercialization plan approved by Biogen, which approval shall not be unreasonably withheld, and (B) any costs incurred by TMC in connection with Commercial Development Activities for the PTCA indication that are agreed to by the parties, which agreement shall not be unreasonably withheld, and provided further that in no event (1) shall the amount offset under this paragraph exceed \$ [REDACTED] in the aggregate or (2) shall the amount of royalties actually paid to Biogen under Section 6.3 for any royalty payment period be less than [REDACTED] of Net Sales. Notwithstanding anything in this Agreement to the contrary, TMC shall not be entitled to any offset under this paragraph (d) in any calendar year in which Net Sales calculated in the manner set forth in Section 6.3 are greater than [REDACTED]. TMC shall offset its costs under this paragraph against royalties due for the calendar year in which the costs are incurred and shall not carry over such costs to offset royalties for any other calendar year. Notwithstanding anything herein to the contrary, TMC shall not be entitled to apply the offsets available under any other paragraph of this Section 6.4 (and shall not carry-over any such offsets)

in any period in which TMC is applying its offset for development costs as set forth in this paragraph (d).

6.5 Sublicense Royalty Income. TMC shall pay to Biogen [REDACTED] percent [REDACTED] of all royalty income ("Sublicense Royalty Income") received by TMC from its Sublicensees with respect to sales of Products.

6.6 Off-label Sales by Sublicensees. In the event that Biogen can reasonably demonstrate a loss in earned royalties from TMC as a result of off-label sales by any of TMC's Sublicensees, TMC shall reimburse Biogen for Biogen's loss of earned royalties up to the amount actually received by TMC from such Sublicensee for such off-label sales.

6.7 Quarterly Payments and Reports. Royalty payments and payments on Sublicense Royalty Income shall be made quarterly (i) within ninety (90) days following the end of the first calendar quarter of Product sales with respect to Sublicense Royalty Income received and Net Sales on sales made during such quarter, (ii) within sixty (60) days following the end of each of the second, third and fourth calendar quarters of Product sales with respect to Sublicense Royalty Income received and Net Sales on sales made during such quarter, and (iii) within forty-five (45) days following the end of each calendar quarter thereafter with respect to Sublicense Royalty Income received and Net Sales on sales made during such quarter. Every payment shall be accompanied by a report setting forth for the relevant quarter the following information:

- (a) Net Sales by TMC and its Affiliates and Distributors, by country;
- (b) Sales by Sublicensees by country (for purposes of calculating the royalty rate);
- (c) Quantity of Product sold, by country, by TMC, its Affiliates, Distributors and Sublicensees;

- (d) Sublicense Royalty Income received by TMC; and
- (e) Total amount payable to Biogen.

Since Net Sales in each calendar year to be used to finally determine the applicable royalty rate for such year will not be known until the end of such year, in order to make the quarterly payments specified under this Section 6.7, TMC shall use a royalty rate which is determined by annualizing the year-to-date Net Sales. As changes in the royalty rate determined using annualized Net Sales occur from one calendar quarter to the next calendar quarter within the same calendar year, in addition to the payment for the calendar quarter, TMC shall make the necessary adjustment in such calendar quarter reflecting the change in the royalty rate applied to Net Sales in the preceding calendar quarter or quarters. Within thirty (30) days of the end of each calendar year, TMC shall calculate the actual royalty rate to which Biogen is entitled based on the actual Net Sales for the year. In the event Biogen has not received its full royalty amount for the year, TMC shall promptly make a balancing payment to Biogen in the amount of the deficit. In the event TMC has paid Biogen more than its full royalty amount for the year, Biogen shall promptly reimburse TMC in the amount of the excess.

6.8 Form of Payment. All payments to be made under this Agreement shall be made in United States dollars by check or wire transfer, at Biogen's option.

6.9 Foreign Exchange. For purposes of computing Net Sales for Product sold in currency other than United States Dollars, such currency shall be converted into United States Dollars using the spot purchase rate published in the Wall Street Journal (New York Edition) for the last day of the calendar quarter for which Net Sales are being calculated.

6.10 Taxes. Any taxes required to be withheld by TMC under the laws of any foreign country for the account of Biogen shall be promptly paid by TMC for and on

behalf of Biogen to the appropriate governmental authority, and TMC shall furnish Biogen with proof of payment of such tax within thirty (30) days following payment. Any such tax actually paid on Biogen's behalf shall be deducted from royalty payments due Biogen. TMC agrees to make all lawful and reasonable efforts to minimize such taxes to Biogen.

6.11 Interest on Payments Past Due. Any amounts due under this Agreement that are not paid when due shall bear interest at the lesser of (i) an annualized rate of two percent over the prime rate then in effect at BankBoston, or (ii) the highest rate permitted by applicable law.

6.12 Books and Records. For a period of three (3) years next following each calendar year, TMC shall keep, and shall use commercially reasonable efforts (which shall include obtaining and enforcing a contractual commitment) to cause each of its Affiliates, Distributors and Sublicensees to keep, full, true and accurate books and records containing all particulars relevant to its sales of Products during such year in sufficient detail to enable Biogen to verify the amounts payable to Biogen under this Agreement. Biogen shall have the right, not more than once during any calendar year, to have the books and records of TMC or any of its Distributors or Sublicensees related to the sales of Products audited by a qualified nationally-recognized, independent accounting firm of Biogen's choosing, during normal business hours upon reasonable notice, for the sole purpose of verifying the accuracy of the amounts paid to Biogen under this Agreement, provided, however, that Sublicensees or Distributors who refuse to submit to an audit on behalf of Biogen despite TMC's commercially reasonable efforts (which shall include enforcing a contractual commitment) to obtain their consent to such audit shall not be bound by the audit obligation set forth in this sentence. In the event that an audit shows that TMC has underpaid Biogen by five percent (5%) or more, then TMC shall pay for all costs of such

audit, otherwise the costs of such audit shall be borne by Biogen. In all cases, TMC shall pay to Biogen any underpaid compensation promptly and with interest at an annualized rate of the prime rate then in effect at BankBoston, plus two percent (2%), and Biogen shall promptly pay to TMC any overpaid compensation. All information and data reviewed in any audit conducted under this Section shall be used only for the purpose of verifying amounts due to Biogen under this Agreement and shall be treated as Confidential Information of TMC subject to the terms of this Agreement.

#### SECTION 7 - PATENTS.

7.1 Prosecution and Maintenance. During the term of this Agreement, TMC shall have responsibility for prosecuting, maintaining and defending the Biogen Patent Rights, and in doing so shall use a level of effort and professional representation consistent with the level of effort and professional representation a mid-size biotechnology company would use to prosecute, maintain and defend its own patent rights. Notwithstanding anything herein to the contrary, TMC shall obtain Biogen's written consent prior to (i) instituting any reissue or reexamination proceedings with respect to any Biogen Patent Rights that are issued patents as of the Effective Date, or (ii) making any strategic decision in any opposition, nullity, reissue or reexamination proceedings involving any Biogen Patent Rights that are issued patents as of the Effective Date, which Biogen consent shall not be unreasonably withheld. TMC shall bear all of the costs of prosecution, maintenance and defense of the Biogen Patent Rights incurred after the Effective Date. TMC shall keep Biogen regularly informed of the status of the Biogen Patent Rights. TMC shall provide copies to Biogen of all filings and correspondence with the patent offices, administrative boards or courts which TMC sends or receives in connection with prosecution,

maintenance and defense of the Biogen Patent Rights. As soon as practical after the Effective Date, Biogen shall provide to TMC a copy of Biogen's existing files on the Biogen Patent Rights. Biogen undertakes to promptly and fully cooperate in, and to provide all information and data and sign any documents reasonably necessary and requested by TMC for the prosecution, maintenance and defense of the Biogen Patents Rights. If TMC decides to abandon or to allow to lapse any Biogen Patent Right, TMC shall inform Biogen at least ninety (90) days prior to the effective date of such decision and Biogen shall be given the opportunity to prosecute such Biogen Patent Right which such Biogen Patent Right shall no longer be subject to this Agreement. Upon termination of TMC's responsibility for prosecuting and maintaining any Biogen Patent Rights, TMC shall promptly deliver to Biogen all files related to the Biogen Patent Rights, and shall take all action and execute all documents reasonably necessary for Biogen to resume prosecution.

## 7.2 Infringement.

(a) TMC and Biogen shall each promptly inform the other in writing of any infringement of the Biogen Patent Rights of which such party has notice and provide the other with any available evidence of infringement.

(b) In the event TMC, alone or with an Affiliate or Sublicensee, wishes to take action in a suit to enforce any Biogen Patent Rights against infringement, TMC may take action and, at its option and expense, join Biogen as a plaintiff. In determining whether to bring an action to enforce any Biogen Patent Rights, TMC shall act in a commercially reasonable manner, giving due consideration to the threat represented by the infringement and the potential risk to the Biogen Patent Rights involved. If within six (6) months after having been notified by Biogen of any alleged infringement or providing notice to Biogen of an alleged infringement, TMC has been unsuccessful in persuading the

alleged infringer to desist and has not brought, and/or is not diligently prosecuting an infringement action, or if TMC notifies Biogen at any time prior thereto of its intention not to bring suit against any alleged infringer, Biogen may take action and, at its option, join TMC as a plaintiff in any suit.

(c) The party which institutes any suit to protect or enforce a Biogen Patent Right shall have sole control of that suit and shall bear the reasonable expenses of the other party, not including legal fees incurred by the other party, in providing any assistance and cooperation as is requested pursuant to this Section. The party initiating or carrying on such legal proceedings shall keep the other party informed of the progress of such proceedings and such other party shall be entitled to counsel in such proceedings but at its own expense.

(d) Any award paid by third parties (whether by way of settlement or otherwise) as the result of any proceedings initiated by TMC under this Section 7 shall first be applied to reimbursement of the unreimbursed legal fees and expenses incurred by either party and then the remainder shall be divided between the parties as follows:

(i) If the amount is based on lost profits, (x) TMC shall receive an amount equal to the damages the court determines it has suffered as a result of the infringement, less the amount of any royalties (and/or payments on Sublicense Royalty Income) that would have been due to Biogen on sales of Product lost by TMC and/or its Affiliates, Distributors and Sublicensees as a result of the infringement had they made such sales; and (y) Biogen shall receive an amount equal to the royalties (and/or payments on Sublicense Royalty Income) that it would have received if such sales had been made by TMC and/or its Affiliates, Distributors and Sublicensees; and

(ii) As to awards other than those based on lost profits,  $\frac{3}{4}$  to TMC and  $\frac{1}{4}$  to Biogen.

(e) Any award paid by third parties (whether by way of settlement or otherwise) as the result of any proceedings initiated by Biogen under this Section 7 shall first be applied to reimbursement of the unreimbursed legal fees and expenses incurred by either party and then shall be divided between the parties,  $\frac{1}{4}$  to TMC and  $\frac{3}{4}$  to Biogen.

7.3 Cooperation. In any suit as either party may institute or control to enforce the Biogen Patent Rights pursuant to this Agreement, the other party agrees, at the request and expense of the party initiating or controlling the suit, to cooperate in all respects, to have its employees testify when requested and to make available relevant records, papers, information, samples, specimens, and the like.

7.4 Third Party Claim. In the event that a third party at any time provides written notice of a claim to, or brings an action, suit or proceeding against a party or such party's Affiliates, Distributors or Sublicensees, claiming infringement of its patent rights or unauthorized use or misappropriation of its Technology based upon an assertion or claim arising out of the development, manufacture, use or sale of Products, such party shall promptly notify the other party of the claim or the commencement of such action, suit or proceeding, enclosing a copy of the claim and/or all papers served.

## SECTION 8 - REPRESENTATIONS, WARRANTIES AND COVENANTS.

8.1 Corporate Action. Each party represents and warrants to the other party that: (i) it is free to enter into this Agreement; (ii) in so doing, it will not violate any other agreement to which it is a party; and (iii) it has taken all corporate action necessary to

authorize the execution and delivery of this Agreement and the performance of its obligations under this Agreement.

8.2 Compliance with Law. Each party covenants and agrees that in conducting activities contemplated under this Agreement, it shall comply with all applicable laws and regulations. Without limiting the generality of the foregoing, TMC covenants and agrees that in conducting activities in connection with the manufacture, use or sale of Product, TMC shall comply with all applicable laws and regulations.

8.3 Right to License. Biogen represents and warrants to TMC that Biogen is the owner or licensee of the Biogen Technology and Biogen Patent Rights and has the right and ability to grant the licenses granted under this Agreement. In addition, Biogen covenants and agrees that it will not enter into any agreement or other arrangement with any third party following the Effective Date that would limit TMC's right and ability to exploit the rights granted by Biogen to TMC under this Agreement.

8.4 Disclaimers. THE REPRESENTATIONS AND WARRANTIES SET FORTH IN THIS SECTION AND IN SECTION 3.5 ARE IN LIEU OF ALL OTHER REPRESENTATIONS AND WARRANTIES NOT EXPRESSLY SET FORTH HEREIN. WITHOUT LIMITING THE GENERALITY OF THE FOREGOING STATEMENT, BIOGEN DISCLAIMS ALL WARRANTIES, WHETHER EXPRESS OR IMPLIED, WITH RESPECT TO BIOGEN TECHNOLOGY, BIOGEN PATENT RIGHTS, THE BIOGEN INVENTORY AND THE UCB MATERIAL, INCLUDING, WITHOUT LIMITATION, ANY REPRESENTATIONS OR WARRANTIES AS TO WHETHER PRODUCT CAN BE SUCCESSFULLY DEVELOPED OR MARKETED, REGARDING THE ACCURACY, PERFORMANCE, UTILITY, RELIABILITY, TECHNOLOGICAL OR COMMERCIAL VALUE, COMPREHENSIVENESS, MERCHANTABILITY OR FITNESS FOR ANY

PARTICULAR PURPOSE WHATSOEVER OF THE BIOGEN TECHNOLOGY, BIOGEN PATENT RIGHTS, BIOGEN INVENTORY OR UCB MATERIAL OR AS TO THE VALIDITY OF THE BIOGEN PATENT RIGHTS OR THAT THE MANUFACTURE, USE, MARKETING OR SALE OF PRODUCTS BY TMC OR ANY OF ITS AFFILIATES, DISTRIBUTORS OR SUBLICENSEES WILL NOT CONSTITUTE AN INFRINGEMENT OF THE INTELLECTUAL PROPERTY RIGHTS OF ANY THIRD PARTY. NEITHER BIOGEN NOR TMC SHALL BE LIABLE FOR SPECIAL, INDIRECT, INCIDENTAL OR CONSEQUENTIAL DAMAGES ARISING OUT OF THIS AGREEMENT WHETHER BASED ON CONTRACT, TORT OR ANY OTHER LEGAL THEORY.

#### SECTION 9 - INDEMNIFICATION.

9.1 Indemnification by TMC. TMC shall defend, indemnify and hold harmless Biogen and its Affiliates and their respective employees, agents, officers, shareholders and directors and each of them (the "Biogen Indemnified Parties") from and against any and all liability, damage, loss, cost or expense of any nature (including reasonable attorneys fees and expenses of litigation) incurred or imposed upon the Biogen Indemnified Parties or any one of them in connection with any claims, suits, actions, demands, proceedings, causes of action or judgments resulting from or arising out of (i) the development, design, testing, production, manufacture, sale, use or promotion of Product by TMC or any of its Affiliates, Sublicensees or Distributors or any of their respective agents or employees; (ii) any other activities carried out by TMC or any of its Affiliates, Sublicensees or Distributors or any of their respective agents or employees, including any failure to comply in any material respect with applicable laws or regulations, or (iii) breach by TMC of any term of this Agreement, except to the extent any such claim results or arises from breach of this

Agreement by Biogen or the negligence or willful misconduct of Biogen or any its Affiliates or any of their respective employees, agents, officers or directors.

9.2 Indemnification by Biogen. Biogen shall defend, indemnify and hold harmless TMC and its Affiliates and their respective employees, agents, officers, shareholders and directors and each of them (the "TMC Indemnified Parties") from and against any and all liability, damage, loss, cost or expense of any nature (including reasonable attorneys fees and expenses of litigation) incurred or imposed upon the TMC Indemnified Parties or any one of them in connection with any claims, suits, actions, demands, proceedings, causes of action or judgments resulting from or arising out of the breach of this Agreement by Biogen or the negligence or willful misconduct of Biogen or any its Affiliates or any of their respective employees, agents, officers or directors.

9.3 Conditions to Indemnification. An indemnified party shall give prompt notice to the indemnifying party (either TMC or Biogen, as the case may be) of any claim for which the indemnified party may seek indemnification under Section 9.1 or 9.2 and, provided that the indemnifying party is not contesting the indemnity obligation, shall permit the indemnifying party to control any litigation relating to such claim and disposition of any such claim, provided that the indemnifying party shall act reasonably and in good faith with respect to all matters relating to the settlement or disposition of any claim as the settlement or disposition relates to the indemnified party, and the indemnifying party shall not settle or otherwise resolve any claim without prior notice to the indemnified party. The indemnified party shall cooperate with the indemnifying party in its defense of any claim for which indemnification is sought under this Section.

9.4 Insurance. At such time as Product is being marketed, TMC shall obtain and shall thereafter maintain, at TMC's sole cost and expense, product liability insurance

for Product naming Biogen as an additional insured. The amount of the insurance coverage obtained under this Section shall be at least [REDACTED], combined single limit, for each single occurrence of bodily injury and/or property damage and the like. TMC shall provide to Biogen copies of each insurance policy obtained under this Section and all renewals of such policies.

#### SECTION 10 - TERMINATION.

10.1 Term. Except as otherwise specifically provided herein and unless sooner terminated pursuant to Sections 10.2 or 10.3, this Agreement and the licenses and rights granted hereunder shall remain in full force and effect until TMC's obligations to pay compensation hereunder terminates in accordance with Sections 6.3 and 6.5. Upon expiration of TMC's obligation to pay royalties and/or a percentage of Sublicense Royalty Income under Sections 6.3 and 6.5 with respect to a specific country as to which TMC's license is then in effect, the license shall be deemed to be fully paid and TMC shall thereafter have a royalty-free right to use the Biogen Patent Rights and Biogen Technology to make, have made, use, import, offer to sell and sell Product in such country.

10.2 Termination for Breach. In addition to any other available remedies, either party shall have the right to terminate this Agreement in the event of a material breach of this Agreement by the other party, provided that the breach is not cured within ninety (90) days after written notice thereof is received from the non-breaching party.

10.3 Termination for Convenience. TMC shall have the right to terminate this Agreement for any reason upon ninety (90) days prior written notice to Biogen.

10.4 Survival of Rights and Obligations. Termination or expiration of the Agreement for any reason shall be without prejudice to any rights which shall have

accrued to the benefit of either party prior to such termination or expiration, including damages arising from any breach hereunder. In addition, Sections 5, 6.12, 9, 10.6, 13, 14 and the last sentence of Section 7.1 shall survive any such termination or expiration.

10.5 Consequence of Termination as to Japan Upon termination under Section 4.5 of the rights and licenses granted to TMC in Japan but not the entire Agreement, (i) TMC shall have no further right or license under this Agreement in Japan, and (ii) TMC shall grant to Biogen and its Affiliates and sublicensees a permanent and irrevocable right of access and reference to all regulatory submissions, including regulatory approvals, applicable to Product in Japan, and shall notify the applicable regulatory authorities of such right no later than thirty (30) days thereafter. If any right of access and reference granted under the preceding sentence is not sufficient to permit Biogen or its sublicensees to file an application for regulatory approval and receive regulatory approval for the sale of Product in Japan, TMC shall within sixty (60) days of receipt of notice from Biogen to that effect, provide Biogen with the complete data package that TMC used in such regulatory submissions, or if none, in regulatory submissions in United States in order to allow Biogen or its Affiliates or sublicensees to conduct clinical trials or file for regulatory approval for the sale of Product in Japan, provided that such data package shall be considered Confidential Information of TMC and shall be subject to Section 5. At the time of any termination of the license granted to TMC in Japan under Section 4.5, TMC and Biogen shall negotiate in good faith a commercially reasonable royalty to be paid to TMC for use of TMC-generated data and access to TMC's regulatory filings related to Product.

10.6 Consequences of Termination of Agreement. If TMC terminates this Agreement under Section 10.3 or if Biogen terminates this Agreement under Section 10.2,

TMC shall, at TMC's expense, return to Biogen all Biogen Technology furnished to TMC by Biogen, including any unused Biogen Inventory and UCB Material, and shall transfer to Biogen all TMC Technology generated in connection with the Product development and commercialization program. In the event Biogen terminates this Agreement under Section 10.2, TMC shall grant to Biogen an exclusive, royalty-free license, with the right to grant sublicenses, to all TMC Patent Rights and TMC Technology related to Product. If TMC terminates this Agreement under Section 10.3, TMC shall grant to Biogen an exclusive license, with the right to grant sublicenses, to TMC Technology and TMC Patent Rights in consideration for which Biogen shall, as its sole obligation to TMC, pay royalties to TMC on sales of Product (i) in indications other than Cardiology Indications if the manufacture, use or sale of the Product in such indication is covered by a claim of a TMC Patent Right other than a claim to an improvement to Peptide or the Semilog Process or (ii) in any indication if marketing approval for Product in such indication was based on phase III clinical data generated by TMC, at a royalty rate to be negotiated in good faith by the parties at the time of termination based on the parties' relative levels of investment in the Product and taking into consideration any damage or delay to the development and commercialization of Product caused by TMC's termination of this Agreement. Upon termination of this Agreement other than by TMC under Section 10.2, TMC shall, at TMC's expense, grant to Biogen an irrevocable right of reference or assign to Biogen, as requested by Biogen, TMC's rights in any regulatory filings related to Product and shall assign to Biogen any trademarks, together with all goodwill associated therewith, used in connection with Product. Upon termination of this Agreement for any reason, TMC shall assign to Biogen, at no cost to Biogen, (i) any regulatory filings and data and information originally assigned by Biogen to TMC, (ii) all of TMC's rights in the CSL Agreement and

the HRI Agreement, and (iii) all of TMC's rights to the HIRULOG trademark, together with all goodwill associated therewith, provided that if this Agreement has been terminated by TMC under Section 10.2, Biogen shall reimburse TMC for its out-of-pocket costs of assigning the trademark, together with all goodwill associated therewith, and regulatory filings to Biogen. Upon termination of this Agreement for any reason, the licenses granted to TMC under Section 2.1 of this Agreement shall terminate and the parties shall have no further rights or obligations under this Agreement except as set forth in Section 10.4. Any matter related to termination with respect to which the parties cannot agree will be referred to binding arbitration pursuant to Section 14.7. Notwithstanding anything in this Section 10.6 to the contrary, neither party shall be prevented from initiating a claim for damages due to a breach of this Agreement by the other party.

#### SECTION 11 - No Hire.

TMC shall not knowingly hire as an employee or employ directly as a consultant any person who is an employee of Biogen at the time of the employment offer from TMC or who has been an employee of Biogen within four (4) years of the date of the employment offer from TMC. Breach of this Section 11 by TMC shall be considered a material breach by TMC of this Agreement. In the event of any breach by TMC of this Section 11, Biogen shall have the right to terminate this Agreement for material breach under Section 10.2, or, in lieu of terminating this Agreement, may elect the following remedy as payment of liquidated damages: (i) immediate payment to Biogen by TMC of [REDACTED] and (ii) an increase of [REDACTED] in the royalty rates applicable to Net Sales of Product under Section 6.3 and the rate applicable to Sublicense Royalty Income under Section 6.5 of this Agreement. Election of the liquidated damages remedy by Biogen shall not be

deemed a waiver and shall not in any way limit Biogen's right to terminate this Agreement for any subsequent breach of this Section or any material breach of any other provision of this Agreement.

## SECTION 12 - TRADEMARKS, PATENT MARKING AND LITERATURE

### 12.1 HIRULOG Trademark.

(a) Biogen hereby assigns to TMC all of Biogen's rights, title and interest in and to the HIRULOG trademark in the Territory, together with all goodwill associated therewith. Biogen shall execute all documents reasonably requested by TMC to effect the foregoing assignment. TMC shall promptly reimburse Biogen for all costs and expenses incurred by Biogen in connection with assignment of the HIRULOG trademark, together with all goodwill associated therewith, to TMC.

