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(54) Title: MULTIMERIC POLYVALENT ANTITHROMBOTIC AGENTS

(57) Abstract

This invention relates to antithrombotic agents and uses thereof. Specifically, the invention relates to chemical moieties that specifically bind to platelets and inhibit their aggregation, including linear and cyclic peptides. The invention provides methods for using these antithrombotic agents to prevent the formation of thrombi at sites in a mammalian body. In particular, the platelet-specific binding moieties including linear and cyclic peptides of the invention are covalently linked to a polyvalent linker moiety, so that the polyvalent linker moiety is covalently linked to a multiplicity of the platelet-specific binding moieties including linear and cyclic peptides. Efficacious antithrombotic agents are thereby provided.

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MULTIMERIC POLYVALENT ANTITHROMBOTIC AGENTS

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to antithrombotic agents and uses thereof. Specifically, the invention relates to compounds that bind to platelets, specifically compounds that bind to the platelet receptor molecule GPIIb/IIIa. Such compounds include peptides and cyclic peptides that bind to platelets and inhibit their aggregation. In particular, the platelet-specific antithrombotic agents of the invention are covalently linked to a polyvalent linker moiety, so that the polyvalent linker moiety is covalently linked to a multiplicity of the platelet-specific moieties, particularly peptides and cyclic peptides, thereby providing the multimeric polyvalent antithrombotic agents of the invention. The invention also provides methods for using such compounds to prevent the formation of thrombi at sites in a mammalian body.

2. Description of the Prior Art

Thrombosis and thromboembolism, in particular deep vein thrombosis (DVT) and pulmonary embolism (PE), are common clinical conditions that are associated with significant morbidity and mortality. It has been estimated that in the U.S. approximately 5 million patients experience one or more episodes of DVT per year and that over 500,000 cases of pulmonary embolism occur, resulting in 100,000 deaths (J. Seabold, Society of Nuclear Medicine Annual Meeting 1990).

In addition, myocardial infarction (heart attack) is usually caused by thrombosis in a coronary artery, often at the site of an atherosclerotic plaque. Conventional therapies for heart attack involve removing such thrombi, either surgically by angioplasty and/or by adminstration of thrombolytic drugs, such as recombinant tissue plasminogen activator or streptokinase. Following such therapy, however, re-stenosis or even re-occlusion of the affected coronary artery frequently occurs due to formation of another thrombus at the site of the original thrombus. Preventing such re-occurrence of coronary artery thrombi is thus an

important goal of all post-infarct therapy.

The physiological processes involved in the formation of thrombi are initiated by the accumulation of platelets at sites of damage or insult to the endothelial cell wall of a blood vessel, such as an atheroschlerotic plaque. Platelets normally accumulate at such sites after stimulation by local mediators signalling the injury. Subsequently, such platelets become aggregated at such sites by binding serum fibrinogen *via* GPIIb/IIIa receptors expressed on the platelet surface. It is with the binding of fibrinogen that such an aggregation of platelets becomes a thrombus.

The amino acid sequence of the fibrinogen molecule recognized by GPIIb/IIIa receptors is the sequence -Arg-Gly-Asp- (RGD), which sequence is present four times in each fibrinogen molecule. Platelet aggregation can be inhibited using fibrinogen antagonists that bind to the GPIIb/IIIa receptors. Thus, compounds that are fibrinogen antagonists and bind to GPIIb/IIIa are useful in preventing thrombosis, particularly in post-angioplasty or post-thrombolytic treatment regimes.

Peptides having the ability to bind to platelets and inhibit their aggregation are known in the prior art.

Ruoslahti & Pierschbacher, U.S. Patent No. 4,578,079 describe peptides of sequence X-Arg-Gly-Asp-R-Y, wherein X and Y are either H or an amino acid, and R is Thr or Cys, the peptides being capable of binding to platelets.

Ruoslahti & Pierschbacher, U.S. Patent No. 4,792,525 describe peptides of sequence Arg-Gly-Asp-X, wherein X is Ser, Thr or Cys, the peptides being capable of binding to platelets.

Pierschbacher *et al.*, 1989, PCT/US88/04403 disclose conformationally-restricted RGD-containing peptides for inhibiting cell attachment to a substratum.

Hawiger *et al.*, 1989, PCT/US89/01742 relates to peptides comprising sequences for two binding sites of a protein.

Nutt *et al.*, 1990, European Patent Application 90202015.5 disclose cyclic RGD peptides that are fibrinogen receptor antagonists.

Nutt *et al.*, 1990, European Patent Application 90202030.4 disclose cyclic

RGD peptides that are fibrinogen receptor antagonists.

Nutt *et al.*, 1990, European Patent Application 90202031.2 disclose cyclic RGD peptides that are fibrinogen receptor antagonists.

Nutt *et al.*, 1990, European Patent Application 90202032.0 disclose cyclic RGD peptides that are fibrinogen receptor antagonists.

Nutt *et al.*, 1990, European Patent Application 90311148.2 disclose cyclic peptides that are fibrinogen receptor antagonists.

Nutt *et al.*, 1990, European Patent Application 90311151.6 disclose cyclic peptides that are fibrinogen receptor antagonists.

Ali *et al.*, 1990, European Patent Application 90311537.6 disclose cyclic peptides that are fibrinogen receptor antagonists.

Barker *et al.*, 1991, PCT/US90/03788 disclose cyclic peptides for inhibiting platelet aggregation.

Maraganore *et al.*, 1991, PCT/US90/04642 disclose a radiolabeled thrombus inhibitor comprising (a) an inhibitor moiety; (b) a linker moiety; and (c) and an "anion binding exosite (ABE)" binding site moiety.

Pierschbacher *et al.*, 1991, PCT/US91/02356 disclose cyclic peptides that are fibrinogen receptor antagonists.

Ojima *et al.*, 1992, 204th Meeting, Amer. Chem. Soc. Abst. 44 disclose synthetic multimeric RDGF peptides useful in inhibiting platelet aggregation.

Although it is possible to prepare cyclic peptides that specifically bind to platelets, some such peptides exhibit low binding site affinity whereby the strength of peptide binding to platelets is insufficient to prevent platelet aggregation and thereby have an antithrombotic effect. Peptides comprised of linear arrays of thrombus-specific peptide binding units have been described in the prior art.

Rodwell *et al.*, 1991, PCT/US91/03116 disclose linear arrays of the peptide sequence RGD.

Alternative arrangements of specific binding peptide units are preferable. The present invention provides multimeric polyvalent antithrombotic agents, one embodiment of which are reagent comprised of a multiplicity of cyclic peptides that specifically bind to platelets and have a sufficient affinity for platelets to

prevent their aggregation. The incorporation of a multiplicity of platelet-specific cyclic peptides in the antithrombotic agents of the invention permits the use of particular platelet-specific cyclic peptides comprising platelet binding sequences whose individual binding affinity might not otherwise be sufficient to produce the desired inhibition of platelet aggregation resulting in an antithrombotic effect *in vivo*, but which have other desireable properties, such as improved *in vivo* stability and half-life, which are evidenced for example by increased retention times at thrombus sites *in vivo*, detected by Tc-99m scintigraphy. Improved inhibition of platelet aggregation by particular platelet-specific binding moieties including peptides and cyclic peptides is achieved using the multimeric polyvalent antithrombotic agents of this invention.

SUMMARY OF THE INVENTION

The present invention provides multimeric polyvalent antithrombotic agents useful for preventing thrombus formation in a mammalian body.

In a first aspect, the invention provides a multimeric polyvalent antithrombotic agent comprising a multiplicity of platelet-binding moieties that are ligands for a GPIIb/IIIa receptor molecule, covalently linked to a polyvalent linking moiety. In a preferred embodiment, such a multimeric polyvalent antithrombotic agent has a molecular weight of less than about 20,000 daltons.

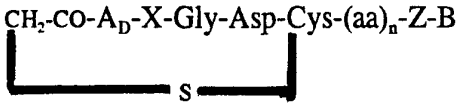
In a second aspect, the invention provides a multimeric polyvalent antithrombotic agent comprising a multiplicity of platelet binding peptides, each peptide comprising an amino acid sequence of 3-100 amino acids, covalently linked to a polyvalent linking moiety, wherein each of the platelet binding peptides has the formula

$$A\text{-}R^1\text{-}X\text{-}Gly\text{-}Asp\text{-}(aa)_n\text{-}Z\text{-}B$$

wherein A is H, an amine protecting group or $(aa)_p$, where $(aa)_p$ is a peptide comprising an amino acid sequence of length p, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and p is an integer from 0 to 97; R^1 is a lipophilic amino acid or H; X is an amino acid capable of being positively charged; $(aa)_n$ is a peptide comprising an amino acid

sequence of length n, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and n is an integer from 0 to 97; Z is either absent or cysteine, isocysteine or homocysteine; B is -OH, $-NH_2$, -SH or $(aa)_m$, wherein $(aa)_m$ is a peptide comprising an amino acid sequence of length m, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and m is an integer from 0 to 94; and the sum of n + m + p is less than or equal to 97. In a preferred embodiment, the lipophilic amino acid is a phenylalanine, tyrosine, tryptophan, valine, leucine or isoleucine residue. Lysine, homolysine, arginine, homoarginine, or L-[*S*-(3-aminopropyl) cysteine] are preferred as amino acid X in the formula. Each of the multiplicity of platelet binding peptides is preferably comprised of 3 to 20 amino acids.

