

SERINE PROTEASE INHIBITORS

This invention relates to serine protease inhibitors and substrates, as well as to the synthesis of such compounds and novel methods and materials for synthesis of boron-containing compounds.

5

Modulation or inhibition of serine protease inhibitors is useful *inter alia* to prevent thrombosis.

The family of serine protease enzymes cleaves peptide bonds by a mechanism
10 involving the catalytic triad of Asp-His-Ser residues in the active site of the enzymes. Serine protease inhibitors have been designed which use functional groups to interact with the triad and thereby block activation of the enzyme substrates. It can be desirable to make inhibitors selective for one target protease. Discussion of the prior art relating to peptide inhibitors can be found in the specification of a UK patent
15 application entitled "Thrombin Inhibitors" filed on the same date as this application and in PCT/GB96/00352. A copy of the specification of the application entitled "Thrombin Inhibitors" is filed herewith. The content of this application does include the subject matter of the application entitled "Thrombin Inhibitors", whose specification the skilled reader may wish to consult, along with various prior
20 documents referred to in that specification. The "Thrombin Inhibitors" specification does not physically form part of the published specification of this application.

Certain serine proteases are known to have a second site or "exosite" for binding to an anionic portion of the substrate. This exosite is often called the anion binding exosite
25 ("ABE").

The amino acid residue which provides the carbonyl group of the scissile bond of a serine protease substrate is designated "P1". Successive amino acid residues on the N-terminal side of residue P1 are designated P2, P3, P4 ... etc; amino acid residues on the
30 C-terminal side of residue P1 are designated P1', P2', P3' In fibrinogen, P1' is glycine and P2' is proline. The protease contains a "specificity pocket" which

This Sheet Has Been Replaced



recognises the side chain of the P1 amino acid. Trypsin-like proteases normally recognise P1 residues with arginine-like or serine-like side chains.

5 The present invention provides novel bifunctional serine protease inhibitors comprising:

- (a) a catalytic site-directed moiety (CSDM) which binds to and inhibits the active site of a serine protease;
- (b) an exosite associating moiety (EAM); and, optionally,
- 10 (c) a connector moiety bonded between the EAM and the CSDM,

the CSDM and the EAM being capable of binding simultaneously to a molecule of the serine protease.

15 In one class of inhibitors, the serine protease is not thrombin. The serine protease is preferably a trypsin-like protease. In any event, the inhibitor does not comprise a thrombin inhibitor in which the connector moiety is bonded to the CSDM as a C-terminal extension thereof, *i.e.* is not a compound as disclosed in US 5196404 and corresponding International application No WO 91/02750.

20

In another aspect, the invention provides a novel method for preparing boron-containing peptides.

25 As used herein, "natural" amino acid means an L-amino acid (or a residue thereof) selected from one of the twenty common or "standard" α -amino acids found in proteins.

30 By "unnatural" amino acid is meant any α -amino acid (or residue thereof) other than the twenty "standard" amino acids. Unnatural amino acids therefore include the D-isomers of natural L- amino acids and amino acids having side chain protecting groups.



The prefixes "D" and "L" are used as normal to indicate amino acids of D- or L-configuration respectively. A "D, L-" prefix indicates a racemic mixture whilst the absence of a prefix indicates that the amino acid can be of either D- or L-configuration, except in the examples where residues are of L-configuration unless
5 otherwise states. For those groups of unspecified configuration in the text which can be of D- or L- configuration, L- configuration is preferred.

Abbreviations and terms prefixed by "boro" indicate amino acids where in the terminal carboxyl group $\text{-CO}_2\text{H}$ has been replaced by a boron functionality.

10

Brief Description of the Drawings

Figure 1 is a Fourier Transform Infra Red (F.t.-I.R.) spectrum of a Merrifield
15 resin

Figure 2 is an F.t.-I.R. spectrum of the same resin after reaction with sodium 2,2-dimethyl-1,3-dioxolane-4-methanolate.

Figure 3 is an F.t.-I.R. spectrum of the reacted resin after treatment with HCl to deprotect the hydroxy groups of the dioxolane.

20 Figure 4 is an F.t.-I.R. spectrum of the deprotected resin after reaction with phenylboronic acid.

Considering now the inventive compounds and processes in more details:

25

The Catalytic Site-Directed Moiety (CSDM)

The catalytic site-directed moiety (CSDM) binds to and inactivates the catalytic site of a serine protease enzyme. The structure of the CSDM is not critical to the invention.
30 It may comprise the amino acid sequence of any known inhibitor of a serine protease catalytic site, for example.

This Sheet Has Been Replaced



One class of CSDMs is included in the following Formula 1:



- 5 wherein aa^1 , aa^2 , and aa^3 represent natural or unnatural acid residues and $(aa^4)_m$ one or more optional amino acid residues linked to the amino group of aa^3 . Alternatively any one or more aa groups may be analogues of amino acid residues in which the α -hydrogen is replaced by a substituent. The sequence of amino acids and/or amino acid analogues binds to the serine protease active site. Suitable sequences are described later
- 10 in this specification. X represents H or a substituent on the N-terminal amino group. Z is $-COOH$ or a C-terminal extension group (carboxy replacement group), for example as known in the art. In preferred compounds Z is a heteroatom acid group, e.g. $-B(OH)_2$, $-P(OH)_2$ or $PO(OH)_2$, or a derivative thereof, for example a carboxylic acid ester, a dioxo-boronate $[-B(Osubstituent)_2]$ or a phosphate $[-PO(Osubstituent)_2]$ or BF_2 .
- 15 Preferred heteroatom analogue groups are $-B(OH)_2$ and $-P(O)(OH)_2$; a less preferred heteroatom analogue group is $S(O)_2OH$. Amongst other possible Z groups there may be mentioned $-CN$, $-COCH_2Cl$ and $-COCH_2F$. In preferred embodiments m is from 0 to 7 and more usually 0 to 5, e.g. 0, 1 or 2, especially 0. Normally, $n=1$.
- 20 In one class of compounds, the $(aa^2)-(aa^1)$ natural peptide linkage is replaced by another linkage (ψ). Additionally or alternatively other natural peptide linkages may be replaced by another linkage.

- Catalytic site inhibitors of serine proteases are well known in the art. A short review
- 25 of serine protease inhibitors, i.e. inhibitors of the serine protease catalytic site, is to be found in EP-B-145441, which patent discloses a class of serine proteases having a C-terminal boron group. Other patent specifications describing serine protease inhibitors include EP 293881, EP 471651 (equivalent to US 5288707), EP 235692, US 4963655 and WO 89/09612 (concerned particularly with inhibitors of Factor
- 30 VII/VIIa in the [TF:VII/VIIa] complex).

This Sheet Has Been Replaced



For inhibitors of trypsin-like enzymes, preferred classes of P1 residues of CSDMs are (i) Arg, Lys and their analogues, and (ii) hydrophobic residues; further description of preferred P1 groups for thrombin which are also for other trypsin-like enzymes may be found in the aforesaid specification entitled "Thrombin Inhibitors" and in PCT/GB96/00352. Chymotrypsin-like serine proteases bind preferentially to CSDMs having phenylalanine-like and alanine-like side chains on the P1 residue. The following table A indicates the most preferred (P4)P3P2 residues for eight particular serine proteases:

10

Table A

Enzyme	Residue Sequence
Thrombin	D-Phe-/substituted D-Phe-/D-Dpa-/Dba-/Pms-/α-Nal-/β-Nal-/TMSal-/Chg-/Phg-/D-Tiq-/para-ether of D-Tyr-/NaSO ₂ -Pro
Factor Xa	IleuGluGly, PyroGluGly, ArgGly, ChaGly, LeuArg
Factor VIIa	L-PhePhe, NalPhe, D-TiqPhe, NalThr, NalPhg
Factor IXa	ValVal
Factor XIa	γ-BzlGluGly, Glu(OBzl)Ala, GlyArg, GlyLys
Factor XIIa	GlnGly
Urokinase	PhePro, GluGly
Protein Ca	LeuSerThr

In each case, a preferred amino acid may be replaced by an analogue thereof.

15 The Exosite Associating Moiety (EAM)

The exosite associating moiety (EAM) is a moiety which binds to an exosite (ABE) of a serine protease. Thrombin has a well defined exosite to which there binds, in addition to a fibrinogen amino acid sequence C-terminal to the thrombin cleavage site, non-substrate ligands of thrombin such as hirudin. Hirudin sequences such as Hir⁵³⁻⁶⁴ have been used in bifunctional peptides named "hirulogs". The hirulogs are described in US 5196404; a further description of thrombin EAMs may be found in the



aforesaid UK patent application entitled "Thrombin Inhibitors". EAMs for thrombin are often termed "anion binding exosite associating moieties" (ABEAMs).

The implications from the crystal structure of FXa (Padmanabhan K et al. "Structure of Human Des (1-45) Factor Xa at 2.2Å Resolution" *J Mol.Biol* 1993, 232, 947-966) are that the sequences 35-41 and 70-81, containing 8 acidic residues constitute a cation binding exosite. The natural polypeptide inhibitors antistasin and ghilanten contain the electropositive cation exosite associating sequences ¹⁰⁸CRPKRKLIPR¹¹⁷ and ¹⁰⁸CKPKRKI.VPR¹¹⁷ respectively.

10

It is clear that serine protease interactions in P' sites (C-terminal to the cleavage point) can be as important to specificity as those at P sites (for example Ding, L.; Coombs, G.S.; Strandberg, L.; Navre, M.; Coreyu, D.R. and Madison, E.L. Origins of the Specificity of Tissue-type Plasminogen Activator, *Proc.Natl.Acad.Sci.* 1995, 92, 7627-7631), and larger ligands need to be compared spanning both sites, as are available rapidly from library screening. Recently (Lawson 1992) has shown that screening of peptide sequences, containing significantly P' as well as P binding units, allows detection of FVIIa/TF activity where existing substrates were too insensitive. Peptide libraries are showing great efficacy for screening for biological activity in a variety of applications (e.g Eichler, 1994) and are useful for the present invention.

15

The invention contemplates that sequences C-terminal to the cleavage point of other serine proteinase substrates form an EAM, as follows:

20

Table B

Enzyme	Natural Substrate	Cleavage Site (—)
Factor Xa	Prothrombin	YIDGR—IVEGSDAEIGMSPWQ
Factor Xa	Prothrombin	AIEGR—TATSEYQTFFNPRTFGS
Factor Xa	Factor VII	SKPQGR—IVGGKVC
Factor VIIa	Factor X	NLTRR—IVGGQECKDGEC

This Sheet Has Been Replaced



Factor IXa	Factor X	NLTR--IVGGQECKDGEC
Factor XIa	Factor IX	SKLTR--AEAVFPDVDYVN
Factor XIa	Factor IX	FNDFTR--VVGGEDAKPGQF
Factor XIIa	Factor XI	KIKPR--IVGGTASVRGE
Factor XIIa	Plasma Kallikrein	KTSTR--IVGGTNSSWGE
Protein C α	Factor VIII	ELR--MKNNEEAEDYDDDLTDSEMD
t-PA/UK	Plasminogen	PKKCPGR--VVGGCVAHPHSWPWQVSLRT

The Connector Moiety

- 5 The compounds of the present invention may contain a connector moiety which interconnects the CSDM and the EAM, the connector moiety being capable of permitting the CSDM and the EAM to bind simultaneously to a molecule of the respective serine proteinase inhibitor. In the case of thrombin, the connector moiety is bonded to the CSDM as an N-terminal extension or as or through a side chain thereof.

10

The connector moiety may be bonded to the CSDM either as a C-terminal extension or, alternatively, as an N-terminal extension or as or through a side chain. However, if the compound is a thrombin inhibitor, the connector moiety may not be a C-terminal extension of the CSDM.

15

Especially if the connector moiety is an N-terminal extension of the CSDM, or if it is comprised in a side chain thereof, it desirably comprises an amino acid sequence containing at least two adjacent Gly residues, e.g. at its N-terminal end. In one class of compounds the connector preferably comprises a peptide "spacer" and a non-peptide

- 20 "linker". A representative connector structure is:



This Sheet Has Been Replaced



- 25 wherein λ represents a non-peptide linker and σ a spacer comprising a sequence of amino acids, λ and σ suitably being joined by a peptide bond. The spacer σ is

preferably linked to the EAM and the linker λ to the CSDM, although compounds in which σ is linked to the CSDM and λ to the EAM form a less preferred embodiment included in the invention.

- 5 The linker is typically a residue of a compound having functional groups to react with the N-terminal amino group of the spacer and a functional group of the CSDM, such as the N-terminal group, for example. A preferred linker, therefore, has two carboxylate groups, e.g. is a dicarboxylic acid which can form amide bonds with the N-terminal amino groups of the CSDM and the spacer. Particularly preferred linkers are a residue
10 of glutaric acid ($\text{HO}_2\text{C}(\text{CH}_2)_3\text{CO}_2\text{H}$) and homologues thereof of the formula $(\text{HO}_2\text{C}(\text{CH}_2)_h\text{CO}_2\text{H})$ wherein h is an integer of 2 or from 4 to 6. The alkylene residue $[-(\text{CH}_2)_{2-6}-]$ may be substituted by one or more substituents which do not sterically hinder the linker, whereby the desirable flexibility of the linker is maintained.
- 15 Less preferably, the linker may comprise for example the residue of another compound having two carboxyl groups whose carbon atoms are separated by from 2 to 6 atoms.

The amino acid sequence of the spacer is not critical to the invention but it preferably comprises at least two adjacent Gly residues, normally at its N-terminal end. The length
20 of the spacer is dependent upon *inter alia* the position on the CSDM to which the linker is attached.