(b) TMC shall maintain and prosecute the HIRULOG trademark in the Territory using efforts and professional representation consistent with the level of effort and professional representation as would be applied by a mid-size biopharmaceutical company in prosecuting and maintaining its own trademarks. TMC shall bear all of the costs of prosecution and maintenance of the HIRULOG trademark after the Effective Date. TMC shall provide copies to Biogen of all filings of trademark applications and all notices of grants which TMC sends or receives related to the HIRULOG trademark. If TMC decides to abandon the HIRULOG trademark or allow the HIRULOG trademark to lapse in any country, TMC shall inform Biogen at least ninety (90) days prior to the effective date of such decision and, at Biogen's request, shall take all reasonable action, at Biogen's expense, to assign the HIRULOG trademark, together with all goodwill associated therewith, back to Biogen in such country.

12.2 Patent Marking. At Biogen's request, TMC shall mark, and shall require its Affiliates or Sublicensees to mark, any and all forms of Product and Product packaging with an appropriate patent marking identifying the issued patents of the Biogen Patent Rights which cover the Product.

12.3 Promotional Literature. At Biogen's request, TMC shall describe its relationship with Biogen in TMC's promotional literature and advertising related to Product. Biogen shall have the right to review any such description prior to use.

### SECTION 13 - PUBLICITY

The parties agree that the public announcement of the execution of this Agreement shall be in the form of a press release mutually agreeable to the parties. Each party shall be entitled to make or publish any public statement concerning this Agreement consistent with the press release or as otherwise mutually agreed by the parties. The terms of this Agreement which are not divulged in the approved press release may not be disclosed except to a government agency as required by law. In any disclosure made to a government agency under the preceding sentence, the disclosing party shall request confidential treatment of the sensitive terms and conditions such as financial terms of this Agreement, and shall provide such confidential treatment request to the other party for review and comment.

### SECTION 14 - GENERAL PROVISIONS.

14.1 Assignment. Neither party shall have the right to assign this Agreement without the prior written consent of the other party, except that either party without the consent of the other party may assign this Agreement to an Affiliate or to a successor in

interest or transferee of all or substantially all of the assets of such party. This Agreement shall be binding upon and inure to the benefit of the parties hereto and their respective successors in interest and permitted assignees. Any such successor or permitted assignee of a party's interest shall in writing expressly assume and agree to be bound by all of the terms and conditions of this Agreement. No assignment shall relieve the assignor of any of its obligations under this Agreement.

14.2 Force Majeure. Neither party shall be liable to the other party for any failure or delay in performance of any obligation under this Agreement if the failure is caused by fire, explosion, flood, earthquake, strike or lockout, embargo, civil commotions, riots, wars, or any similar cause beyond such party's reasonable control, provided that the party claiming this exception has exerted all reasonable efforts to avoid or remedy such event and provided such event does not extend for more than six (6) months.

14.3 Independent Parties. The relationship between Biogen and TMC is that of independent contractors. Biogen and TMC are not and shall not be deemed to be joint venturers, partners, principal and agent, master and servant, employer or employee, and have no relationship other than as independent contracting parties. Neither party shall have the authority to bind or obligate the other party in any manner except as may be expressly provided herein or authorized in writing.

14.4 Entire Agreement. This Agreement sets forth the entire agreement and understanding between the parties as to the subject matter hereof and all prior agreements negotiations, representations and understandings, including a certain letter of intent dated February 7, 1997, are superseded hereby. No amendments, modifications or supplements to this Agreement may be made, except by means of a written document which is signed by authorized representatives of both parties.

14.5 Severability. If any provision of this Agreement is found by a court to be void, invalid or unenforceable, the same shall either be reformed to comply with applicable law or stricken if not so conformable, so as not to affect the validity or enforceability of this Agreement, except if the principal intent of this Agreement is frustrated by such reformation or deletion in which case this Agreement shall terminate.

14.6 Governing Law. This Agreement shall be construed and enforced in accordance with the laws of the Commonwealth of Massachusetts without reference to its choice-of-law principles.

14.7 Dispute Resolution. Any dispute arising out of or relating to this Agreement or to a breach thereof, including its interpretation, performance or termination, may be submitted by a party for resolution by binding arbitration. The arbitration shall be conducted by three (3) arbitrators. Each party shall select one arbitrator to serve on an arbitration panel to decide the issue. The arbitrator selected by a party shall not be a past or present employee of or consultant to such party or of any Affiliate or Sublicensee of such party. The arbitrators selected by the parties shall, within ten (10) days of their selection, select a third member to serve on the panel. If the arbitrators selected by the parties cannot, within ten (10) days of their selection, agree on a third member, the parties shall request that the American Arbitration Association ("AAA") select the third member who shall not be a past or present employee of or consultant to either party or of any Affiliate or Sublicensee of either party. Each party shall then have thirty (30) days from the date the panel is complete to submit to the panel and to the other party a written statement presenting such party's position on the issue. The panel shall, within thirty (30) days after receipt of both parties statements, hold a joint meeting on the issue at which each party will have an opportunity to make a presentation and to respond to the other party's presentation. Within fifteen (15) days of the conclusion of the meeting, the panel

shall render its decision in writing. The decision of the panel shall be final and binding on the parties. Each party shall bear its own costs in connection with the arbitration proceedings, including the costs of the arbitrator selected by it. The costs of the third arbitrator will be shared equally. The arbitration shall be held in the Commonwealth of Massachusetts and conducted under the rules of the AAA, except as otherwise expressly provided in this Section.

14.8 Headings. The headings in this Agreement have been included for convenience only, and shall not be used to construe the meaning of this Agreement.

14.9 Waiver. Failure of a party to enforce its rights under this Agreement shall not constitute a waiver of that right or the ability to later assert that right relative to the particular situation involved or to terminate this Agreement as a result of any subsequent default or breach.

14.10 Notices. Any notices given pursuant to this Agreement shall be in writing and shall be deemed delivered upon the earlier of (i) when received at the address set forth below, or (ii) three (3) business days after mailed by certified or registered mail postage prepaid and properly addressed, with return receipt requested, (iii) one (1) business day after being sent by a reputable nationwide overnight courier service, or (iv) when sent, if sent, by facsimile, as confirmed by certified or registered mail or by overnight courier. Notices shall be delivered to the respective parties as indicated:

If to Biogen:

Biogen, Inc.  
14 Cambridge Center  
Cambridge, MA 02142  
Telephone: (617) 679-2000  
Fax: (617) 679-2617

with a copy to Vice President - General Counsel

If to TMC:

The Medicines Company  
One Cambridge Center  
Cambridge, MA 02142  
Telephone: (617) 225-9099  
Fax: (617) 225-2397

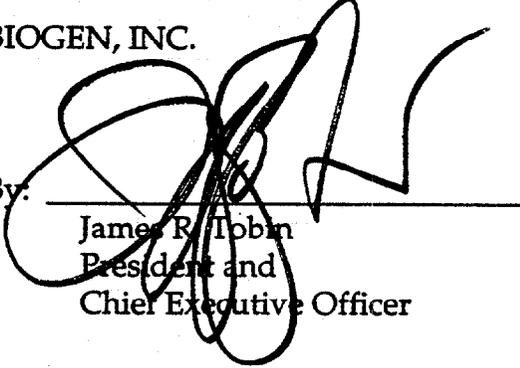
with a copy to President

14.11 Counterparts. This Agreement may be executed in any number of separate counterparts, each of which shall be deemed to be an original, but which together shall constitute one and the same instrument.

IN WITNESS WHEREOF, the parties have executed this Agreement as of the date set forth above.

BIOGEN, INC.

By: \_\_\_\_\_

  
James R. Tobin  
President and  
Chief Executive Officer

THE MEDICINES COMPANY

By: \_\_\_\_\_

  
Clive A. Meanwell  
President and  
Chief Executive Officer

## **APPENDIX A**

### **Patent Rights**

## HIRULOG PATENTS AND APPLICATIONS

REVISED APPENDIX

DKT. NO.	REL.	COUNTRY	APPLICATION NO.	APP. DATE	STA	PAT. NO.	PAT. DATE
B135	DIV	U. S. A.	08/439297	11MY1995	F		
B135	CIP	U. S. A.	07/549388	06JL1990	G	5196404	23MR1993
B135	CIP	U. S. A.	07/834259	10FE1992	G	5433940	18JL1995
B135	CIP	AUSTRALIA	62841/90	17AU1990	G	652125	06DE1994
B135	CIP	AUSTRIA	90912754.0	17AU1990	F		
B135	CIP	BELGIUM	90912754.0	17AU1990	F		
B135	CIP	BULGARIA	98566	24FE1994	F		
B135	CIP	CANADA	2065150	17AU1990	F		
B135	CIP	DENMARK	90912754.0	17AU1990	F		
B135	CIP	EUROPEAN PA	90912754.0	17AU1990	G	489070	24AP1996
B135	CIP	FINLAND	920672	17AU1990	F		
B135	CIP	FRANCE	90912754.0	17AU1990	F		
B135	CIP	GREAT BRITA	90912754.0	17AU1990	F		
B135	CIP	WEST GERMAN	90912754.0	17AU1990	F		
B135	CIP	HUNGARY	473/92	17AU1990	F		
B135	CIP	HUNGARY	P/P00684	30JE1995	G	211158	30AU1995
B135	CIP	ITALY	90912754.0	17AU1990	F		
B135	CIP	JAPAN	2-512078	17AU1990	F		

## HIRULOG PATENTS AND APPLICATIONS

DKT. NO.	REL.	COUNTRY	APPLICATION NO.	APP. DATE	STA	PAT. NO.	PAT. DATE
B135	CIP	KOREA SOUTH	92-700364	17AU1990	F		
B135	CIP	LUXEMBOURG	90912754.0	17AU1990	F		
B135	CIP	MEXICO	923196	24JE1992	G	183498	09DE1996
B135	CIP	NETHERLANDS	90912754.0	17AU1990	F		
B135	CIP	NORWAY	92.0616	17AU1990	F		
B135	CIP	SINGAPORE	9603269-3	17AU1990	F		
B135	CIP	SPAIN	90912754.0	17AU1990	F		
B135	CIP	SWEDEN	90912754.0	17AU1990	F		
B135	CIP	SWITZERLAND	90912754.0	17AU1990	F		
B135 REEXAM	X	U. S. A.	90/003511	27JL1994	F		
B154	ORG	U. S. A.	07/623611	07DE1990	G	5242810	07SE1993
B159	ORG	U. S. A.	07/652929	08FE1991	G	5240913	31AU1993
B159	DIV	U. S. A.	07/924549	31JL1992	G	5425936	20JE1995
B159	DIV	U. S. A.	08/431678	02MY1995	G	5514409	07MY1996
B159	ORG	AUSTRALIA	13621/92	03FE1992	G	659828	03FE1995
B159	DIV	BULGARIA	98558	24FE1994	F		
B159	ORG	BULGARIA	98559	24FE1994	F		
B159	ORG	CANADA	2079778	03FE1992	F		

## HIRULOG PATENTS AND APPLICATIONS

<u>DKT.NO.</u>	<u>REL.</u>	<u>COUNTRY</u>	<u>APPLICATION.NO.</u>	<u>APP. DATE</u>	<u>STA</u>	<u>PAT.NO.</u>	<u>PAT. DATE</u>
B159	ORG	EUROPEAN PA	92905748.7	03FE1992	F		
B159	ORG	FINLAND	924503	03FE1992	F		
B159	ORG	HUNGARY	P9203500	03FE1992	F		
B159	ORG	KOREA SOUTH	92-702485	03FE1992	F		
B159	ORG	MEXICO	923194	24JE1992	F		
B159	ORG	NEW ZEALAND	241557	07FE1992	G	241557	17MR1997
B159	ORG	NORWAY	92.3889	03FE1992	F		
B168	FWC	U. S. A.	08/328388	24OC1994	G	5446131	29AU1995

LICENSE AGREEMENT

This Agreement, made and entered into this 6<sup>th</sup> day of June 1990, by and between Biogen, Inc. a Massachusetts corporation, of 14 Cambridge Center, Cambridge, MA 02142 ("BIOGEN") and Health Research, Inc., a not-for-profit corporation of 1683 Empire State Plaza, Albany, New York 12237 ("HRI").

WHEREAS, HRI is a co-owner with BIOGEN of the Licensed Patent Rights (as herein defined); and

WHEREAS, BIOGEN desires to become exclusively licensed to HRI's rights in the Licensed Patent Rights.

NOW, THEREFORE, for good and valuable consideration and upon the mutual covenants and promises hereinafter set forth, the parties agree as follows:

1. DEFINITIONS

1.1 "Affiliates" shall mean any corporation, partnership, or other business organization which directly or indirectly controls, is controlled by, or is under common control with BIOGEN. For purposes of this Agreement, "control" shall mean the holding directly or indirectly of fifty percent (50%) or more of the voting stock or other ownership interest of the corporation or business entity involved.

1.2 "Licensed Patent Rights" shall mean the co-owned BIOGEN and HRI patent application listed in Exhibit A, attached hereto, and any foreign counterpart patent applications and any patents which issue therefrom, together with any extensions, reissues, renewals, divisions, continuations or continuations-in-part thereof, and any other co-owned BIOGEN and HRI patent applications or patents describing or arising out of the inventions covered by the patent application listed in Exhibit A.

1.3 "Licensed Product(s)" shall mean any product which falls within the scope of a claim of the Licensed Patent Rights or is made in whole or in part in accordance with a process which falls within the scope of a claim of the Licensed Patent Rights.

1.4 "Net Sales" shall mean the gross invoice price of Licensed Product(s) sold in any country by BIOGEN or its Affiliates

ATG

to any party other than sublicensees, less deductions for (i) any sales taxes, excise taxes and duties, (ii) packaging, shipping, handling and insurance charges, (iii) allowances and adjustments for spoiled, damages, outdated or returned Licensed Product(s), and (iv) trade discounts, to the extent such deductions are actually billed or credited to the customer. Sales or transfers of Licensed Product(s) between BIOGEN and its Affiliates shall not be deemed Net Sales unless BIOGEN or its Affiliates are the end users of the Licensed Product(s).

ATG

1.5 "Sublicense Income" shall mean the royalty income actually received by BIOGEN or its Affiliates from the sale of Licensed Product(s) by BIOGEN's sublicensees, less deductions for any withholding or other taxes. It is agreed that royalty income shall not include payments BIOGEN or its Affiliates receive from the supply of material, equipment, know-how, technical information and the like to BIOGEN's sublicensees, and shall not include the sales or transfers of Licensed Product(s) between BIOGEN or its Affiliates and the sublicensees unless the sublicensees are the end users of the Licensed Product(s).

2. LICENSE GRANT

2.1 Subject to the terms and conditions hereinafter set forth, HRI hereby grants to BIOGEN and its Affiliates a worldwide license, with the right to grant sublicensees, under the Licensed Patent Rights to make, have made, use, sell, and have sold Licensed Product(s). Such license shall be exclusive except that HRI reserves the right to use the Licensed Patent Rights for noncommercial research and educational purposes.

3. PAYMENTS

3.1 In consideration for the contribution to the scientific and technical developments relating to Licensed Products by the HRI, BIOGEN shall pay the following non-refundable fees to HRI:

- (a) [REDACTED] within thirty (30) days of execution of this Agreement.
- (b) [REDACTED] within thirty (30) days of BIOGEN's submission of the first Investigational New Drug application to the United States Food and Drug Administration ("FDA") for a Licensed Product.
- (c) [REDACTED] within thirty (30) days of Biogen's submission of the first New Drug Application to the FDA for a Licensed Product.

- (d) [REDACTED], within thirty (30) days of the FDA's approval of Biogen's first New Drug Application for a Licensed Product.

3.2 In consideration for the rights granted by HRI to BIOGEN hereunder, BIOGEN shall pay to HRI:

- (a) A minimum annual royalty of [REDACTED] beginning with the calendar year following the first sale of Licensed Product.
- (b) A royalty on the cumulative annual Net Sales of Licensed Product according to the following schedule:

<u>Cumulative Annual Net Sales</u>	<u>Royalty Percentage</u>
------------------------------------	---------------------------

[REDACTED]	[REDACTED]
[REDACTED]	[REDACTED]
[REDACTED]	[REDACTED]

- (c) A royalty of [REDACTED] of the Sublicense Income received by BIOGEN and its Affiliates.

3.3 BIOGEN shall be entitled to credit any minimum annual royalty paid to HRI in a calendar year pursuant to Section 3.2(a) against any royalties earned in that same calendar year and due to HRI pursuant to Section 3.2(b).

3.4 Unless this Agreement is terminated pursuant to Article 9, BIOGEN shall, within sixty (60) days after the last days of June and December in each year during the term of this Agreement, provide HRI with an accounting of Net Sales and/or Sublicense Income for the immediately preceding six (6) month period (the Royalty Period). Further, BIOGEN shall provide HRI with an accounting of the royalties due with respect to the preceding six (6) month period and shall, at the time when it delivers such account, make payment of the amount of royalty payment due under this Article 3.

3.5 BIOGEN shall keep (or cause to be kept) and maintain complete and accurate records of its Net Sales and Sublicense Income in accordance with generally accepted accounting procedures. Such records shall be accessible for review by HRI or by an independent certified public accountant selected and paid for by HRI and acceptable to BIOGEN (which acceptance shall not be unreasonably withheld), not more than once a year at any reasonable time during business hours within one (1) year after the end of the royalty period to which such records relate, for the purpose of verifying any royalty due thereon. The individual conducting such

review shall disclose to HRI only information relating to the accuracy of the records kept and the payments made, shall be under a duty to keep confidential any other information gleaned from such records.

3.6 All monies to be paid to HRI shall be made and computed in United States Dollars, and BIOGEN shall use its reasonable efforts to convert royalty payments payable on Net Sales and Sublicense Income in any country foreign to United States Dollars; provided, however, that if conversion to and transfer of Dollars cannot be made by BIOGEN in any country for any reason, BIOGEN may pay such sums in the currency of the country in which such sales are made, deposited in HRI's name in a bank designated by HRI in any such country. The rate of exchange of local currencies to United States Dollars shall be at the rate of exchange in force on the last business day of the Royalty Period as reported by The Wall Street Journal.

3.7 If BIOGEN or its Affiliates or their sublicensees, in order to operate under or exploit the licenses granted under Article 2 of this Agreement in any country, is required to make any payment (including, but not limited to, royalties, up-front payments, option fees or license fees) to one or more third parties to obtain a license or similar right in the absence of which the Licensed Products could not be used, manufactured or sold in such country without violating the property, patent or other right of a third party, BIOGEN may deduct from royalties otherwise payable to HRI an amount equal to such payments made during the same Royalty Period to such third party, provided that (a) the royalties paid to HRI for such Royalty Period shall not be reduced on any payment date by more than fifty percent (50%) and (b) BIOGEN provides HRI with evidence, reasonably satisfactory to HRI, of such third-party payments.

#### 4. COMMERCIALIZATION

4.1 BIOGEN agrees to use reasonable commercial efforts to research and develop, obtain regulatory approval and commercialize Licensed Product(s) in the United States and agrees to provide HRI with written annual reports on such efforts.

4.2 BIOGEN shall not use the name of HRI, the New York State Department of Health or New York State in any advertising or promotional sales literature without the prior written consent of HRI, except that BIOGEN may state that it is licensed by HRI under the Licensed Patents Rights.

#### 5. WARRANTIES

5.1 Each party represents and warrants that it has the full power and authority to enter into this Agreement and that entering

into this Agreement does not breach any existing agreements already signed by that party.

5.2 Nothing herein contained shall be construed by either party hereto as a representation or warranty that the exercise of the licensed rights will not constitute an infringement of the intellectual property rights of third parties.

5.3 BIOGEN agrees that HRI shall have no responsibility, or liability with respect to any Licensed Product and agrees to hold HRI, the New York State Department of Health and New York State harmless from any and all damages, losses, costs and expenses which they incur as a result of any action, claim or demand as a result of activities by BIOGEN, its Affiliates and sublicensees arising out of or relating to Licensed Product.

5.4 BIOGEN, its Affiliates and its sublicensee(s) agree to comply with all regulations and safety standards of government agencies such as the Consumer Product Safety Act and the Food and Drug Act.

## 6. PATENTS

6.1 BIOGEN shall be responsible for, and bear the costs of, the filing, prosecution, issuance, enforcement, defense and maintenance of the Licensed Patent Rights, *except as provided for in section 6.2 below.*

6.2 BIOGEN and HRI shall promptly notify the other in writing of any actual or threatened infringement of any Licensed Patent Rights, and shall at the same time provide the other with any available evidence of infringement. The parties shall then discuss what action, if any, each parties believes should be taken in the matter.

(a) In the event BIOGEN, alone or with an Affiliate or sublicensee, wishes to take action in a suit to enforce or defend any Licensed Patent Rights, BIOGEN may take action and, at its option, join HRI as a plaintiff. BIOGEN, alone or with its Affiliates and sublicensees, shall exercise control over such action and shall bear all costs thereof, including, but not limited to attorney's fees; provided that HRI may, if it so desires, be represented by counsel of its own selection, the fees for which counsel shall be paid by HRI. Any recovery from such action shall be retained by BIOGEN or shared by BIOGEN, its Affiliates or sublicensees.

(b) In the event BIOGEN does not take action in a suit to enforce or defend any Licensed Patent Rights and HRI wishes to take action, HRI may take action and, at its option, join BIOGEN as a plaintiff. HRI shall exercise control over such action and shall bear all costs thereof, including, but not limited to

attorney's fees; provided that BIOGEN may, if it so desires, be represented by counsel of its own selection, the fees for which counsel shall be paid by BIOGEN. Any recovery from such action shall be retained by HRI.

6.3 In any suit as either party may institute or control to enforce or defend the Licensed Patent Rights pursuant to this Agreement, the other party hereto agrees, at the request and expense of the party initiating or controlling the suit, to cooperate in all respects, to have its employees testify when requested and to make available relevant records, papers, information, samples, specimens, and the like.

6.4 Neither party may enter into a settlement or consent judgment or other voluntary and final disposition of any suit effecting the Licensed Patent Rights without the consent of the other party, which consent shall not be unreasonably withheld. Notwithstanding the foregoing, the party instituting or controlling (as the case may be) any suit referred to in this Article 6 shall have the right to settle any claims for infringement upon such terms and conditions as it, in its sole discretion, shall determine (including through the granting of a sublicense by BIOGEN).

6.5 In the event an infringement or infringements by third parties of the Licensed Patent Rights significantly affects BIOGEN's sales of Licensed Product(s) by capturing [REDACTED] of Biogen's market share, and neither HRI nor BIOGEN elect to bring an infringement suit against such infringer, the royalties hereunder payable by BIOGEN pursuant to Article 2 shall be reduced by [REDACTED] of the sums otherwise payable; provided, however, that BIOGEN presents information to HRI showing the loss of market share and that such infringer has refused to enter into a royalty bearing, sublicensing agreement with BIOGEN on terms reasonably acceptable to BIOGEN.

6.6 In the event that one or more patents, or particular claims therein (which read on the Licensed Product) within the Licensed Patent Rights expire, or are abandoned, or are declared invalid or by a court of last resort, or by lower court from whose decree no appeal is taken, or certiorari is not granted within the period allowed therefor, then such patents or particular claims shall, as of the date of expiration or abandonment or final decree of invalidity as the case may be, cease to be included within the License Patent Rights for the purpose of this Agreement. HRI agrees to renegotiate in good faith with BIOGEN a reasonable royalty rate under the remaining Licensed Patent Rights which are unexpired and in effect, and under which BIOGEN desires to retain a license if BIOGEN can demonstrate that subsequent to such

expiration, invalidity or abandonment of patents or particular claims (but not all of the Licensed Patent Rights) BIOGEN's market share of Licensed Products has been reduced by more than [REDACTED]

## 7. DURATION AND TERMINATION

7.1 The license granted hereunder shall continue until expiration of the last remaining patent granted from the Licensed Patent Rights, unless earlier terminated in accordance with this Article.

7.2 The royalty obligations in each country shall end on a country-by-country basis upon expiration of the patent granted from the Licensed Patent Rights in such country.

7.3 BIOGEN shall have the right to terminate this Agreement upon ninety (90) days prior written notice to HRI and upon payment of a termination fee equal to the minimum annual royalty set forth in Section 3.2(a) of this Agreement.