In another aspect, the invention provides multimeric polyvalent antithrombotic agents, each comprising a multiplicity of platelet binding cyclic peptides, each peptide comprising an amino acid sequence of 5-100 amino acids, covalently linked to a polyvalent linking moiety, wherein each of the platelet binding cyclic peptides has the formula



wherein A_D is a lipophilic D-amino acid; X is an amino acid capable of being positively charged; $(aa)_n$ is a peptide comprising an amino acid sequence oflength n, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and n is an integer from 0 to 95; Z is either absent or cysteine, isocysteine or homocysteine; and B is -OH, $-NH_2$, -SH, or $(aa)_m$, wherein (aa) is any amino acid, wherein when the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, or where $(aa)_m$ is a peptide comprising an amino acid sequence of length m, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and m is an integer from 0 to 95, and the sum of n + m is $\leq$ 95. In a preferred embodiment, the lipophilic D-amino acid is selected from the group consisting of phenylalanine, tyrosine, tryptophan, valine, leucine and isoleucine. Lysine,

homolysine, arginine, homoarginine, or L-[*S*-(3-aminopropyl)cysteine] are preferred as amino acid X in the formula. In another preferred embodiment, each of the multiplicity of platelet binding cyclic peptides is comprised of 5 to 20 amino acids.

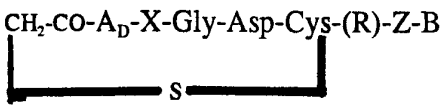
The invention also provides a multimeric polyvalent antithrombotic agent comprising a multiplicity of platelet binding peptides, each peptide comprising an amino acid sequence of 3-100 amino acids, covalently linked to a polyvalent linking moiety, wherein each of the platelet binding peptides has the formula

$$A\text{-}R^1\text{-}X\text{-}Gly\text{-}Asp\text{-}(R)\text{-}Z\text{-}B$$

wherein A is H, an amine protecting group or $(aa)_p$, where $(aa)_p$ is a peptide comprising an amino acid sequence of length p, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and p is an integer from 0 to 97; R^1 is a lipophilic amino acid or H; X is an amino acid capable of being positively charged; (R) is a substituted or unsubstituted linear or branched chain lower alkyl group having 1-20 carbon atoms or a substituted or unsubstituted phenyl, aryl, polycyclic or heterocyclic group, optionally comprising at least one heteroatom selected from the group consisting of O, S, and N; Z is either absent or cysteine, isocysteine or homocysteine; and B is -OH, $-NH_2$, -SH, or $(aa)_m$, wherein $(aa)_m$ is a peptide comprising an amino acid sequence of length m, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and m is an integer from 0 to 97; and the sum of m + p is less than or equal to 97. In a preferred embodiment, the lipophilic amino acid is selected from the group consisting of phenylalanine, tyrosine, tryptophan, valine, leucine and isoleucine. Lysine, homolysine, arginine, homoarginine, or L-[*S*-(3-aminopropyl)cysteine] are preferred as amino acid X in the formula. In another preferred embodiment, each of the multiplicity of platelet binding cyclic peptides is comprised of 3 to 20 amino acids.

In yet another aspect, the invention provides multimeric polyvalent antithrombotic agents, each comprising a multiplicity of platelet binding cyclic peptides, each peptide comprising an amino acid sequence of 5-100 amino acids, covalently linked to a polyvalent linking moiety, wherein each of the platelet

binding cyclic peptides has the formula



wherein A_D is a lipophilic D-amino acid; X is an amino acid capable of being positively charged; (R) is a substituted or unsubstituted linear or branched chain lower alkyl group having 1 to 20 carbon atoms, or a substituted or unsubstituted phenyl, aryl, polycyclic or heterocyclic group optionally comprising at least one heteroatom selected from the group consisting of O, S, and N; Z is either absent or cysteine, isocysteine or homocysteine; and B is -OH, $-NH_2$, -SH, or $(aa)_m$, wherein $(aa)_m$ is a peptide comprising an amino acid sequence of length m, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and m is an integer from 0 to 95. In a preferred embodiment, the lipophilic D-amino acid is selected from the group consisting of phenylalanine, tyrosine, tryptophan, valine, leucine and isoleucine. Lysine, homolysine, arginine, homoarginine, or L-[*S*-(3-aminopropyl)cysteine] are preferred as amino acid X in the formula. In another preferred embodiment, each of the multiplicity of platelet binding cyclic peptides is comprised of 5 to 20 amino acids.

In each of the linear peptides comprising a multimeric polyvalent antithrombotic agent of the invention the amino terminus may be protected by an amine protecting group. Preferred amine protecting groups include but are not limited to aliphatic or aromatic acyl groups comprising lower alkyl having 1 to 6 carbon atoms, or phenyl or phenyl substituted with lower alkyl, hydroxy, lower alkoxy, carboxy, or lower alkoxycarbonyl groups.

In each of the cyclic peptides of the invention having an amino acid residue (aa) comprising a cysteine, the sulfur atom of such a cysteine residue may be protected by a thiol protecting group. Preferably, such thiol protecting groups have the formula

$$-CH_2-NH-CO-R$$

wherein R is a lower alkyl having 1 to 6 carbon atoms, 2-,3-,4-pyridyl, phenyl,

or phenyl substituted with lower alkyl, hydroxy, lower alkoxy, carboxy, or lower alkoxycarbonyl.

The multimeric polyvalent antithrombotic agents of the invention comprise polyvalent linking moieties. Such polyvalent linking moieties are comprised of at least 2 linker functional groups capable of covalently bonding to the platelet binding moieties and linear and cyclic peptides comprising the antithrombotic agents of the invention. Preferably, at least 2 of the linker functional groups are identical, and most preferably, the linker functional groups are primary or secondary amines, hydroxyl groups, carboxylic acid groups or thiol-reactive groups. In a preferred embodiment, the polyvalent linking moieties are comprised of a multiplicity of polyvalent linking moieties covalently linked to form a branched polyvalent linking moiety. Preferred polyvalent linking moieties are *bis*-maleimidomethylether, *tris*(maleimidoethyl)amine and derivatives thereof, which form *bis*-succinimidylmethylether and *tris*(succinimidylethyl)amine peptide conjugates.

Each peptide-containing embodiment of the invention is comprised of a sequence of amino acids. The term amino acid as used in this invention is intended to include all L- and D- amino acids, naturally occurring and otherwise. Reagents comprising platelet-binding peptides provided by the invention include but are not limited to peptides comprising the following sequences, including the following cyclic peptides:

CH$_3$CO.Y$_D$RGDC

CH$_3$CO.Y$_D$RGDCWGG

CH$_3$CO.Y$_D$RGDCFGG

CH$_3$CO.Y$_D$RGDCGGG

CH$_3$CO.Y$_D$RGDCGG

CH$_3$CO.Y$_D$.Apc.GDCGGG

CH$_3$CO.Y$_D$KGDCGGG

GGRGDS

GGRGDGGRGDS
GGRGDGGRGDGGRGDS
KRARGDDMDDY
RRRRRRRRRGD
GRGDVK
GRGDV
GRGDVRGDFK
GRGDVRGDF
GGGRGDF
RGD
GRGDGG
GGRGDF
GGGRGDF
GRGDGGGG
RGDF
G.Apc.GDV.Apc.GDFKCamide
SYNRGDSTC

CH.CO.Y_D.Apc.GDCGGC$_{Acm}$GC$_{Acm}$GGCamide

CH.CO.Y_D.Apc.GDCKGC$_{Acm}$GC$_{Acm}$GGCamide

Single-letter abbreviations for amino acids can be found in G. Zubay, *Biochemistry* (2d. ed.), 1988 (MacMillen Publishing: New York) p.33; subscript "D" means a D-amino acid; Apc= L-[*S*-(3-aminopropyl)cysteine]. It will be understood by those with skill in the art that the sequences underlined in the above structures and in all other structures included herein represent a covalent bond between the the first and the last so underlined molecules, and further that when cysteine is either the first or the last underlined molecule, such a covalent bond will be to the sidechain sulfer atom of said cysteine.

This invention provides methods for preparing peptides of the invention by chemical synthesis *in vitro*. In a preferred embodiment, peptides are synthesized by solid phase peptide synthesis.

The invention also provides a method for preventing thrombosis within a mammalian body. This method comprises administering an effective therapeutic amount of a multimeric polyvalent antithrombotic agent of the invention to an animal in a pharmaceutical carrier.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred

embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the experiment described in Example 4.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides multimeric, polyvalent compounds which inhibit platelet aggregation and thus exhibit antithrombotic properties in a mammalian body, comprising a multiplicity of platelet-specific binding moieties, including peptides and cyclic peptides having an amino acid sequence of 3-100 amino acids, covalently linked to a polyvalent linking moiety.