A further description of connector moieties suitable for peptide thrombin inhibitors can be found in the aforesaid UK patent application entitled "Thrombin Inhibitors" filed on
25 the same day as the present application.

The connector moiety may have one or more natural amide bonds replaced by other linkages.

30

SYNTHESIS

This Sheet Has Been Replaced



The compounds of the invention can be prepared by using, for example, generally known methods for peptide synthesis and for coupling peptides. In an exemplary method, the novel compounds are made by a solid phase synthetic technique.

5 Solid phase synthesis is a technique familiar to peptide chemists and detailed elucidation is therefore not required here. An introduction to the technique may be found in "The Chemical Synthesis of Peptides", John Jones, Clarendon Press, Oxford, England, 1991. The principle of conventional solid phase synthesis is that an amino acid or peptide coupled to a solid phase is reacted with an amino acid which is protected against
10 reaction with itself and, after coupling with the solid phase-linked amino acid, is deprotected for reaction with a further amino acid protected against reaction with itself. These steps are repeated as often as necessary.

One solid phase synthesis technique is the Fmoc technique (Fmoc =
15 fluorenylmethylcarbonyl). In Fmoc chemistry (also known generally as the 'Sheppard approach'), the carboxy terminus of a peptide (or an amino acid) is coupled to a resin bead via a linker which is terminated by a reactive function. The resin bead itself is typically polystyrene (PS), though other solids have been used that have suitable swelling characteristics in solvent, since it is now known that the peptide chain grows in
20 the pores on the inside of the bead. An example of an alternative solid is the polyamide called Kiesulguhr.

The linker can be many things, but we prefer to use PEG (i.e. a polyethylene glycol linker), which has an alcohol function.
25

The terminus of the linker, typically called a 'handle', depends on the desired product, but for Fmoc chemistry will be a moiety such that it can finally be cleaved by acid. The most common terminus (which we have used) is HMBA or para-hydroxymethylbenzoic acid linker. The HMBA is esterified onto the PEG, and then the peptide or amino acid
30 (with Fmoc on its N-terminus) is reacted to give also an ester link to the HMBA. The ester links are then cleavable by acid. The Fmoc protecting group is base labile and typically removed by a secondary base (e.g. piperidine) and the resulting free amino



group is reacted with a selected Fmoc-protected amino acid; the amino acid sequence is extended by repetition of these steps.

Another solid phase synthetic approach is the Boc technique (Boc =
5 tertiarybutyloxycarbonyl). The resin used in Boc chemistry (also known more generally
as the Merrifield method) is often divinylbenzyl based, for instance a 'Wang' resin has
chloromethyl benzene co-polymerised to 2% divinylbenzene. The chloromethyl
benzene group is reacted with an amino acid or peptide whose amino group is protected
by Boc, to give a link to the resin. The link to the resin is typically cleaved (very
10 carefully!) by dry, liquid HF. This is described as 'vigorous' acidolysis. The Boc
protecting group is acid labile and typically cleaved by TFA, prior to reaction of the
resultant free amino group with a selected Boc-protected amino acid; as with Fmoc
chemistry, the amino acid sequence is extended by repetition of these steps.

15 The two classical methods of solid phase peptide synthesis (Sheppard and Merrifield),
therefore, involve coupling amino acids via their carboxy-termini or their derivatives
to a solid resin particle, then sequentially coupling new amino acids (via their
activated carboxy termini) to via the N-termini generated.

20 Alternatively recent reports have shown coupling to the resin via the N-termini, for
example via an acid labile benzyloxycarbonyl linkage, subsequent liberating of the
carboxy termini, activating of these and coupling of amino acids via their N-termini,
the carboxy termini of the amino acids temporarily being protected. (Sharma, R.P.;
Jones, D.A.; Broadbridge, R.J.; Corina, D.L. and Akhtar, M. A Novel Method of
25 Solid Phase Synthesis Of Peptide Analogues, in Innovation and Perspectives in Solid
Phase Synthesis, ed., R.Epton, 1994, Mayflower Worldwide Limited, Birmingham,
page 353-356; Letsinger, R.L. and Kornet, M.J. J.Amer.Chem.Soc., 1963, 85, 3045.)

Such N-terminal coupling methods may be used in making the products of the
30 invention. In one embodiment the CSDM, including any directly attached amino
acid(s), is synthesised by N-terminal coupling. This technique is especially useful if
the CSDM has C-terminal heteroatom group; in this method the resin bound peptide



chain made using N-terminal coupling is derivatized to activate its carboxy termini, then a free α -aminoboronate ester or acid is coupled to the resin bound sequence. Finally the peptide boronate (comprising the CSDM) is cleaved from the resin by strong acid (e.g. HF or TFA) prior to being joined to the remainder of the final product.

When synthesising compounds whose CSDM contains a P1-P2 non-natural amide bond, it is convenient to premake as intermediates the binding subsite affinity moiety [X-(aa⁴)_m-(aa³)_n-(aa²) of Formula I] and the specificity pocket affinity moiety with its attached C-terminal group [(aa¹)-Z of Formula I]. The two intermediates contain suitable functional groups to react together to form the target non-natural amide bond [ψ] and are caused or allowed to react together to form the compound (or a precursor thereof to undergo one or more further functional group transformations).

Suitable synthetic techniques for making peptides containing a non-amide bond ψ are described in PCT/GB96/00352.

We have unexpectedly successfully synthesised peptide boronate esters using solid phase chemistry. For example, in an exemplary process for synthesising a serine proteinase inhibitor in which the connector moiety and its attached EAM form an N-terminal extension of the CSDM, the EAM is prepared by Fmoc solid phase peptide chemistry, e.g. using an Fmoc-polyamide continuous flow method. A suitable solid phase for this purpose is the pre-derivatised solid support Fmoc-Leu-PEG-PS. The peptide-conjugated resin is subsequently treated with, for example, glutaric anhydride, one carboxyl group of which reacts with the N-terminal amino group of the EAM. A pre-synthesised peptide boronate CSDM is reacted with the resin/peptide/glutaric acid conjugate to form the final compound, which is cleaved from the resin, for example by treatment with 100% TFA.

Another method of using peptide boronate esters in solid phase chemistry is a completely novel technique in which boronic acids [-B(OH)₂] are directly esterified onto diols coupled to a resin. Chain extension is continued from the amino group of the



amino acid by, for example, standard Fmoc chemistry. The boronic acid ester is cleaved from the resin by acid (e.g. TFA) to give the peptide boronate [peptide-B(OH)₂], or by transesterification, for example, by concentrated solution of a hindered diol, such as pinanediol, for example.

5

The invention therefore includes a method of making a compound of the invention, comprising performing the following steps to make a target amino acid sequence:

- 10 (i) providing a solid phase having coupled thereto functional groups capable of reacting with an amino group or, preferably, with a carboxyl group or a reactive derivative thereof;
- (ii) causing the amino or carboxyl group (which may be in the form of a reactive derivative thereof) of a terminal amino acid of an amino acid sequence
15 of a compound of the invention selectively to react with said functional groups;
- (iii) coupling the amino acid sequentially following, in the target sequence, the sequentially preceding amino acid coupled to the solid phase to said preceding amino acid; and
20
- (iv) repeating step (iii) as often as necessary.

In step (i), the functional groups coupled to a solid phase may be on a moiety which is incorporated in the end product compound, e.g. may be an amino group (which may be
25 derivatised) of an amino acid coupled directly or indirectly to the solid phase.

One or more additional steps may be, and often are, included in the method to obtain the compound of the invention. Thus, preferred methods include, when desired, a step (v) of coupling a said sequentially following amino acid of a step (iii) to said preceding
30 amino acid of the step through a compound having two functional groups capable of reacting with an amino group, whereby one of said functional groups becomes bonded



to the amino group of said preceding amino acid and the other to the amino group of said following amino acid.

5 The sequentially following amino acid of a step (iii) may be part of a larger moiety, e.g. of an amino acid sequence optionally containing a replacement for a natural peptide bond.

10 In the method, any one or more carboxylate groups reacted with an amino group may be in the form of a reactive carbonyl-containing derivative thereof, such as an activated carboxyl group, for example an acid anhydride.

15 Before use, the final compound of the solid phase synthesis is cleaved from the solid phase, for example in a manner known *per se*. The cleaved compound may be subjected to one or more further chemical reactions before the end product compound is obtained.

20 In a preferred embodiment, the terminal amino acid reacted with the functional groups attached to the solid phase is the C-terminal amino acid of the EAM and step (iii) is repeated to couple successive amino acids of the EAM sequence and successive amino acids of any contiguous connector peptide, to form an uninterrupted amino acid sequence.

25 The final amino acid of the uninterrupted amino acid sequence coupled to the solid phase may be reacted with a compound having two carboxylate groups or reactive derivatives thereof, for example the anhydride of a dicarboxylic acid, to bond one of the two carboxylates to the amino group of the final amino acid. The unreacted carboxylate or carboxylate derivative is typically reacted with the amino group of an amino acid, which is normally the N-terminal amino acid of the CSDM. In this latter case, the amino acid may already be bonded to the remainder of the CSDM, i.e. the CSDM may be separately made in whole (or in part) for joining to the unreacted carboxylate (derivative). The compound having two carboxylate groups is preferably a linker as
30 described above.



In some preferred methods, there is used a preformed CSDM having a heteroatom group in place of a C-terminal carboxy group. The heteroatom group is preferably a boronate or boronate derivative as described above.

5 An amino acid or other moiety reacted with the solid phase material (the solid phase and any attached molecules) desirably has all its reactive functional groups which could interfere with the synthesis protected, other of course than the group to be reacted with the solid phase material. Any protected functional group of the reacted amino acid or moiety which is subsequently itself to be reacted is deprotected before it is subjected to
10 reaction.

A first preferred method, therefore, comprises:

(i) providing a solid phase having coupled thereto functional groups capable
15 of reacting with a carboxyl group or a reactive derivative thereof;

(ii) contacting the solid phase with the C-terminal amino acid of an EAM, the amino acid having a protected amino group and optionally a derivatised carboxy group, and causing or allowing the carboxy groups of the amino acid
20 molecules to react with the functional groups of the solid phase;

(iii) deprotecting the amino groups of the reacted amino acid, whereby the solid phase becomes provided with free amino groups;

25 (iv) repeating steps (ii) and (iii) with successive amino acids of the EAM and optionally of a contiguous spacer peptide to form on the solid phase an amino acid sequence from the C-terminal of the EAM to, at the free end of the sequence, the N-terminal of the spacer;

30 (v) contacting the solid phase with a linker compound having two carboxyl groups or reactive residues thereof, and causing or allowing linker carboxy

This Sheet Has Been Replaced



groups or reactive carboxy residues to react with the N-terminal amino groups of the spacer sequence:

5 (vi) contacting the solid phase having the linker compound coupled thereto with the N-terminal amino acid of a CSDM sequence and causing or allowing the amino groups of the amino acid molecules to react with the carboxy groups or reactive carboxy residues of the linker compound, the N-terminal amino acid of the CSDM sequence optionally being part of a complete CSDM:

10 (vii) if necessary, repeating steps (ii) and (iii) with successive amino acids of the CSDM to complete the CSDM sequence: and

(viii) cleaving the resultant compound from the functional groups of the solid phase.

15

A second preferred method comprises:

(i) providing a solid phase having coupled thereto functional groups capable of reacting with a carboxyl group or a reactive derivative thereof;

20

(ii) contacting the solid phase with the C-terminal amino acid of an EAM, the amino acid having a protected amino group and optionally a derivatised carboxy group, and causing or allowing the carboxy groups of the amino acid molecules to react with the functional groups of the solid phase:

25

(iii) deprotecting the amino group of the reacted amino acid, whereby the solid phase becomes provided with free amino groups;

30

(iv) repeating steps (ii) and (iii) with successive amino acids of the EAM to form on the solid phase an amino acid sequence from the C-terminal of the EAM to, at the free end of the sequence, the N-terminal of the EAM;

This Sheet Has Been Replaced



- (v) contacting the solid phase with a linker compound having two carboxyl groups or reactive residues thereof, and causing or allowing linker carboxy residues to react with the N-terminal amino groups of the EAM:
- 5 (vi) optionally contacting the solid phase having the linker compound coupled thereto with the N-terminal amino acid of a peptide spacer sequence and causing or allowing the amino groups of the amino acid molecules to react with the carboxy groups or reactive carboxy residues of the linker compound:
- 10 (vii) optionally repeating steps (ii) and (iii) with successive amino acids of the spacer and then repeating step (ii) with the N-terminal amino acid of a CSDM sequence, the N-terminal amino acid of the CSDM sequence optionally being part of a complete CSDM:
- 15 (viii) if necessary, repeating steps (ii) and (iii) with successive amino acids of the CSDM to complete the CSDM sequence; and
- (ix) cleaving the resultant compound from the functional groups of the solid phase.

20

In either of the preceding methods the synthesised compound is preferably cleaved from the solid phase by acid.

25 The preceding methods preferably involve the use of a CSDM amino acid or amino acid sequence (e.g. a complete CSDM) having a C-terminal boron group.

In the first and second preferred methods the functional groups coupled to a solid phase may be part of a moiety which is incorporated in the end product compound, e.g. may be an amino group (which may be derivatised) of an amino acid coupled directly or
30 indirectly to the solid phase.

This Sheet Has Been Replaced



In one class of processes of the invention, the functional groups coupled to the solid phase are part of an amino acid boronate which is incorporated in the end product compound, *i.e.* the solid phase has coupled thereto a diol to which is bound an amino acid boronate.