7.4 HRI shall have the right to terminate this Agreement: (a) in the event BIOGEN materially breaches this Agreement or fails to account for or pay royalties or minimum royalties as herein provided, provided, however, that if BIOGEN cures the said breach or default within ninety (90) days of notice, this license shall continue in full force and effect; and (b) immediately upon written notice to BIOGEN in the event of bankruptcy, liquidation or insolvency of BIOGEN.

7.5 Upon any termination of this Agreement nothing herein shall be construed to release either party of any obligation matured prior to the effective date of such termination, and BIOGEN may after the effective date of such termination sell all Licensed Product(s) that it may have on hand at the date of termination provided that it pays the royalties as provided in this Agreement.

## 8. NOTICES

8.1 Any notice required or permitted to be given hereunder shall be sent in writing by registered or certified airmail, postage prepaid, return receipt requested, or by telecopier, air courier or hand delivery, addressed to the party to whom it is to be given as follows:

If to BIOGEN:

BIOGEN, INC.  
14 Cambridge Center  
Cambridge, MA 02142  
Telecopier: 617 491 1228  
Attention: Vice President -  
Marketing and Business

With a copy to:

BIODEN, INC.  
14 Cambridge Center  
Cambridge, MA 02142  
Telecopier: 617 491 1228  
Attention: Vice President-  
General Counsel

If to HRI:

HEALTH RESEARCH INC.  
1683 Empire State Plaza  
Albany, NY 12237  
Telecopier: (518)474-4434  
Attention: Director of Operations

or to such other address or addresses as may from time to time be given in writing by either party to the other pursuant to the terms hereof.

8.2 Any notice sent pursuant to this Article shall be deemed delivered within 5 days if sent by airmail and within 24 hours if sent by air courier or hand delivery.

## 9. ARBITRATION

9.1 The parties desire to avoid and settle without litigation future disputes which may arise between them relative to this Agreement. Accordingly, the parties agree to engage in good faith negotiations to resolve any such disputes. In the event they are unable to resolve any such dispute through negotiation, then such dispute shall be submitted to arbitration in accordance with the Rules of the American Arbitration Association (hereinafter "Rules") then in effect and the award rendered by the arbitrators shall be binding as between the parties and judgment on such award may be entered in any court having jurisdiction thereof, provided, however, that with respect to any matter in dispute concerning royalties due and payable by one party, such party shall have previously exercised its rights to have an auditor examine the records of the other party pursuant to Article 3 herein before proceeding, and further provided that a dispute relating to the payments set forth in Paragraphs 3.1 and 3.2 of this Agreement which arises out of a contention regarding the interpretation or validity of the Licensed Patent Rights shall not be submitted to arbitration .

9.2 Three neutral arbitrators shall be appointed by the American Arbitration Association in accordance with ~~Section 12 of~~ such Rules, and at least one of such arbitrators shall be an attorney-at-law, and all decisions and awards shall be made by majority of them except for decisions relating to discovery and disclosures as set forth in Paragraph 9.3 hereof.

9.3 Notice of a demand for arbitration of any dispute subject to arbitration by one party shall be filed in writing with the other party and with the American Arbitration Association. The parties agree that after any such notice has been filed, they shall, before the hearing thereof, make discovery and disclosure of all matters relevant to such dispute. Discovery and disclosure shall be completed no later than ninety (90) days after filing of such notice of arbitration unless extended upon a showing of good cause by either party to the arbitration. The arbitrators may consider any material which is relevant to the subject matter of such dispute even if such material might also be relevant to an issue or issues not subject to arbitration hearing.

9.4 In the event a patent which is the subject matter of an award rendered by the arbitrators is subsequently determined to be invalid or unenforceable in a judgment rendered by a court of competent jurisdiction from which no appeal can or has been taken, such award may be modified by a court of competent jurisdiction upon application by any party to the arbitration. Any such modification shall govern the rights and obligations between the parties from the date of such modification.

#### 10. MISCELLANEOUS PROVISIONS

10.1 Neither party shall assign this Agreement without the written consent of the other party which consent shall not be unreasonably withheld; provided, however, that either party, without such consent, may assign or sell the same to an affiliate or in connection with the transfer or sale of all or substantially all of its business or in the event of its merger, consolidation, or joint venture with another company. Each assignee shall assume all obligations of its assignor under this Agreement. No assignment shall relieve either party of responsibility for the performance of any accrued obligations which such party then has hereunder.

10.2 This Agreement constitutes the entire understanding between the parties and may not be varied except by a written document signed by both parties.

10.3 This Agreement shall be construed, governed, interpreted, and applied in accordance with the laws of the Commonwealth of Massachusetts, except that questions affecting the validity, construction, and effect of any foreign patent shall be determined by the laws of the country in which the patents were granted.

10.4 The provisions of this Agreement are severable, and in the event that any of the provisions of this Agreement are determined to be invalid or unenforceable under any controlling

body of law, such invalidity or unenforceability shall not in any way affect the validity or enforceability of the remaining provisions hereof.

IN WITNESS WHEREOF, the parties hereto have hereunder set their hands and seals and duly executed this License Agreement the day and year first written above.

BIOGEN, INC.

HEALTH RESEARCH INC.

By: Alan Tuck

By: [Signature]

Name: Alan Tuck

Name: Lee J. VanDeCarr

Title: Vice President - Marketing & Business Development

Title: Sec/Treasurer

Date: 28 May 1990

Date: JUN 4 1990

**EXHIBIT A**

**Licensed Patent Rights**

1. United States patent application Serial No. 395,482 filed August 18, 1989, entitled "Novel Inhibitors of Thrombin"

AMENDMENT NO. 1 TO LICENSE AGREEMENT

This Amendment No. 1 to License Agreement is made as of this 1st day of April, 1996 by and between Biogen, Inc., a Massachusetts corporation with its principal offices located at 14 Cambridge Center, Cambridge, Massachusetts, 02142 ("Biogen") and Health Research, Inc., a not-for-profit corporation with offices located at 66 Hackett Boulevard, Albany, New York 12209 ("HRI").

Biogen and HRI are parties to a certain License Agreement dated as of June 4, 1990 ("License Agreement") under which HRI has granted to Biogen a license to HRI's rights in certain jointly-owned patents. Biogen and HRI would like to amend Section 10.1 of the License Agreement regarding assignment and sublicensing. Therefore, they agree as follows:

Section 1. Section 10.1 of the License Agreement shall be amended to read in its entirety as follows:

10.1 Neither party shall assign this Agreement without the written consent of the other party, which consent shall not be unreasonably withheld; provided, however, that either party without such consent, may assign or sell the same to an affiliate or in connection with the transfer or sale of all or substantially all of its business or in the event of its merger, consolidation or joint venture with another company. Except as provided below, each assignee shall assume all obligations of its assignor.

Each party shall give the other party prior written notice of any assignment for which consent is required hereunder. If a party providing notice of a proposed assignment has not received written objection to the assignment from the other party within ten (10) days after receipt of the notice, the other party shall be deemed to have consented to the assignment for purposes of this Section.

Biogen is co-owner of the Licensed Patent Rights, and Biogen may decide to retain its rights and obligations under Sections 6.1 through 6.4 of this Agreement notwithstanding the assignment of its other rights and obligations to a third party. However, whether or not Biogen decides to retain such rights and obligations, Biogen's assignee, as a condition to such assignment, shall be obligated to pay (1) the applicable non-refundable fees set forth in Section 3.1 (if they

have not previously been paid by Biogen), and (2) the royalties set forth in Section 3.2 of this Agreement.

If, in connection with an assignment, Biogen is a sublicensee of its assignee, HRI shall be entitled to receive (i) royalties on its assignee's Net Sales pursuant to Sections 3.2(a) and (b), and (ii) pursuant to Section 3.2(c), royalties on the Sublicense Income received by the assignee from sales by Biogen.

Each party shall properly notify the other party in writing of any completed assignment. Except as expressly provided above, upon assumption by assignee of all or part of this Agreement in accordance with this Section 10.1, the obligations assumed by the assignee shall cease in their entirety to be obligations of the assignor, except that no assignment shall relieve either party of its responsibility to the other party for the performance of any obligations accrued prior to the date of the assignment.

Section 2. The reference in the sixth line of Section 6.5 of the License Agreement to "Article 2" shall be changed to "Article 3."

IN WITNESS WHEREOF, the parties have executed this Amendment as of the date and year first above written.

BIOGEN, INC.

HEALTH RESEARCH, INC.

By: Kenneth M. Bates

By: Lee J. VanDeCarr

Name: Kenneth M. Bates

Name: Lee J. VanDeCarr, Sec/Treas, HRI

Title: VP Marketing & Sales

Title: Lee J. VanDeCarr, Sec/Treas, HRI



US005196404A

**United States Patent** [19]  
**Maraganore et al.**

[11] **Patent Number:** **5,196,404**  
[45] **Date of Patent:** **Mar. 23, 1993**

- [54] **INHIBITORS OF THROMBIN**
- [75] **Inventors:** John M. Maraganore, Concord, Mass.; John W. Fenton, II, Malden Bridge; Tomi Kline, New York, both of N.Y.
- [73] **Assignees:** Biogen, Inc., Cambridge, Mass.; Health Research, Inc., Albany, N.Y.
- [21] **Appl. No.:** 549,388
- [22] **Filed:** Jul. 6, 1990

**Related U.S. Application Data**

- [63] Continuation-in-part of Ser. No. 395,482, Aug. 18, 1989, abandoned.
- [51] **Int. Cl.<sup>5</sup>** ..... A61K 37/02; C07K 7/08; C07K 7/10
- [52] **U.S. Cl.** ..... 514/13; 514/12; 514/14; 530/326; 530/327; 530/325; 530/324; 623/11
- [58] **Field of Search** ..... 514/12, 13, 14; 530/326, 324, 325, 327; 623/11

**References Cited**

**FOREIGN PATENT DOCUMENTS**

- 276014 7/1988 European Pat. Off. .
- 333356 9/1989 European Pat. Off. .
- 341607 11/1989 European Pat. Off. .
- WO/9119734 12/1991 PCT Int'l Appl. .... 514/13

**OTHER PUBLICATIONS**

- Maraganore, J. et al., *Biochemistry*, 29: 7095-7101, Aug. 1990.
- DiMaio, J. et al., *JBC*, 265 (35): 21698-21703, 1990 (Dec. 15).
- Kettner, C. et al., *JBC*, 256 (24): 15106-15114, 1984.
- Bone, R. et al., *Biochemistry*, 26: 7609-7614, 1987.
- Liang, T. et al., *Biochemistry*, 26: 7603-7608, 1987.
- Hortin, G. et al., *JBC*, 265 (11): 6866-6871, Jun. 1991.
- Tsiang, M. et al., *Biochemistry*, 29: 10602-10612, 1990.
- Khai, T. et al., *Cell*, 64: 1057-1068, Mar. 1991.
- Krstenansky, J. et al., *Thrombosis Research*, 52: 137-141, 1988.

- Krstenansky, J. et al., *Thrombosis Research*, 54: 319-325, 1989.
- Scharf, M. et al., *FEBS Lett*, 255 (1): 105-110, Sep. 1989.
- Krstenansky, J. et al., *Throm. and Haemo*, 63: 208-214, 1990.
- S. Bajusz et al., "Inhibition of Thrombin and Trypsin by Tripeptide Aldehydes", *Int. J. Peptide Protein Res.*, 12, pp. 217-221 (1978).
- W. Bode et al., "The Refined 1.9 Å Crystal Structure of Human . . .", *Embo J.*, 8, pp. 3467-3475 (1989).
- A. Falanga et al., "Isolation and Characterization of Cancer Procoagulant: . . .", *Biochemistry*, 24, pp. 5558-5567 (1985) [Falanga I].
- A. Falanga et al., "A New Procoagulant in Acute Leukemia", *Blood*, 71, pp. 870-875 (1988) [Falanga II].
- J. W. Fenton II, "Regulation of Thrombin Generation and Function", *Semin. Thromb. Hemost.*, 14, pp. 229-235 (1988) [Fenton I].
- J. W. Fenton II, "Thrombin Bioregulatory Functions", *Adv. Clin. Enzymol.*, 6, pp. 186-193 (1988) [Fenton II].
- J. W. Fenton II et al., "Thrombin Anion-binding Exosite Interactions . . .", *Ann. New York Acad. Sci.*, 556, pp. 158-165 (1989) [Fenton III].

(List continued on next page.)

*Primary Examiner*—Lester L. Lee  
*Assistant Examiner*—Susan M. Perkins  
*Attorney, Agent, or Firm*—James F. Haley, Jr.; Andrew S. Marks; Margaret A. Pierri

[57] **ABSTRACT**

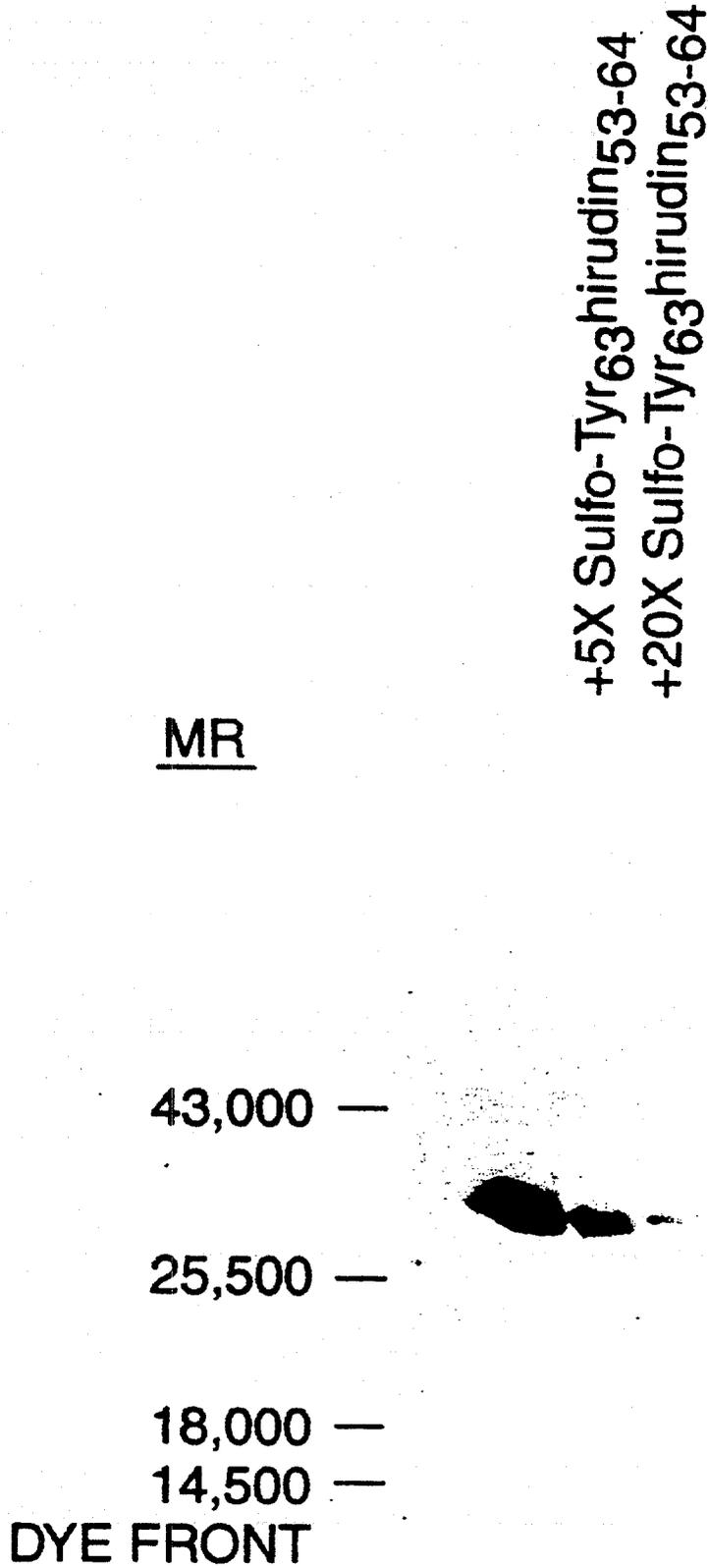
This invention relates to novel biologically active molecules which bind to and inhibit thrombin. Specifically, these molecules are characterized by a thrombin anion-binding exosite association moiety (ABEAM); a linker portion of at least 18 Å in length; and a thrombin catalytic site-directed moiety (CSDM). This invention also relates to compositions, combinations and methods which employ these molecules for therapeutic, prophylactic and diagnostic purposes.

37 Claims, 13 Drawing Sheets

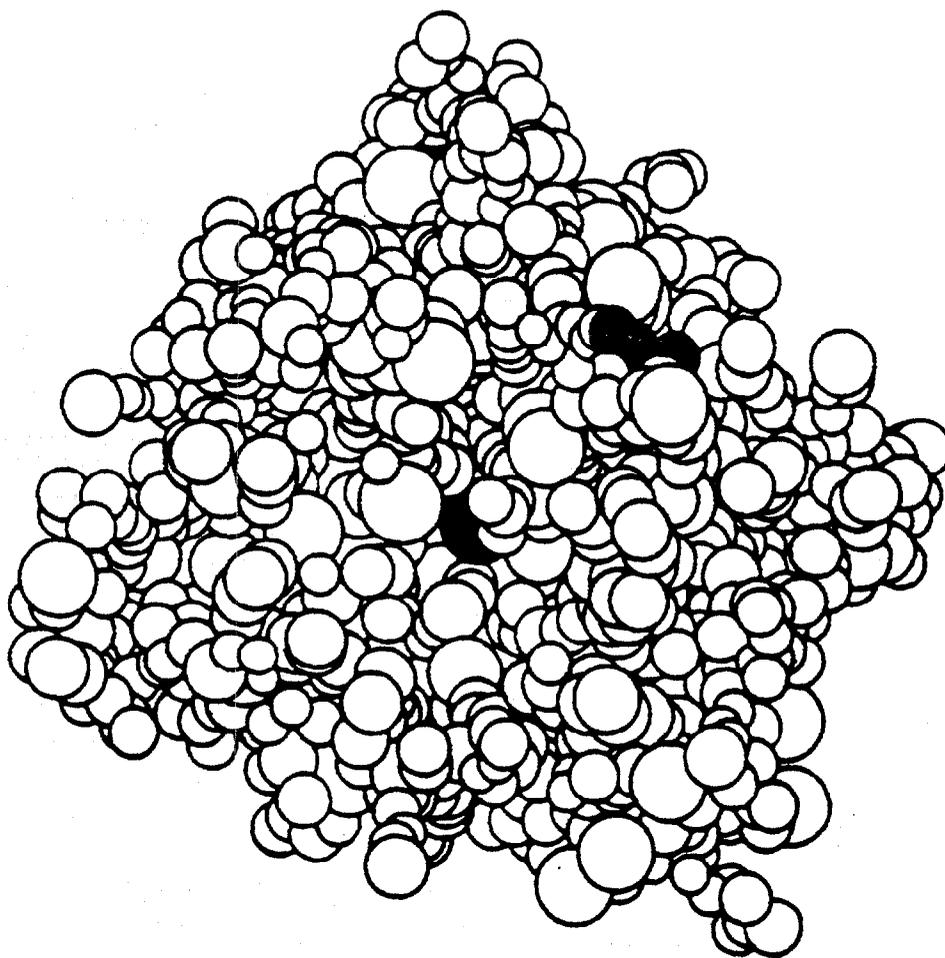
## OTHER PUBLICATIONS

- B. Furie et al., "Computer-Generated Models of Blood Coagulation Factor Xa, . . .", *J. Biol. Chem.*, 257, pp. 3875-3882 (1982).
- S. G. Gordon et al., "Cysteine Proteinase Procoagulant From Amnion-Chorion", *Blood*, 66, pp. 1261-1265 (1985).
- D. Gurwitz et al., "Thrombin Modulates and Reverses Neuroblastoma . . .", *Proc. Natl. Acad. Sci. USA*, 86, pp. 3440-3444 (1988).
- S. R. Hanson et al., "Interruption of Acute Platelet-dependent Thrombosis . . .", *Proc. Natl. Acad. Sci. USA*, 85, pp. 3184-3188 (1988).
- C. Kettner et al., "D-Phe-Pro-ArgCH<sub>2</sub>Cl-A Selective Affinity Label for Thrombin", *Thromb. Res.*, 14, pp. 969-973 (1979).
- S. Konno et al., "Analysis of the Secondary Structure of Hirudin and . . .", *Arch. Biochem. Biophys.*, 267, pp. 158-166 (1988).
- J. L. Krstenansky et al., "Anticoagulant Peptides: Nature of the Interaction . . .", *J. Med. Chem.*, 30, pp. 1688-1691 (1987) [Krstenansky I].
- J. L. Krstenansky et al., "Antithrombin Properties of C-Terminus of . . .", *FEBS Lett.*, 211, pp. 10-16 (1987) [Krstenansky II].
- J. M. Maraganore et al., "Anticoagulant Activity of Synthetic Hirudin Peptides", *J. Biol. Chem.*, 264, pp. 8692-8698 (May 1989).
- S. R. Stone et al., "Kinetics of the Inhibition of Thrombin by Hirudin", *Biochemistry*, 25, pp. 4622-4628 (1986).
- Krstenansky et al., *FEBS Letters*. Jan., 1987. 211(1): 10-16.
- Rose et al., *Advances In Protein Chemistry*. 1985, pp. 1 and 20-45.