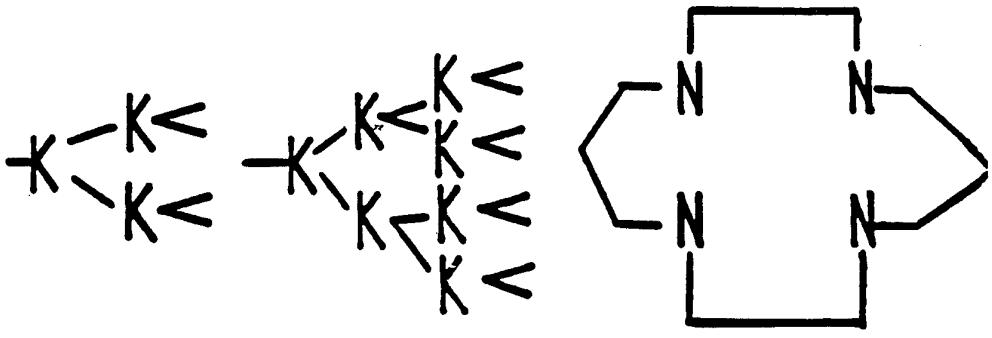
For purposes of this invention, the term "multimeric" is defined to describe compounds having multiple copies of a platelet-binding moeity that is a ligand for a platelet GPIIb/IIIa receptor molecule. Exemplary of such platelet binding moieties are peptides and cyclic peptides having an amino acid sequence comprising the sequence -Arg-Gly-Asp- (RGD), in linear or polyvalent arrays. For the purposes of this invention, the term "polyvalent" is defined to describe compounds in which a multiplicity of platelet-specific binding moieties, including peptides and cyclic peptides having an amino acid sequence having specific platelet-binding properties, exemplified by the sequence -Arg-Gly-Asp- (RDG), are covalently linked to a moiety having at least 2 functional groups, each capable of covalent linkage to platelet-binding moieties of the invention.

The polyvalent multimeric antithrombotic agents of the invention have advantageous properties that make them preferable to the linearly multimeric peptides known in the art. In particular, the antithrombotic agents of the invention exhibit IC_{50} values of platelet aggregation inhibition (i.e., the concentration of each agent at which platelet aggregation is reduced by 50%) lower than the IC_{50} values for platelet aggregation inhibiting agents known in the art.

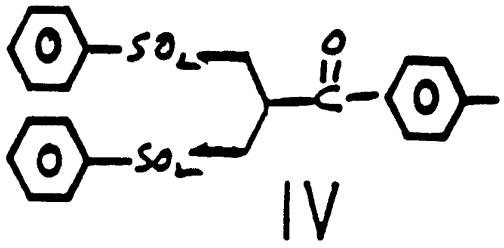
In addition, such multimeric polyvalent antithrombotic agents of the invention exhibit increased retention times at thrombus sites *in vivo*, as detected

by technetium-99m (Tc-99m) scintigraphy. These results suggest that the multimeric polyvalent antithrombotic agents of the invention are capable of remaining in contact with thrombi or at sites of thrombus formation for longer times than compounds known in the art. This indicates that the multimeric polyvalent antithrombotic agents of this invention bind to platelets with higher avidity than compounds known in the art.

Polyvalent linking moieties provided by the invention are comprised of at least 2 linker functional groups capable of covalently bonding to platelet-specific moieties, including linear and cyclic peptides. Such functional groups include but are not limited to primary and secondary amines, hydroxyl groups, carboxylic acid groups and thiol reactive groups. Polyvalent linking moieties are comprised of preferably at least three functional groups capable of being covalently linked to platelet-specific moeties, including linear and cyclic peptides. Preferred polyvalent linking moieties include amino acids such as lysine, homolysine, ornithine, aspartic acid and glutamic acid; linear and cyclic amines and polyamines; polycarboxylic acids; activated thiols; and thiol-reactive reagents such as di- and tri-maleimides. Also preferred are embodiments wherein the polyvalent linking moieties comprise a multiplicity of polyvalent linking moieties covalently linked to form a branched polyvalent linking moiety. For the purposes of this invention, the term "branched" polyvalent linking moieties is intended to include but are not limited to polyvalent linking moieties having formula:



I II III



IV

Most preferred polyvalent linking moieties are *bis*-maleimidomethylether, *tris*(maleimidoethyl)amine and derivatives thereof, which form *bis*-succinimidylmethylether and *tris*(succinimidylethyl)amine peptide conjugates. Those with skill in this art will recognize that the polyvalent linking moieties are properly denoted as being "succinimidyl" moieties when they are referred to as the chemical components of the reagents of the invention, and as being "maleimido" moieties when they are referred to as the chemical compounds used to synthesize said reagents.

Peptides of the present invention can be chemically synthesized *in vitro*. Peptides of the present invention can generally advantageously be prepared on an amino acid synthesizer.

The peptides provided by the invention are preferably administered intravenously in any conventional medium for intravenous injection such as an aqueous saline medium. Such medium may also contain conventional pharmaceutical adjunct materials such as, for example, pharmaceutically acceptable salts to adjust the osmotic pressure, buffers, preservatives and the like. Among the preferred media are normal saline and other isotonic buffers.

The peptides provided by the invention may also be administered by subcutaneous or intra-muscular injection or by mouth.

The antithrombotic agents of the invention, and methods for making and using these compounds, are more fully illustrated in the following Examples. These Examples illustrate certain aspects of the above-described invention and advantageous results thereof. These Examples are shown by way of illustration and not by way of limitation.

EXAMPLE 1
Synthesis of TMEA [*tris*(2-maleimidoethyl)amine]

tris(2-aminoethyl)amine (1.49 mL, 10 mmol) dissolved in 50 mL saturated aqueous sodium bicarbonate and cooled in an ice bath, was treated with *N*-carbomethoxymaleimide (4.808 g, 31 mmol). The mixture was stirred for 30 min on ice and then for another 30 min at room temperature. The mixture was then partitioned between dichloromethane and water, dried over magnesium sulfate, filtered and evaporated to give 3.442 g of product. Reverse phase thin-layer chromatography (RP-TLC) yielded essentially 1 spot (R_f= 0.63 in 1:1 acetonitrile : 0.5 M sodium chloride). 3.94 mmol (1.817g) of this product was dissolved in 20 mL tetrahydrofuran and 20 mL saturated sodium bicarbonate and mixed for 2 h. The reaction mixture was then partitioned between ethyl acetate and water. The organic phase was washed with saturated sodium chloride, dried over magnesium sulfate, and filtered. The ethyl acetate solution was diluted with hexanes and cooled. Solid TMEA was collected by filtration and dried to a yield of 832 mg. Chemical analysis of the product confirmed its identity as TMEA as follows:

^{1}H NMR ($CDCl_3$): 2.65 (tr. 2 H), 3.45 (tr.2 H). 6.64 (s. 2 H).

^{13}C NMR ($CDC1_3$), 35.5, 51.5, 133.9, 170.4.

EXAMPLE 2
Solid Phase Peptide Synthesis

Solid phase peptide synthesis (SPPS) was typically carried out on a 0.25 millimole (mmole) scale using an Applied Biosystems Model 431A Peptide Synthesizer and using 9-fluorenylmethyloxycarbonyl (Fmoc) amino-terminus protection, coupling with dicyclohexyl- carbodiimide/hydroxybenzotriazole or 2-(1H-benzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/ hydroxybenzotriazole (HBTU/HOBT), and using *p*-hydroxymethyl-phenoxymethylpolystyrene (HMP) resin for carboxyl-terminus acids or Rink amide resin for carboxyl-terminus amides. Resin-bound products were routinely cleaved using a solution containing trifluoroacetic acid (TFA) and various amounts of dichloromethane (DCM), water, thioanisole, ethanedithiol, and triethylsilane

(TES), typically a solution of TFA : DCM : H_2O : TES, prepared in ratios of 50 : 50 : 5 : 2 for 0.5 - 1.5 h at room temperature.

Where appropriate, N-terminal acetyl groups were introduced by treating the free N-terminal amino peptide bound to the resin with 20% v/v acetic anhydride in N-methyl- pyrrolidinone (NMP) for 30 min. Where appropriate, 2-chloroacetyl or 2-bromoacetyl groups were introduced either by using the appropriate 2-haloacetic acid as the last residue to be coupled during SPPS, or by treating the N-terminus free amino acid peptide bound to the resin with either the 2-haloacetic acid/diisopropylcarbodiimide/*N*-hydroxysuccinimide in NMP or the 2-haloacetic anhydride/diisopropylethylamine in NMP.

Where appropriate, HPLC-purified 2-haloacetylated peptides were cyclized by stirring an 0.1-1.0 mg/mL solution in phosphate or bicarbonate buffer or ammonia solution (pH 8.0), which may also contain 0.5-1.0 mM EDTA, for 0.5-48 h followed by acidification with acetic acid, lyophilization and HPLC purification.

Where appropriate, TSEA adducts were prepared by reacting single thiol-containing peptides (1 to 10 mg/mL in aqueous buffer, pH 7, with or without added acetonitrile) with 0.33 molar equivalents of TMEA [*tris*(2-maleimidoethyl)amine] added in 0.2 to 1 mL dimethylformamine (DMF) at room temperature for approximately 1 to 2 hours. The products were purified by HPLC.

Where appropriate, BSME adducts were prepared by reacting single thiol-containing peptides (1 to 10 mg/mL in aqueous buffer, pH 7, with or without added acetonitrile) with 0.5 molar equivalents of BMME (*bis*-maleimidomethylether) pre-dissolved in DMF at room temperature for approximately 0.5 to 2 hours. The solution was concentrated and the product were purified by HPLC.