5

In another class of methods, an amino acid whose side chain has an amino or carboxyl group is coupled to a solid phase through the carboxyl group or amino group to the side chain. Chain extension is carried out from one of the functional groups of the amino acid, for example Fmoc synthesis from the amino group. The other functional group is then reacted with some other constituent part of the end product, for example an amino acid boronate (to form the PI residue of the CSDM). Solid phase synthesis of boron-containing peptides is, however, of applicability to any such peptides, and not only to the serine protease inhibitors of the invention.

15

Solid Phase Synthesis of Boron-Containing Compounds

Peptide boronates are a well established class of compounds which have hitherto been made by solution chemistry. Thus, peptide serine protease inhibitors are known in which the C-terminal carboxy group is replaced by a boronic acid group or a derivative thereof. Representative compounds are of the Formula II:

20



This Sheet Has Been Replaced



25 wherein:

$(aa)_k$ represents a sequence of amino acids (*e.g.* as in Formula I); R^2 and R^3 are each independently selected from halogen, $-OH$, $-OR^4$ and $-NR^4R^5$, where R^4 and R^5 are each independently a group of the formula $R^6(CO)_u-$, wherein u is 0 or 1; R^6 is H or an optionally halogenated alkyl, aryl or arylalkyl group containing up to $(10 - u)$ carbon atoms and optionally substituted by one or more groups selected from $-OH$, $R^7(CO)_vO-$

30

and $R^7(CO)_v$, wherein v is 0 or 1; R^7 is C_1 - C_{6-v} alkyl, or is an aryl, alkylaryl, arylalkyl or alkylarylalkyl group containing up to $(10-v)$ carbon atoms,

or R^2 and R^3 taken together represent a residue of a diol or a dithiol.

5

Such peptide boronates are described, for example, in WO 92/07869 (equivalent to USSN 08/317,387), EP 0471651 (which corresponds to US 5288707) and USSN 08/240,606, the disclosures of which are incorporated herein by reference.

10 As already indicated, we have now surprisingly found that boron-containing peptides may be synthesised by solid phase chemistry without serious degradation. One aspect of the invention, therefore, is the use of a boron-containing amino acid analogue in peptide synthesis using solid phase chemistry, especially Fmoc chemistry (also known as the "Sheppard approach").

15

In another aspect, there is provided a method of making a peptide or a peptide-containing compound, comprising performing the following steps to make a target amino acid sequence:

- 20 (i) providing a solid phase having coupled thereto functional groups;
- (ii) causing a compound reactive with the functional groups selectively to react therewith, the reacted compound having a functional group capable of reacting with an amino group or with a carboxyl group or a reactive derivative thereof;
- 25 (iii) causing an amino or carboxyl group (which may be in the form of a reactive derivative thereof) of a terminal amino acid of a target amino acid sequence selectively to react with said functional groups of the reacted compound;
- 30

This Sheet Has Been Replaced



- (iv) coupling the amino acid sequentially following, in the target sequence, the sequentially preceding amino acid coupled to the solid phase to said preceding amino acid;
- 5 (v) repeating step (iv) as often as necessary; and
- (vi) cleaving a solid phase-linked compound prepared using steps (i)-(iv) from the solid phase by the action of an acid or base.
- 10 characterised in that a compound comprising a boronic acid group $[-B(OH)_2]$ or a derivative thereof, especially an ester, is incorporated in the solid phase-linked compound prior to its cleavage from the solid phase.
- 15 The method of the invention for making a peptide or peptide-containing compound may comprise a step of coupling a peptide to a solid phase-linked compound prepared by previous steps of the process, optionally as a step (iv) of the process [i.e. a step (iv) of the process in which the sequentially following amino acid is part of a peptide].
- 20 The method of the invention for making a peptide or peptide-containing compound may comprise a step of coupling to a solid phase-linked compound prepared by previous steps of the process a compound other than an amino acid or peptide, such as, for example, a compound having two carboxyl groups for use in linking an amino group of a solid phase-linked peptide with an amino group of a peptide, peptide analogue or amino acid in the liquid phase. Of course, any other liquid phase
- 25 compound capable of reacting with an amino group or, as the case may be, a carboxyl group could thus be linked to the solid phase-linked peptide. Diamines are useful for interconnecting moieties having carboxyl groups (e.g. in the form of reactive derivatives thereof). Extension of a solid phase-linked peptide by a dicarboxylic acid, especially glutaric acid, is useful in the preparation of bifunctional serine protease
- 30 inhibitors having a CSDM joined, typically through its N-terminal, to an EAM through a connector moiety comprising a peptide spacer portion and dicarboxylic acid residue linker portion.



A solid phase-linked compound whose free end terminates with a dicarboxylic acid residue may be further extended by reaction of the free carboxyl residue (optionally in the form of a derivative thereof) with the amino group of an amino acid, *e.g.* a dicarboxylic acid residue coupled to a solid phase-linked EAM-spacer moiety may be reacted with an amino acid of a CSDM, for example the N-terminal amino acid of a CSDM.

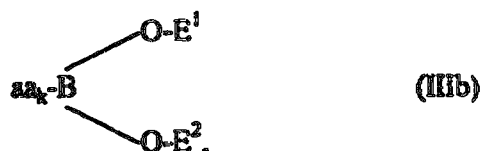
Step (ii) of the method may comprise reacting an amino group or an optionally derivatised carboxy group of an amino acid with a functional group coupled directly or indirectly to a solid phase, as part of conventional solid phase peptide synthesis, for example. The amino or carboxy group coupled to the solid phase is often the terminal amino or carboxy group but in some embodiments is a functional group of a side chain, such as the side chain carboxyl group of glutaric acid, for example; the C-terminal carboxy group of the amino acid attached to the solid phase through its side chain may be replaced by the boronic acid residue $[-B(OH)_2]$ or an ester thereof.

The method may comprise N-terminal coupling in SPPS, in which the carboxy terminus of a resin bound peptide is coupled to a free α -aminoboronate acid or ester, prior to acid cleavage of the resultant product from the resin.

In another embodiment, step (ii) comprises reacting an amino acid or peptide boronic acid or ester



or



where E^1 and E^2 represent boronic ester-forming residues or may together form a single residue,

This Sheet Has Been Replaced



with a diol coupled to the solid phase. The technique of linking a boron atom (*e.g.* as part of an amino acid or peptide boronic acid or ester) to a solid phase through hydroxy groups coupled (directly or indirectly) to the solid phase is novel and forms an aspect of the invention.

5

If the compound comprising a boronic acid or ester is an amino acid boronate used in step (ii) in either of the preceding embodiments, the solid phase synthetic method may be used to make a peptide boronate inhibitor of a serine protease catalytic site, optionally in the synthesis of a bifunctional serine protease inhibitor.

10

Thus, boronic acids can be directly esterified onto diol-containing resins, and then chain extension continued from the N-terminal end by, for example, standard Fmoc-chemistry. Subsequently the boronic acid ester can be cleaved, either by mineral acids, to give the free boronic acids [peptide-B(OH)₂], or by transesterification, *e.g.* by a concentrated solution of a diol, especially a hindered diol such as pinanediol, for example.

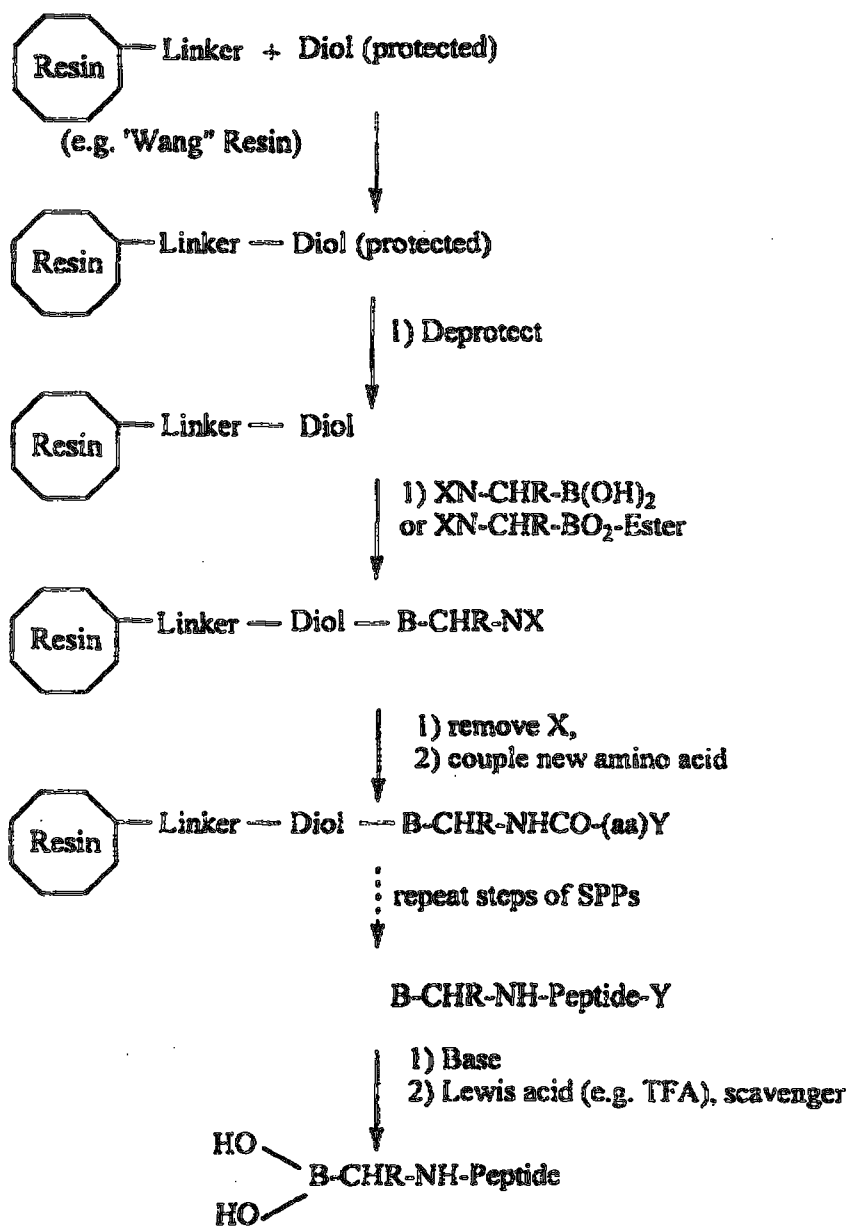
The literature describes ways of preparing diol-containing solid phase resins, which can be derivatised by aldehydes and are suitable also to be derivatised by boronic acids/esters (*e.g.* Xu,Z.-H., McArthur,C.R and Leznoff,C.C. 'The monoblocking of symmetrical Diketones on Insoluble Polymer Supports', *Can.J.Chem.*, 1993, 61,1405-1409. and Leznoff,C.C. and Sywanyk,W. 'Use of Polymer Supports in Organic Synthesis.9. Synthesis of Unsymmetrical Carotenoids on Solid Phase', *J.Org.Chem.*, 1997, 42, 3203-3205).

25

A general procedure is as follows:

This Sheet Has Been Replaced





The diol is a compounds having two or more alcoholic hydroxy groups.

X and Y are protecting groups.

R is the side chain of an amino acid boronate/boronic acid.

- 5 Typically, the resin is washed after each step. In suitable embodiments the diol is not protected before it is reacted with the resin.

A more specific procedure is set out below:

This Sheet Has Been Replaced



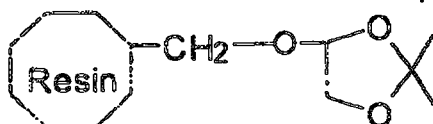
23



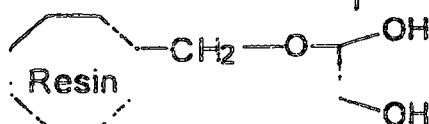
(e.g. 'Wang' Resin)

(Sodium salt of Solketal)

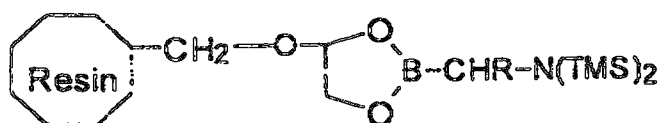
- 1) 24h, 2 fold excess sodium salt
- 2) wash



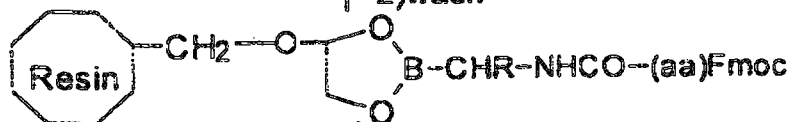
- 1) Mineral acid
- 2) wash



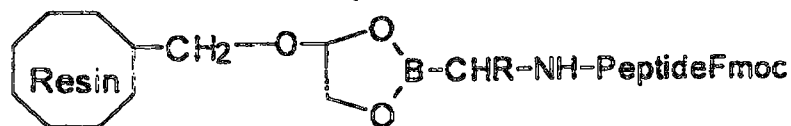
- 1) $(\text{TMS})_2\text{-N-CHR-B(OH)}_2$
or $(\text{TMS})_2\text{-N-CHR-BO}_2\text{-Ester}$
- 2) wash off excess



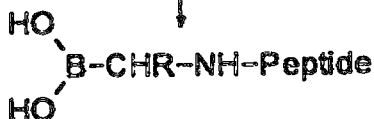
- 1) Fmoc(aa)-OH, I-BuCOCl , Morpholine, Et₃N.
or Fmoc(aa)-OH, activating agent, $(\text{Bu})_4\text{NF}$.
- 2) wash



repeat steps of SPPS.



- 1) Base
- 2) Lewis acid (e.g. TFA), scavenger.



TMS = Trimethylsilyl

SPPS = solid phase peptide synthesis

This Sheet Has Been Replaced



The invention therefore opens the way to the preparation of peptide boronates by solid phase chemistry, for example in preparing a library of peptide boronates by a combinatorial method.