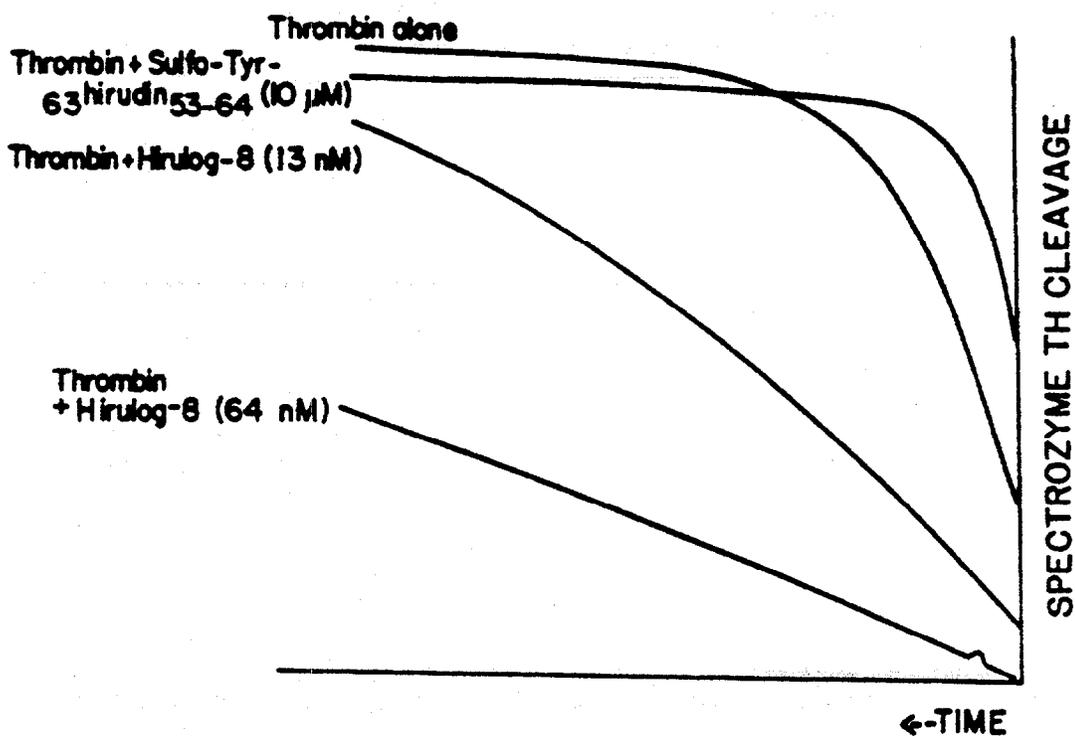
FIG. 1



**FIG. 2**



**FIG. 3A**



**FIG. 3B**

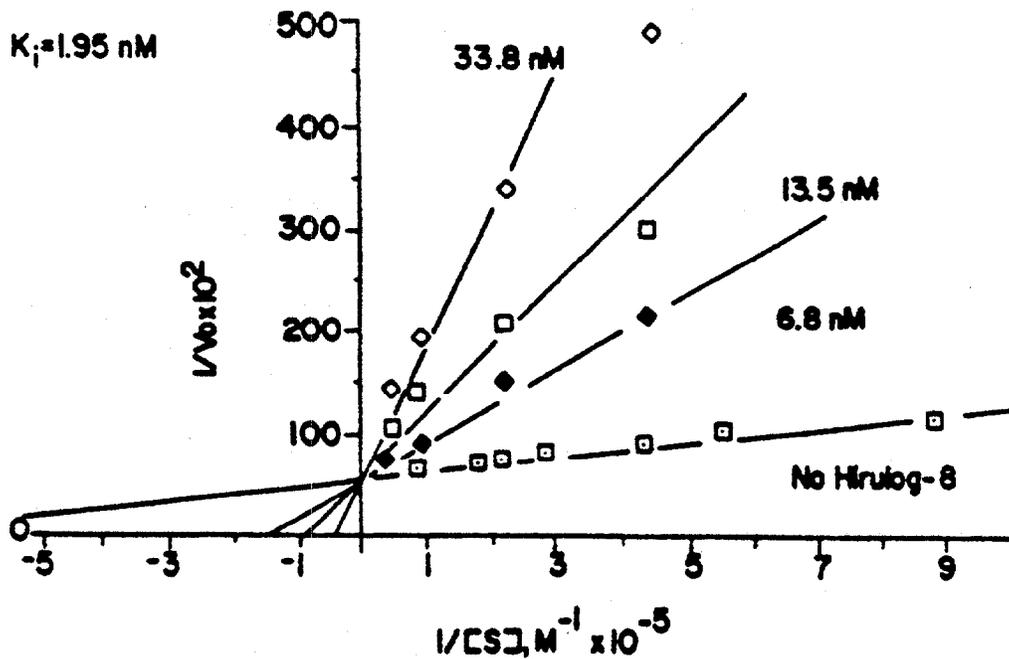


FIG. 4

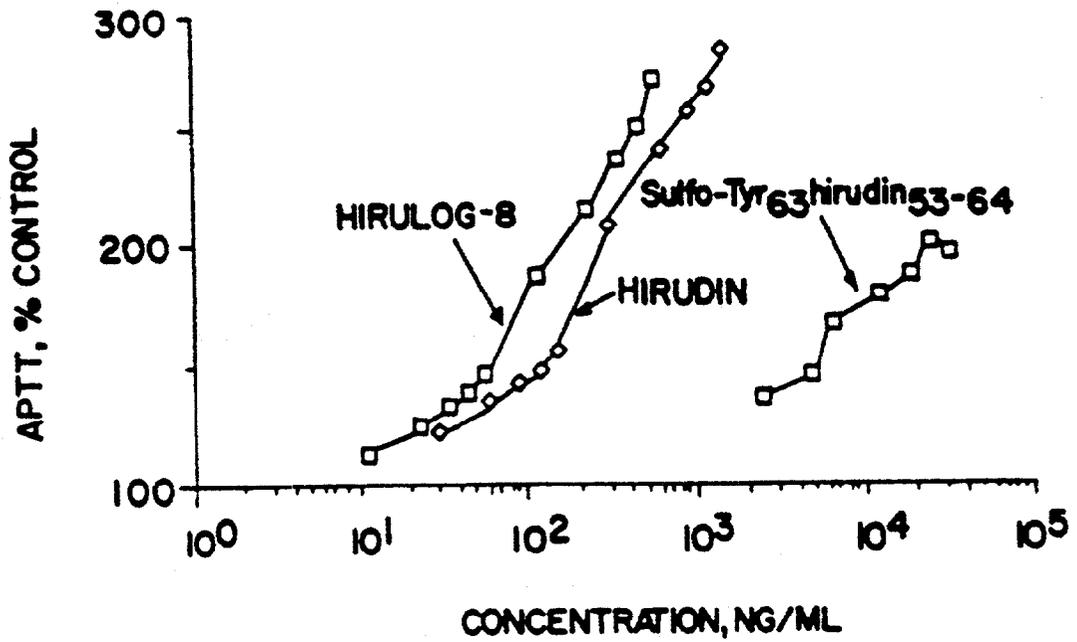
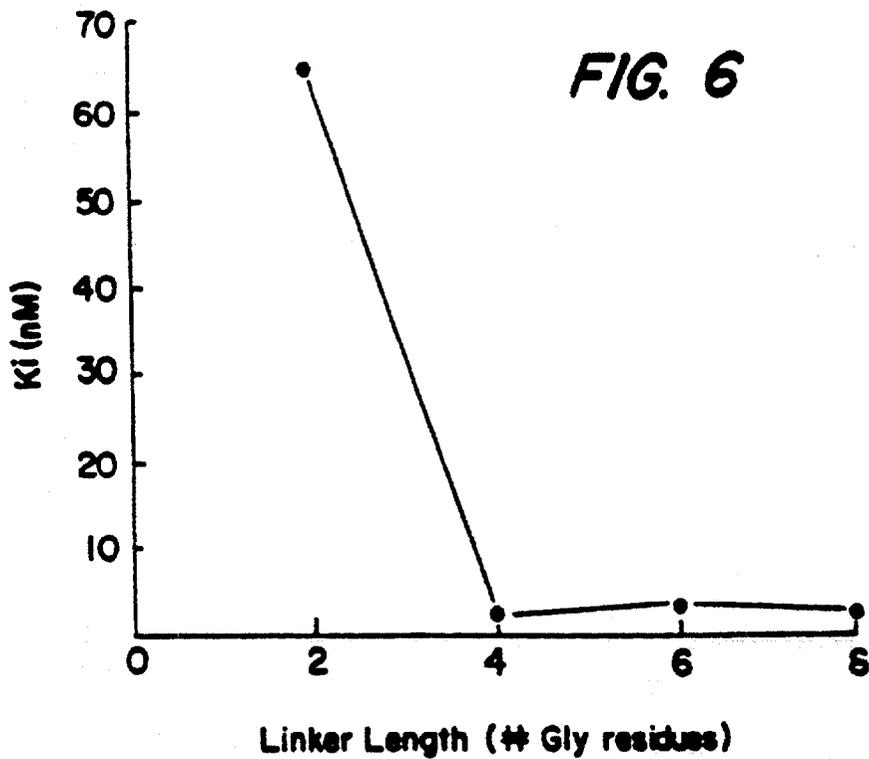


FIG. 6



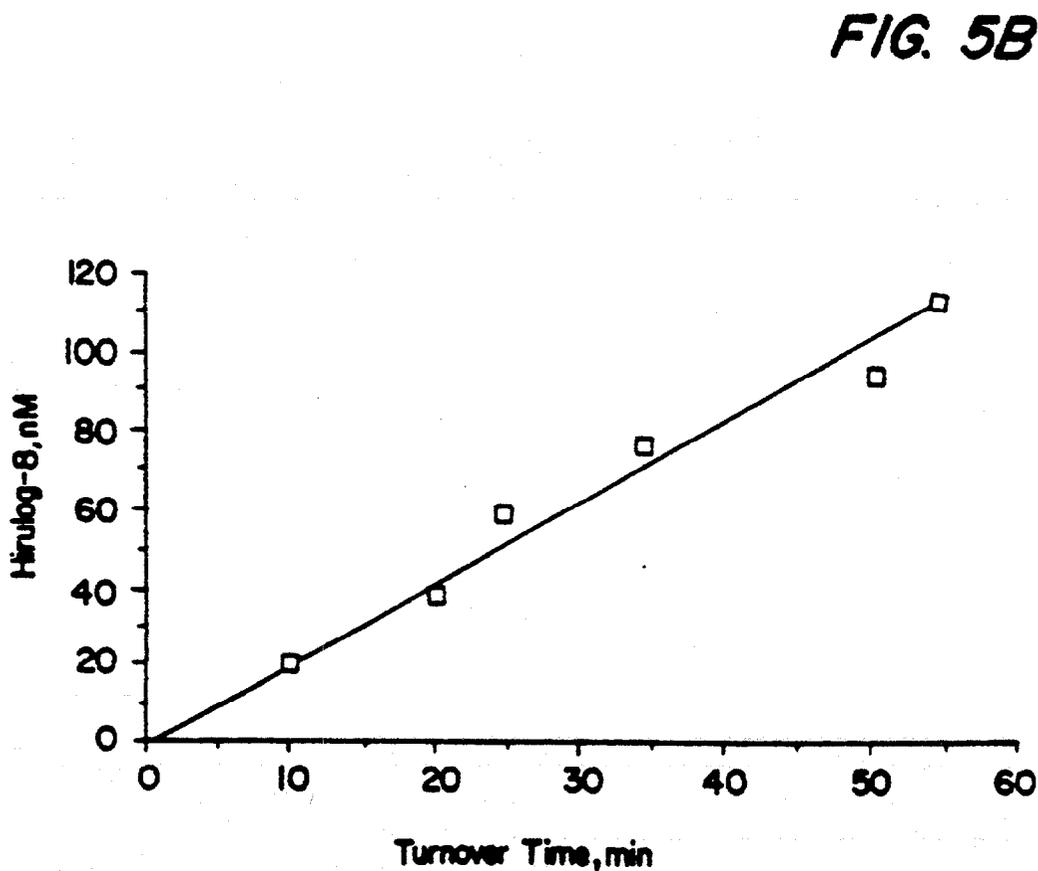
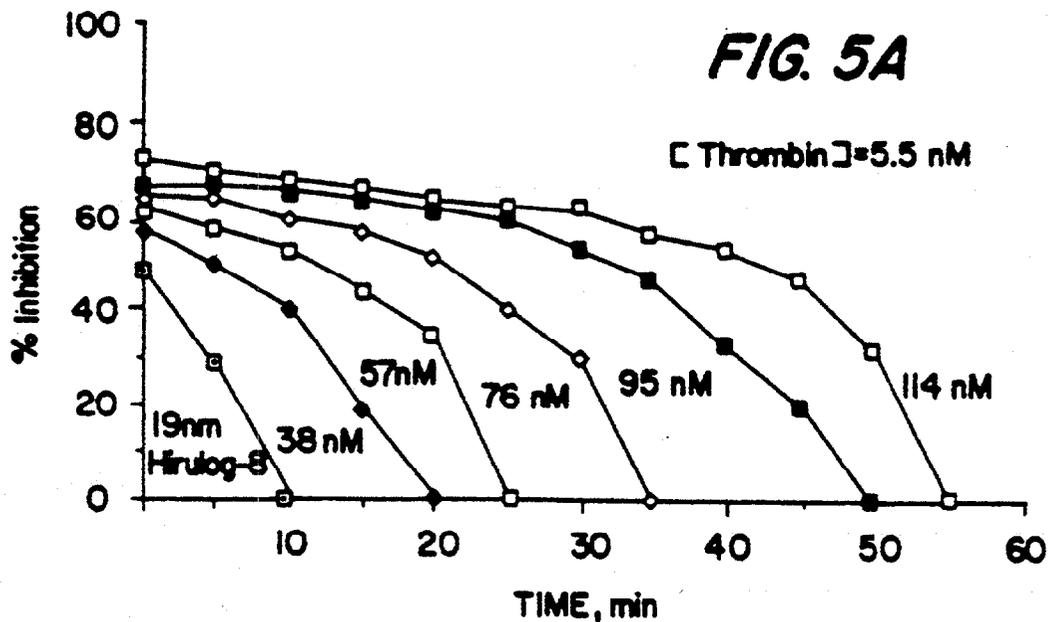


FIG. 7

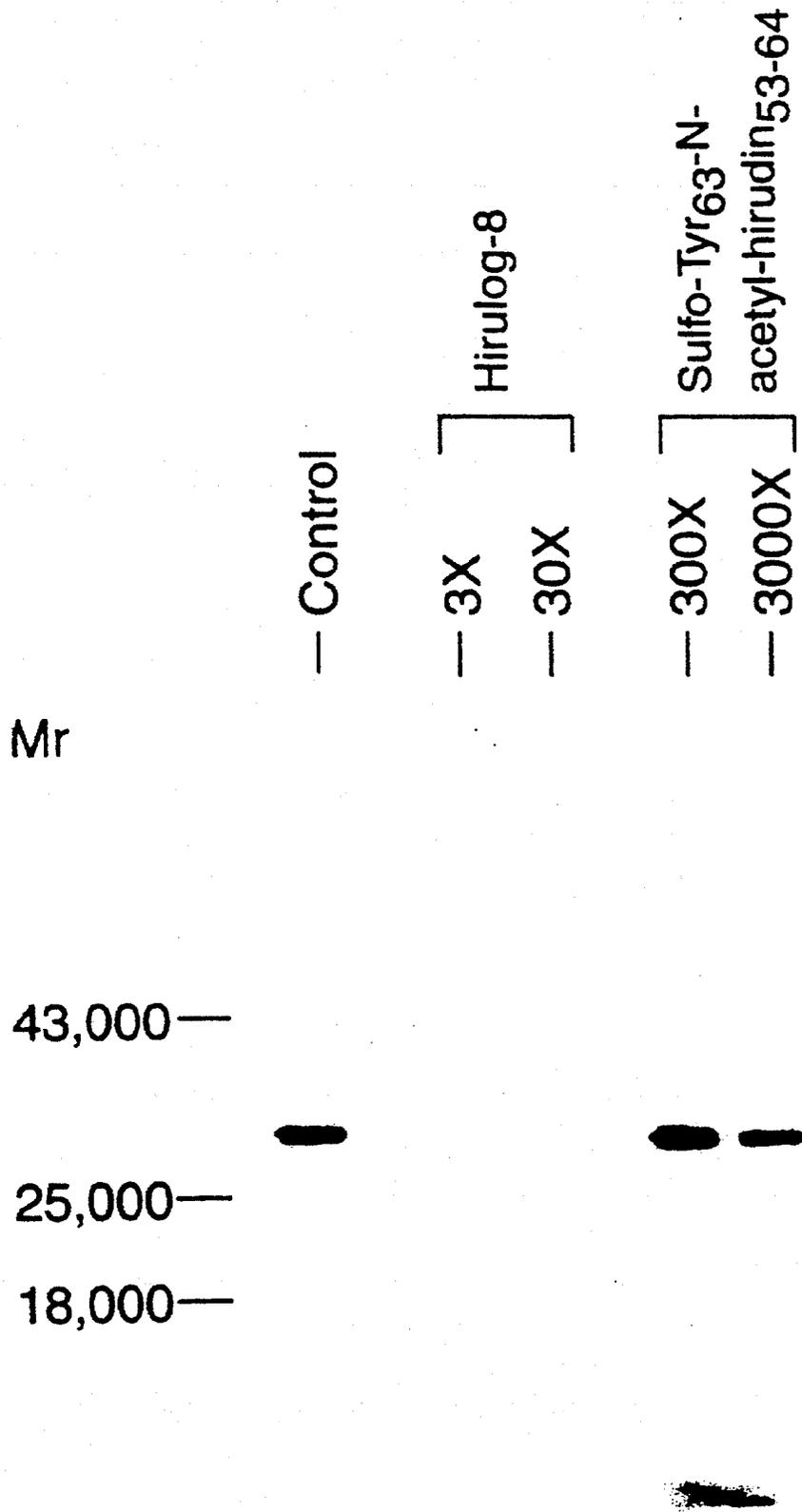


FIG. 8

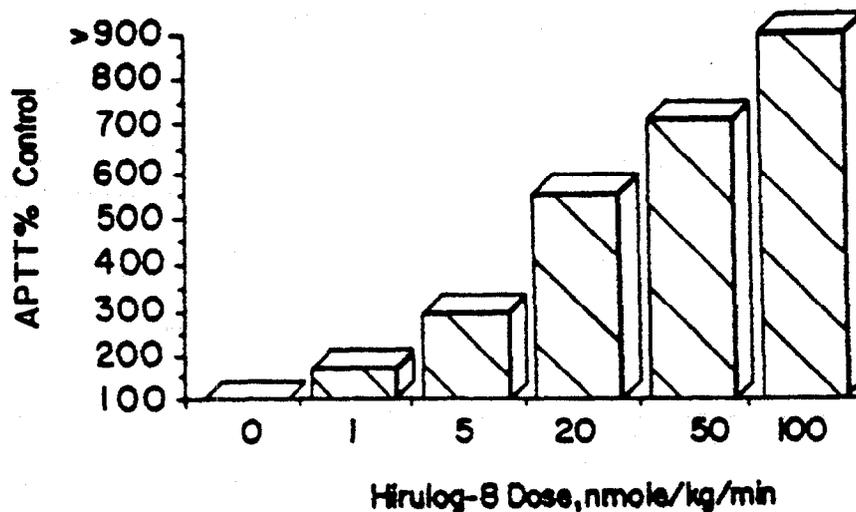
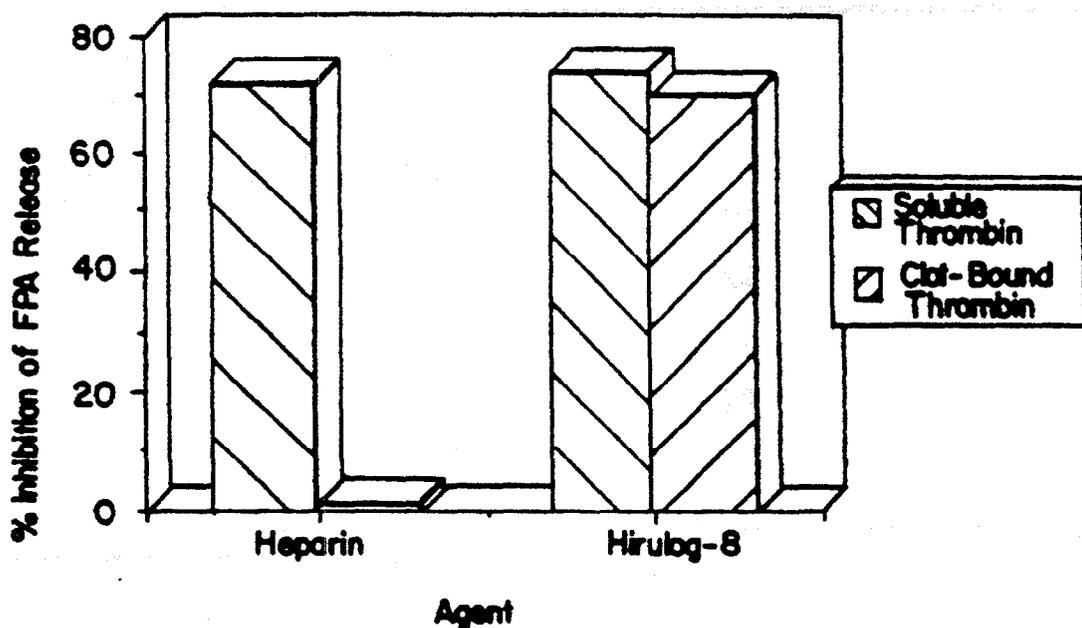


FIG. 9



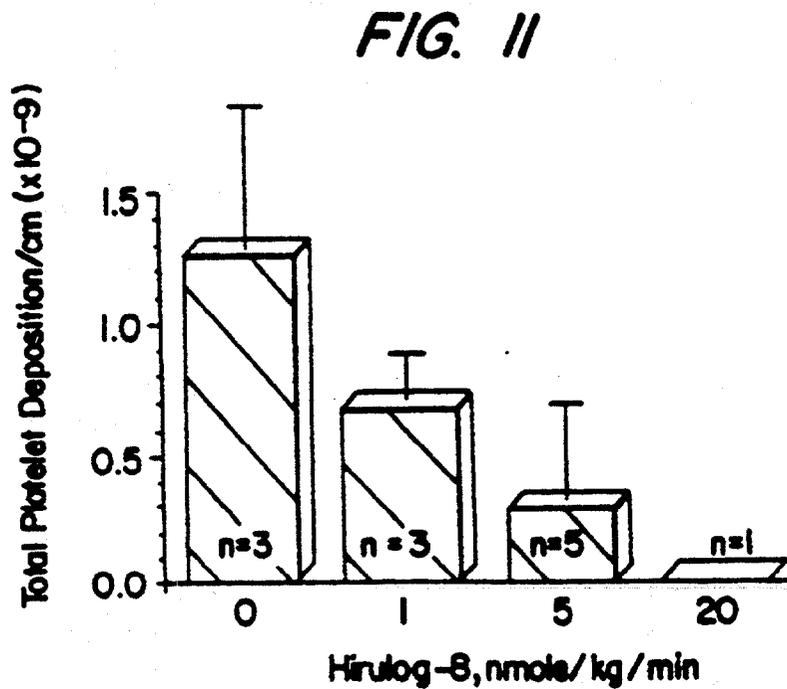
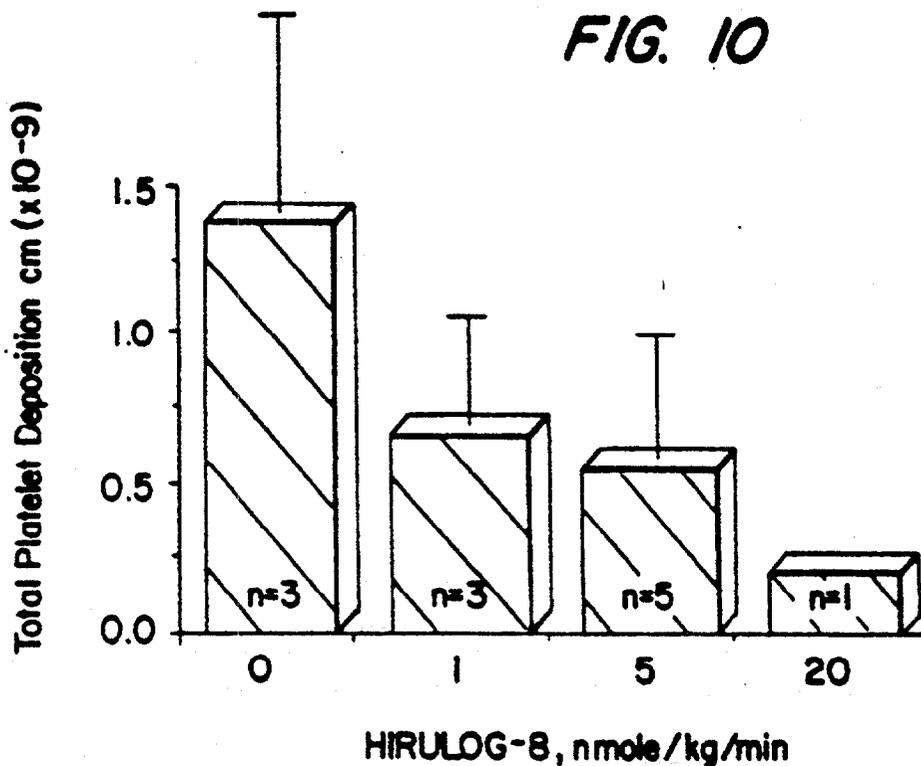