Where appropriate, BAT-BS adducts were prepared by reacting single thiol-containing peptide (at concentrations of 2 to 50 mg/mL peptide in 50mM sodium phosphate (pH 7)/ acetonitrile or THF) with 0.5 molar equivalents of BAT-BM (*N*-[2-(*N'*,*N'*-*bis*(2-maleimidoethyl)aminoethyl)]-N^9-(*t*-butoxycarbonyl)-

N^6,N^9-*bis*(2-methyl-2-triphenylmethylthiopropyl)-6,9-diazanonanamide; *see* co-pending U.S. Patent Application 08/044,825, incorporated by reference) pre-dissolved in acetonitrile or THF, at room temperature for approximately 1-18h. The solution was then evaporated to dryness and [BAT-BS]-peptide conjugates deprotected by treatment with 10mL TFA and 0.2mL triethylsilane for 1h. The solution was concentrated, the product adducts precipitated with ether, and then purified by HPLC.

Crude peptides were purified by preparative high pressure liquid chromatography (HPLC) using a Waters Delta Pak C18 column and gradient elution using 0.1% trifluoroacetic acid (TFA) in water modified with acetonitrile. Acetonitrile was evaporated from the eluted fractions which were then lyophilized. The identity of each product was confirmed by fast atom bombardment mass spectroscopy (FABMS). Illustrative peptides are shown in Table I.

TABLE I

Peptides	MW[a]	HPLC[b]
(CH$_3$CO.Y$_D$.Apc.GDCGGC$_{Acm}$GC$_{Acm}$GGCamide)$_2$-BSME	3021[1]	12.4
(CH$_3$CO.Y$_D$.Apc.GDCGGC$_{Acm}$GC$_{Acm}$GGCamide)$_3$-TSEA	4563[2]	12.8
(CH$_3$CO.Y$_D$.Apc.GDCKGC$_{Acm}$GC$_{Acm}$GGCamide)$_2$-BSME	3163[2]	11.6
(CH$_3$CO.Y$_D$.Apc.GDCGGC$_{Acm}$GC$_{Acm}$GGCamide)$_2$-BSH	3062[1]	14.3
(CH$_3$CO.Y$_D$.Apc.GDCGGC$_{Acm}$GC$_{Acm}$GGCamide)$_2$-[BAT-BS]	3409[2]	13.0
(CH$_3$CO.Y$_D$.Apc.GDCKGC$_{Acm}$GC$_{Acm}$GGCamide)$_2$-[BAT-BS]	3552[1]	13.1

a Molecular weight determined by 1: fast atom bombardment mass spectroscopy (MH^+) or 2: electrospray mass spectroscopy (M)

b HPLC methods [in R_T(min)]:
solvent A = 0.1% CF3COOH/H_2O
solvent B_{90} = 0.1% CF_3COOH/90% CH_3CN/H_2O
solvent flow rate = 1 mL/min

Waters column = Waters DeltaPure RP-18, 5μ, 150mm x 3.9mm analytical column

Conditions: 10% A to 40% B_{90} in 20 min

Single-letter abbreviations for amino acids can be found in G. Zubay, *Biochemistry* (2d. ed.), 1988 (MacMillen Publishing: New York) p.33; Acm = acetamidomethyl; Apc = L-[*S*-(3-aminopropyl)cysteine]; Y_D = D-tyrosine; BSME = *bis*-succinimidylmethylether;TSEA = *tris*(succinimidylethyl)amine;[BAT-BS] = N-[2-(N',N'-bis(2-succinimidoethyl)aminoethyl]-N^6,N^9-bis(2-mercapto-2-methylpropyl)-6,9-diazanonanamide; BSH = 1,6-*bis*-succinimidylhexane

Peptides are linked to BSME, TSEA or [BAT-BS] linkers *via* the free thiol moiety of the unprotected cysteine residue (C) in each peptide.

EXAMPLE 3

Platelet Aggregation Inhibition Assays

Platelet aggregation studies were performed essentially as described by Zucker (1989, Methods in Enzymol. 169: 117-133). Briefly, platelet aggregation was assayed with or without putative platelet aggregation inhibitory compounds using fresh human platelet-rich plasma, comprising 300,000 platelets per microlitre. Platelet aggregation was induced by the addition of a solution of adenosine diphosphate to a final concentration of 15 micromolar, and the extent of platelet aggregation monitored using a Bio/Data aggregometer (Bio/Data Corp., Horsham, PA). The concentrations of platelet aggregation inhibitory compounds used were varied from 0.1 to 500 μg/mL. The concentration of inhibitor that reduced the extent of platelet aggregation by 50% (defined as the IC_{50}) was determined from plots of inhibitor concentration *versus* extent of platelet aggregation. An inhibition curve for peptide RGDS was determined for each batch of platelets tested as a positive control.

The results of these experiments are shown in Tables II and III. In Table II, the compounds tested are as follows:

P96 = **GRGDV**$C_{Acm}GC_{Acm}$amide
P143 = CH$_2$CO-Y_D**RGDC**$GGC_{Acm}GC_{Acm}$amide
P154 = CH$_2$CO-Y_D**ApcGDC**$GGGC_{Acm}GC_{Acm}$amide
P353 = CH$_2$CO-Y_D**ApcGDC**$GGC_{Acm}GC_{Acm}GGC_{NES}$amide
P474 = (CH$_2$CO-Y_D**ApcGDC**$GGC_{Acm}GC_{Acm}$GGC-amide)$_2$-BSH
P476 = (CH$_2$CO-Y_D**ApcGDCK**$GC_{Acm}GC_{Acm}$GGC-amide)$_2$-[BAT-BS]
P280 = (CH$_2$CO-Y_D**ApcGDC**$GGC_{Acm}GC_{Acm}$GGC-amide)$_2$-BSME
P357 = (CH$_2$CO-Y_D**ApcGDC**$GGC_{Acm}GC_{Acm}$GGC-amide)$_2$-[BAT-BS]
P317 = (CH$_2$CO-Y_D**ApcGDC**$GGC_{Acm}GC_{Acm}$GGC-amide)$_3$-TSEA

(Abbreviations used herein are the same as the abbreviations used in Table I; chemical structures representing peptides P474, P476, P280, P357 and P317 are shown on pages 21-23).

These results demonstrate that the IC_{50} decreases for cyclic peptides as compared with linear ones, and is even less for polyvalent peptide agents as compared with monovalent peptide agents. These results illustrate the efficacy of the multimeric polyvalent antithrombotic agents of the invention at reducing platelet aggregation.

Table III shows the IC_{50} values of the agents of the invention compared with some of the most potent antithrombotic agents known in the prior art. The compounds tested as shown in Table III are as follows (RGDS is given as a positive control):

Cytogen Pac-8 = *acetyl*SYG**RGDVRGD**FKCTCCA
Monsanto = $H_2NC(N=H)NH(CH_2)_7CO$-GDF[1]
Rhone-Poulenc-Rorer = $H_2NC(N=H)NH(CH_2)_5CO$-Sar.DV[2]
Genentech = (*1*-naphthyl)CHCO-**GRGDC**[3]
Diatech P280 = (CH_2CO-Y_D**ApcGDC**GG$C_{Acm}GC_{Acm}$GGC-amide)$_2$-BSME
SmithKline Beckman = *acetyl***C**$R_{(N\text{-}Me)}$**GD**Penamide[4]
Merck = *acetyl*CN-ββDiMeTzl(***p-amino***)**FGDC**amide[5]
Diatech P317 = (CH_2CO-Y_D**ApcGDC**GG$C_{Acm}GC_{Acm}$GGC-amide)$_3$-TSEA

(Pen = L-penicillamine; Sar = sarcosine; other abbreviations are as in Table I. References for these peptides are all taken from the Proceedings of the 12th American Peptide Symposium, held in Cambridge, MA on June 16-21, 1991: [1]: Tjoeng *et al.*, Abst. LTh8; [2]: Klein *et al.*, Abst. P-492; [3]: Burnier *et al.*, Abst. LTh9; [4]: Ali *et al.*, Abst. P-471; [5]: Nutt *et al.*, Abst. LF11).

These results demonstrate that the multimeric polyvalent antithrombotic agents P280 and P317 provided by the invention have a capacity to inhibit platelet aggregation with efficiencies comparable to and in some cases better than the most potent peptides known in the prior art.