5 The invention includes a method for making a compound comprising a peptide boronic acid or peptide boronate ester, the method comprising:

(i) providing a solid phase having coupled thereto alcoholic hydroxy groups:

10

(ii) causing an amino acid boronic acid or peptide boronic acid to react with the hydroxy groups whereby the boronic acid residue becomes esterified to the solid phase:

15

(iii) causing the carboxyl group of the amino acid sequentially following, in the end product peptide boronic acid or boronate ester, selectively to react with the amino group of the sequentially preceding amino acid coupled to the solid phase:

20

(iv) repeating step (iii) as often as necessary:

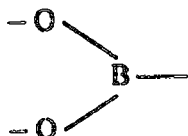
(v) cleaving the resultant peptide boronate from the resin.

the method optionally comprising one or more further steps to make said compound.

25

The alcoholic hydroxy groups coupled to the solid phase are preferably arranged such that pairs of the groups can be bonded to a boron atom, i.e. such that a boron atom can be di-esterified by them:

30



This Sheet Has Been Replaced



SUBSTITUTE SHEET (RULE 26)

In some embodiments the hydroxy groups are in a 1,2-arrangement (i.e. on adjacent carbons); in other embodiments they are spaced apart on chains (e.g. a residue of $\text{NH}(\text{CH}_2\text{CH}_2\text{OH})_2$).

5 Preferably each amino acid coupled to the solid phase has a protected amino group and step (iii) comprises deprotecting the amino group of the sequentially preceding amino acid. Preferably the cleavage of step (v) is performed with acid or by transesterification.

10 More generally, there is provided the use in solid phase synthesis of a boronic acid residue attached to the solid phase through hydroxy group residues.

Also provided is a method for making a compound comprising a boron atom, the method comprising:

15

(i) providing a solid phase having coupled thereto alcoholic hydroxy groups;

20 (ii) causing a boronic acid or boronate ester to react with the hydroxy groups whereby the boronic acid residue becomes esterified to the solid phase; and

(iii) performing one or more further steps to make said compound.

25 The alcoholic hydroxy groups are preferably arranged as described above.

In other aspects, there are provided a solid phase material having coupled thereto boronic acid residues through hydroxy groups, as well as a solid phase material having coupled thereto a moiety of the Formula IV:

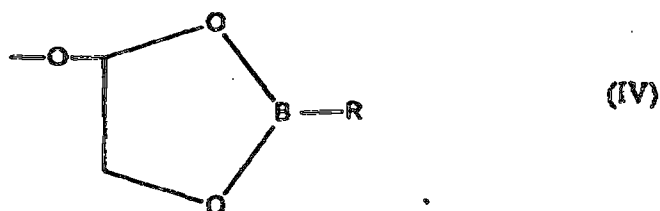
30

This Sheet Has Been Replaced

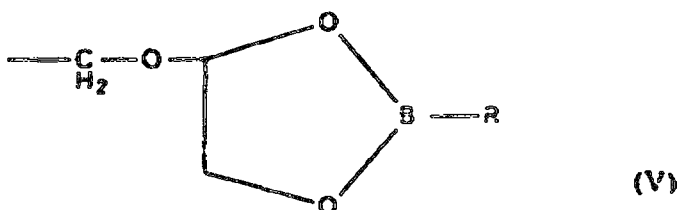


SUBSTITUTE SHEET (RULE 26)

26



wherein R is a residue bonded to the boron atom, and is usually an organic moiety. Residue R is in one class of material free of functional groups reactive with alcoholic hydroxy groups (but the material may contain such functional groups in protected form, e.g. prior to deprotection). In another class of material, such functional groups are unprotected, protecting groups having previously been removed. R is typically an organic moiety having one or more functional groups to enable R to undergo a chemical reaction; any protectable functional groups may be protected. In one class of materials, the solid phase has coupled thereto a moiety of Formula V:



One or both of the hydrogen atoms of the $-\text{CH}_2-$ group may be replaced by other groups compatible with the use of the material, e.g. alkyl groups (for example methyl or butyl).

In a yet further class of materials, the left hand oxygen of Formula IV is part of an ester.

A first class of solid phase synthetic methods comprises performing the following steps to make a target amino acid sequence:

- (i) providing a solid phase having coupled thereto functional groups capable of reacting with a carboxyl group;



- (ii) causing a compound reactive with the functional groups and comprising an amino group protected by a base-labile protecting group to react with the functional groups to form an acid labile bond;
- (iii) deprotecting the amino group with a base;
- 5 (iv) causing the carboxyl group of an amino acid whose amino group is protected by a base-labile protecting group to react with the deprotected amino group resulting from step (iii);
- (v) deprotecting the protected amino acid with a base;
- (vi) causing the amino acid sequentially following, in the target sequence, the sequentially preceding amino acid coupled to the solid phase to react with the deprotected amino group of the sequentially preceding amino acid, the sequentially following amino acid having its amino group protected by a base-labile protecting group;
- 10 (vii) deprotecting the protected amino acid group with base;
- (viii) repeating steps (vi) and (viii) as often as necessary; and
- 15 (ix) cleaving the acid labile bond with acid or by transesterification.

characterised in that a compound comprising a boronic acid group $[-B(OH)_2]$ or a derivative thereof, especially an ester, is incorporated in the solid phase-linked compound prior to cleavage of the acid labile bond.

20

As described above in relation to the method of making a peptide or peptide-containing compound, step (ii) of the method may comprise reacting an optionally derivatised carboxy group of an amino acid with a functional group coupled directly or indirectly to a solid phase, for example by a method known *per se* in solid phase chemistry. The amino acid may be the compound comprising a boronic acid or ester group, i.e. an amino acid boronic acid or ester. Alternatively, step (ii) may comprise reacting the compound comprising a boronic acid or ester group in the form of an amino acid or peptide boronic acid or ester with a diol coupled to the solid phase. In either case that an amino acid (or peptide) boronic acid or ester is used, the process is suitable for making a peptide boronate inhibitor of a serine protease catalytic site, optionally in the synthesis of a bifunctional serine protease inhibitor.

25

30



The other variants described above of the method of making a peptide or peptide-containing compound are applicable to said first class of solid phase synthetic methods.

5

A second class of solid phase synthetic methods comprises performing the following steps to make a target amino acid sequence:

10

(i) providing a solid phase having coupled thereto functional groups capable of reacting with a carboxyl group;

(ii) causing a compound reactive with the functional groups and comprising an amino group protected by an acid-labile protecting group to react with the functional groups, to form a base labile bond;

(iii) deprotecting the amino group with an acid;

15

(iv) causing the carboxyl group of an amino acid whose amino group is protected by an acid-labile protecting group to react with the deprotected amino group resulting from step (iii);

(v) deprotecting the protected amino acid with an acid;

20

(vi) causing the amino acid sequentially following, in the target sequence, the sequentially preceding amino acid coupled to the solid phase to react with the deprotected amino group of the sequentially preceding amino acid, the sequentially following amino acid having its amino group protected by an acid-labile protecting group;

(vii) deprotecting the protected amino acid group with acid;

25

(viii) repeating steps (vi) and (viii) as often as necessary; and

(ix) cleaving the base labile bond with base or by transesterification,

30

characterised in that a compound comprising a boronic acid group $[-B(OH)_2]$ or a derivative thereof, especially ester, is incorporated in the solid phase-linked compound prior to cleavage of the acid labile bond.



The above-described variants of the first class of solid phase synthetic methods are applicable also to the second class.

As described above in relation to the method of making a peptide or peptide-containing compound, methods of the first and second classes of solid phase synthetic methods may comprise a step of coupling to a solid phase-linked compound prepared by previous steps of the process a compound other than an amino acid or peptide.

10 Synthesis Generally

The compounds of the invention do not have to contain boron, although they may do so. The non-boron containing compounds may also be prepared by solid phase synthesis. Some compounds of the invention have a natural peptide bond replaced by another linking group. Further information on methods suitable for the synthesis of all the compounds of the invention may be found in the aforesaid patent application filed today and entitled "Thrombin Inhibitors".

20 USE

The novel compounds according to the present invention are useful as inhibitors or substrates of serine proteases, e.g. thrombin, and may be used *in vitro* or *in vivo* for diagnostic and mechanistic studies of such enzymes. More generally, the novel peptides may be useful for research or synthetic purposes. Furthermore, because of their inhibitory action, the inhibitors are useful in the prevention or treatment of diseases caused by an excess of thrombin or another serine proteases in a regulatory system particularly a mammalian system, e.g. the human or animal body, for example control of the coagulation system. The pharmaceutically useful compounds have a pharmaceutically acceptable group as any N-terminal substituent (X).



The anti-thrombotic compounds of the invention may be employed when an anti-thrombogenic agent is needed. Generally, these compounds may be administered orally or parenterally to a host in an effective amount to obtain an anti-thrombogenic effect. In the case of larger mammals such as humans, the compounds may be administered alone
5 or in combination with one or more pharmaceutical carriers or diluents at a dose of from 0.02 to 10mg/Kg of body weight and preferably 1-100mg/Kg, to obtain the anti-thrombogenic effect, and may be given as a single dose or in divided doses or as a sustained release formulation. When an extracorporeal blood loop is to be established for a patient, 0.1-10mg/Kg may be administered intravenously. For use with whole
10 blood, from 1-100 mg per litre may be provided to prevent coagulation.

Pharmaceutical diluents or carriers for human or veterinary use are well known and include sugars, starches and water, and may be used to make acceptable formulations of pharmaceutical compositions (human or veterinary) containing one or more of the
15 subject peptides in the required pharmaceutically appropriate or effective amount or concentration. The pharmaceutical formulations may be in unit dosage form. Formulations of the compounds include tablets, capsules, injectable solutions and the like.

20 The anti-coagulant compounds of the invention may also be added to blood for the purpose of preventing coagulation of the blood in blood collecting or distribution containers, tubing or implantable apparatus which comes in contact with blood.

Advantages enabled by the compounds of the invention include oral activity, rapid onset
25 of activity and low toxicity. In addition, these compounds may have special utility in the treatment of individuals who are hypersensitive to compounds such as heparin or other known inhibitors of thrombin or other serine proteases.

The methods of the invention are useful for the synthesis of serine protease inhibitors
30 and other compounds. They are useful in combinatorial chemistry.



The invention will be further described and illustrated by the Examples which now follow.

5 EXAMPLES

In the examples, amino acid residues are of L-configuration unless otherwise stated.



10



GlyGlyGln(Tyr⁶³)Hir⁵¹⁻⁶⁴ which has the amino acid formula H-Gly-Gly-Gln-His-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Tyr-Leu-OH, was prepared by solid phase peptide chemistry on a Milligen[®] 9050 PepSynthesizer using an Fmoc-polyamide continuous flow method and proprietary 9050 Plus on column monitoring software. Pre-derivatised solid support, Fmoc-Leu-PEG-PS (1.6g, 0.22meq/g) was used throughout; Fmoc-Leu-PEG-PS comprises polyethylene glycol derivatised polystyrene with HMBA linker. Fmoc groups were removed using 20% piperidine in DMF. Fmoc-amino acids (4 equiv.) as their pentafluorophenyl esters with side chain protection where appropriate (e.g. Fmoc-L-Tyr(tBu)OPfp, Fmoc-L-Glu(tBu)OPfp, Fmoc-L-Asp(tBu)OPfp, Fmoc-L-Asn(Trt)OPfp and Fmoc-His(boc)OPfp, were coupled sequentially. Once the required peptide sequence was complete the N-terminal Fmoc group was removed using 20% piperidine in DMF. A positive ninhydrin test indicated that the Fmoc group had been removed. The peptide-conjugated resin was subsequently decanted on a filter and washed 'off line' with dichloromethane, methanol and dichloromethane before being dried *in-vacuo* for a few hours.

25



30

The peptide obtained in Example 1a was suspended in DMF (5ml) and treated with glutaric anhydride (300mg) and 4-methyl-morpholine (200mg) in a round bottomed

This Sheet Has Been Replaced



flask (25ml). The reaction mixture was swirled overnight. The resin was washed with DMF, DCM and MeOH, and then dried *in-vacuo* overnight to obtain the target compound.

5 c. H-D-Phe-ProBoroBpgOPin

H-D-Phe-ProBoroBpgOPin was prepared by adding a 40% solution of HBr in acetic acid (20ml) to Cbz-D-Phe-Pro-BoroBpgOPin (2g) in a round bottomed flask (100ml) fitted with a septum and flushed with nitrogen. The flask was swirled to effect complete
10 dissolution of the protected tripeptide. When the gas evolution ceased after approximately 30 minutes, anhydrous ether (200ml) was added and the reaction mixture was stored in a refrigerator for 4 hours. The reaction mixture was filtered, the residue was dissolved in EtOH (1ml) and dry ether was added to precipitate the produce (800mg) as a white solid (M+H), 516; Tlc (C/M/A, 95/5/3), Rf=0.05.

15

d. [-D-PheProBoroBpgOPin]CO(CH₂)₃COGly₂Gln(Tyr⁶³)Hir⁵¹⁻⁶⁴

To synthesise [-D-PheProBoroBpgOPin]CO(CH₂)₃COGly₂Gln(Tyr⁶³)Hir⁵¹⁻⁶⁴, the dry resin HOCO(CH₂)₃COGly₂Gln(Tyr⁶³)Hir⁵¹⁻⁶⁴ was suspended in DMF (10ml), before
20 IBTU (129mg, 0.4mmol) and H-D-Phe-ProBoroBpgOPin (230mg, 0.4mmol) were added to the reaction mixture. After 5 minutes stirring, triethylamine (40mg, 0.04mmol) was added and the flask left stirring overnight.