FIG. 12

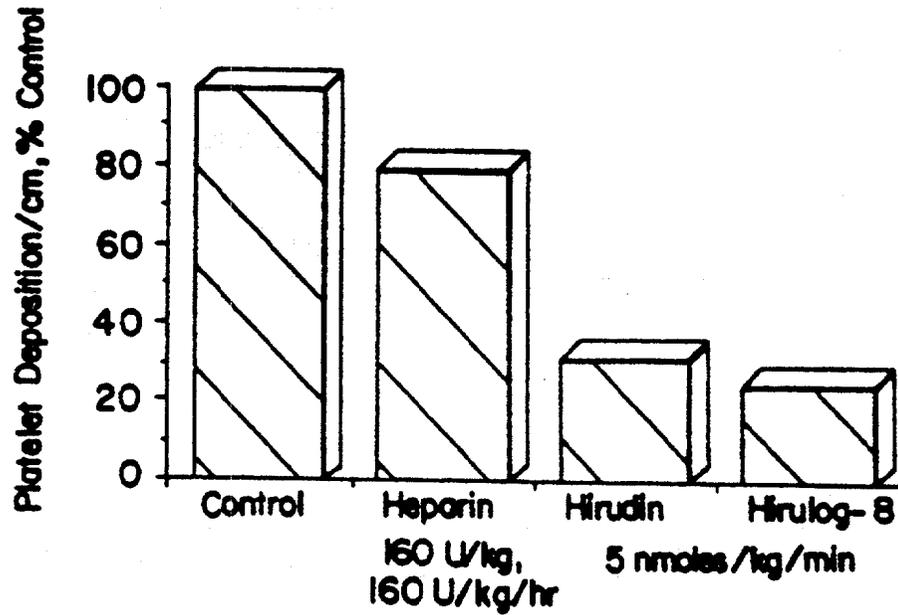
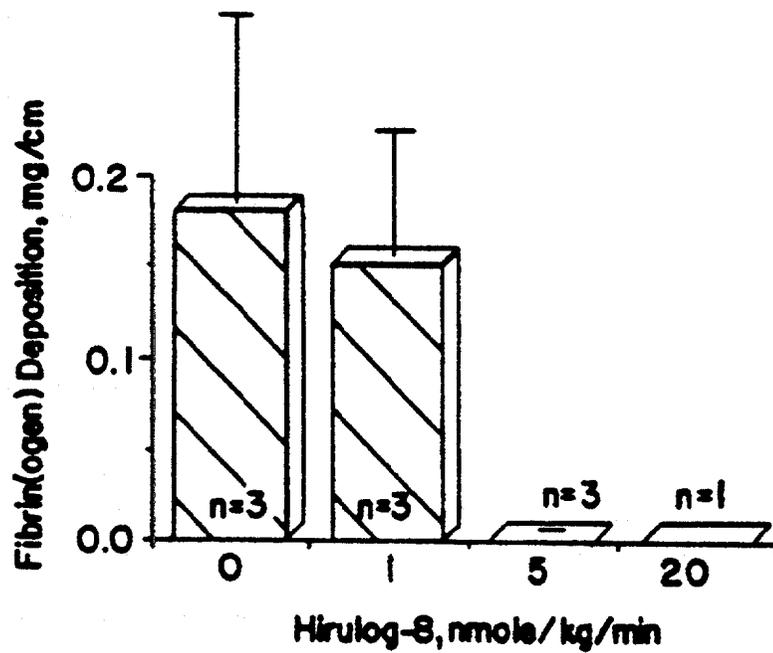
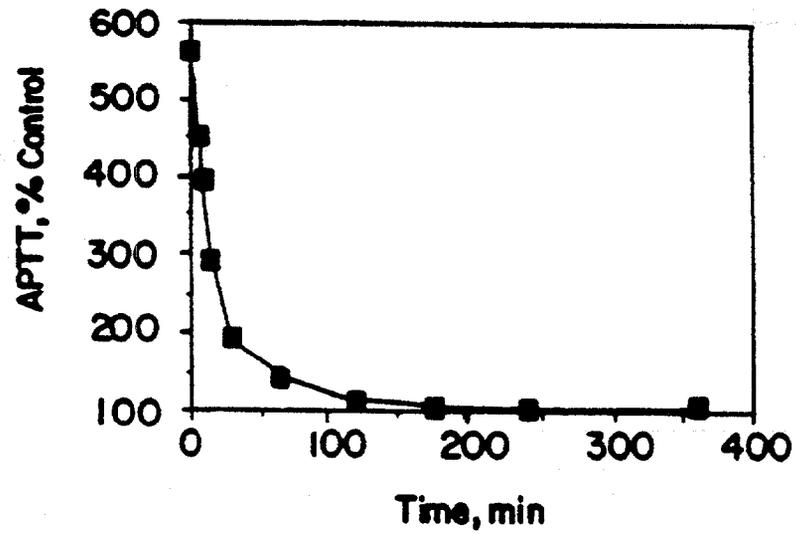


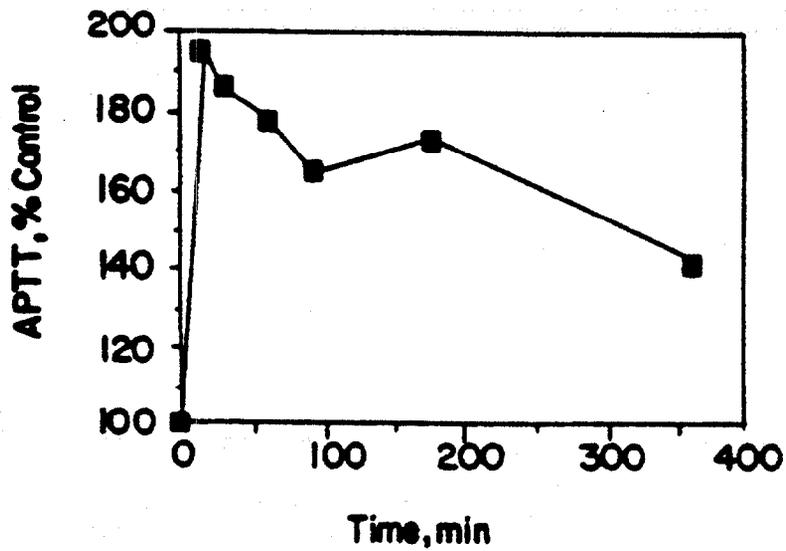
FIG. 13



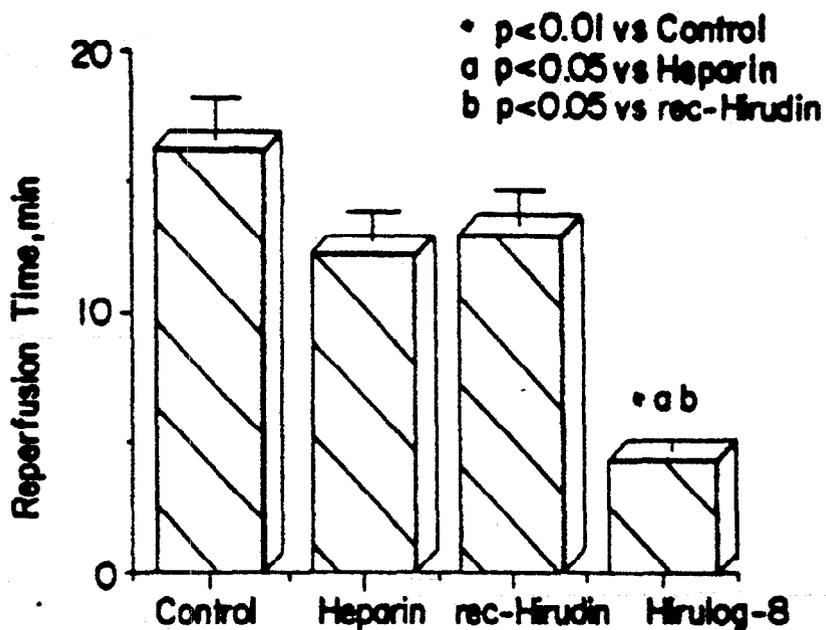
**FIG. 14**



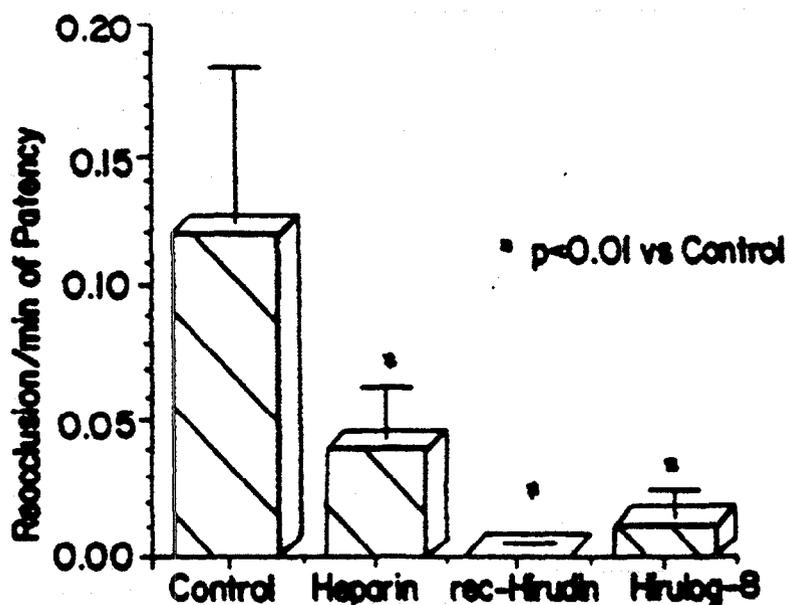
**FIG. 15**



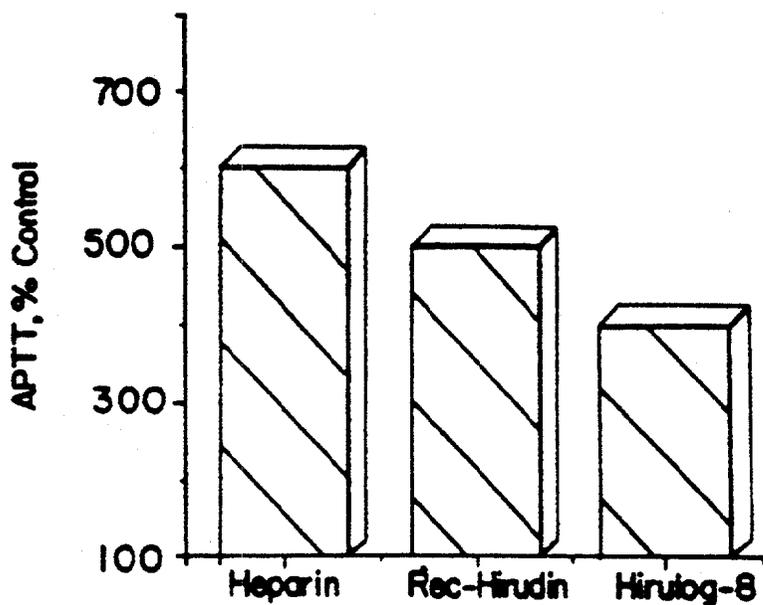
**FIG. 16**



**FIG. 17**



**FIG. 18**



**FIG. 19**

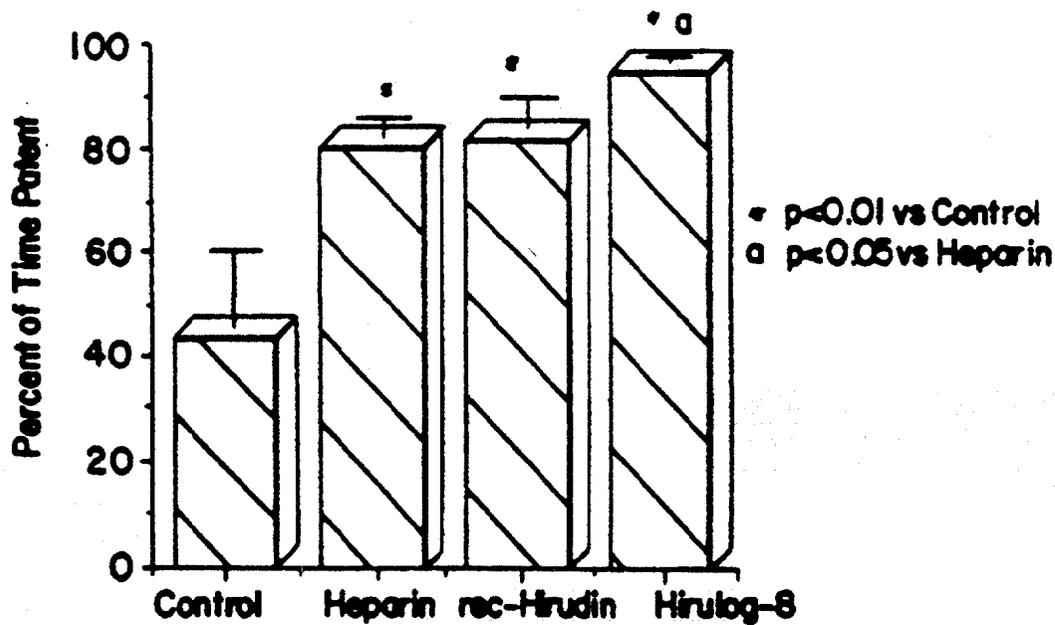
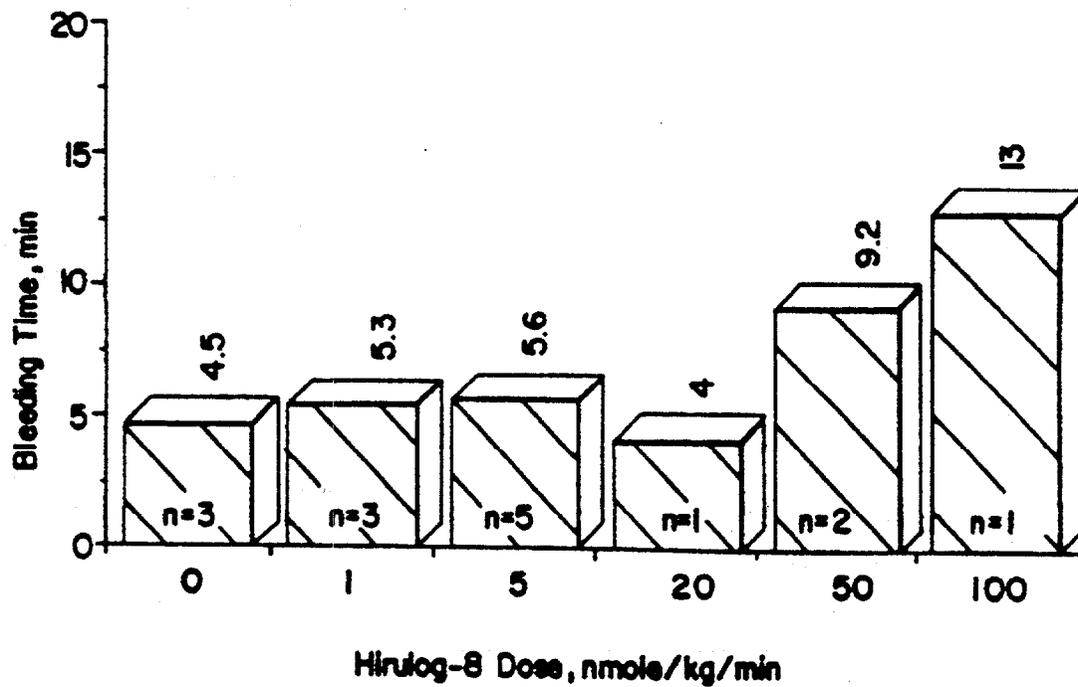


FIG. 20



## INHIBITORS OF THROMBIN

## CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of copending United States patent application Ser. No. 395,482 filed August 18, 1989, now abandoned.

## TECHNICAL FIELD OF INVENTION

This invention relates to novel biologically active molecules which bind to and inhibit thrombin. Specifically, these molecules are characterized by a thrombin anion-binding exosite associating moiety (ABEAM); a linker portion of at least 18 Å in length; and a thrombin catalytic site-directed moiety (CSDM). This invention also relates to compositions, combinations and methods which employ these molecules for therapeutic, prophylactic and diagnostic purposes.

## BACKGROUND ART

Acute vascular diseases, such as myocardial infarction, stroke, pulmonary embolism, deep vein thrombosis, peripheral arterial occlusion, and other blood system thromboses constitute major health risks. Such diseases are caused by either partial or total occlusion of a blood vessel by a blood clot, which contains fibrin and platelets.

Current methods for the treatment and prophylaxis of thrombotic diseases involve therapeutics which act in one of two different ways. The first type of therapeutic inhibits thrombin activity or thrombin formation, thus preventing clot formation. These drugs also inhibit platelet activation and aggregation. The second category of therapeutic accelerates thrombolysis and dissolves the blood clot, thereby removing it from the blood vessel and unblocking the flow of blood [J. P. Cazenave et al., *Agents Action*, 15, Suppl., pp. 24-49 (1984)].

Heparin, a compound of the former class, has been widely used to treat conditions, such as venous thromboembolism, in which thrombin activity is responsible for the development or expansion of a thrombus. Although effective, heparin produces many undesirable side effects, including hemorrhaging and thrombocytopenia. This has led to a search for a more specific and less toxic anticoagulant.

Hirudin is a naturally occurring polypeptide which is produced by the blood sucking leech *Hirudo medicinalis*. This compound, which is synthesized in the salivary gland of the leech, is the most potent natural inhibitor of coagulation known. Hirudin prevents blood from coagulating by binding tightly to thrombin ( $K_d=2 \times 10^{-11}M$ ) in a 1:1 stoichiometric complex [S. R. Stone and J. Hofsteenge, "Kinetics of the Inhibition of Thrombin by Hirudin", *Biochemistry*, 25, pp. 4622-28 (1986)]. This, in turn, inhibits thrombin from catalyzing the conversion of fibrinogen to fibrin (clot), as well as inhibiting all other thrombin-mediated processes [J. W. Fenton, II, "Regulation of Thrombin Generation and Functions", *Semin. Thromb. Hemost.*, 14, pp. 234-40 (1988)].

The actual binding between hirudin and thrombin is a two-step process. Initially, hirudin binds to a "low" affinity site on the thrombin molecule ( $K_d=1 \times 10^{-8}M$ ) which is separate from the catalytic site. This binding involves interaction of structure from the C-terminus of hirudin with an "anion-binding exosite" (ABE) in

thrombin [J. W. Fenton, II et al., "Thrombin Anion Binding Exosite Interactions with Heparin and Various Polyanions", *Ann. New York Acad. Sci.*, 556, pp. 158-65 (1989)]. Following the low affinity binding, the hirudin-thrombin complex undergoes a conformational change and hirudin then binds to the "high" affinity site on thrombin [S. Kono et al., "Analysis of Secondary Structure of Hirudin and the Conformational Change Upon Interaction with Thrombin", *Arch. Biochem. Biophys.*, 267, pp. 158-66 (1988)]. This latter site corresponds to the active site of thrombin.

The isolation, purification and chemical composition of hirudin are known in the art [P. Walsmann and F. Markwardt, "Biochemical and Pharmacological Aspects of the Thrombin Inhibitor Hirudin", *Pharmazie*, 36, pp. 653-60 (1981)]. More recently, the complete amino acid sequence of the polypeptide has been elucidated [J. Dodt et al., "The Complete Covalent Structure of Hirudin: Localization of the Disulfide Bonds", *Biol. Chem. Hoppe-Seyler*, 366, pp. 379-85 (1985); S. J. T. Mao et al., "Rapid Purification and Revised Amino Terminal Sequence of Hirudin: A Specific Thrombin Inhibitor of the Blood-Sucking Leech", *Anal. Biochem.*, 161, pp. 514-18 (1987); and R. P. Harvey et al., "Cloning and Expression of a cDNA Coding for the Anti-Coagulant Hirudin from the Bloodsucking Leech, *Hirudo medicinalis*", *Proc. Natl. Acad. Sci. USA*, 83, pp. 1084-88 (1986)].

At least ten different isomeric forms of hirudin have been sequenced and have been shown to differ slightly in amino acid sequence [D. Tripiet, "Hirudin: A Family of Iso-Proteins. Isolation and Sequence Determination of New Hirudins", *Folia Haematol.*, 115, pp. 30-35 (1988)]. All forms of hirudin comprise a single polypeptide chain protein containing 65 or 66 amino acids in which the amino terminus primarily comprises hydrophobic amino acids and the carboxy terminus typically comprises polar amino acids. More specifically, all forms of hirudin are characterized by an N-terminal domain (residues 1-39) stabilized by three disulfide bridges in a 1-2, 3-5, and 4-6 half-cysteinyl pattern and a highly acidic C-terminal segment (residues 40-65). In addition, the C-terminal segment of hirudin is characterized by the presence of a tyrosine residue at amino acid position 63 which is sulfated.

In animal studies, hirudin, purified from leeches, has demonstrated efficacy in preventing venous thrombosis, vascular shunt occlusion and thrombin-induced disseminated intravascular coagulation. In addition, hirudin exhibits low toxicity, little antigenicity and a very short clearance time from circulation [F. Markwardt et al., "Pharmacological Studies on the Antithrombotic Action of Hirudin in Experimental Animals", *Thromb. Haemost.*, 47, pp. 226-29 (1982)].

In an effort to create a greater supply of hirudin, attempts have been made to produce the polypeptide through recombinant DNA techniques. The presence of an O-sulfated tyrosine residue on native hirudin and the inability of microorganisms to perform a similar protein modification made the prospect of recombinant production of biologically active hirudin highly speculative. The observation that desulfatohirudins were almost as active as their sulfated counterparts [U.S. Pat. No. 4,634,302], however, led the way to the cloning and expression of hirudin in *E. coli* [European patent applications 158,564, 168,342 and 171,024] and yeast [European patent application 200,655]. Despite these advances,

hirudin is still moderately expensive to produce and it is not widely available commercially.

Recently, efforts have been made to identify peptide fragments of native hirudin which are also effective in prolonging clotting times. An unsulfated 21 amino acid C-terminal fragment of hirudin, N-acetylhirudin<sub>45-65</sub>, inhibits clot formation in vitro. In addition, several other smaller, unsulfated peptides corresponding to the C-terminal 11 or 12 amino acids of hirudin (residues 55-65 and 54-65) have also demonstrated efficacy in inhibiting clot formation in vitro [J. L. Krstenansky et al., "Antithrombin Properties of C-terminus of Hirudin Using Synthetic Unsulfated N-acetyl-hirudin<sub>45-65</sub>", *FEBS Lett.* 211, pp. 10-16 (1987)]. Such peptide fragments, however, may not be fully satisfactory to dissolve blood clots in on-going therapy regimens because of low activity. For example, N-acetyl-hirudin<sub>45-65</sub> has a specific activity four orders of magnitude lower than native hirudin.

In addition to catalyzing the formation of a fibrin clot, thrombin has several other bioregulatory roles [J. W. Fenton, II, "Thrombin Bioregulatory Functions", *Adv. Clin. Enzymol.* 6, pp. 186-93 (1988)]. For example, thrombin directly activates platelet aggregation and release reactions. This means that thrombin plays a central role in acute platelet-dependent thrombosis [S. R. Hanson and L. A. Harker, "Interruption of Acute Platelet-Dependent Thrombosis by the Synthetic Antithrombin

D-Phenylalanyl-L-Prolyl-L-Arginyl-chloromethylketone", *Proc. Natl. Acad. Sci. USA*, 85, pp. 3184-88 (1988)]. Thrombin can also directly activate an inflammatory response by stimulating the synthesis of platelet activating factor (PAF) in endothelial cells [S. Prescott et al., "Human Endothelial Cells in Culture Produce Platelet-Activating Factor (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) When Stimulated With Thrombin", *Proc. Natl. Acad. Sci. USA*, 81, pp. 3534-38 (1984)]. PAF is exposed on the surface of endothelial cells and serves as a ligand for neutrophil adhesion and subsequent degranulation [G. M. Vercolletti et al., "Platelet-Activating Factor Primes Neutrophil Responses to Agonists: Role in Promoting Neutrophil-Mediated Endothelial Damage", *Blood*, 71, pp. 1100-07 (1988)]. Alternatively, thrombin may promote inflammation by increasing vascular permeability which can lead to edema [P. J. Del Vecchio et al., "Endothelial Monolayer Permeability To Macromolecules", *Fed. Proc.*, 46, pp. 2511-15 (1987)]. Reagents which block the active site of thrombin, such as hirudin, interrupt the activation of platelets and endothelial cells [C. L. Knupp, "Effect of Thrombin Inhibitors on Thrombin-Induced Release and Aggregation", *Thrombosis Res.*, 49, pp. 23-36 (1988)].

Thrombin has also been implicated in promoting cancer, based on the ability of its native digestion product, fibrin, to serve as a substrate for tumor growth [A. Falanga et al., "Isolation and Characterization of Cancer Procoagulant: A Cysteine Proteinase from Malignant Tissue", *Biochemistry*, 24, pp. 5558-67 (1985); S. G. Gordon et al., "Cysteine Proteinase Procoagulant From Amnion-Chorion", *Blood*, 66, pp. 1261-65 (1985); and A. Falanga et al., "A New Procoagulant in Acute Leukemia", *Blood*, 71, pp. 870-75 (1988)]. And thrombin has been implicated in neurodegenerative diseases based on its ability to cause neurite retraction [D. Gurwitz et al., "Thrombin Modulates and Reverses Neuroblastoma Neurite Outgrowth", *Proc. Natl. Acad. Sci. USA*, 85, pp. 3440-44 (1988)]. Therefore, the ability to regulate the in

vivo activity of thrombin has many important clinical implications.

Despite the developments to date, the need still exists for a molecule that effectively inhibits thrombin function in clot formation, platelet activation and various other thrombin-mediated processes and which can be produced inexpensively and in commercially feasible quantities.