TABLE II
INHIBITION OF PLATELET AGGREGATION

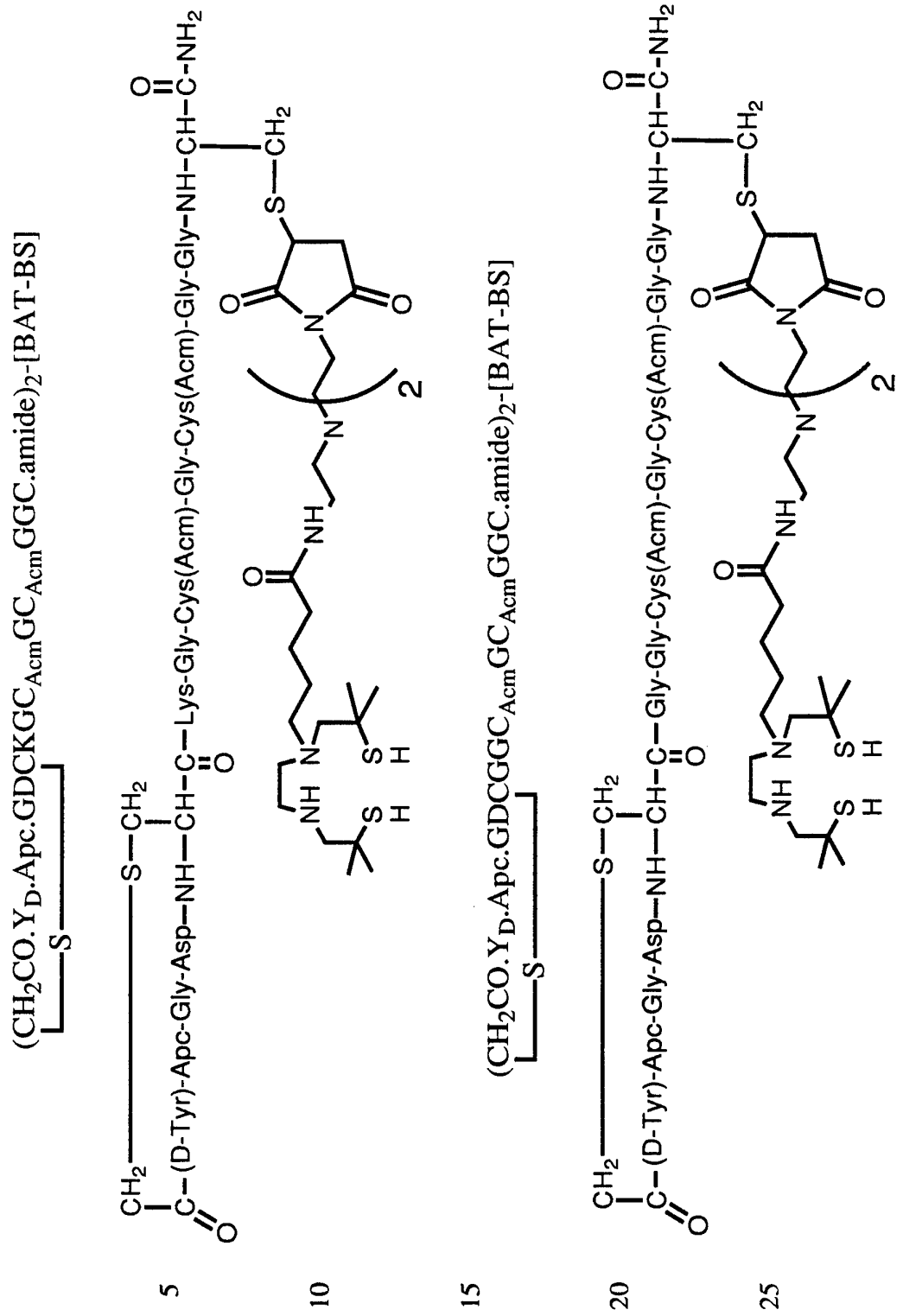
Peptide Code	Type	Structure	$IC_{50}(\mu M)$
P96	linear, monomer	**GRGDV**$C_{Acm}GC_{Acm}$amide	121
P143	cyclic, monomer	CH$_2$CO-Y_D**RGDC**$GGC_{Acm}GC_{Acm}$amide	1.3
P154	R analog, cyclic, monomer	CH$_2$CO-Y_D**ApcGDC**$GGGC_{Acm}GC_{Acm}$amide	0.30
P353	R analog, cyclic, monomer	CH$_2$CO-Y_D**ApcGDC**$GGC_{Acm}GC_{Acm}GC_{NES}$amide	0.27
P474	R analog, cyclic, dimer	(CH$_2$CO-Y_D**ApcGDC**$GGC_{Acm}GC_{Acm}$GGC-amide)$_2$-BSH	0.14
P476	R analog, cyclic, dimer	(CH$_2$CO-Y_D**ApcGDC**K$GC_{Acm}GC_{Acm}$GGC-amide)$_2$-[BAT-BS]	0.093
P280	R analog, cyclic, dimer	(CH$_2$CO-Y_D**ApcGDC**$GGC_{Acm}GC_{Acm}$GGC-amide)$_2$-BSME	0.083
P357	R analog, cyclic, dimer	(CH$_2$CO-Y_D**ApcGDC**$GGC_{Acm}GC_{Acm}$GGC-amide)$_2$-[BAT-BS]	0.079
P317	R analog, cyclic, trimer	(CH$_2$CO-Y_D**ApcGDC**$GGC_{Acm}GC_{Acm}$GGC-amide)$_3$-TSEA	0.036

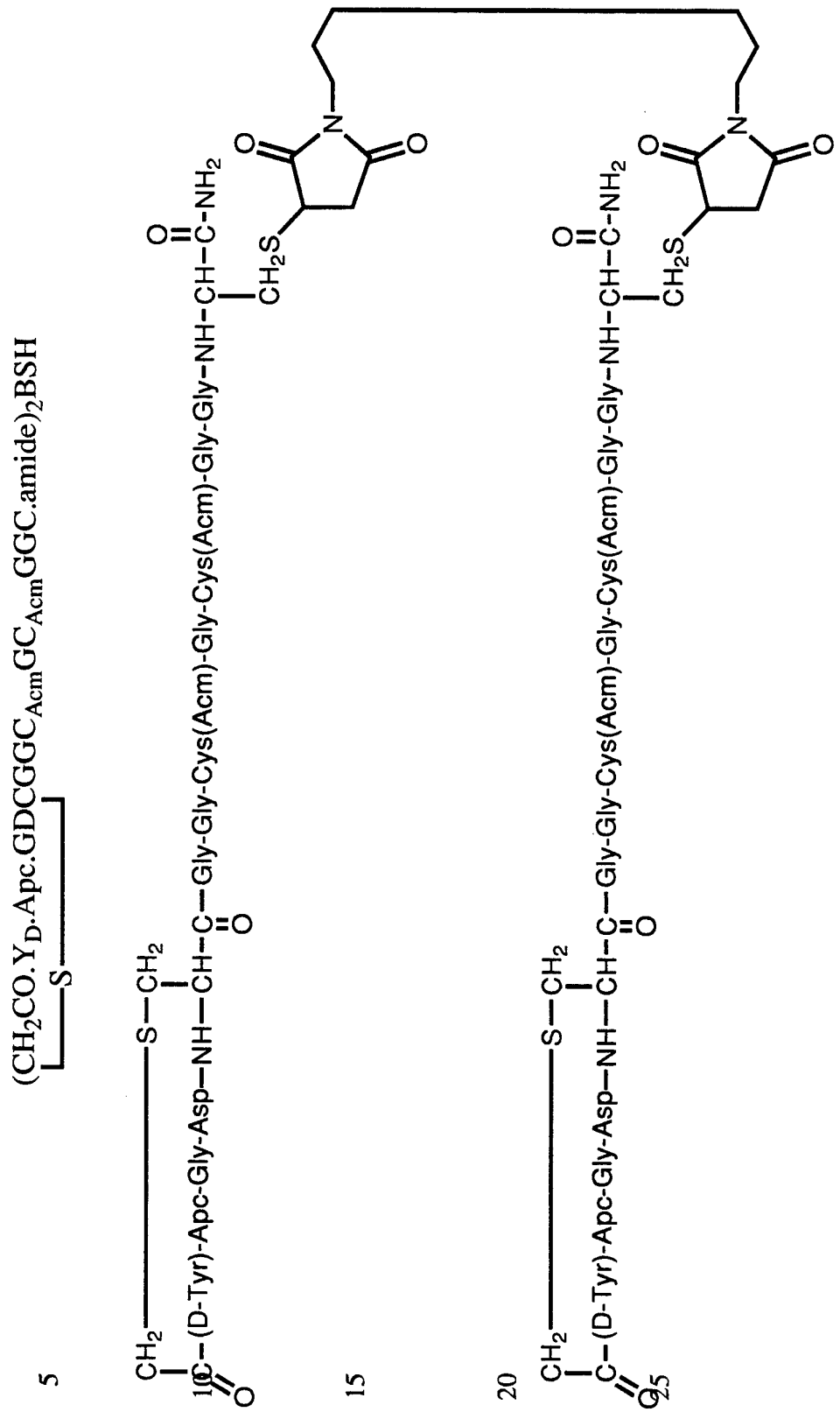
TABLE III

INHIBITION OF PLATELET AGGREGATION

Peptide	$IC_{50}(\mu M)$
RGDS	140
Cytogen Pac-8	12
Monsanto	1.6
Rhone-Poulenc Rorer	0.63
Genentech	0.15
Diatech P280	0.083
SmithKline Beckman	0.08
Diatech P317	0.036
Merck	0.022
echistatin	0.03