The fully protected peptide resin was washed with dichloromethane, methanol and
25 dichloromethane and then dried under vacuum. Cleavage from the resin with simultaneous deprotection of side chain protecting groups was achieved by treating the resin with 100% TFA for two hours. TFA was removed and the free peptide with a C-terminal carboxylic acid was generated by precipitation with cold dry ether. The crude peptide was collected by filtration and washed with further portions of ether.

30

Purification of the crude peptide was carried out by reversed-phase HPLC using a Vydac[™] C-18 preparative column (TP silica, 10µm, 25mm x 300mm). The column was



eluted with a 30-90% linear gradient of solvent A (0.1% TFA in water) and solvent B (0.1 TFA in acetonitrile). The column eluants were monitored at 230nm, and fractions were collected appropriately. The purity of the products were determined by analytical RP-HPLC and mass spectrometry.

5

2. Cbz-D-Phe-Pro- ψ (CO₂)-borochethylglycine pinanediol

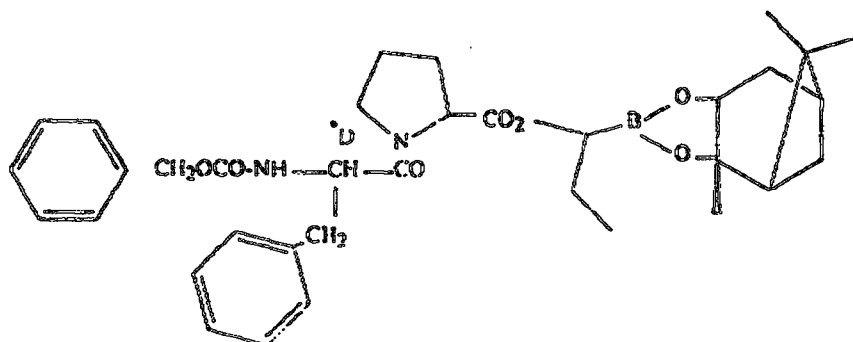
a. Cbz-D-Phe-Pro- ψ (CO₂)-BoroEtg pinanediol

10

1-Chloroethane-pinanediol boronate ester (0.321g, 1.25x10⁻³mol) added with stirring to Cbz-D-Phe-Pro-OH (0.6g, 1.52x10⁻³mol). When the addition had been completed, DBU (0.23g, 1.52mmol) in CH₂Cl₂ was added to the mixture and allowed to stir at room temperature, before being left to stir for an extended period at 4°C before workup. The opaque liquid was washed with HCl (0.1M, 2x50 ml), NaHCO₃ (1%, 50ml). The organic layer was dried by vigorous stirring over anhydrous MgSO₄, and filtered off, to remove the desiccant. The filtrate was concentrated under reduced pressure on a rotary evaporator, to afford a thick, viscous residue. Preliminary examination by ¹H N.M.R. showed the required crude product. The crude sample was dissolved in a small amount of MeOH, applied to the sephadex[®] LH20 column, and then eluted with a pump using the same solvents. The elution profile was followed with the aid of a U.V. lamp (226nm) and recorder. The void volume, fraction 1-6, and a further bulk volume were collected. From the shape of the chromatogram, it was deemed that fractions 1-6 would be the most likely fractions in which the tripeptide may be found. The fractions were concentrated individually to afford clear slightly coloured viscous residues. One fraction containing the bulk of the material when placed under high vacuum was later afforded as a slightly crystalline product (0.269 yield of 35%). N.M.R., FABMS (Fast Atom Bombardment Mass Spectrometry) and C, H, N were very strong (good) indicators that the compound has been formed.

30





b. H-Phe-Pro-ψ(CO₂)-BoroEtg pinanediol

5 Cbz-D-Phe-Pro-ψ(CO₂)-BoroEtg pinanediol (from Example 2a) was dissolved in MeOH (30ml) and treated with 10% Pd/C. and purged with argon with stirring. the flask evacuated and pumped with H₂ with stirring for 5H. Ninhydrin staining indicated deprotected product on TLC. The solution was purged with argon for 10 min. filtered and concentrated under reduced pressure to afford a thick black oil, which was dissolved
 10 in CHCl₃, filtered and concentrated. ¹H N.M.R. of the crude product indicated no protected product. The residue from above was chromatographed on a Sephadex LH20 chromatography column. ¹H (60 MHz) N.M.R. showed that the isolated compound displayed many of the characteristics expected on the basis of the putative structure. 122mg of the free amino boronate ester was isolated.

15

3. H-Phe-L-Glu-BoroBpgOPin

a. Fmoc-L-Glu(PEG-PS)OH

20

Tetrakis(triphenylphosphine) palladium(0) [PdP(Ph₃)₄] (1g) was dissolved under Ar in a solution of CH₂Cl₂ containing 5% acetic acid and 2.5% N-methylmorpholine (30ml). This mixture was transferred under Ar to a flask containing Fmoc-L-Glu(PEG-PS)OAl (1.6g). The resin was left to stand for 2 hours with occasional gentle
 25 agitation. The resin was filtered on a sintered glass funnel and washed with 0.5%



diisopropylethylamine and sodium diethyldithiocarbamate (0.5%w/w) in DMF (300ml) to remove the catalyst.

b. Fmoc-L-Glu(PEG-PS)NHBoroBpgOPin

5

The dry resin Fmoc-L-Glu(PEG-PS)OH (1.5g) was suspended in DMF(10ml) under Ar. TBTU (129mg, 0.4mmol) and NH₂BoroBpgOPin (165mg, 0.5mmol) were added to the reaction mixture. After 5 minutes stirring, triethylamine (40mg, 0.4mmol) was added and the flask left stirring overnight. The resin was washed with 10 dichloromethane, methanol and dichloromethane and then dried under vacuum.

c. H-Phe-L-Glu(PEG-PS)NHBoroBpgOPin

H-Phe-L-Glu(PEG-PS)NHBoroBpgOPin was prepared by solid phase chemistry on a 15 Milligen 9050 peptide synthesiser

Fmoc group was removed from the solid support Fmoc-L-Glu(PEG-PS)NHBoroBpgOPin using 20% piperidine in DMF. Fmoc-Phe-OPfp was coupled to the free N-terminus.

20 The protected peptide resin was washed with dichloromethane, methanol and dichloromethane and then dried under vacuum.

d. H-Phe-L-Glu-BoroBpgOPin

25 Cleavage of the peptide from the resin was achieved by treating the resin with 100% TFA for two hours. TFA was removed and the free peptide H-Phe-Glu-NH-BoroBpgOPin was generated by precipitation with cold dry ether. The crude peptide was collected by filtration and washed with further portions of ether.

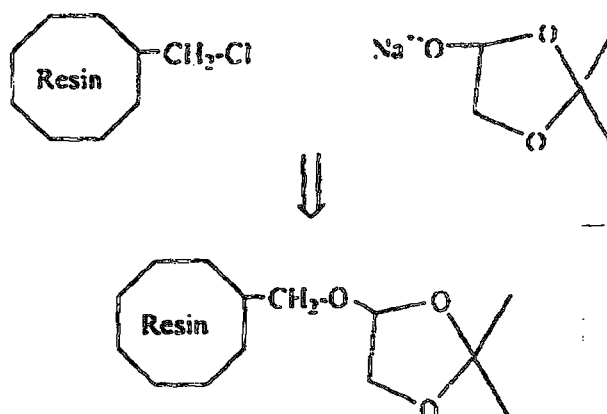
30 Purification of the crude peptide was carried out by reversed-phase HPLC using a Vydac C-18 preparative column (TP silica:particle size 10mm; 25mmX300mm). The column was eluted with a 30-90% linear gradient of solvent A(0.1%TFA in water)



and solvent B(0.1%TFA in acetonitrile). The column eluants were monitored at 230nm, and fractions were appropriately collected. The purity of the products were determined by analytical RP-HPLC and mass spectrometry. Product H-Phe-Glu-NH-BoroBpgOPin, was obtained in 17% yield, 34mg. ES-MS: 626 [M+Na]; retention time analytical HPLC (4x250mm, Vydac, C-18 techsphere), eluted by 10-60% MeCN with 0.1% TFA in water with 0.1% TFA over 25 minutes, gave Rt 23.1 minutes.

4. Attachment of Boronic Acid to Merrifield Resin

a. Derivatisation of Resin with protected Diol (2,2-dimethyl-1,3-dioxolane-4-methanol)



This Sheet Has Been Replaced

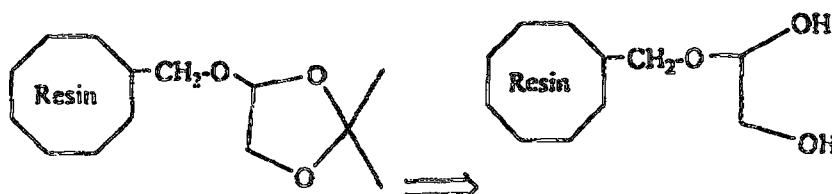


Na (solid, 8g) is added to 2,2-dimethyl-1,3-dioxolane-4-methanol (240ml), under argon gas, and the mixture stirred until it gives a clear solution. Merrifield Resin (Sigma, 1.1 MeQ. Cl per gram, 20g) is added and the mixture stirred overnight, then heated at 80°C for 24h.

Derivatised resin was collected by filtration, washed by 1,4-dioxane (1L), water (3x500ml), and MeOH:water (1:1, 3x500ml), MeOH (3x500ml) and dry ether (3x500ml). An infra red spectrum was obtained by powdering of 1.5-2mg of resin with KBr (dry, 300mg) and compacting into a disc, then scanning on a Perkin® 1600 Fourier Transform I.R. The derivatised resin (Fig. 2) compared to Merrifield resin (Fig. 1) shows distinct stretching signals 1050 to 1150cm⁻¹(s) for ether stretching frequencies

characteristic of a five membered ring; and dialkyl ether stretching at 1060 to 1150cm⁻¹(s) for alkyl-alkyl stretching.

b. Deprotection

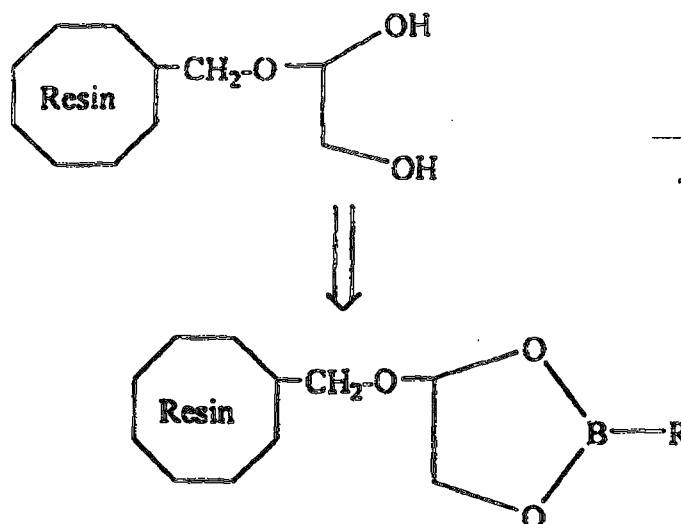


5

The derivatised resin was mixed with HCl (1.5M, 250ml) and 1,4-dioxane (250ml) and the suspension stirred and heated at 80°C. After 72h the resin was washed by water (500ml), MeOH (500ml), DCM (500ml) and Et₂O (500ml), then dried in the air. F.t.-I.R. spectrum of the resin shows distinct O-H stretching frequencies at 3400-3550cm⁻¹ (s), and a main peak at 3413.6 (Fig. 3); this peak is substantially larger than the signal at 2917.6cm⁻¹. In comparison the ether (Fig. 2) and Merrifield resin show only a weak ≈ 3400cm⁻¹ signal for background moisture.

10

c. Reaction of the Derivatised Resin with a boronic acid



This Sheet Has Been Replaced



15

The diol resin (5g, 5.5mmol of diol) was suspended in THF (dry, 500ml) and phenylboronic acid (3.35g, 27.5mmol, 5 equivalents), and 4Å sieves (dried at 150°C).

After stirring under argon overnight, the resin was filtered under argon in a closed system, washed by THF (500ml) and dried under vacuum. Ft-L.R. (Fig. 4) shows a strong signal at 1026cm^{-1} (aryl-alkyl stretching frequency) for the phenyl ring and a weak signal at 3417cm^{-1} (compared to Fig 3, for the starting diol).

5

Ref. Leznoff, C.C. and Wong, J.Y., The use of Polymer Supports in Organic Synthesis. III. Selective Chemical Reactions on One Aldehyde Group of Symmetrical Dialdehydes.. Can.J.Chem.. 1973, 51, 3756-3764.

10

*

*

*

Analytical and Activity Data

15

The following Table 1 contains activity data relating to the invention. In the Table, the designation "Z" denotes benzyloxycarbonyl and "NHir" refers to normal hirudin. "NHir49-64(des-S)" refers to the amino acid sequence from amino acid 49 to amino acid 64 of normal hirudin in which the native Tyr(OSO₃H)⁶¹ is replaced by Tyr.

20

The compounds listed in Table 1 were prepared by the same or analogous methods to the compounds of the preparation Examples 1 and 2 above or, in the case of intermediates, were obtained from sources.

25

The following techniques were employed for activity measurement:

Plasma thrombin time (TT)

A volume of 150 μ l of citrated normal human plasma and 20 μ l of buffer or sample were warmed at 37°C for 1 min. Coagulation was started by adding 150 μ l of freshly prepared bovine thrombin (5NIHu/ml saline) and the coagulation time was recorded on a coagulometer.