#### SUMMARY OF THE INVENTION

The present invention solves the problems enumerated above by providing molecules which mimic the action of hirudin by binding to both the low affinity anion-binding exosite (ABE) and the catalytic site of  $\alpha$ -thrombin. These molecules are more potent than hirudin and, therefore, they may be administered to patients in dosages which are comparatively lower than those required in hirudin-based therapy regimens. The molecules of this invention may be utilized in compositions and methods for inhibiting any thrombin-mediated or thrombin-associated function or process. Pharmaceutical compositions containing these molecules, as well as methods of treatment or prophylaxis of vascular diseases, inflammatory responses, carcinomas, and neurodegenerative diseases using them are also part of the present invention. These molecules may also be employed in compositions and methods for ex vivo imaging, for storing and treating extracorporeal blood and for coating invasive devices. And the molecules of this invention may be administered to a patient in combination with a fibrinolytic agent to increase the efficacy of a given dose of that agent or to lower the dose of that agent required for a given effect, such as dissolving a blood clot.

Due to their high potency and the fact that they may be prepared by chemical synthesis techniques, the molecules of the present invention may be prepared inexpensively, in commercially feasible amounts. Moreover, because the molecules of the present invention are significantly smaller than hirudin, they are less likely to stimulate an undesirable immune response in patients treated with them. Accordingly, the use of these thrombin inhibitors is not limited to the treatment of acute disease. These molecules may also be utilized in therapy for chronic thromboembolic diseases, such as atherosclerosis and restenosis following angioplasty. The molecules of the present invention may also be utilized in a variety of other applications in place of natural or recombinant hirudin.

As will be appreciated from the disclosure to follow, the molecules, compositions and methods of this invention are useful in the treatment and prevention of various diseases attributed to the undesirable effects of thrombin, as well as for diagnostic purposes.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts an autoradiograph of an SDS-polyacrylamide gel demonstrating the binding of DNFB-[<sup>35</sup>S]-Sulfo-Tyr<sub>63</sub>hirudin<sub>54-64</sub> to human  $\alpha$ -thrombin in the presence or absence of Sulfo-Tyr<sub>63</sub>-N-acetyl-hirudin<sub>53-64</sub>.

FIG. 2 depicts a three-dimensional model of human  $\alpha$ -thrombin.

FIG. 3, panel A, depicts the effects of Hirulog-8 and Sulfo-Tyr<sub>63</sub>hirudin<sub>53-64</sub> on the cleavage of Spectrozyme TH by human  $\alpha$ -thrombin.

FIG. 3, panel B, depicts a Lineweaver-Burke plot of the cleavage of Spectrozyme TH by human  $\alpha$ -thrombin

in the presence or absence of either Hirulog-8 or Sulfo-Tyr<sub>63</sub>hirudin<sub>53-64</sub>.

FIG. 4 depicts the effect of varying concentrations of Hirulog-8, hirudin, or Sulfo-Tyr<sub>63</sub>-N-acetyl-hirudin<sub>53-64</sub> on the activated partial thromboplastin time of normal human serum.

FIG. 5, panel A, depicts the time course for cleavage of varying concentrations of Hirulog-8 by human  $\alpha$ -thrombin.

FIG. 5, panel B, depicts the relationship between Hirulog-8 concentration and the duration of inhibition of Spectrozyme TH hydrolysis by human  $\alpha$ -thrombin.

FIG. 6 depicts the effect of linker length of the thrombin inhibitors of this invention on the inhibition of thrombin-catalyzed hydrolysis of Spectrozyme TH.

FIG. 7 depicts the inhibitory effects of varying concentrations of Hirulog-8 or Sulfo-Tyr<sub>63</sub>-N-acetyl-hirudin<sub>53-64</sub> on the modification of thrombin by <sup>14</sup>C-DFP.

FIG. 8 depicts the in vivo effect of varying doses of Hirulog-8 on APTT in baboons.

FIG. 9 depicts the comparative inhibitory effects of Hirulog-8 or heparin on the hydrolysis of fibrinogen by soluble or clot-bound thrombin.

FIG. 10 depicts the in vivo effects of varying doses of Hirulog-8 on platelet deposition on an endarterectomized segment of baboon aorta.

FIG. 11 depicts the in vivo effects of varying doses of Hirulog-8 on platelet deposition on a segment of collagen-coated tubing inserted into a baboon.

FIG. 12 depicts the comparative in vivo effects of heparin, hirudin and Hirulog-8 on platelet deposition on a segment of collagen-coated tubing inserted into a baboon AV shunt.

FIG. 13 depicts the in vivo effects of varying doses of Hirulog-8 on fibrin deposition on a segment of collagen-coated tubing inserted into a baboon AV shunt.

FIG. 14 depicts the change in APTT over time following intravenous bolus injection of baboons with Hirulog-8.

FIG. 15 depicts the change in APTT over time following subcutaneous injection of baboons with Hirulog-8.

FIG. 16 depicts the comparative in vivo effects of tissue plasminogen activator together with either saline, heparin, hirudin or Hirulog-8 on reperfusion time in a rat model.

FIG. 17 depicts the comparative in vivo effects of tissue plasminogen activator together with either saline, heparin, hirudin or Hirulog-8 on reocclusion time in a rat model.

FIG. 18 depicts the comparative in vivo effects of tissue plasminogen activator together with either saline, heparin, hirudin or Hirulog-8 on APTT in a rat model.

FIG. 19 depicts the comparative in vivo effects of tissue plasminogen activator together with either saline, heparin, hirudin or Hirulog-8 on vessel patency in a rat model.

FIG. 20 depicts the effect of varying doses of Hirulog-8 on bleeding times in a baboon model.

#### DETAILED DESCRIPTION OF THE INVENTION

The following common abbreviations of the amino acids are used throughout the specification and in the claims:

Orn - ornithine	Gly - glycine
Ala - alanine	Val - valine
Leu - leucine	Ile - isoleucine
Pro - proline	Phe - phenylalanine
Trp - tryptophan	Met - methionine
Ser - serine	Thr - threonine
Cys - cysteine	Tyr - tyrosine
Asn - asparagine	Gln - glutamine
Asp - aspartic acid	Glu - glutamic acid
Lys - lysine	Arg - arginine
His - histidine	Nle - norleucine
Hyp - hydroxyproline	Pgl - phenylglycine
Ac - acetyl	Suc - succinyl
BOC - tert.Butoxycarbonyl	Tos - paraToluenesulfonyl
Cbz - Carbobenzyloxy	D-Ala - D-alanine
3,4,-dehydroPro - 3,4,- dehydroproline	Sar - sarcosine (N-methylglycine)
Tyr(OSO <sub>3</sub> H) - tyrosine sulfate	Tyr(SO <sub>3</sub> H) - tyrosine sulfonate
3-, 5-diiodoTyr - 3-,5- diiodotyrosine	

The term "any amino acid" as used herein includes the L-isomers of the naturally occurring amino acids, as well as other "non-protein"  $\alpha$ -amino acids commonly utilized by those in the peptide chemistry arts when preparing synthetic analogs of naturally occurring amino peptides. The naturally occurring amino acids are glycine, alanine, valine, leucine, isoleucine, serine, methionine, threonine, phenylalanine, tyrosine, tryptophan, cysteine, proline, histidine, aspartic acid, asparagine, glutamic acid, glutamine,  $\gamma$ -carboxyglutamic acid, arginine, ornithine and lysine. Examples of "non-protein"  $\alpha$ -amino acids include norleucine, norvaline, alloisoleucine, homoarginine, thiaproline, dehydroproline, hydroxyproline (Hyp), homoserine, cyclohexylglycine (Chg),  $\alpha$ -amino-n-butyric acid (Aba), cyclohexylalanine (Cha), aminophenylbutyric acid (Pba), phenylalanines substituted at the ortho, meta, or para position of the phenyl moiety with one or two of the following: a (C<sub>1</sub>-C<sub>4</sub>) alkyl, a (C<sub>1</sub>-C<sub>4</sub>) alkoxy, halogen or nitro groups or substituted with a methylenedioxy group;  $\beta$ -2- and 3-thienylal-alanine,  $\beta$ -2- and 3-furanylalanine,  $\beta$ -2-, 3- and 4-pyridylalanine, B-(benzothienyl-2- and 3-yl)alanine, B-(1- and 2-naphthyl)alanine, O-alkylated derivatives of serine, threonine or tyrosine, S-alkylated cysteine, S-alkylated homocysteine, O-sulfate, O-phosphate and O-carboxylate esters of tyrosine, 3- and 5-sulfonyl tyrosine, 3- and 5-carbonyl tyrosine, 3- and 5-phosphonyl tyrosine, 4-methylsulfonyl tyrosine, 4-methylphosphonyl tyrosine, 4-phenylacetic acid, 3,5-diiodotyrosine, 3- and 5-nitrotyrosine,  $\epsilon$ -alkyl lysine, delta-alkyl ornithine, and the D-isomers of the naturally occurring amino acids.

The term "patient" as used in this application refers to any mammal, especially humans.

The term "anionic amino acid" as used herein means a meta, para or ortho, mono- or di-substituted phenylalanine, cyclohexylalanine or tyrosine containing a carboxyl, phosphoryl or sulfonyl moiety, as well as S-alkylated cysteine, S-alkylated homocysteine,  $\gamma$ -carboxyglutamic acid,  $\epsilon$ -alkyl lysine, delta-alkyl ornithine, glutamic acid, and aspartic acid. Examples of anionic amino acids are phosphothreonine, phosphoserine, phosphotyrosine, 3-, 4-, or 5-sulfonyl tyrosine, 3-methyl phosphonyltyrosine and 3-methyl sulfonyltyrosine.

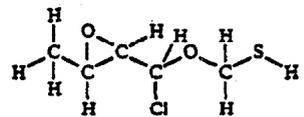
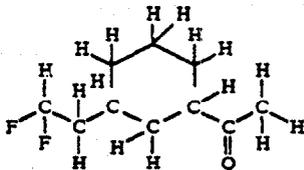
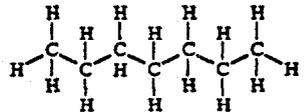
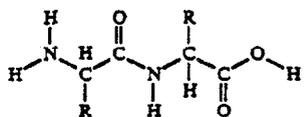
The terms "catalytic site", "active site" and "active site pocket" as used herein, each refer to any or all of the following sites in thrombin: the substrate binding or "S<sub>1</sub>" site; the hydrophobic binding or "oily" site; and

the site where cleavage of a substrate is actually carried out ("charge relay site").

The term "N<sup>orn</sup>" as used herein, refers to the side chain nitrogen of ornithine. The term "N<sup>arg</sup>" refers to any of the side chain nitrogens of arginine. The term "N<sup>α</sup>" refers to the α-amino group of an amino acid. And the term "psi" as used in the specification and claims, refers to the replacement of an amide bond with the atoms designated in brackets, according to the nomenclature described in J. Rudinger, In *Drug Design*, Vol. II, E. J. Ariens, ed., Academic Press, New York, p. 319 (1971).

The term "backbone chain" as used herein, refers to the portion of a chemical structure that defines the smallest number of consecutive bonds that can be traced from one end of that chemical structure to the other. The atomic components that make up a backbone chain may comprise any atoms that are capable of forming bonds with at least two other atoms.

For example, each of the following chemical structures is characterized by a backbone chain of 7 atoms (the atoms which comprise the backbone chain are indicated in boldface):



The term "calculated length" as used in this application, refers to a predicted measurement derived by summing up the bond lengths between the atoms which comprise the backbone chain. Bond lengths between any two given atoms are well known in the art [see, for example, *CRC Handbook of Chemistry and Physics*, 65th Edition, R. C. Weist, ed., CRC Press, Inc., Boca Raton, Fla., pp. F-166-70 (1984)].

The present invention relates to molecules which bind to and inhibit thrombin. These molecules are characterized by three domains: a catalytic site-directed moiety ("CSDM"), a linker region, and an anion binding exosite associating moiety ("ABEAM").

According to the present invention, the first domain, CSDM, binds to the catalytic site of thrombin located at or near about Ser-195 and inhibits or retards the amidolytic or estereolytic activity of thrombin. Preferably, CSDMs of the present invention are selected from one of three general groups: those which bind reversibly to thrombin and are slowly cleaved; those which bind reversibly to thrombin and cannot be cleaved; and those which bind irreversibly to thrombin. Reversible inhibi-

tors bind to the active site of thrombin through non-covalent interactions, such as ionic bonds, hydrophobic interactions or hydrogen bonding. Irreversible CSDMs form covalent bonds with thrombin.

According to a preferred embodiment, the CSDM which binds reversibly to thrombin and is slowly cleaved has the formula:



wherein X is hydrogen or is characterized by a backbone chain consisting of from 1 to 35 atoms; A<sub>1</sub> is Arg, Lys or Orn; A<sub>2</sub> is a non-amide bond; A<sub>3</sub> is characterized by a backbone chain consisting of from 1 to 9 atoms; and Y is a bond.

The non-amide bond component according to this embodiment may be formed by chemically modifying an amide bond. This may be achieved by methods well known in the art [M. Szelke et al., "Potent New Inhibitors of Human Renin", *Nature*, 299, pp. 555-57 (1982); D. H. Coy et al., "Facile Solid Phase Preparation of Proteins Containing the CH<sub>2</sub>-NH Peptide Bond Isostere and Application to the Synthesis of Somatostatin (SRIF) Octapeptide Analogues", *Peptides* 1986, D. Theodoropoulos, Ed., Walter DeGruyter & Co., Berlin, pp. 143-46 (1987)]. When a non-amide bond is formed in this manner, it is preferable that the chemical modification be performed prior to the addition of the dipeptide containing this bond to the other components of CSDM or to the rest of the thrombin inhibitor molecule. In this manner, the dipeptide A<sub>1</sub>-A<sub>2</sub>-A<sub>3</sub> is added en bloc, in a single synthesis step, to the rest of the molecule.

According to a more preferred embodiment, A<sub>1</sub> is Arg and A<sub>3</sub> is Pro, D-Pro or Sar. In this embodiment A<sub>2</sub> is a naturally occurring imide bond, which is slowly cleaved by thrombin. This avoids having the necessity of pre-forming the non-amide bond and allows A<sub>1</sub> and A<sub>3</sub> to be added to the rest of the molecule sequentially rather than en bloc.

As set forth above, CSDMs according to this invention may bind irreversibly to thrombin. Examples of irreversible CSDMs include, but are not limited to, general serine proteinase inhibitors, such as phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), tosylpropylchloromethylketone (TPCK) and tosyllysylchloromethylketone (TLCK); heterocyclic protease inhibitors, such as isocoumarins; thrombin-specific inhibitors, such as D-Phe-Pro-Arg-CHCl<sub>2</sub> (PPACK); and transition state analogues, such as difluoroketomethylene.

According to another preferred embodiment of the present invention, non-cleavable, reversible CSDMs consist of the formula:



wherein C<sub>1</sub> is a derivative of Arg, Lys or Orn characterized by a reduced carboxylate moiety or a carboxylate moiety that is displaced from the α-carbon by a chemical structure characterized by a backbone chain of from 1 to 10 atoms; C<sub>2</sub> is a non-cleavable bond; and X, Y and A, are as defined previously. Examples of C<sub>1</sub> components are β-homoarginine; arginine containing a reduced carboxylate moiety, such as Arg[psiCH<sub>2</sub>NH]; β-homolysine and β-homoomithine.

Other non-cleavable, reversible CSDMs that may be employed in the thrombin inhibitors of this invention

are benzamide, DAPA, NAPAP and argatroban (argipidine).

For those thrombin inhibitors of this invention which have CSDM regions characterized by an A<sub>2</sub> or C<sub>2</sub> bond, the term "P<sub>1</sub>-P<sub>1</sub>" sequence as used herein, refers to the two chemical structures joined by said bond.

The X component of CSDM, which does not participate in actually binding to the catalytic site, can be of unlimited length and variable make-up. However, for practical purposes and reduced cost of synthesis, X is preferably characterized by a backbone chain consisting of from 1 to 35 atoms and does not exceed a calculated length of 36 Å. It is preferred that X be a peptide, most preferably, D-Phe-Pro. This most preferable embodiment allows the X component to fit into a groove in thrombin that is adjacent to the active site [S. Bajusz et al., "Inhibition of Thrombin and Trypsin by Tripeptide Aldehydes", *Int. J. Peptide Protein Res.*, 12, pp. 217-21 (1978); C. Kettner et al., "D-Phe-Pro-Arg-CH<sub>2</sub>Cl—A Selective Affinity Label for Thrombin", *Thromb. Res.*, 14, pp. 969-73 (1979)]. This allows the CSDM component and therefore the molecules of the present invention, to bind to thrombin with an advantageously high degree of affinity and optimal specificity.

According to the present invention, the second component of the thrombin inhibitors of this invention is a linker region. Because the role of this portion of the molecule is to provide a bridge between the CSDM and the ABEAM, it is the length of the linker, rather than its structure, that is of prime importance. The calculated length of the backbone chain which characterizes the linker must be at least about 18 Å—the distance between the catalytic site and the anion binding exosite of thrombin—and less than about 42 Å.

The backbone chain of the linker may comprise any atoms which are capable of bonding to at least two other atoms. Preferably, the backbone chain consists of any chemically feasible combination of atoms selected from oxygen, carbon, nitrogen and sulfur. Those of skill in the art are aware of what combination of the above backbone chain atoms falls within the required length based on known distances between various bonds [see, for example, R. T. Morrison and R. N. Boyd, *Organic Chemistry*, 3rd Edition, Allyn and Bacon, Inc., Boston, Mass. (1977)]. According to a preferred embodiment, the linker is a peptide which comprises the amino acid sequence Gly-Gly-Gly-Asn-Gly-Asp-Phe. Preferably, the amino acid bound to the ABEAM component is Phe.

The third domain of the thrombin inhibitors of this invention is the ABEAM which binds to the anion binding exosite of thrombin. Preferably the ABEAM has the formula:



wherein W is a bond; B<sub>1</sub> is an anionic amino acid; B<sub>2</sub> is any amino acid; B<sub>3</sub> is Ile, Val, Leu, Nle or Phe; B<sub>4</sub> is Pro, Hyp, 3,4-dehydroPro, thiazolidine-4-carboxylate, amino acid; B<sub>5</sub> is an anionic amino acid; B<sub>6</sub> is a lipophilic amino acid selected from the group consisting of Tyr, Trp, Phe, Leu, Nle, Ile, Val, Cha, Pro, or a dipeptide consisting of one of these lipophilic amino acids and any amino acid; B<sub>7</sub> is a bond or a peptide containing from one to five residues of any amino acid; and Z is a carboxy terminal residue selected from OH, C<sub>1</sub>-C<sub>4</sub> alkoxy, amino, mono- or di-(C<sub>1</sub>-C<sub>4</sub>) alkyl substituted amino or benzylamino.

Peptides which are homologous to the carboxy terminal portion of hirudin have been shown to bind to the anion binding exosite on thrombin [copending U.S. patent application Ser. No. 314,756 and J. M. Maragano et al., "Anticoagulant Activity of Synthetic Hirudin Peptides", *J. Biol. Chem.*, 264, pp. 8692-98 (1989); both of which are herein incorporated by reference].

According to a preferred embodiment of this invention, ABEAM is homologous to amino acids 56-64 of hirudin, i.e., B<sub>1</sub> is Glu; B<sub>2</sub> is Glu; B<sub>3</sub> is Ile; B<sub>4</sub> is Pro; B<sub>5</sub> is Glu; B<sub>6</sub> is Glu; B<sub>7</sub> is Tyr-Leu, Tyr(SO<sub>3</sub>H)-Leu or Tyr(OSO<sub>3</sub>H)-Leu, or (3,5-diiodoTyr)-Leu; B<sub>8</sub> is a bond; and Z is OH. It should be noted that native hirudin contains Tyr(OSO<sub>3</sub>H) at position 63. However, carboxy terminal hirudin peptides which contain Tyr(SO<sub>3</sub>H) have identical anticoagulant activity as those which contain the native Tyr(OSO<sub>3</sub>H) [see copending U.S. patent application Ser. No. 314,756].

Other ABEAM components within the scope of this invention may comprise those portions of any molecule known to bind to the anion binding site of thrombin.

These include amino acids 1675-1686 of Factor V, amino acids 272-285 of platelet glycoprotein Ib, amino acids 426-444 of thrombomodulin, amino acids 245-259 of prothrombin 2 and amino acids 30 to 44 of fibrinogen A<sub>α</sub> chain. In addition, the ABEAM component may be selected from any of the hirudin peptide analogues described by J. L. Krstenansky et al., "Development of MDL-28,050, A Small Stable Antithrombin Agent Based On A Functional Domain of the Leech Protein, Hirudin", *Thromb. Haemostas.*, 63, pp. 208-14 (1990).

The preferred thrombin inhibitors of this invention are termed Hirulogs, and are described in the subsequent examples. The most preferred Hirulogs are Hirulog-8, Hirulog-12, Hirulog-18a, Hirulog-18b and Hirulog-33. Hirulog-8, -12 and -33 are reversible thrombin inhibitors that are slowly cleaved. Hirulog-18a and -18b are reversible inhibitors which are not cleaved.

The thrombin inhibitors of the present invention may be synthesized by various techniques which are well known in the art. These include enzymatic cleavage of natural or recombinant hirudin, recombinant DNA techniques, solid-phase peptide synthesis, solution-phase peptide synthesis, organic chemical synthesis techniques, or a combination of these techniques. The choice of synthesis technique will, of course, depend upon the composition of the particular inhibitor. In a preferred embodiment of this invention, the thrombin inhibitor is entirely peptidic and is synthesized by solid-phase peptide synthesis techniques, solution-phase peptide synthesis techniques or a combination thereof which constitute the most cost-efficient procedures for producing commercial quantities of these molecules.

When "non-protein" amino acids are contained in the thrombin inhibitor molecule, they may be either added directly to the growing chain during peptide synthesis or prepared by chemical modification of the complete synthesized peptide, depending on the nature of the desired "non-protein" amino acid. Those of skill in the chemical synthesis art are well aware of which "non-protein" amino acids may be added directly and which must be synthesized by chemically modifying the complete peptide chain following peptide synthesis.

The synthesis of those thrombin inhibitors of this invention which contain both non-amino acid and peptidic portions is preferably achieved by a mixed heterologous/solid phase technique. This technique involves the solid-phase synthesis of all or most of the

peptide portion of the molecule, followed by the addition of the non-amino acid components which are synthesized by solution phase techniques. The non-amino acid may be coupled to the peptidic portion via solid-phase or solution-phase methods. Similarly, any remaining peptidic portions may also be added via solid-phase or solution phase methods.

The molecules of the present invention display potent anticoagulant activity. This activity may be assayed *in vitro* using any conventional technique. Preferably, an assay for anticoagulant activity involves direct determination of the thrombin-inhibitory activity of the molecule. Such techniques measure the inhibition of thrombin-catalyzed cleavage of colorimetric substrates or, more preferably, the increase in thrombin times or increase in activated partial thromboplastin times of human plasma. The latter assay measures factors in the "intrinsic" pathway of coagulation. Alternatively, the assay employed may use purified thrombin and fibrinogen to measure the inhibition of release of fibrinopeptides A or B by radioimmunoassay or ELISA.

The antiplatelet activity of the molecules of this invention may also be measured by any of a number of conventional platelet assays. Preferably, the assay will measure a change in the degree of aggregation of platelets or a change in the release of a platelet secretory component in the presence of thrombin. The former may be measured in an aggregometer. The latter may be measured using RIA or ELISA techniques specific for the secreted component.

The molecules of the present invention are useful in compositions, combinations and methods for the treatment and prophylaxis of various diseases attributed to thrombin-mediated and thrombin-associated functions and processes. These include myocardial infarction, stroke, pulmonary embolism, deep vein thrombosis, peripheral arterial occlusion, restenosis following arterial injury or invasive cardiologic procedures, acute or chronic atherosclerosis, edema and inflammation, various cell regulatory processes (e.g. secretion, shape changes, proliferation), cancer and metastasis, and neurodegenerative diseases.

The thrombin inhibitors of the present invention may be formulated using conventional methods to prepare pharmaceutically useful compositions, such as the addition of a pharmaceutically acceptable carrier. These compositions and the methods employing them may be used for treating or preventing thrombotic diseases in a patient.