(CH2CO.YD.Apc.GDCGGCAcmGCAcmGGC.amide)3TSEA
S
CH2
S
CH2
C
O
(D-Tyr)-Apc-Gly-Asp
NH
CH
C
O
Gly-Gly-Cys(Acm)-Gly-Cys(Acm)-Gly-Gly
NH
CH
O
C
NH2
CH2S
O
NCH2CH2
N
O
3
(CH2CO.YD.Apc.GDCGGCAcmGCAcmGGC.amide)2BSME
S
CH2
S
CH2
C
O
(D-Tyr)-Apc-Gly-Asp
NH
CH
C
O
Gly-Gly-Cys(Acm)-Gly-Cys(Acm)-Gly-Gly
NH
CH
O
C
NH2
CH2S
O
NCH2
O
O
2


(CH2CO.YD.Apc.GDCKGCAcmGCAcmGGC.amide)2-[BAT-BS]
(D-Tyr)-Apc-Gly-Asp-NH-CH-C-Lys-Gly-Cys(Acm)-Gly-Cys(Acm)-Gly-NH-CH-C-NH2
(CH2CO.YD.Apc.GDCGGCAcmGCAcmGGC.amide)2-[BAT-BS]
(D-Tyr)-Apc-Gly-Asp-NH-CH-C-Gly-Gly-Cys(Acm)-Gly-Cys(Acm)-Gly-NH-CH-C-NH2


(CH2CO.YD.Apc.GDCGGCAcmGCAcmGGC.amide)2BSH
S
CH2
S
CH2
C
O
(D-Tyr)-Apc-Gly-Asp-NH-CH-C-Gly-Gly-Cys(Acm)-Gly-Cys(Acm)-Gly-Gly-NH-CH-C-NH2
O
CH2S
N
O
O

EXAMPLE 4
Demonstration of Antithrombotic Activity *In Vivo*

The Folts model of coronary thrombosis (Folts *et al.*, 1976, Circulation 54: 365-370) was used to demonstrate the antithrombotic activity of P280 *in vivo*. The Folts model is a largely platelet dependent, fibrin independent model which is characterized by the cyclic flow reductions (CFRs) produced by alternating gradual vessel occlusion by platelet-rich thrombus and abrupt restoration of flow caused by dislodgement of the thrombus. This model has been used to demonstrate the antithrombotic activity of potent GPIIb/IIIa binding peptides derived from snake venom [*e.g.* Bush *et al.*, 1989, Circulation 80: 11-23 (Abst)].

Three male mongrel dogs (12-19 kg) were anesthetized with sodium pentobarbital (30 mg/kg i.v.), intubated with a cuffed endotracheal tube and respirated on room air delivered by a Harvard respirator. Catheters were placed in the jugular and femoral veins to deliver drugs and fluids. A catheter was also placed in the carotid artery to measure arterial blood pressure (ABP). A left thoracotomy was made, exposing the heart, which was tented in a pericardial cradle. A segment of the left anterior descending (LAD) or left circumflex coronary artery (LCX) was gently isolated and a pulsed Doppler flow probe was placed on the coronary artery just proximal to the intended site of stenosis. In addition to ABP, pulsatile and mean Doppler flow and limb lead II electrocardiogram (ECG) were recorded on a Grass Physiological recorder. After obtaining control measurements of the parameters indicated above, the vessel's inner surface was denuded by squeezing together apposing sides of the artery and thereafter a rigid plastic constrictor was placed on the artery. This resulted in the development of an occlusive thrombus within approximately 3 to 7 minutes. After approximately one hour of sustained CFRs, peptide P280, dissolved in 10% propylene glycol/water, was infused into a free-running i.v. saline drip at a rate of 10, 30 or 100 μg/kg/min.

In all three dogs, infusion of P280 abolished the CFRs. The results from one of the dogs are shown in Figure 1. Panel A shows CFRs in dog coronary arteries before administration of P280. Panel B shows partial reduction of CFRs upon administration of 10 μg/kg/min of P280, and Panel C shows complete

abolition of CFRs in this dog on administration of 30 μg/kg/min of P280. The reversability of the effects of P280 administration is shown in Panel D, where return of the first occlusive thrombus in the dog coronary arteries is indicated by detection of resumed CFRs. Comparable results were attained by using infused doses of 100 μg/kg/min in the other two dogs. In all animals the CFRs returned within approximately 30 to 50 minutes after stopping the P280 infusion, as is expected for low molecular weight GPIIb/IIIa binding peptides of this type. These experiments demonstrated that P280, as an example of peptides of this invention, is an effective antithrombotic agent.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

WHAT WE IS CLAIM IS:

1. A multimeric polyvalent antithrombotic agent comprising a multiplicity of platelet-binding moieties that are ligands for a platelet GPIIb/IIIa receptor molecule, covalently linked to a polyvalent linking moiety wherein the molecular weight of the multimeric polyvalent antithrombotic agent is less than about 20,000 daltons.

2. A multimeric polyvalent antithrombotic agent according to Claim 1 wherein the polyvalent linking moiety is comprised of at least 2 linker functional groups capable of covalently bonding to the platelet-binding moieties and wherein at least 2 of the linker functional groups are identical.

3. A multimeric polyvalent antithrombotic agent according to Claim 2 wherein the linker functional groups of the polyvalent linking moiety are primary or secondary amines, hydroxyl groups, carboxylic acid groups or thiol-reactive groups.

4. A multimeric polyvalent antithrombotic agent according to Claim 1 wherein the polyvalent linking moiety is comprised of a multiplicity of polyvalent linking moieties covalently linked to form a branched polyvalent linking moiety.

5. A multimeric polyvalent antithrombotic agent according to Claim 1 wherein the polyvalent linking moiety is *bis*-succinimidylmethylether, *tris*(succinimidylethyl)amine or a derivative thereof.

6. A multimeric polyvalent antithrombotic agent according to Claim 1 wherein the multiplicity of platelet-binding moieties that are ligands for a platelet GPIIb/IIIa receptor molecule are each covalently linked to the polyvalent linking moiety by a functional linker group, said functional linker group selected from the group consisting of primary or secondary amines, hydroxyl groups, carboxylic acid groups and thiol groups.

7. A method for preventing thrombosis within a mammalian body comprising administering an effective therapeutic amount of the multimeric polyvalent antithrombotic agent of Claim 1 in a pharmaceutical carrier.

8. A multimeric polyvalent antithrombotic agent comprising a

multiplicity of platelet binding peptides, each peptide comprising an amino acid sequence of 3-100 amino acids, covalently linked to a polyvalent linking moiety, wherein each of the platelet binding peptides has the formula

$$A\text{-}R^1\text{-}X\text{-}Gly\text{-}Asp\text{-}(aa)_n\text{-}Z\text{-}B$$

wherein A is H, an amine protecting group or $(aa)_p$, where $(aa)_p$ is a peptide comprising an amino acid sequence of length p, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and p is an integer from 0 to 97;
R^1 is a lipophilic amino acid or H;
X is an amino acid capable of being positively charged;
$(aa)_n$ is a peptide comprising an amino acid sequence of length n, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and n is an integer from 0 to 97;
Z is absent or cysteine, isocysteine or homocysteine;
B is -OH, $-NH_2$, -SH, or $(aa)_m$, wherein (aa) is a peptide comprising an amino acid sequence of length m, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, or where $(aa)_m$ is a peptide comprised of a multiplicity of amino acids, and m is an integer from 0 to 97; and
$n + m + p \leq 97$,

wherein the molecular weight of the multimeric polyvalent antithrombotic agent is less than about 20,000 daltons.

9. The multimeric polyvalent antithrombotic agent of Claim 8 wherein the lipophilic amino acid is selected from the group consisting of phenylalanine, tyrosine, tryptophan, valine, leucine and isoleucine.

10. The multimeric polyvalent antithrombotic agent of Claim 8 wherein X is an amino acid that is lysine, homolysine, arginine, homoarginine, or L-[*S*-(3-aminopropyl)cysteine].

11. The multimeric polyvalent antithrombotic agent of Claim 8 wherein each of the multiplicity of platelet binding peptides is comprised of 3 to 20 amino acids.

12. The multimeric polyvalent antithrombotic agent of Claim 8 wherein each protected cysteine has a thiol protecting group of formula

$$-CH_2\text{-}NH\text{-}CO\text{-}R$$

wherein R is a lower alkyl having 1 to 6 carbon atoms, 2-,3-,4-pyridyl, phenyl,

or phenyl substituted with lower alkyl, hydroxy, lower alkoxy, carboxy, or lower alkoxycarbonyl.

13. The multimeric polyvalent antithrombotic agent of Claim 8 wherein the polyvalent linking moiety is comprised of at least 2 linker functional groups capable of covalently bonding to platelet binding peptides and wherein at least 2 of the linker functional groups are identical.

14. The multimeric polyvalent antithrombotic agent of Claim 13 wherein the linker functional groups of the polyvalent linking moiety are primary or secondary amines, hydroxyl groups, carboxylic acid groups or thiol-reactive groups.

15. The multimeric polyvalent antithrombotic agent of Claim 8 wherein the polyvalent linking moiety is comprised of a multiplicity of polyvalent linking moieties covalently linked to form a branched polyvalent linking moiety.

16. The multimeric polyvalent antithrombotic agent of Claim 8 wherein the polyvalent linking moiety is *bis*-succinimidylmethylether, *tris*(succinimidylethyl)amine or a derivative thereof.