30

This Sheet Has Been Replaced



A phosphate buffer, pH7.8, containing 0.1% bovine serum albumine and 0.02% sodium azide was used. The samples were dissolved in DMSO and diluted with the buffer. When no inhibitor was used DMSO was added to the buffer to the same concentration
 5 as that used in the samples. The inhibitor concentrations were plotted against the thrombin times in a semilogarithmic graph from which the inhibitor concentration that caused a doubling (40 sec) of the thrombin time was determined.

Determination of K_i

10 The inhibition of human α -thrombin was determined by the inhibition of the enzyme catalysed hydrolysis of three different concentrations of the chromogenic substrate S-2238

200 μ l of sample or buffer and 50 μ l of S-2238 were incubated at 37°C for 1 min and
 15 50 μ l of human α -thrombin (0.25 NIH μ /ml) was added. The initial rate of inhibited and uninhibited reactions were recorded at 4.5nm. The increase in optical density was plotted according to the method of Lineweaver and Burke. The K_m and apparent K_m were determined and K_i was calculated using the relationship.

$$20 \quad V = \frac{V_{max}}{1 + \frac{K_m}{[S]} + \frac{[I]}{K_i}}$$

The buffer used contained 0.1M sodium phosphate, 0.2M NaCl, 0.5% PEG and 0.02%
 25 sodium azide, adjusted to pH 7.5 with orthophosphoric acid.

The samples consist of the compound disclosed in DMSO.

The reader is referred to Dixon, M and Webb, E. C., "Enzymes", third edition, 1979,
 30 Academic Press, the disclosure of which is incorporated herein by reference, for a further description of the measurement of K_i .

SUBSTITUTE SHEET (RULE 26)

This Sheet Has Been Replaced



Table 1

	Ki μ M	TT μ M
98 GGGDFEPIPL	n/e	100
99 [-BoroBrOPin]CO(CH ₂) ₃ COGGGDFEPIPL	n/e	58
105 GGGGDFEPIPL	n/e	94.9
106 GGGGGDFEPIPL	n/e	15
107 [-BoroBrOPin]OC(CH ₂) ₃ COGGGGDFEPIPL	n/e	58.4
108 [-BoroBrOPin]OC(CH ₂) ₃ COGGGGGDFEPIPL	n/e	63.4
114 GGNSHNDGDFEEIPEEYL Hir ⁹⁹⁻⁶¹	0.613	2
121 HO ₂ C(CH ₂) ₃ COGGGGGDFEPIPL	0.738	30.9
128 [-L-PheProBoroValOPin]OC(CH ₂) ₃ COG ₄ DFEPIPL	n/e 11.7	27.1
129 [-D-PheProBoroValOPin]OC(CH ₂) ₃ COG ₄ DFEPIPL	16.4	33.5
137 [-D-Phe-ProBoroEtgOPin]OC(CH ₂) ₃ COG ₄ DFEPIPL	1.23	10.2
166 [-D-Phe-ProBoroBpgOPin]OC(CH ₂) ₃ COG ₃ NHir ⁹⁹⁻⁶¹ (des-S)	0.000649	0.029
167 Hir ⁹⁹⁻⁶¹ (des-S)	n/e 0.155	N.T.
175 [-D-Phe-ProBoroBpgOPin]OC(CH ₂) ₃ COG ₄ NHir ⁹⁹⁻⁶¹ (des-S)	0.00211	0.065
176 Z-D-Phe-Pro-BoroBpgOPin + Hir ⁹⁹⁻⁶¹	0.0218	0.636
182 [-D-Phe-ProBoroCegOPin]OC(CH ₂) ₃ COGPGGNHir ⁹⁹⁻⁶¹ (des-S)	0.00271	N.T.
184 HO ₂ C(CH ₂) ₃ COGPGGNHir ⁹⁹⁻⁶¹ (des-S)	12.7	56.7
185 [-D-Phe-ProBoroCegOPin]OC(CH ₂) ₃ COGPG ₃ NHir ⁹⁹⁻⁶¹ (des-S)	n/e 0.75	7.36
186 [-D-Phe-Pro-BoroCegOPin]OC(CH ₂) ₃ COGPG ₃ NHir ⁹⁹⁻⁶¹ (des-S)	n/e 0.9	4.61
267 [-PheProBoroCegPin]OC(CH ₂) ₃ COG ₄ (EDFEPIPL)	0.762	4.9
268 [-Pg] ¹⁸ (OEt) ₂ OC(CH ₂) ₃ COG ₄ (EDFEPIPL)	n/e 88.8	66.5
[-D-PheProBoroIrgOPin]OC(CH ₂) ₃ COG ₄ NHir ⁹⁹⁻⁶¹ (des-S)	N.T.	N.T.

n/e = no effect

5 n/e 11.7 = no effect up to a concentration of 11.7 μ M

N.T. = not tested

This Sheet Has Been Replaced



**CLAIMS**

1. A method of making a peptide or a peptide-containing compound, comprising performing the following steps to make a target amino acid sequence:

5

(i) providing a solid phase having coupled thereto functional groups;

10

(ii) causing a compound reactive with the functional groups selectively to react therewith, the reacted compound having a functional group capable of reacting with an amino group or with a carboxyl group or a reactive derivative thereof;

15

(iii) causing the amino or carboxyl group (which may be in the form of a reactive derivative thereof) of a terminal amino acid of a target amino acid sequence selectively to react with said functional groups of the reacted compound;

20

(iv) coupling the amino acid sequentially following, in the target sequence, the sequentially preceding amino acid coupled to the solid phase to said preceding amino acid;

This Sheet Has Been Replaced



(v) repeating step (iv) as often as necessary; and

25

(vi) cleaving a solid phase-linked compound prepared using steps (i)-(iv) from the solid phase by the action of an acid or base.

30

characterised in that a compound comprising a boronic acid group $[-B(OH)_2]$ or an ester or other derivative thereof is incorporated in the solid phase-linked compound prior to its cleavage from the solid phase.

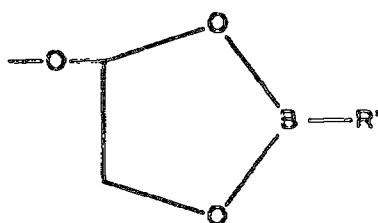
2. A method of claim 1 in which step (ii) comprises reacting an amino group or an optionally derivatised carboxy group of an amino acid with a functional group



coupled directly or indirectly to a solid phase as part of conventional solid phase peptide synthesis, for example.

3. A method of claim 1 in which the compound comprising a boronic acid group
5 is an amino acid boronic acid, a peptide boronic ester or a boronate ester of either and step (ii) comprises reacting the amino acid or peptide boronic acid or ester with a diol coupled to the solid phase, e.g. to form a solid phase having coupled thereto, suitably via a linker, a moiety of the formula:

10



- wherein R' is a residue of a natural or unnatural amino acid or of an analogue thereof,
15 the analogue optionally having its amino group replaced by an alternative functional group capable of forming a linkage other than a natural peptide linkage and/or its α -hydrogen atom replaced.

4. A method for making a compound comprising a peptide boronic acid or
20 peptide boronate ester, the method comprising:

- (i) providing a solid phase having coupled thereto alcoholic hydroxy groups;
- 25 (ii) causing an amino acid boronic acid or peptide boronic acid to react with the hydroxy groups whereby the boronic acid residue becomes esterified to the solid phase;
- 30 (iii) causing the carboxyl group of the amino acid sequentially following, in the end product peptide boronic acid or boronate ester, selectively to react with the amino group if the sequentially preceding amino acid coupled to the solid phase;





(iv) repeating step (iii) as often as necessary;

(v) cleaving the resultant peptide boronate from the resin,

5

the method optionally comprising one or more further steps to make said compound.

5. A method of claim 4, wherein each amino acid coupled to the solid phase has a protected amino group and step (iii) comprises deprotecting the amino group of the sequentially preceding amino acid and/or the cleavage of step (v) is performed with acid or by transesterification.

10

6. The use of amino acid or peptide boronic acids or boronate esters in the solid phase synthesis of peptide-containing compounds.

15

7. The use in solid phase synthesis of a boronic acid residue attached to the solid phase through hydroxy residues.

8. A method for making a compound comprising a boron atom, the method comprising:

20

(i) providing a solid phase having coupled thereto alcoholic hydroxy groups;

25 (ii) causing a boronic acid or boronate ester to react with the hydroxy groups whereby the boronic acid residue becomes esterified to the solid phase; and

(iii) performing one or more further steps to make said compound.

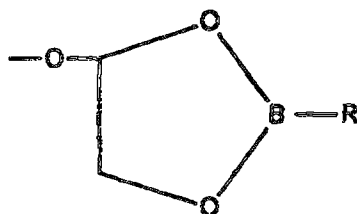
30

9. A solid phase material having coupled thereto boronic acid residues through hydroxy groups.





10. A solid phase material having coupled thereto a moiety of the formula:



wherein R is a residue bonded to the boron atom and the oxygen through which the moiety is bonded to the solid phase is optionally bonded directly to an alkylene residue, which may optionally be inertly substituted, or to a carbonyl group.

11. A bifunctional serine protease inhibitor comprising:

- (a) a catalytic site-directed moiety (CSDM) which binds to and inhibits the active site of a serine protease;
- (b) an exosite associating moiety (EAM); and, optionally,
- (c) a connector moiety bonded between the EAM and the CSDM.

the CSDM and the EAM being capable of binding simultaneously to a molecule of the serine protease, provided that the inhibitor is not a thrombin inhibitor.

12. An inhibitor of claim 11, in which the CSDM has a P1 residue which is an Arg, Lys or an analogue thereof or is hydrophobic and has a (P4)P3P2 residue selected from:

Residue Sequence
D-Phe-/substituted D-Phe-/D-Dpa-/Dba-/Pms-/α-Nal-/β-Nal- /TMSal-/Chg-/Phg-/D-Tiq-/para-ether of D-Tyr-/NaSO ₂ -Pro IleuGluGly, PyroGluGly, ArgGly, ChaGly, LeuArg L-PhePhe, NalPhe, D-TiqPhe, NalThr, NalPhg

This Sheet Has Been Replaced





ValVal
γ -BzlGluGly, Glu(OBzl)Ala, GlyArg, GlyLys
GlnGly
PhePro, GluGly
LeuSerThr

13. An inhibitor of claim 11 or claim 12, in which the EAM is an amino acid sequence selected from:

YIDGR--IVEGSDAEIGMSPWQ
AIEGR--TATSEYQTFFNPRTFGS
SKPQGR--IVGGKVC
NLTRR--IVGGQECKDGEC
NLTRR--IVGGQECKDGEC
SKLTR--AEAVFPDVDYVN
FNDFTR--VVGGEDAKPGQF
KIKPR--IVGGTASVRGE
KTSTR--IVGGTNSSWGE
ELR--MKNNEEAEDYDDDLTDSEMD
PKKCPGR--VVGGCVAHPHSWPWQVSLRT

5

14. An inhibitor of any of claims 1 to 3, wherein the connector moiety is of the formula:



10

wherein λ is a residue of a non-peptide linker of the formula $\text{HO}_2\text{C}(\text{CH}_2)_h\text{CO}_2\text{H}$, wherein h is from 2 to 6, and σ is a peptide spacer comprising at least two adjacent Gly residues.





15. A method of making an inhibitor of any of claims 11 to 14 comprising performing the following steps to make a target amino acid sequence:
- 5 (i) providing a solid phase having coupled thereto functional groups capable of reacting with an amino group or, preferably, with a carboxyl group or a reactive derivative thereof;
 - 10 (ii) causing the amino or carboxyl group of a terminal amino acid of the target amino acid sequence selectively to react with said functional groups, the carboxyl group optionally being in the form of a reactive derivative thereof;
 - 15 (iii) coupling the amino acid sequentially following, in the target sequence, the sequentially preceding amino acid coupled to the solid phase to said preceding amino acid; and
 - (iv) repeating step (iii) as often as necessary.
16. A method of claim 15 which further includes a step (v) of coupling a said sequentially following amino acid of a step (iii) to said preceding amino acid of the step
- 20 through a compound having two functional groups capable of reacting with an amino group, whereby one of said functional groups becomes bonded to the amino group of said preceding amino acid and the other to the amino group of said following amino acid.
- 25 17. A method of claim 14 or claim 15, wherein the terminal amino acid reacted with the functional groups attached to the solid phase is the C-terminal amino acid of the EAM and step (iii) is repeated to couple successive amino acids of the EAM sequence and successive amino acids of any contiguous connector peptide.
- 30 18. A method of any of claims 15 to 17, wherein the final amino acid of the uninterrupted amino acid sequence coupled to the solid phase is reacted with a compound having two carboxylate groups or reactive derivatives thereof, and the





unreacted carboxylate or carboxylate derivative is typically reacted with the amino group of an amino acid which is the N-terminal amino acid of the CSDM.

19. A method of claim 18, wherein said N-terminal amino acid is already bonded to the remainder of the CSDM and the CSDM has a heteroatom group in place of a C-terminal carboxy group.

20. A pharmaceutical formulation comprising an inhibitor of any of claims 1 to 4 formulated for use as a human or veterinary pharmaceutical, the formulation optionally including a pharmaceutically acceptable diluent, excipient or carrier.

21. A method of treating by therapy or prophylaxis a bodily disease or disorder capable of treatment by inhibition of a serine protease, comprising administering, e.g. orally or parenterally, to a human or animal patient a therapeutically or prophylactically effective amount of an inhibitor of any of claims 1 to 4.