According to an alternate embodiment of the present invention, the thrombin inhibitors may be employed in combinations, compositions, and methods for treating thrombotic disease, and for decreasing the dosage of a thrombolytic agent required to establish reperfusion or prevent reocclusion in a patient. Additionally, the thrombin inhibitors of this invention may be used in combinations, compositions, and methods for decreasing reperfusion time or increasing reocclusion time in a patient treated with a thrombolytic agent. These combinations and compositions comprise a pharmaceutically effective amount of a thrombin inhibitor of the present invention and a pharmaceutically effective amount of a thrombolytic agent.

In these combinations and compositions, the thrombin inhibitor and the thrombolytic agent work in a complementary fashion to dissolve blood clots, resulting in decreased reperfusion times and increased reocclusion times in patients treated with them. Specifically, the

thrombolytic agent dissolves the clot, while the thrombin inhibitor prevents newly exposed, clot-entrapped or clot-bound thrombin from regenerating the clot. The use of the thrombin inhibitor in the combinations and compositions of this invention advantageously allows the administration of a thrombolytic reagent in dosages previously considered too low to result in thrombolytic effects if given alone. This avoids some of the undesirable side effects associated with the use of thrombolytic agents, such as bleeding complications.

Thrombolytic agents which may be employed in the combinations and compositions of the present invention are those known in the art. Such agents include, but are not limited to, tissue plasminogen activator purified from natural sources, recombinant tissue plasminogen activator, streptokinase, urokinase, prourokinase, anisolated streptokinase plasminogen activator complex (AS-PAC), animal salivary gland plasminogen activators and known, biologically active derivatives of any of the above.

The term "combination" as used herein, includes a single dosage form containing at least one thrombin inhibitor of this invention and at least one thrombolytic agent; a multiple dosage form, wherein the thrombin inhibitor and the thrombolytic agent are administered separately, but concurrently; or a multiple dosage form wherein the two components are administered separately, but sequentially. In sequential administration, the thrombin inhibitor may be given to the patient during the time period ranging from about 5 hours prior to about 5 hours after administration of the thrombolytic agent. Preferably, the thrombin inhibitor is administered to the patient during the period ranging from 2 hours prior to 2 hours following administration of the thrombolytic agent.

Alternatively, the thrombin inhibitor and the thrombolytic agent may be in the form of a single, conjugated molecule. Conjugation of the two components may be achieved by standard cross-linking techniques well known in the art. The single molecule may also take the form of a recombinant fusion protein, if both the thrombin inhibitor and the thrombolytic agent are peptidic.

Various dosage forms may be employed to administer the compositions and combinations of this invention. These include, but are not limited to, parenteral administration, oral administration and topical application. The compositions and combinations of this invention may be administered to the patient in any pharmaceutically acceptable dosage form, including those which may be administered to a patient intravenously as bolus or by continued infusion, intramuscularly—including paravertebrally and periarticularly—subcutaneously, intracutaneously, intra-articularly, intrasynovially, intrathecally, intra-lesionally, periostally or by oral, nasal, or topical routes. Such compositions and combinations are preferably adapted for topical, nasal, oral and parenteral administration, but, most preferably, are formulated for parenteral administration.

Parenteral compositions are most preferably administered intravenously either in a bolus form or as a constant infusion. If the thrombin inhibitor is being used as an antiplatelet compound, constant infusion is preferred. If the thrombin inhibitor is being used as an anticoagulant, a subcutaneous or intravenous bolus injection is preferred. For parenteral administration, fluid unit dose forms are prepared which contain a thrombin inhibitor of the present invention and a sterile vehicle. The thrombin inhibitor may be either suspended or

dissolved, depending on the nature of the vehicle and the nature of the particular thrombin inhibitor. Parenteral compositions are normally prepared by dissolving the thrombin inhibitor in a vehicle, optionally together with other components, and filter sterilizing before filling into a suitable vial or ampule and sealing. Preferably, adjuvants such as a local anesthetic, preservatives and buffering agents are also dissolved in the vehicle. The composition may then be frozen and lyophilized to enhance stability.

Parenteral suspensions are prepared in substantially the same manner, except that the active component is suspended rather than dissolved in the vehicle. Sterilization of the compositions is preferably achieved by exposure to ethylene oxide before suspension in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of its components.

Tablets and capsules for oral administration may contain conventional excipients, such as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, and wetting agents. The tablet may be coated according to methods well known in the art. Suitable fillers which may be employed include cellulose, mannitol, lactose and other similar agents. Suitable disintegrants include, but are not limited to, starch, polyvinylpyrrolidone and starch derivatives, such as sodium starch glycolate. Suitable lubricants include, for example, magnesium stearate. Suitable wetting agents include sodium lauryl sulfate.

Oral liquid preparations may be in the form of aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or another suitable vehicle before use. Such liquid preparations may contain conventional additives. These include suspending agents; such as sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethylcellulose, aluminum stearate gel or hydrogenated edible fats; emulsifying agents which include lecithin, sorbitan monooleate, polyethylene glycols, or acacia; non-aqueous vehicles, such as almond oil, fractionated coconut oil, and oily esters; and preservatives, such as methyl or propyl p-hydroxybenzoate or sorbic acid.

Compositions formulated for topical administration may, for example, be in aqueous jelly, oily suspension or emulsified ointment form.

The dosage and dose rate of the thrombin inhibitor will depend on a variety of factors, such as the size of the patient, the specific pharmaceutical composition used, the object of the treatment, i.e., therapy or prophylaxis, the nature of the thrombotic disease to be treated, and the judgment of the treating physician.

According to the present invention, a preferred pharmaceutically effective daily dose of the thrombin inhibitor of this invention is between about 1  $\mu\text{g}/\text{kg}$  body weight of the patient to be treated ("body weight") and about 5 mg/kg body weight. In combinations containing a thrombolytic agent, a pharmaceutically effective daily dose of the thrombolytic is between about 10% and 80% of the conventional dosage range. The "conventional dosage range" of a thrombolytic agent is the daily dosage used when that agent is employed in a monotherapy. [*Physician's Desk Reference* 1989, 43rd Edition, Edward R. Barnhart, publisher]. That conventional dosage range will, of course, vary depending on the thrombolytic agent employed. Examples of conventional dosage ranges are as follows: urokinase—500,000

to 6,250,000 units/patient; streptokinase—140,000 to 2,500,000 units/patient; tPA—0.5 to 5.0 mg/kg body weight; ASPAC—0.1 to 10 units/kg body weight.

Most preferably, the therapeutic and prophylactic compositions of the present invention comprise a dosage of between about 10  $\mu\text{g}/\text{kg}$  body weight and about 500  $\mu\text{g}/\text{kg}$  body weight of the thrombin inhibitor. Most preferred combinations comprise the same amount of the thrombin inhibitor and between about 10% and about 70% of the conventional dosage range of a thrombolytic agent. It should also be understood that a daily pharmaceutically effective dose of either the thrombin inhibitors of this invention or the thrombolytic agent present in combinations of the invention, may be less than or greater than the specific ranges cited above.

Once improvement in the patient's condition has occurred, a maintenance dose of a combination or composition of this invention is administered, if necessary. Subsequently, the dosage or the frequency of administration, or both, may be reduced, as a function of the symptoms, to a level at which the improved condition is retained. When the symptoms have been alleviated to the desired level, treatment should cease. Patients may, however, require intermittent treatment upon any recurrence of disease symptoms.

According to an alternate embodiment of this invention, thrombin inhibitors may be used in compositions and methods for coating the surfaces of invasive devices, resulting in a lower risk of clot formation or platelet activation in patients receiving such devices. Surfaces that may be coated with the compositions of this invention include, for example, prostheses, artificial valves, vascular grafts, stents and catheters. Methods and compositions for coating these devices are known to those of skill in the art. These include chemical cross-linking or physical adsorption of the thrombin inhibitor-containing compositions to the surfaces of the devices.

According to a further embodiment of the present invention, thrombin inhibitors may be used for *ex vivo* thrombus imaging in a patient. In this embodiment, the thrombin inhibitor is labelled with a radioisotope. The choice of radioisotope is based upon a number of well-known factors, for example, toxicity, biological half-life and detectability. Preferred radioisotopes include, but are not limited to,  $^{125}\text{I}$ ,  $^{123}\text{I}$  and  $^{111}\text{In}$ . Techniques for labelling the thrombin inhibitor are well known in the art. Most preferably, the radioisotope is  $^{123}\text{I}$  and the labelling is achieved using  $^{123}\text{I}$ -Bolton-Hunter Reagent. The labelled thrombin inhibitor is administered to a patient and allowed to bind to the thrombin contained in a clot. The clot is then observed by utilizing well-known detecting means, such as a camera capable of detecting radioactivity coupled to a computer imaging system. This technique also yields images of platelet-bound thrombin and meizothrombin.

This invention also relates to compositions containing the thrombin inhibitors of this invention and methods for using such compositions in the treatment of tumor metastases. The efficacy of the thrombin inhibitors of this invention for the treatment of tumor metastases is manifested by the inhibition of metastatic growth. This is based upon the presence of a procoagulant enzyme in certain cancer cells. This enzyme activates the conversion of Factor X to Factor Xa in the coagulation cascade, resulting in fibrin deposition which, in turn, serves as a substrate for tumor growth. By inhibiting fibrin deposition through the inhibition of thrombin, the molecules of the present invention serve as effective anti-

metastatic tumor agents. Examples of metastatic tumors which may be treated by the thrombin inhibitors of this invention include, but are not limited to, carcinoma of the brain, carcinoma of the liver, carcinoma of the lung, osteocarcinoma and neoplastic plasma cell carcinoma.

The invention also relates to methods and compositions employing the above-described thrombin inhibitors to inhibit thrombin-induced endothelial cell activation. This inhibition includes the repression of platelet activation factor (PAF) synthesis by endothelial cells. These compositions and methods have important applications in the treatment of diseases characterized by thrombin-induced inflammation and edema, which is thought to be mediated by PAF. Such diseases include, but are not limited to, adult respiratory distress syndrome, septic shock, septicemia and reperfusion damage.

Early stages of septic shock include discrete, acute inflammatory and coagulopathic responses. It has previously been shown that injection of baboons with a lethal dose of live *E. coli* leads to marked declines in neutrophil count, blood pressure and hematocrit. Changes in blood pressure and hematocrit are due in part to the generation of a disseminated intravascular coagulopathy (DIC) and have been shown to parallel consumption of fibrinogen [F. B. Taylor et al., "Protein C Prevents the Coagulopathic and Lethal Effects of *Escherichia coli* infusion in the Baboon", *J. Clin. Invest.*, 79, pp. 918-25 (1987)]. Neutropenia is due to the severe inflammatory response caused by septic shock which results in marked increases in tumor necrosis factor levels. The thrombin inhibitors of this invention may be utilized in compositions and methods for treating or preventing DIC in septicemia and other diseases.

This invention also relates to the use of the above-described thrombin inhibitors, or compositions comprising them, as anticoagulants for extracorporeal blood. As used herein, the term "extracorporeal blood" includes blood removed in line from a patient, subjected to extracorporeal treatment, and then returned to the patient in such processes as dialysis procedures, blood filtration, or blood bypass during surgery. The term also includes blood products which are stored extracorporeally for eventual administration to a patient and blood collected from a patient to be used for various assays. Such products include whole blood, plasma, or any blood fraction in which inhibition of coagulation is desired.

The amount or concentration of thrombin inhibitor in these types of compositions is based on the volume of blood to be treated or, more preferably, its thrombin content. Preferably, an effective amount of a thrombin inhibitor of this invention for preventing coagulation in extracorporeal blood is from about 1 µg/60 ml of extracorporeal blood to about 5 mg/60 ml of extracorporeal blood.

The thrombin inhibitors of this invention may also be used to inhibit clot-bound thrombin, which is believed to contribute to clot accretion. This is particularly important because commonly used anti-thrombin agents, such as heparin and low molecular weight heparin, are ineffective against clot-bound thrombin.

Finally, the thrombin inhibitors of this invention may be employed in compositions and methods for treating neurodegenerative diseases. Thrombin is known to cause neurite retraction, a process suggestive of the rounding in shape changes of brain cells and implicated

in neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease.

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting this invention in any manner.

#### EXAMPLE 1

##### Synthesis Of Sulfo-Tyr<sub>63</sub>hirudin<sub>54-64</sub>

Sulfo-Tyr<sub>63</sub>hirudin<sub>54-64</sub> has the amino acid formula: H-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(OSO<sub>3</sub>)-Leu-OH. We prepared this peptide by solid-phase peptide synthesis employing an Applied Biosystems 430 A Peptide Synthesizer (Applied Biosystems, Foster City, Calif.).

Specifically, we reacted 0.259 meq of BOC-O-Leu resin (1% DVB resin) sequentially with 2 mmoles of protected amino acids. Following 10 cycles of synthesis, we deprotected the peptide and uncoupled it from the DVB resin by treatment with anhydrous HF: p-cresol: ethyl methyl sulfate (10:1:1, v/v/v). The peptide was further purified on a Vydac C<sub>18</sub> HPLC reverse phase column (22 mm x 25 cm) which had previously been equilibrated in 0.1% TFA in water. Prior to applying the peptide to the column, we dissolved it in 2.0 ml of 0.1% TFA in water. If necessary, an additional 1 ml of 6M guanidinium chloride was added to the sample to increase solubility. After we applied the sample, the column was developed with a linear gradient of increasing acetonitrile (0-80%) in 0.1% TFA over 45 minutes at a flow rate of 4.0 ml/min. The effluent stream was monitored at 229 nm and fractions were collected manually.

We sulfated the resulting purified peptide at the single tyrosine residue using standard methodology [T. Nakahara et al., "Preparation of Tyrosine-O-[<sup>35</sup>S]Sulfated Cholecystokinin Octapeptide From A Non-Sulfated Precursor Peptide", *Anal. Biochem.*, 154, pp. 194-99 (1986)]. Sulfo-Tyr<sub>63</sub>hirudin<sub>54-64</sub> was then purified away from other peptides and reaction components by reverse-phase HPLC employing a Vydac C<sub>18</sub> column (4.6 x 25 cm) and an Applied Biosystems liquid chromatographic system. The column was equilibrated in a 0.1% TFA/water solvent and developed with a linear gradient of increasing acetonitrile concentration from 0 to 35% over 90 minutes at a flow rate of 0.8 ml/min with a 0.085% TFA-containing solvent. Fractions were assayed for absorbance at 214 nm.

#### EXAMPLE 2

##### Crosslinking Of Human Thrombin With Sulfo-Tyr<sub>63</sub>-Dinitrofluorobenzyl-hirudin<sub>54-64</sub>

We prepared Sulfo-Tyr<sub>63</sub>-dinitrofluorobenzyl-hirudin<sub>54-64</sub> (Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>54-64</sub>) by reacting Sulfo-Tyr<sub>63</sub>hirudin<sub>54-64</sub> (2.0 mg; prepared as in Example 1) with a stoichiometric quantity of difluorodinitrobenzene (Pierce Chemical Co., Rockford, Ill.) in dimethylformamide (DMF) for 18 hours at room temperature. We then subjected the sample to analytical HPLC separation employing an Applied Biosystems 150 A Liquid Chromatographic System and a Brownlee RP-300 C<sub>3</sub> column (0.46 x 10 cm) to determine the extent of derivatization. The column was equilibrated in 0.1% TFA in water (solvent A) and developed with a 0-50% linear gradient of 0.085% TFA/70% acetonitrile (solvent B) over 45 min and then a 50-100% linear

gradient of solvent B over 15 min. We used a constant flow rate of 1.0 ml/minute.

The effluent stream was monitored at 214 nm and 310 nm for absorbance. Peptide derivatized with the difluorodinitrobenzene reagent absorbs at 310 nm. We found that the above-described reaction produced Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>53-64</sub> at 15-30% yield. Following synthesis, Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>53-64</sub> was stored in the same dimethylformamide solvent at -20° C. for up to 1 month.

We reacted a 10-fold molar excess of Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>53-64</sub> with human  $\alpha$ -thrombin (12.5 mg) for 18 hr at room temperature in a phosphate-buffered saline. We determined the extent of cross-linking by analyzing the reaction mixture on an SDS-polyacrylamide gel. SDS-PAGE showed a decrease in the relative mobility of the  $\alpha$ -thrombin band reflective of an increase in molecular weight of 1000-2000 daltons (Da). This shift is consistent with cross-linking of thrombin with Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>54-64</sub> at a single site.

We confirmed that formation of a covalent complex between Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>54-64</sub> and human thrombin is specific by using [<sup>35</sup>S]-Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>54-64</sub> was prepared essentially as described above using H<sub>2</sub>[<sup>35</sup>S]O<sub>4</sub> instead of H<sub>2</sub>SO<sub>4</sub> in the Nakahara sulfation procedure [see also, copending U.S. patent application Ser. Nos. 164,178, 251,150, 280,618, and 314,756, and J. M. Maraganore et al., "Anticoagulant Activity of Synthetic Hirudin Peptides", *J. Biol. Chem.*, 264, pp. 8692-98 (1989) all of which are herein incorporated by reference].

We reacted [<sup>35</sup>S]-Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>54-64</sub> with human  $\alpha$ -thrombin, either in the presence or absence of a 5- or 20-fold molar excess (over the concentration of thrombin) of Sulfo-Tyr<sub>63</sub>-N-acetyl-hirudin<sub>3-64</sub> (prepared as in Example 1 with the addition of N-acetyl asparagine as a final step in peptide synthesis). Following incubation at room temperature for 18 hrs, we subjected the mixture to SDS-PAGE and autoradiography. The results (FIG. 1) showed that [<sup>35</sup>S]-labeled peptide was incorporated into the band which represents thrombin and that the presence of cold, unlabeled hirudin peptide attenuated the magnitude of covalent complex formation to <10%. Thus, reaction of Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>53-64</sub> with thrombin results in the 1:1 stoichiometric binding of the hirudin peptide at a specific binding site.

In order to identify the site on thrombin where Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>53-64</sub> binds, thrombin/Sulfo-Tyr<sub>63</sub>-dinitrobenzyl(DNB)-hirudin<sub>53-64</sub> complex (1.0 mg) was applied to a Sephadex G-50 column (1.5×45 cm) which was equilibrated and developed with 7M urea, 20 mM Tris, pH 7.5. This chromatography removed any unreacted Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>53-64</sub>. A peak containing thrombin/Sulfo-Tyr<sub>63</sub>-DNB-hirudin<sub>53-64</sub> was isolated in the void volume fractions, pooled and reduced by the addition of 10  $\mu$ l of  $\beta$ -mercaptoethanol.

Following reduction, we S-carboxymethylated the complex using iodoacetic acid as previously described [J. M. Maraganore et al., "A New Class of Phospholipases A<sub>2</sub> with Lysine in Place of Aspartate-49", *J. Biol. Chem.* 259, pp. 13839-43 (1984)]. The reduced, S-alkylated protein was then dialyzed extensively against 3% acetic acid at room temperature. Following dialysis, we digested the complex with pepsin (2% w/v) for 4 hr at 37° C. Peptic fragments of reduced, S-carboxyme-

thylated thrombin/Sulfo-Tyr<sub>63</sub>-DNB-hirudin<sub>54-64</sub> were purified by reverse-phase HPLC using an Aquapore Rp-300 C<sub>1</sub> column (0.46×10 cm). The column was equilibrated in 0.1% TFA in water and developed with a gradient of increasing 0.085% TFA/70% acetonitrile (0-60%) over 80 minutes at a flow rate of 1.0 ml/min. The effluent stream was monitored for absorbance at both 214 and 310 nm. Fractions of 10 ml were collected automatically. HPLC separation of peptic fragments allowed resolution of a single major peak of both 214 and 310 nm-absorbing material. Because of its far UV absorbance, this fragment contained the bound Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>54-64</sub>.

We then subjected the fragment to automated Edman degradation with an Applied Biosystems 470A gas-phase sequencer equipped with a 900A data system. Phenylthiohydantoin (PTH) amino acids were analyzed on-line using an Applied Biosystems 120A PTH analyzer and a PTH-C<sub>18</sub> column (2.1×220 mm). Shown below is a table of repetitive yields from the sequence analysis:

Cycle	Amino Acid	pmoles
1	Lys	858.5
2	Glu	629.2
3	Thr	357.6
4	Trp	276.3
5	Thr	289.0
6	Ala	474.4
7	Asn	369.0
8	Val	490.7
9	Gly	296.1
10	(x)	(-)
11	Gly	267.2
12	Gln	208.8
13	Pro	103.5
14	Ser	21.6
15	Val	23.3

The peptide sequence was found to correspond to residues 144-154 of human  $\alpha$ -thrombin [J. W. Fenton, II., "Thrombin Active Site Regions" *Semin. Thromb. Hemostasis*, 12, pp. 200-08 (1986)]. Peptic cleavages occurred at a Leu-Lys and Val-Leu bond, consistent with the specificity of this enzyme.

In the course of sequence analysis, the amino acid corresponding to Lys-149 (cycle 10) could not be identified or quantitated. This probably resulted from derivatization of the  $\epsilon$ -NH<sub>2</sub> group of this amino acid with the dinitrofluorobenzyl moiety of Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>54-64</sub>. Thus, Lys-149 is the major site where Sulfo-Tyr<sub>63</sub>-DNFB-hirudin<sub>54-64</sub> reacts with  $\alpha$ -thrombin.

### EXAMPLE 3

Design Of A Thrombin Inhibitor Capable Of Blocking The Catalytic Site And Binding To The Anion Binding Exosite

Carboxy terminal hirudin peptides effectively block thrombin-catalyzed fibrinogen hydrolysis, but not chromogenic substrate hydrolysis [J. M. Maraganore et al., *J. Biol. Chem.*, 264, pp. 8692-98 (1989)]. In addition, hirudin peptides do not neutralize thrombin-catalyzed activation of Factors V and VIII [J. W. Fenton, II, et al., "Hirudin Inhibition by Thrombin", *Angio. Archiv. Biol.*, 18, p. 27 (1989)].

Hirudin peptides, such as Sulfo-Tyr<sub>63</sub>-N-acetyl-hirudin<sub>53-64</sub>, exhibit potent inhibitory effects toward thrombin-induced platelet activation in vitro [J. A. Jakubowsky and J. M. Maraganore, "Inhibition of

Thrombin-Induced Platelet Activities By A Synthetic 12 Amino Acid Residue Sulfated Peptide (Hirugen)", *Blood*, p. 1213 (1989)]. Nevertheless, a thrombin inhibitor capable of blocking the active site may be required for inhibition of platelet thrombosis in vivo, if activation of Factors V and VIII are rate-limiting steps. This conclusion is warranted from results obtained with the irreversible thrombin inhibitor (D-Phe)-Pro-Arg-CH<sub>2</sub>Cl [S. R. Hanson and L. A. Harker, "Interruption of Acute Platelet-Dependent Thrombosis by the Synthetic Antithrombin D-Phenylalanyl-L-Prolyl-L-Arginyl Chloromethyl Ketone", *Proc. Natl. Acad. Sci. USA*, 85, pp. 3184-88 (1988)] and other reversible thrombin inhibitors [J. F. Eidt et al., "Thrombin is an Important Mediator of Platelet Aggregation in Stenosed Canine Coronary Arteries with Endothelial Injury", *J. Clin. Invest.*, 84, pp. 18-27 (1989)].

Using the above knowledge that the NH<sub>2</sub>-terminus of hirudin peptides is proximal to Lys-149, we employed a three-dimensional model of thrombin (FIG. 2) [B. Furie, et al., "Computer-Generated Models of Blood Coagulation Factor Xa, Factor IXa, and Thrombin Based Upon Structural Homology with Other Serine Proteases", *J. Biol. Chem.*, 257, pp. 3875-82 (1982)] to design an agent which: 1) binds to the anion binding exosite of thrombin; and, 2) is capable of blocking the active site pocket of thrombin and inhibiting the function of catalytic residues contained therein.

We determined that the minimal distance from the ε-NH<sub>2</sub> of Lys-149 to the β-hydroxylate of Ser-195 is 18-20 Å. Based on a 3 Å/amino acid residue length, we calculated that at least about 4-7 amino acids would be required to link a hirudin peptide, such as Sulfo-Tyr<sub>63</sub> hirudin<sub>53-64</sub>, to a domain comprising an active-site inhibitor structure. The composition of the linker was designed to be glycine. Glycine was chosen in order to engineer the greatest flexibility of a linker for these preliminary investigations. It should be understood, however, that other, more rigid biopolymer linkers may also be employed.