17. A multimeric polyvalent antithrombotic agent according to Claim 8 wherein the multiplicity of platelet-binding peptides are each covalently linked to the polyvalent linking moiety by a functional linker group, said functional linker group selected from the group consisting of primary or secondary amines, hydroxyl groups, carboxylic acid groups and thiol groups.

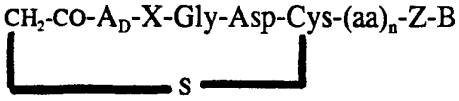
18. The multimeric polyvalent antithrombotic agent according to Claim 8 wherein the platelet-binding peptides are chemically synthesized *in vitro*.

19. The multimeric polyvalent antithrombotic agent according to Claim 18 wherein the platelet-binding peptides are synthesized by solid phase peptide synthesis.

20. A method for preventing thrombosis within a mammalian body comprising administering an effective therapeutic amount of the multimeric polyvalent antithrombotic agent of Claim 8 in a pharmaceutical carrier.

21. A multimeric polyvalent antithrombotic agent comprising a multiplicity of platelet binding peptides, each peptide comprising an amino acid

sequence of 5-100 amino acids, covalently linked to a polyvalent linking moiety, wherein each of the platelet binding cyclic peptides has the formula



wherein A_D is a lipophilic D-amino acid;
X is an amino acid capable of being positively charged;
$(aa)_n$ is a peptide comprising an amino acid sequence of length n, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and n is an integer from 0 to 95;
Z is absent or cysteine, isocysteine or homocysteine;
B is -OH, $-NH_2$, -SH, or $(aa)_m$, wherein $(aa)_m$ is a peptide comprising an amino acid sequence of length m, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and m is an integer from 0 to 95; and
$n + m \leq 95$,

wherein the molecular weight of the multimeric polyvalent antithrombotic agent is less than about 20,000 daltons.

22. The multimeric polyvalent antithrombotic agent of Claim 21 wherein the lipophilic D-amino acid is selected from the group consisting of phenylalanine, tyrosine, tryptophan, valine, leucine and isoleucine.

23. The multimeric polyvalent antithrombotic agent of Claim 21 wherein X is an amino acid that is lysine, homolysine, arginine, homoarginine, or L-[*S*-(3-aminopropyl)cysteine].

24. The multimeric polyvalent antithrombotic agent of Claim 21 wherein each of the multiplicity of platelet binding cyclic peptides is comprised of 5 to 20 amino acids.

25. The multimeric polyvalent antithrombotic agent of Claim 21 wherein each protected cysteine has a thiol protecting group of formula

$$-CH_2-NH-CO-R$$

wherein R is a lower alkyl having 1 to 6 carbon atoms, 2-,3-,4-pyridyl, phenyl, or phenyl substituted with lower alkyl, hydroxy, lower alkoxy, carboxy, or lower alkoxycarbonyl.

26. The multimeric polyvalent antithrombotic agent of Claim 21

wherein the polyvalent linking moiety is comprised of at least 2 linker functional groups capable of covalently bonding to platelet binding cyclic peptides and wherein at least 2 of the linker functional groups are identical.

27. The multimeric polyvalent antithrombotic agent of Claim 26 wherein the linker functional groups of the polyvalent linking moiety are primary or secondary amines, hydroxyl groups, carboxylic acid groups or thiol-reactive groups.

28. The multimeric polyvalent antithrombotic agent of Claim 21 wherein the polyvalent linking moiety is comprised of a multiplicity of polyvalent linking moieties covalently linked to form a branched polyvalent linking moiety.

29. The multimeric polyvalent antithrombotic agent of Claim 21 wherein the polyvalent linking moiety is *bis*-succinimidylmethylether, *tris*(succinimidylethyl)amine or a derivative thereof.

30. A multimeric polyvalent antithrombotic agent according to Claim 21 wherein the multiplicity of platelet-binding peptides are each covalently linked to the polyvalent linking moiety by a functional linker group, said functional linker group selected from the group consisting of primary or secondary amines, hydroxyl groups, carboxylic acid groups and thiol groups.

31. The multimeric polyvalent antithrombotic agent according to Claim 21 wherein the platelet-binding cyclic peptides are chemically synthesized *in vitro*.

32. The multimeric polyvalent antithrombotic agent according to Claim 31 wherein the platelet-binding cyclic peptides are synthesized by solid phase peptide synthesis.

33. A composition of matter having the formula

$(\underline{CH_2CO.Y_D.Apc.GDC}KGC_{Acm}GC_{Acm}GGCamide)_2$-BSME

$(\underline{CH_2CO.Y_D.Apc.GDC}GGC_{Acm}GC_{Acm}GGCamide)_2$-BSME

or

$(\underline{CH_2CO.Y_D.Apc.GDC}GGC_{Acm}GC_{Acm}GGCamide)_3$-TSEA.

34. A method for preventing thrombosis within a mammalian body comprising administering an effective therapeutic amount of the multimeric polyvalent antithrombotic agent of Claim 21 in a pharmaceutical carrier.

35. A multimeric polyvalent antithrombotic agent comprising a multiplicity of platelet binding peptides, each peptide comprising an amino acid sequence of 3-100 amino acids, covalently linked to a polyvalent linking moiety, wherein each of the platelet binding peptides has the formula

$$A\text{-}R^1\text{-}X\text{-}Gly\text{-}Asp\text{-}(R)\text{-}Z\text{-}B$$

wherein A is H, an amine protecting group or $(aa)_p$, where $(aa)_p$ is a peptide comprising an amino acid sequence of length p, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and p is an integer from 0 to 97;
R^1 is a lipophilic amino acid or H;
X is an amino acid capable of being positively charged;
(R) is a substituted or unsubstituted linear or branched chain lower alkyl group having 1-20 carbon atoms or a substituted or unsubstituted phenyl, aryl, polycyclic or heterocyclic group, optionally comprising at least one heteroatom selected from the group consisting of O, S, and N;
Z is absent or cysteine, isocysteine or homocysteine;
B is -OH, $-NH_2$, -SH, or $(aa)_m$, wherein $(aa)_m$ is a peptide comprising an amino acid sequence of length m, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and m is an integer from 0 to 97; and
$m + p \leq 97$,

wherein the molecular weight of the multimeric polyvalent antithrombotic agent is less than about 20,000 daltons.

36. The multimeric polyvalent antithrombotic agent of Claim 35 wherein the lipophilic amino acid is selected from the group consisting of phenylalanine, tyrosine, tryptophan, valine, leucine and isoleucine.

37. The multimeric polyvalent antithrombotic agent of Claim 35 wherein X is an amino acid that is lysine, homolysine, arginine, homoarginine, or L-[*S*-(3-aminopropyl)cysteine].

38. The multimeric polyvalent antithrombotic agent of Claim 35 wherein each of the multiplicity of platelet binding peptides is comprised of 3 to 20 amino acids.

39. The multimeric polyvalent antithrombotic agent of Claim 35 wherein each protected cysteine has a thiol protecting group of formula

$$-CH_2\text{-}NH\text{-}CO\text{-}R$$

wherein R is a lower alkyl having 1 to 6 carbon atoms, 2-,3-,4-pyridyl, phenyl, or phenyl substituted with lower alkyl, hydroxy, lower alkoxy, carboxy, or lower alkoxycarbonyl.

40. The multimeric polyvalent antithrombotic agent of Claim 35 wherein the polyvalent linking moiety is comprised of at least 2 linker functional groups capable of covalently bonding to platelet binding peptides and wherein at least 2 of the linker functional groups are identical.

41. The multimeric polyvalent antithrombotic agent of Claim 40 wherein the linker functional groups of the polyvalent linking moiety are primary or secondary amines, hydroxyl groups, carboxylic acid groups or thiol-reactive groups.

42. The multimeric polyvalent antithrombotic agent of Claim 35 wherein the polyvalent linking moiety is comprised of a multiplicity of polyvalent linking moieties covalently linked to form a branched polyvalent linking moiety.

43. The multimeric polyvalent antithrombotic agent of Claim 35 wherein the polyvalent linking moiety is *bis*-succinimidylmethylether, *tris*(succinimidylethyl)amine or a derivative thereof.

44. A multimeric polyvalent antithrombotic agent according to Claim 35 wherein the multiplicity of platelet-binding peptides are each covalently linked to the polyvalent linking moiety by a functional linker group, said functional linker group selected from the group consisting of primary or secondary amines, hydroxyl groups, carboxylic acid groups and thiol groups.

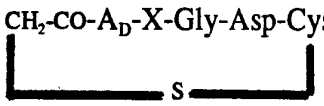
45. The multimeric polyvalent antithrombotic agent according to Claim 35 wherein the platelet-binding peptides are chemically synthesized *in vitro*.

46. The multimeric polyvalent antithrombotic agent according to Claim 45 wherein the platelet-binding peptides are synthesized by solid phase peptide synthesis.

47. A method for preventing thrombosis within a mammalian body comprising administering an effective therapeutic amount of the multimeric polyvalent antithrombotic agent of Claim 35 in a pharmaceutical carrier.