This Sheet Has Been Replaced

1/2

FIG. 1

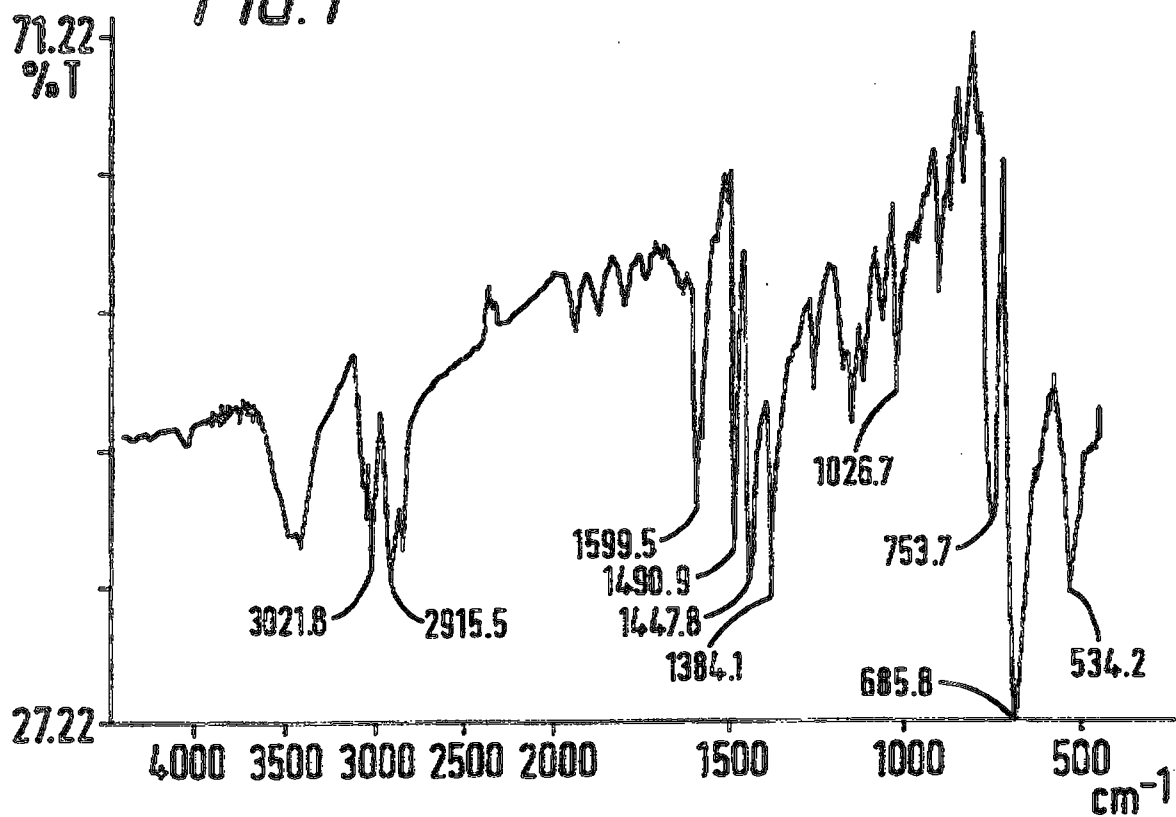
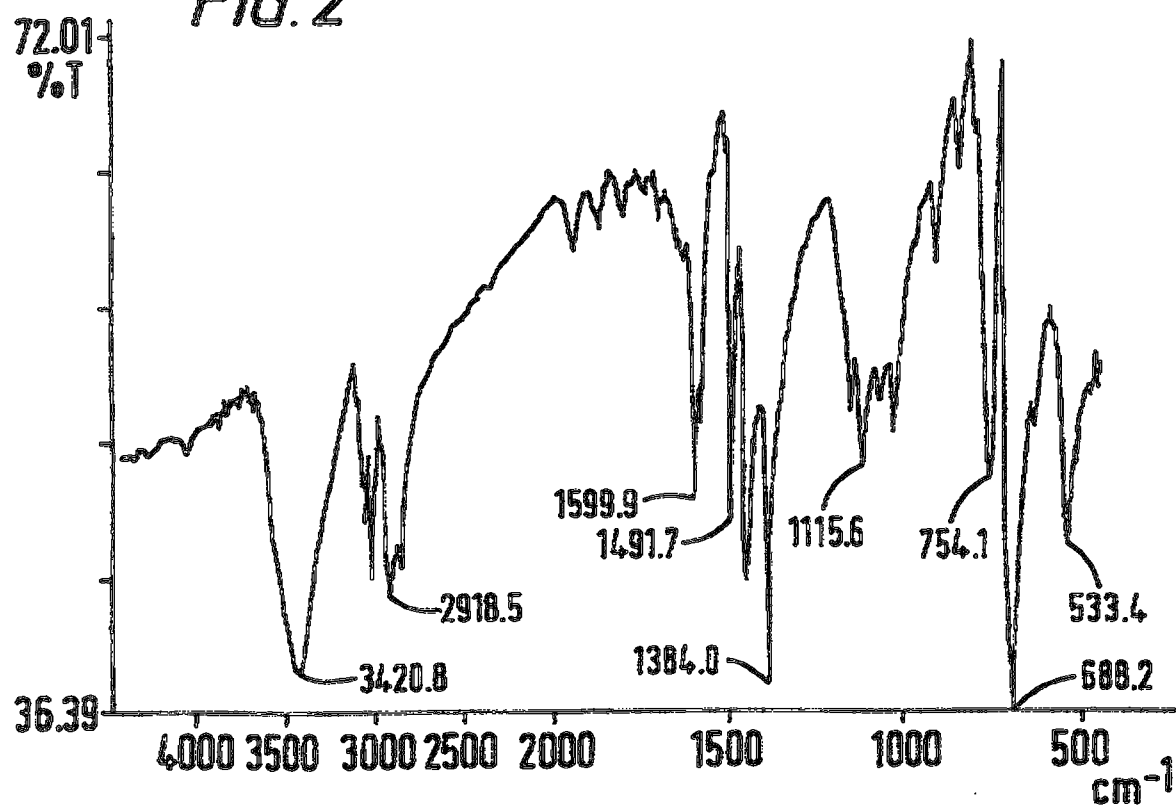


FIG. 2



2/2

FIG. 3

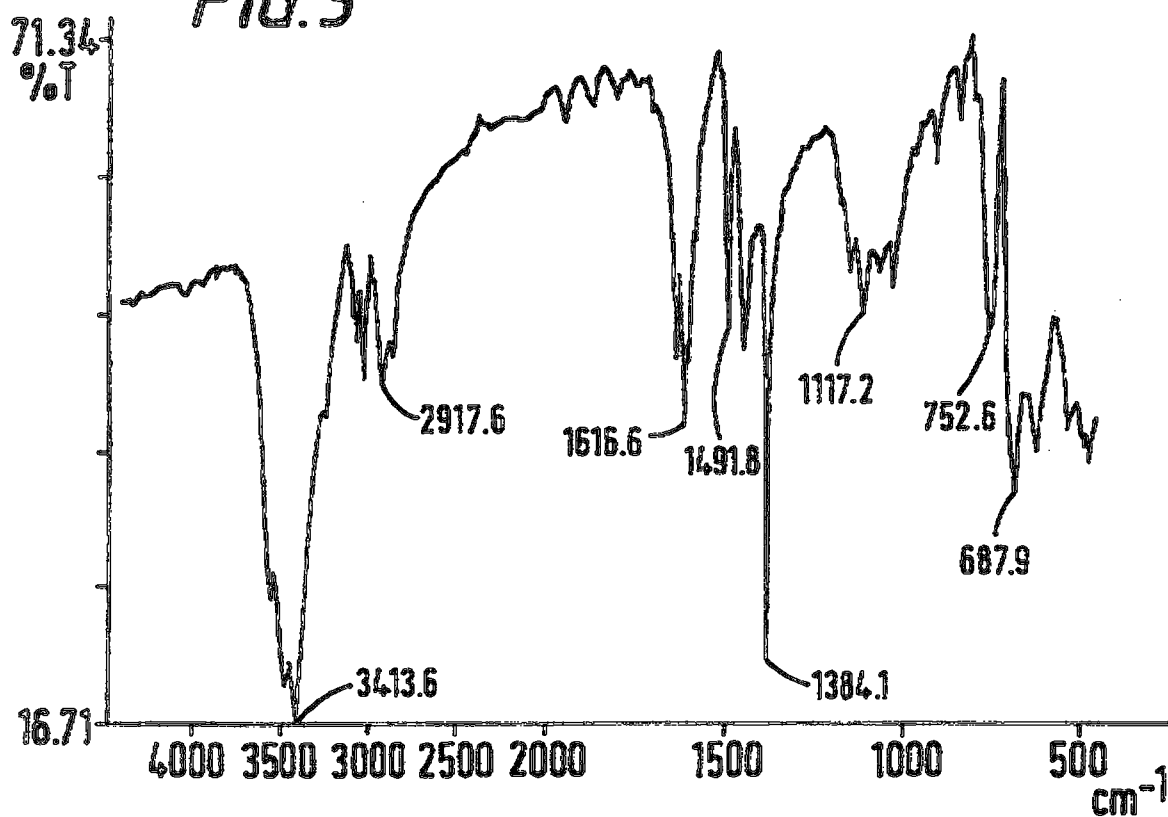
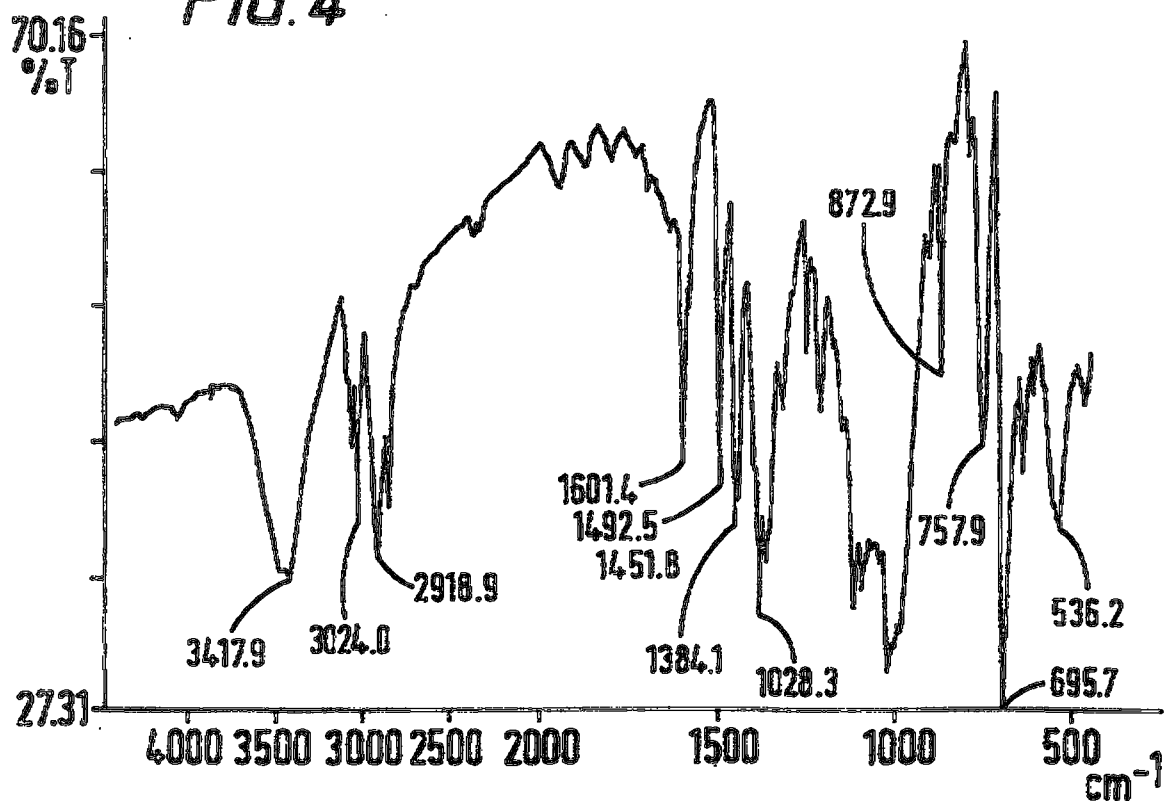


FIG. 4



SOLID PHASE SYNTHESIS OF BORON-CONTAINING COMPOUNDS

This invention relates to novel methods and materials for synthesis of boron-containing compounds, for example bifunctional serine protease inhibitors.

- 5 Modulation or inhibition of serine protease inhibitors is useful *inter alia* to prevent thrombosis.

The family of serine protease enzymes cleaves peptide bonds by a mechanism involving the catalytic triad of Asp-His-Ser residues in the active site of the enzymes.

- 10 Serine protease inhibitors have been designed which use functional groups to interact with the triad and thereby block activation of the enzyme substrates. It can be desirable to make inhibitors selective for one target protease.

Certain serine proteases are known to have a second site or "exosite" for binding to an anionic portion of the substrate. This exosite is often called the anion binding exosite ("ABE").

The amino acid residue which provides the carbonyl group of the scissile bond of a serine protease substrate is designated "P1". Successive amino acid residues on the N-terminal side of residue P1 are designated P2, P3, P4 ... etc; amino acid residues on the C-terminal side of residue P1 are designated P1', P2', P3' In fibrinogen, P1' is glycine and P2' is proline. The protease contains a "specificity pocket" which recognises the side chain of the P1 amino acid. Trypsin-like proteases normally recognise P1 residues with arginine-like or serine-like side chains.