We chose the sequence (D-Phe)-Pro-Arg-Pro as the active site inhibitor because thrombin exhibits specificity for Arg as the P<sub>1</sub> amino acid in the cleavage of substrates. A Pro following the Arg yields a bond that is cleaved very slowly by thrombin. We designed alternate peptides by replacing Pro (following the P<sub>1</sub> Arg) with a sarcosyl- or N-methyl-alanine amino acid or by chemical reduction of an Arg-Gly scissile bond.

#### EXAMPLE 4

##### Synthesis Of Hirulog-8

Hirulog-8 has the formula: H-(D-Phe)-Pro-Arg-Pro-(Gly)<sub>4</sub>-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH. We synthesized Hirulog-8 by conventional solid-phase peptide synthesis employing an Applied Biosystems 430 A Peptide Synthesizer. This peptide was synthesized using BOC-L-Leucine-O-divinylbenzene resin. Additional t-BOC-amino acids (Peninsula Laboratories, Belmont, Calif.) used included BOC-O-2,6-dichlorobenzyl tyrosine, BOC-L-glutamic acid (7-benzyl ester), BOC-L-proline, BOC-L-isoleucine, BOC-L-phenylalanine, BOC-L-aspartic acid (B-benzyl ester), BOC-glycine, BOC-L-asparagine, BOC-L-phenylalanine, and BOC-L-arginine. In order to achieve higher yields in synthesis, the (Gly)<sub>4</sub> linker segment was attached in two cycles of manual addition of BOC-glycylglycine (Beckman Biosciences, Inc., Philadelphia, Pa.). After completion of synthesis, the

peptide was fully deprotected and uncoupled from the divinylbenzene resin by treatment with anhydrous HF: p-cresol: ethylmethyl sulfate (10:1:1, v/v/v). Following removal from the resin, the peptide was lyophilized to dryness.

Crude Hirulog-8 was purified by reverse-phase HPLC employing an Applied Biosystems 151A liquid chromatographic system and a Vydac C<sub>18</sub> column (2.2×25 cm). The column was equilibrated in 0.1% TFA/water and developed with a linear gradient of increasing acetonitrile concentration from 0 to 80% over 45 minutes in the 0.1% TFA at a flow-rate of 4.0 ml/min. The effluent stream was monitored for absorbance at 229 nm and fractions were collected manually. We purified 25-30 mg of crude Hirulog-8 by HPLC and recovered 15-20 mg of pure peptide.

We confirmed the structure of purified Hirulog-8 by amino acid and sequence analyses. Amino acid hydrolysates were prepared by treating the peptide with 6N HCl, in vacuo, at 110° C. for 24 hrs. We then analyzed the hydrolysates by ion-exchange chromatography and subsequent ninhydrin derivatization/detection using a Beckman 6300 automated analyzer. We performed sequence analysis using automated Edman degradation on an Applied Biosystems 470A gas-phase sequencer equipped with a Model 900A data system. Phenylthiohydantoin (PTH) amino acids were analyzed on-line using an Applied Biosystems 120A pTH-analyzer and a PTH-C<sub>18</sub> column (2.1×220 mm).

#### EXAMPLE 5

##### Synthesis Of Hirulog-9

Hirulog-9 has the formula: H-(D-Phe)-Pro-Arg-L-Pro-(Gly)<sub>4</sub>-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH. We synthesized this peptide in the same manner as that described in Example 4 using BOC-D-proline (Peninsula Laboratories) at cycle 15 in lieu of BOC-L-proline. Purification and characterization were performed as described in Example 4.

#### EXAMPLE 6

##### Synthesis Of Hirulog-10

Hirulog-10 has the formula: H-(D-Phe)-Pro-Arg-Sar-(Gly)<sub>5</sub>-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH. The peptide was synthesized as in Example 4 using BOC-sarcosine (Sigma Chemical Co., St. Louis, Mo.) at cycle 16. Purification and characterization were performed as described in Example 4.

#### EXAMPLE 7

##### Synthesis Of Hirulog-11

Hirulog 11 has the formula: H-(D-Phe)-Pro-Arg-Pro-(Gly)<sub>4</sub>-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-(3,5-diiodoTyr)-Leu-OH. This peptide is synthesized as in Example 4 using BOC-3,5-diiodo-L-tyrosine (Sigma) at cycle 2. Purification and characterization is performed as described in Example 4.

#### EXAMPLE 8

##### Synthesis Of Hirulog-12

Hirulog 12 has the formula: H-(D-Phe)-Pro-Arg-Pro-(Gly)<sub>4</sub>-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(OSO<sub>3</sub>)-Leu-OH. This peptide is synthesized by reacting 1.0 mg of Hirulog-8 in dimethylformamide (80 μl) with dicyclohexylcarbodiimide solution (1.25 g/ml, 0.007 ml) and concentrated sulfuric acid (0.5 μl) at 0° C.

for 10 minutes. The reaction is stopped by addition of water (1.0 ml).

The reaction mixture may be subjected to reverse-phase HPLC employing an Applied Biosystems 150A Liquid Chromatographic System and an Aquapore RP-300 C<sub>8</sub> column (0.46 × 10 cm). The column is equilibrated in solvent A (0.1% TFA/water) and developed with an increasing concentration of solvent B (0.085% TFA/70% acetonitrile) from 0 to 50% over 45 minutes at a flow-rate of 1.0 ml/min. The effluent stream is monitored for absorbance at 214 nm.

Purified Hirulog-12 is then neutralized to pH 7 by adding 0.1N NaOH. It is then lyophilized and reconstituted in phosphate-buffered saline.

#### EXAMPLE 9

##### Inhibition Of Thrombin-Catalyzed Hydrolysis Of A p-Nitroanilide Synthetic Substrate By Hirulog-8

We next analyzed the effects of Hirulog-8 on the human α-thrombin-catalyzed hydrolysis of Spectrozyme TH (tosyl-Gly-Pro-Arg-p-nitroanilide; American Diagnostica, New York, NY). Specifically, we measured the initial rate velocities in the presence or absence of Hirulog-8 over a range of substrate concentrations from 2.2 to 22 μM. The thrombin-catalyzed rate was monitored in a Cary 19 spectrophotometer at 405 nm and recorded continuously as a function of time. Kinetics were performed at room temperature (25 ± 1° C.) in a 0.05M Tris, pH 7.5, 0.1M NaCl buffer.

For a typical enzyme reaction, 1.0 ml of buffer was added to both the sample and reference cuvettes. Thrombin (3.2 × 10<sup>-9</sup>M, final concentration) and Hirulog-8 (0.4 × 10<sup>-8</sup>M) were added to the sample cuvette prior to addition of Spectrozyme TH (2.2-22 μM). Immediately following addition of substrate, the contents of the sample cuvette were mixed by use of a plastic pipette. The reaction was monitored spectrophotometrically for 5-15 minutes.

Initial rate velocities at each substrate concentration were expressed as moles Spectrozyme TH hydrolyzed/sec/mole thrombin. This was determined during the initial linear phase of the reaction (≤ 15% total hydrolysis of substrate) by measuring the slope of the hydrolytic reaction. Lineweaver-Burke plots were constructed accordingly, by plotting the inverse of the initial velocity against the inverse of the substrate concentration. The results showed that human α-thrombin-catalyzed hydrolysis of Spectrozyme TH had a V<sub>max</sub> = 17 moles hydrolyzed/sec/mole thrombin and a K<sub>M</sub> at 1.19 × 10<sup>-6</sup>M. FIG. 3, panels A and B, demonstrates that increasing concentrations of Hirulog-8 led to significant, dose-dependent increases in the K<sub>M</sub>, with slight increases in the V<sub>max</sub> for Spectrozyme TH hydrolysis. Therefore, the inhibition of the thrombin-catalyzed reaction by Hirulog-8 was carried out by mixed competitive/non-competitive components with respect to Spectrozyme TH hydrolysis. The K<sub>i</sub> of Hirulog-8 for α-thrombin was determined using the equation:

$$\left( \frac{V_{max}}{K_M \text{ inhibited}} \right) = \left( \frac{V_{max}}{K_M \text{ uninhibited}} \right) \times \left( 1 + \frac{[\text{Hirulog-8}]}{K_i} \right)$$

where

$$\left( \frac{V_{max}}{K_M \text{ inhibited}} \right)$$

is the slope of the thrombin-catalyzed reaction in the presence of Hirulog-8; [Hirulog-8] is the molar concentration of peptide;

$$\left( \frac{V_{max}}{K_M \text{ uninhibited}} \right)$$

is the thrombin-catalyzed reaction in the absence of inhibitor; and K<sub>i</sub> is the molar inhibitory constant for Hirulog-8 with human α-thrombin. The K<sub>i</sub> for Hirulog-8 was calculated to be 1.95 ± 0.11 × 10<sup>-9</sup>M.

#### EXAMPLE 10

##### Specificity Of Hirulog-8 For The Hirudin-Peptide Binding Site And Active Site Of Human α-Thrombin

Hirulog-8 was designed as an analogue that binds human α-thrombin via its hirudin peptide binding site while blocking thrombin's catalytic site. We tested the ability of Hirulog-8 to perform these functions by various studies described below.

The kinetics of Hirulog-8 inhibition of human γ-thrombin were studied essentially as described above in Example 9 for human α-thrombin. The γ-thrombin-catalyzed reaction toward Spectrozyme TH demonstrated a V<sub>max</sub> = 7.14 moles hydrolyzed/sec/mole thrombin and K<sub>M</sub> = 1.1 × 10<sup>-6</sup>M. These results confirm that γ-thrombin, a proteolytic form of thrombin, exhibits nearly complete catalytic competence, although this form essentially lacks clotting activity [S. D Lewis et al., "Catalytic Competence of Human α- and γ-Thrombins in the Activation of Fibrinogen and Factor XIII", *Biochemistry*, 26, pp. 7597-7603 (1987)]. The inhibition of γ-thrombin by Hirulog-8 was examined over a range of peptide concentrations from 2.7 × 10<sup>-8</sup> to 6.8 × 10<sup>-6</sup>M. As shown below, Hirulog-8 exhibited an increased K<sub>i</sub> of 3 orders of magnitude relative to α-thrombin. This high K toward γ-thrombin is due to the absence of an intact anion binding exosite (ABE) in γ-thrombin [J. W. Fenton, II, et al., "Anion-Binding Exosite of Human α-Thrombin and Fibrin(ogen) Recognition", *Biochemistry*, 27, pp. 7106-12 (1988)]. γ-thrombin is formed by proteolysis of the B-chain of α-thrombin at Lys-149 and Arg-78.

The inhibition of human α-thrombin by Hirulog-8 was significantly reduced in the presence of Sulfo-Tyr<sub>63</sub>-N-acetyl-hirudin<sub>53-64</sub> at concentrations of 2.6 × 10<sup>-6</sup>M to 129 × 10<sup>-5</sup>M. This is because Sulfo-Tyr<sub>63</sub>-N-acetyl-hirudin<sub>53-64</sub> competes with Hirulog-8 for binding to the ABE of thrombin.

This was also demonstrated by the addition of phenylmethylsulfonyl-α-thrombin ("PMS-α-thrombin"; 18 nM, final) to reactions of Hirulog-8 with human α-thrombin. The addition of this modified thrombin resulted in a substantial decrease in the ability of Hirulog-8 to inhibit α-thrombin. PMS-α-thrombin has an intact ABE, but is covalently derivatized at its active site. This modified thrombin sequesters the Hirulog-8 in the reaction mix and therefore reduces the amount of peptide available to inhibit intact, catalytically-active human α-thrombin.

We also performed studies of the effect of salt concentrations on the  $K_1$  of Hirulog-8 for thrombin as described above in Example 9. We measured the  $K_1$  in the presence or absence of Hirulog-8 ( $11.5 \times 10^{-9} M$ ) in buffers containing 0.1, 0.25, and 0.5M NaCl. As shown in the table below, inhibition of  $\alpha$ -thrombin by Hirulog-8 increased at lower salt concentrations. This result confirmed that the interaction of the highly anionic hirudin peptide moiety of Hirulog-8 with the positively-charged site surrounding Lys-149 of thrombin is essential for Hirulog-8 inhibition of thrombin-catalyzed hydrolysis of Spectrozyme TH.

Enzyme	Conditions	Hirulog-8, $K_1$ , nM
Human $\alpha$ -thrombin	0.05M Tris, pH 7.5	1.95
Human $\gamma$ -thrombin	0.1 M NaCl (Buffer) Buffer	1,080
Human $\alpha$ -thrombin	Buffer + 2.6 $\mu M$ Sulfo-Tyr <sub>63</sub> -N-acetyl-hirudin <sub>53-64</sub>	25.5
Human $\alpha$ -thrombin	Buffer + 12.9 $\mu M$ Sulfo-Tyr <sub>63</sub> -N-acetyl-hirudin <sub>53-64</sub>	>2,000
Human $\alpha$ -thrombin	Buffer + PMS- $\alpha$ -thrombin	9.90
Human $\alpha$ -thrombin	0.05 M Tris, pH 7.5 0.25 M NaCl	2.09
Human $\alpha$ -thrombin	0.05 M Tris, pH 7.5, 0.5 M NaCl.	3.72

#### EXAMPLE 11

##### Anticoagulant Activity Of Hirulog-8: Comparison To Hirudin And Sulfo-Tyr<sub>63</sub>-N-Acetyl-hirudin<sub>53-64</sub>

We studied the anticoagulant activity of Hirulog-8 using pooled, normal human plasma (George King Biomedical, Overland Park, Kan.) and a Coag-A-Mate XC instrument (General Diagnostics, Organon Technica, Oklahoma City, Okla.). Activity was monitored using the activated partial thromboplastin time (APTT) assay with  $CaCl_2$  and phospholipid solutions obtained from the manufacturer. Hirulog-8, hirudin, or Sulfo-Tyr<sub>63</sub>-N-acetyl-hirudin<sub>53-64</sub> was then added to the APTT determination wells at a final concentrations of 10 to 32,300 ng/ml in a total volume of 25  $\mu l$  prior to addition of 100  $\mu l$  of plasma.

The control APTT (absence of inhibitor) was 29.6 sec (mean,  $n=8$ , SEM  $<0.5\%$ ). FIG. 4 shows the results of these dose-dependency studies. Hirulog-8 was 2 to 3 times more potent than hirudin and 100 to 150 times more potent than Sulfo-Tyr<sub>63</sub>-N-acetyl-hirudin<sub>53-64</sub>. Both Hirulog-8 and hirudin increased the APTT of plasma to values which were too high to be measured. This is in contrast to Sulfo-Tyr<sub>63</sub>-N-acetyl-hirudin<sub>53-64</sub>, which exhibited a saturable dose-response in the APTT to 200-250% of control values [J. M. Maraganore et al., *J. Biol. Chem.*, 264, pp. 8692-98, (1989)]. This result showed that Hirulog-8 can block the active site of thrombin in plasma, as well as in vitro in chromogenic assays, in a manner similar to hirudin.

#### EXAMPLE 12

##### Inhibition Of Thrombin Induced Platelet Activation By Hirulog-8

Thrombin-induced platelet activation studies are performed at 37° C. using a Biodata PAP<sub>4</sub> Platelet Aggregometer. Platelet-rich plasma (PRP) is obtained from normal, healthy, volunteers who have not taken any

medication altering platelet function for at least one week prior to study. PRP is prepared as described by J. A. Jakubowski et al., "Modification of Human Platelet by a Diet Enriched in Saturated or Polyunsaturated Fat", *Atherosclerosis*, 31, pp. 335-44 (1978). Varying concentrations of Hirulog-8 (0-500 ng/ml in 50  $\mu l$  water) are added to 0.4 ml of pre-warmed (37° C.) PRP. One minute later, we add human  $\alpha$ -thrombin to the platelet suspension to a final concentration of 0.2, 0.25 or 0.5 units/ml total assay volume. Aggregation is monitored as an increase in light transmission for 5 minutes following the addition of thrombin. We then calculate % Inhibition as  $(\% \text{ aggregation}_{\text{sample}} / (\% \text{ aggregation}_{\text{control}})) \times 100$ . This study shows that Hirulog-8 blocks thrombin-induced platelet activation in vitro.

#### EXAMPLE 13

##### Use Of Hirulog-8 In Thrombus Imaging

Hirulog-8 is modified by covalent attachment of an <sup>123</sup>I-containing chemical group. Specifically, Hirulog-8 (as prepared in Example 4) is reacted with <sup>123</sup>I-Bolton Hunter Reagent (New England Nuclear, Boston, Mass.) in 0.1M sodium borate, pH 9.0. The <sup>123</sup>I-labelled molecule (with a specific activity of  $>5 \mu Ci/\mu g$ ) is then desalted on a Biogel P2 column which is equilibrated in a phosphate-buffered saline.

Ex vivo imaging of experimental thrombi is performed essentially as described by T. M. Palabrica et al., "Thrombus Imaging in a Primate Model with Antibodies Specific for an External Membrane Protein of Activated Platelets", *Proc. Natl. Acad. Sci. USA*, 86, pp. 1036-40 (1989). Specifically, imaging is performed in baboons using an external Ticoflex shunt between the femoral artery and femoral vein. An experimental thrombus is formed by placement of a segment of pre-clotted Dacron graft in the shunt. <sup>123</sup>I-labelled thrombin inhibitor is injected in the venous portion of the Ticoflex shunt. Serial anterior images are then obtained for 0.5 to 1 hour using an Ohio Nuclear Series 100 Gamma Camera with a PDP-11/34 computer. The kinetics of <sup>123</sup>I-thrombin inhibitor uptake by the graft and the blood pool are derived from the radionuclide images thus obtained.

The same technique may be used to obtain ex vivo images of a deep venous thrombus caused by stasis in the femoral vein of baboons. Because <sup>123</sup>I-Hirulog-8 binds to thrombin with high specificity, the use of this molecule allows precise ex vivo images of thrombi. Also, the small size of Hirulog-8, in contrast to native hirudin or antibodies to thrombin, provides the potential that the radiolabelled thrombin inhibitor will yield images of platelet-bound thrombin and meizothrombin, as well as thrombin contained in the fibrin clot.

#### EXAMPLE 14

##### Anti-Metastatic Activity of Thrombin Inhibitors

The anti-metastatic activity of the thrombin inhibitors of this invention, preferably Hirulog-8, is assayed using sarcoma T241 cells [L. A. Liotta et al., *Nature*, 284, pp. 67-68 (1980)] and syngeneic C57BL/6 mice (Jackson Laboratory, Bar Harbor, Me.). The mice are injected either intravenously or subcutaneously with 0-250 g/kg of Hirulog-8, prepared as in Example 4, followed by injection with  $10^4$ - $10^6$  T241 tumor cells. After 15 days, the animal is sacrificed and lung tumor colonies are quantitated. Anti-metastatic activity of Hirulog-8 is measured as percent reduction in tumor

colonies compared to placebo-treated control mice. Hirulog-8 demonstrates anti-metastatic activity in this assay.

#### EXAMPLE 15

##### Inhibition Of Endothelial Cells By A Thrombin Inhibitor

The ability of the thrombin inhibitors of this invention to prevent thrombin-induced synthesis of platelet activating factor (PAF) is assayed using cultured human umbilical vein endothelial cells (HUVECs). HUVECS are extracted from human umbilical cords by collagenase digestion according to established procedures [M. A. Gimborne, Jr., "Culture of Vascular Endothelium", *Prog. Hemost. Thromb.*, 3, pp. 1-28 (1976)]. HUVECs are grown to confluence in a 96-well microtiter plate in the presence of [<sup>3</sup>H]-acetate. Cells cultured in this manner produce [<sup>3</sup>H]-acetyl-PAF, which may be quantitated by extraction of HUVEC membrane phospholipids.

Hirulog-8 (0-1 µg/ml) is added to the [<sup>3</sup>H]-acetate loaded HUVECs 1 minute prior to the addition of thrombin (final concentration of 1 U/ml). Cells are incubated for 5 minutes and the supernatant is then removed. Medium containing 0.1% gelatin, 50 mM acetic acid in methanol (2:1 v/v) is then added to the HUVECs. PAF is then extracted and quantified using conventional techniques [T. M. McIntyre et al., "Cultured Endothelial Cells Synthesize Bot Platelet-Activating Factor and Prostacyclin in Response to Histamine, Bradykinin and Adenosine Triphosphate", *J. Clin. Invest.*, 76, pp. 271-80 (1985)]. The IC<sub>50</sub> values are then calculated. Hirulog-8 inhibits the synthesis of PAF by HUVECs in this assay.

The effect of Hirulog-8 on thrombin-induced polymorphonuclear leukocyte (PMN) adhesion to HUVECs may be demonstrated as follows. HUVECs are grown to confluence in MEM containing 1% fetal calf serum in 24-well cluster plates. The medium is then removed, the cells are washed two times with fresh, serum-free medium and incubated in the same medium for 10-30 minutes at 37° C. to remove serum products. PMNs (2.5 × 10<sup>6</sup> in 1 ml), which are pre-equilibrated at 37° C., are then added to each well. The PMNs are allowed to settle onto the HUVEC monolayer for 2 minutes. Hirulog-8 (5 µg/ml) or saline is added to each well, immediately followed by the addition of α-thrombin (0.1 or 1 U/ml). The cells are incubated for 5 minutes at 37° C., washed twice and then examined by phase-contrast microscopy. Adherent PMNs are counted directly. Samples incubated with Hirulog-8 have significantly fewer adherent PMNs than those treated with saline.

#### EXAMPLE 16

##### Synthesis Of Hirulog-13

Hirulog-13 has the formula: H-(D-Phe)-Pro-Arg-Pro-(Gly)<sub>2</sub>-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH. We synthesized, purified and characterized this peptide essentially as described in Example 4, except that only one cycle of BOC-glycylglycine was employed to produce the diglycine segment.

#### EXAMPLE 17

##### Synthesis Of Hirulog-14

Hirulog-14 has the formula: H-(D-Phe)-Pro-Arg-Pro-(Gly)<sub>5</sub>-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-

Tyr-Leu-OH. Hirulog-14 was synthesized, purified and characterized using methods described in Example 4, except that one cycle of BOC-glycine addition was employed following the two cycles of BOC-glycylglycine addition to produce the pentaglycine segment.

#### EXAMPLE 18

##### Synthesis Of Hirulog-15

Hirulog-15 has the formula: H-(D-Phe)-Pro-Arg-Pro-(Gly)<sub>6</sub>-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH. Hirulog-15 was synthesized, purified and characterized using methods described in Example 4, except that three cycles of BOC-glycylglycine addition were employed to prepare the hexaglycine segment.

#### EXAMPLE 19

##### Synthesis Of Hirulog-16

Hirulog-16 has the formula: H-(D-Phe)-Pro-Arg-Pro-(Gly)<sub>8</sub>-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH. Hirulog-16 was prepared, purified and characterized as described in Example 4, except that four cycles of BOC-glycylglycine addition were used to prepare the octaglycine segment.

#### EXAMPLE 20

##### Synthesis Of Hirulog-17

Hirulog-17 has the formula: H-(D-Phe)-Pro-Arg-Pro-Gly-Gly-Glu-Gly-His-Gly-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH. Hirulog-17 was synthesized essentially as described in Example 4, except that a Gly-Gly-Glu-Gly-His-Gly replaced the Gly<sub>4</sub> segment present in Hirulog-8. This sequence was added on to the growing peptide chain by the consecutive additions of BOC-glycine, BOC-L-histidine, BOC-glycine, BOC-L-glutamic acid and BOC-glycylglycine at cycles 13-17 of synthesis. Purification and characterization were performed as described in Example 4.

#### EXAMPLE 2

##### Synthesis Of Hirulog-18a, -18b And -18c

Hirulog-18a has the formula: H-(D-Phe)-Pro-(β-homoarginine)-(Gly)<sub>5</sub>-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH. Hirulog-18b has the formula: H-(D-Phe)-Pro-(β-homoarginine)-Pro-(Gly)<sub>4</sub>-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH. Hirulog-18c has the formula: H-(D-Phe)-Pro-(β-homoarginine)-Val-(Gly)<sub>4</sub>-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH. We synthesized Hirulog-18a using a mixed homogeneous/solid-phase procedure. Residues 5-20 were prepared by solid-phase synthesis, as described in Examples 4 and 17. The resulting resin-linked intermediate was reacted with a BOC-β-homoarginine-Gly protected intermediate, which was synthesized in the multi-step reaction scheme depicted below and described immediately thereafter.