48. A multimeric polyvalent antithrombotic agent comprising a

multiplicity of platelet binding cyclic peptides, each peptide comprising an amino acid sequence of 5-100 amino acids, covalently linked to a polyvalent linking moiety, wherein each of the platelet binding cyclic peptides has the formula



wherein A_D is a lipophilic D-amino acid;
X is lysine, homolysine, arginine, homoarginine, or L-[*S*-(3-aminopropyl)cysteine];
(R) is a substituted or unsubstituted linear or branched chain lower alkyl group or a substituted or unsubstituted phenyl, aryl, polycyclic or heterocyclic group, optionally comprising at least one heteroatom selected from the group consisting of O, S, and N;
Z is absent or cysteine, isocysteine or homocysteine; and
B is -OH, $-NH_2$, -SH, or $(aa)_m$, wherein $(aa)_m$ is a peptide comprising an amino acid sequence of length m, wherein whenever the amino acid is cysteine, said cysteine may be protected at its sidechain sulfur atom, and m = 0-95,

wherein the molecular weight of the multimeric polyvalent antithrombotic agent is less than about 20,000 daltons.

49. The multimeric polyvalent antithrombotic agent of Claim 48 wherein the lipophilic D-amino acid is selected from the group consisting of phenylalanine, tyrosine, tryptophan, valine, leucine and isoleucine.

50. The multimeric polyvalent antithrombotic agent of Claim 48 wherein X is an amino acid that is lysine, homolysine, arginine, homoarginine, or L-[*S*-(3-aminopropyl)cysteine].

51. The multimeric polyvalent antithrombotic agent of Claim 48 wherein each of the multiplicity of platelet binding cyclic peptides is comprised of 5 to 20 amino acids.

52. The multimeric polyvalent antithrombotic agent of Claim 48 wherein the polyvalent linking moiety is comprised of at least 2 linker functional groups capable of covalently bonding to platelet-binding cyclic peptides and wherein at least 2 of the linker functional groups are identical.

53. The multimeric polyvalent antithrombotic agent of Claim 52

wherein the linker functional groups of the polyvalent linking moiety are primary or secondary amines, hydroxyl groups, carboxylic acid groups or thiol-reactive groups.

54. The multimeric polyvalent antithrombotic agent of Claim 48 wherein the polyvalent linking moiety is comprised of a multiplicity of polyvalent linking moieties covalently linked to form a branched polyvalent linking moiety.

55. The multimeric polyvalent antithrombotic agent of Claim 48 wherein the polyvalent linking moiety is *bis*-succinimidylmethylether, *tris*(succinimidylethyl)amine or a derivative thereof.

56. A multimeric polyvalent antithrombotic agent according to Claim 48 wherein the multiplicity of platelet-binding peptides are each covalently linked to the polyvalent linking moiety by a functional linker group, said functional linker group selected from the group consisting of primary or secondary amines, hydroxyl groups, carboxylic acid groups and thiol groups.

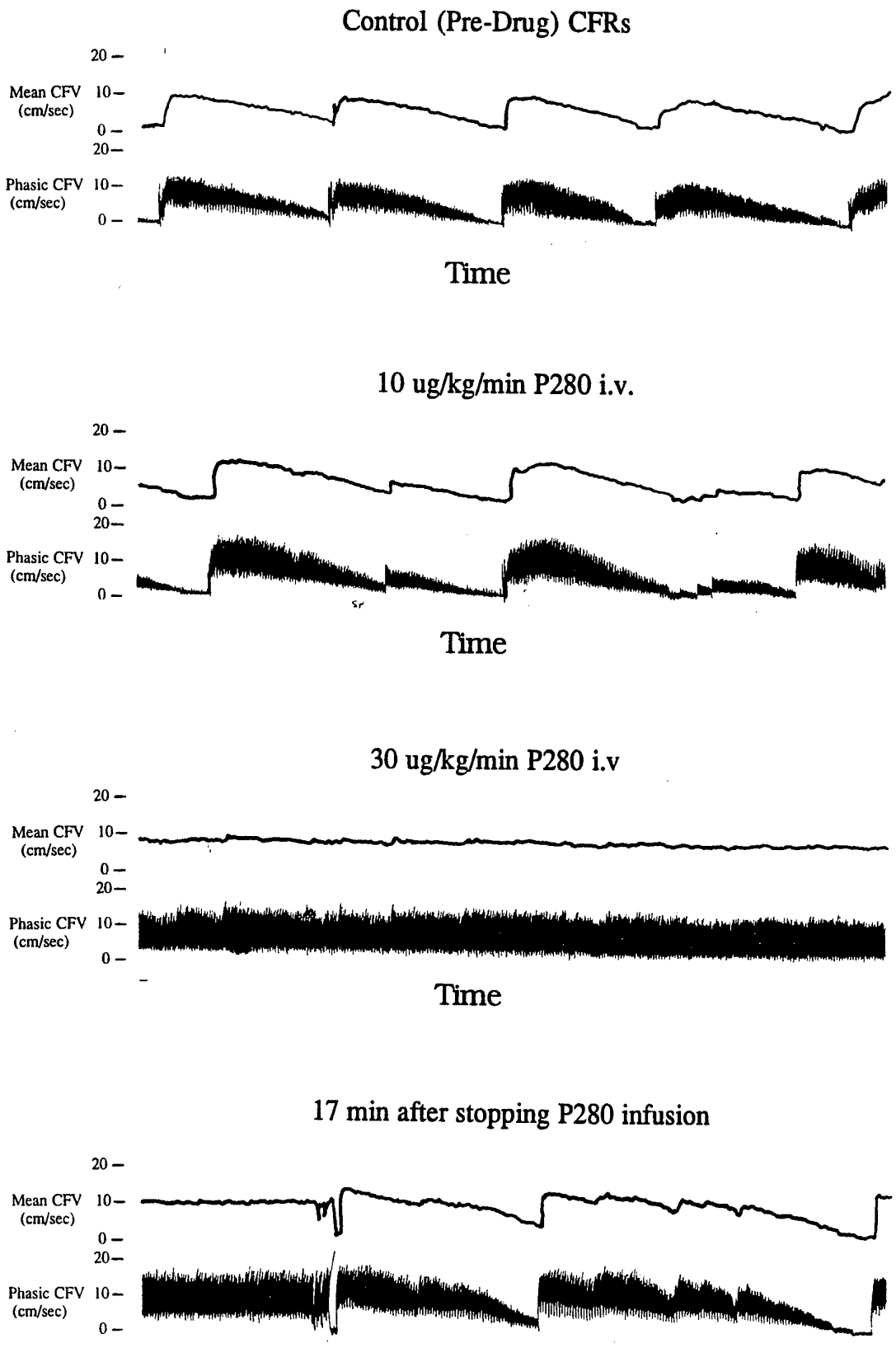
57. The multimeric polyvalent antithrombotic agent according to Claim 48 wherein the platelet-binding cyclic peptides are chemically synthesized *in vitro*.

58. The multimeric polyvalent antithrombotic agent according to Claim 57 wherein the platelet-specific cyclic peptides are synthesized by solid phase peptide synthesis.

59. A method for preventing thrombosis within a mammalian body comprising administering an effective therapeutic amount of the multimeric polyvalent antithrombotic agent of Claim 48 in a pharmaceutical carrier.

60. A composition of matter that is *tris*(maleimidoethyl)amine.

Figure 1


Control (Pre-Drug) CFRs
20
Mean CFV 10
(cm/sec)
0
20
Phasic CFV 10
(cm/sec)
0
Time
10 ug/kg/min P280 i.v.
20
Mean CFV 10
(cm/sec)
0
20
Phasic CFV 10
(cm/sec)
0
Time
30 ug/kg/min P280 i.v
20
Mean CFV 10
(cm/sec)
0
20
Phasic CFV 10
(cm/sec)
0
Time
17 min after stopping P280 infusion
20
Mean CFV 10
(cm/sec)
0
20
Phasic CFV 10
(cm/sec)
0

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 93/09387

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07K15/00 C07D207/452 A61K37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K C07D A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 503 301 (FUJI PHOTO FILM CO., LTD.) 16 September 1992 see the whole document ---	1-59
X	EP,A,0 482 649 (FUJI PHOTO FILM CO., LTD.) 29 April 1992 see the whole document ---	1-59
X	WO,A,90 06767 (LA JOLLA CANCER RESEARCH FOUNDATION) 28 June 1990 see the whole document ---	1-59
X	EP,A,0 446 071 (HYBRITECH INC.) 11 September 1991 see example 2 ---	60
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 1994	04 -03- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Masturzo, P

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF MEDICINAL CHEMISTRY. vol. 35, no. 9 , 1 May 1992 , WASHINGTON US pages 1563 - 1572 J C CHERONIS ET AL. 'A new class of bradykinin antagonists; synthesis and in vitro activity of bissuccinimidoalkane peptide dimers' see compound 41b, page 1569, Table VIII	60

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09387

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark :Although claims 7, 20, 34, 47, 59 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compounds.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Intern ...al Application No
PCT/US 93/09387

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0503301	16-09-92	JP-A-	5186499	27-07-93
EP-A-0482649	29-04-92	JP-A-	4217700	07-08-92
WO-A-9006767	28-06-90	AU-B-	628910	24-09-92
		AU-A-	4828590	10-07-90
		CA-A-	2006060	20-06-90
		EP-A-	0449973	09-10-91
EP-A-0446071	11-09-91	US-A-	5091542	25-02-92
		AU-B-	638313	24-06-93
		AU-A-	7272491	12-09-91
		US-A-	5262524	16-11-93