25

In one aspect, the invention provides a novel method for preparing boron-containing peptides or compounds containing such peptides. The invention is not restricted as to the identity of the end product but is exemplified by reference to the synthesis of bifunctional serine protease inhibitors comprising:

30

- (a) a catalytic site-directed moiety (CSDM) which binds to and inhibits the active site of a serine protease;
- (b) an exosite associating moiety (EAM); and, optionally,

REPLACEMENT SHEETS	SG
☒ Description : paged /... to paged /...	10
☒ Claim(s) : paged /... to paged /...	28
☐ Drawing(s) : paged /... to paged /...	...
☐ Abstract : paged /... to paged /...	...
DATE OF RECEIPT 10/11/99	

REPLACEMENT SHEET
10 NOV 1999

(c) a connector moiety bonded between the EAM and the CSDM,

the CSDM and the EAM being capable of binding simultaneously to a molecule of the serine protease.

5

Peptide boronates are a well established class of compounds which have hitherto been made by solution chemistry. Thus, peptide serine protease inhibitors are known in which the C-terminal carboxy group is replaced by a boronic acid group or a derivative thereof. Representative compounds are of the Formula II:

10



wherein:

15 (aa)_x represents a sequence of amino acids (eg as in Formula I); R² and R³ are each independently selected from halogen, -OH, OR⁴ and -NR⁴R⁵, where R⁴ and R⁵ are each independently a group of the formula R⁶(CO)_u-, wherein u is 0 or 1; R⁶ is H or an optionally halogenated alkyl, aryl or arylalkyl group containing up to (10 - u) carbon atoms and optionally substituted by one or more groups selected from -OH,
20 R⁷(CO)_vO- and R⁷(CO)_v-, wherein v is 0 or 1; R⁷ is C₁-C_{6-v} alkyl, or is an aryl, alkylaryl, arylalkyl or alkylarylalkyl group containing up to (10-v) carbon atoms,

or R² and R³ taken together represent a residue of a diol or a dithiol.

25 Such peptide boronates are described, for example, in WO 92/07869 (equivalent to USSN 08/317,387), EP 0471651 (which corresponds to US 5288707) and USSN 08/240,606, the disclosures of which are incorporated herein by reference.

30 We have now surprisingly found that boron-containing peptides may be synthesised by solid phase chemistry without serious degradation. One aspect of the invention, therefore, is the use of a boron-containing amino acid analogue in peptide synthesis using solid phase chemistry, especially Fmoc chemistry (also known as the "Sheppard approach").

REPLACEMENT SHEET

10 NOV 1999

In another aspect, there is provided a method of making a peptide or a peptide-containing compound, comprising performing the following steps to make a target amino acid sequence:

5

(i) providing a solid phase having coupled thereto functional groups;

(ii) causing a compound reactive with the functional groups selectively to react therewith, the reacted compound having a functional group capable of reacting
10 with an amino group or with a carboxyl group or a reactive derivative thereof;

(iii) causing an amino or carboxyl group (which may be in the form of a reactive derivative thereof) of a terminal amino acid of a target amino acid sequence selectively to react with said functional groups of the reacted compound;
15

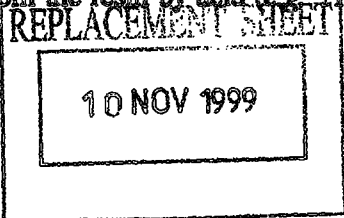
(iv) coupling the amino acid sequentially following, in the target sequence, the sequentially preceding amino acid coupled to the solid phase to said preceding amino acid;

20 (v) repeating step (iv) as often as necessary; and

(vi) cleaving a solid phase-linked compound prepared using steps (i)-(iv) from the solid phase by the action of an acid or base,

25 characterised in that a compound comprising a boronic acid group $[-B(OH)_2]$ or a derivative thereof, especially an ester, is incorporated in the solid phase-linked compound prior to its cleavage from the solid phase.

30 A particular method of using peptide boronate esters in solid phase chemistry is a completely novel technique in which boronic acids $[-B(OH)_2]$ are directly esterified onto diols coupled to a resin. Chain extension is continued from the amino group of the amino acid by, for example, standard Fmoc chemistry. The boronic acid ester is cleaved from the resin by acid (e.g. TFA) to give the peptide boronate [peptide- $B(OH)_2$], or by



10 NOV 1999

transesterification, for example, by concentrated solution of a hindered diol, such as pinanediol, for example.

As used herein, "natural" amino acid means an L-amino acid (or a residue thereof)
5 selected from one of the twenty common or "standard" α -amino acids found in proteins.

By "unnatural" amino acid is meant any α -amino acid (or residue thereof) other than
the twenty "standard" amino acids. Unnatural amino acids therefore include the D-
10 isomers of natural L- amino acids and amino acids having side chain protecting groups.

The prefixes "D" and "L" are used as normal to indicate amino acids of D- or L-
configuration respectively. A "D, L-" prefix indicates a racemic mixture whilst the
15 absence of a prefix indicates that the amino acid can be of either D- or L-
configuration, except in the examples where residues are of L-configuration unless
otherwise states. For those groups of unspecified configuration in the text which can
be of D- or L- configuration, L- configuration is preferred.

20 Abbreviations and terms prefixed by "boro" indicate amino acids where in the
terminal carboxyl group $-\text{CO}_2\text{H}$ has been replaced by a boron functionality.

Brief Description of the Drawings

25 Figure 1 is a Fourier Transform Infra Red (F.t.-I.R.) spectrum of a Merrifield resin

Figure 2 is an F.t.-I.R. spectrum of the same resin after reaction with sodium 2,2-dimethyl-1,3-dioxolane-4-methanolate.

Figure 3 is an F.t.-I.R. spectrum of the reacted resin after treatment with HCl
30 to deprotect the hydroxy groups of the dioxolane.

Figure 4 is an F.t.-I.R. spectrum of the deprotected resin after reaction with phenylboronic acid.

10 NOV 1999

Considering now the inventive processes in more detail:

5 Solid phase synthesis is a technique familiar to peptide chemists and detailed elucidation is therefore not required here. An introduction to the technique may be found in "The Chemical Synthesis of Peptides", John Jones, Clarendon Press, Oxford, England, 1991. The principle of conventional solid phase synthesis is that an amino acid or peptide coupled to a solid phase is reacted with an amino acid which is protected against reaction with itself and, after coupling with the solid phase-linked amino acid, is deprotected for reaction with a further amino acid protected against reaction with itself.

10 These steps are repeated as often as necessary.

One solid phase synthesis technique is the Fmoc technique (Fmoc = fluorenylmethylcarbonyl). In Fmoc chemistry (also known generally as the 'Sheppard approach'), the carboxy terminus of a peptide (or an amino acid) is coupled to a resin bead via a linker which is terminated by a reactive function. The resin bead itself is typically polystyrene (PS), though other solids have been used that have suitable swelling characteristics in solvent, since it is now known that the peptide chain grows in the pores on the inside of the bead. An example of an alternative solid is the polyamide called Kiesulguhr.

15

20

The linker can be many things, but we prefer to use PEG (i.e. a polyethylene glycol linker), which has an alcohol function.

The terminus of the linker, typically called a 'handle', depends on the desired product, but for Fmoc chemistry will be a moiety such that it can finally be cleaved by acid. The most common terminus (which we have used) is HMBA or para-hydroxymethylbenzoic acid linker. The HMBA is esterified onto the PEG, and then the peptide or amino acid (with Fmoc on its N-terminus) is reacted to give also an ester link to the HMBA. The ester links are then cleavable by acid. The Fmoc protecting group is base labile and typically removed by a secondary base (e.g. piperidine) and the resulting free amino group is reacted with a selected Fmoc-protected amino acid; the amino acid sequence is extended by repetition of these steps.

25

30

10 NOV 1999

Another solid phase synthetic approach is the Boc technique (Boc = tertiarybutyloxycarbonyl). The resin used in Boc chemistry (also known more generally as the Merrifield method) is often divinylbenzyl based, for instance a 'Wang' resin has chloromethyl benzene co-polymerised to 2% divinylbenzene. The chloromethyl benzene group is reacted with an amino acid or peptide whose amino group is protected by Boc, to give a link to the resin. The link to the resin is typically cleaved (very carefully!) by dry, liquid HF. This is described as 'vigorous' acidolysis. The Boc protecting group is acid labile and typically cleaved by TFA, prior to reaction of the resultant free amino group with a selected Boc-protected amino acid; as with Fmoc chemistry, the amino acid sequence is extended by repetition of these steps.

The two classical methods of solid phase peptide synthesis (Sheppard and Merrifield), therefore, involve coupling amino acids via their carboxy-termini or their derivatives to a solid resin particle, then sequentially coupling new amino acids (via their activated carboxy termini) to via the N-termini generated.

Alternatively recent reports have shown coupling to the resin via the N-termini, for example via an acid labile benzyloxycarbonyl linkage, subsequent liberating of the carboxy termini, activating of these and coupling of amino acids via their N-termini, the carboxy termini of the amino acids temporarily being protected. (Sharma, R.P.; Jones, D.A.; Broadbridge, R.J.; Corina, D.L. and Akhtar, M. A Novel Method of Solid Phase Synthesis Of Peptide Analogues, in *Innovation and Perspectives in Solid Phase Synthesis*, ed., R.Epton, 1994, Mayflower Worldwide Limited, Birmingham, page 353-356; Letsinger, R.L. and Kornet, M.J. *J.Amer.Chem.Soc.*, 1963, 85, 3045.)

The invention provides a method of making a peptide or a peptide-containing compound, comprising performing the following steps to make a target amino acid sequence:

- (i) providing a solid phase having coupled thereto functional groups;

(ii) causing a compound reactive with the functional groups selectively to react therewith, the reacted compound having a functional group capable of reacting with an amino group or with a carboxyl group or a reactive derivative thereof;

5

(iii) causing an amino or carboxyl group (which may be in the form of a reactive derivative thereof) of a terminal amino acid of a target amino acid sequence selectively to react with said functional groups of the reacted compound;

10

(iv) coupling the amino acid sequentially following, in the target sequence, the sequentially preceding amino acid coupled to the solid phase to said preceding amino acid;

15

(v) repeating step (iv) as often as necessary; and

(vi) cleaving a solid phase-linked compound prepared using steps (i)-(iv) from the solid phase by the action of an acid or base,

20 characterised in that a compound comprising a boronic acid group $[-B(OH)_2]$ or a derivative thereof, especially an ester, is incorporated in the solid phase-linked compound prior to its cleavage from the solid phase.

25 The method of the invention for making a peptide or peptide-containing compound may comprise a step of coupling a peptide to a solid phase-linked compound prepared by previous steps of the process, optionally as a step (iv) of the process [*i.e.* a step (iv) of the process in which the sequentially following amino acid is part of a peptide].

30 The method of the invention for making a peptide or peptide-containing compound may comprise a step of coupling to a solid phase-linked compound prepared by previous steps of the process a compound other than an amino acid or peptide, such as, for example, a compound having two carboxyl groups for use in linking an amino group of a solid phase-linked peptide with an amino group of a peptide, peptide

10 NOV 1999

analogue or amino acid in the liquid phase. Of course, any other liquid phase compound capable of reacting with an amino group or, as the case may be, a carboxyl group could thus be linked to the solid phase-linked peptide. Diamines are useful for interconnecting moieties having carboxyl groups (e.g. in the form of reactive derivatives thereof). Extension of a solid phase-linked peptide by a dicarboxylic acid, especially glutaric acid, is useful in the preparation of bifunctional serine protease inhibitors having a CSDM joined, typically through its N-terminal, to an EAM through a connector moiety comprising a peptide spacer portion and dicarboxylic acid residue linker portion.

10

A solid phase-linked compound whose free end terminates with a dicarboxylic acid residue may be further extended by reaction of the free carboxyl residue (optionally in the form of a derivative thereof) with the amino group of an amino acid, e.g. a dicarboxylic acid residue coupled to a solid phase-linked EAM-spacer moiety may be reacted with an amino acid of a CSDM, for example the N-terminal amino acid of a CSDM.

15

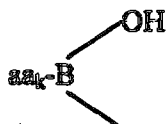
Step (ii) of the method may comprise reacting an amino group or an optionally derivatised carboxy group of an amino acid with a functional group coupled directly or indirectly to a solid phase, as part of conventional solid phase peptide synthesis, for example. The amino or carboxy group coupled to the solid phase is often the terminal amino or carboxy group but in some embodiments is a functional group of a side chain, such as the side chain carboxyl group of glutaric acid, for example; the C-terminal carboxy group of the amino acid attached to the solid phase through its side chain may be replaced by the boronic acid residue $[-B(OH)_2]$ or an ester thereof.

25

The method may comprise N-terminal coupling in SPPS, in which the carboxy terminus of a resin bound peptide is coupled to a free α -aminoboronate acid or ester, prior to acid cleavage of the resultant product from the resin.

30

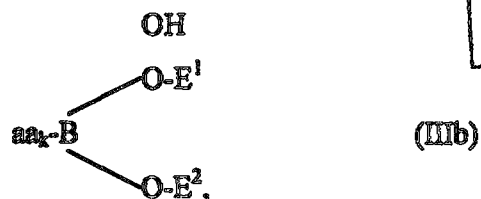
In another embodiment, step (ii) comprises reacting an amino acid or peptide boronic acid or ester



(IIIa)

9

or



5

where E^1 and E^2 represent boronic ester-forming residues or may together form a single residue,

10 with a diol coupled to the solid phase. The technique of linking a boron atom (*e.g.* as part of an amino acid or peptide boronic acid or ester) to a solid phase through hydroxy groups coupled (directly or indirectly) to the solid phase is novel and forms an aspect of the invention.

15 If the compound comprising a boronic acid or ester is an amino acid boronate used in step (ii) in either of the preceding embodiments, the solid phase synthetic method may be used to make a peptide boronate inhibitor of a serine protease catalytic site, optionally in the synthesis of a bifunctional serine protease inhibitor.

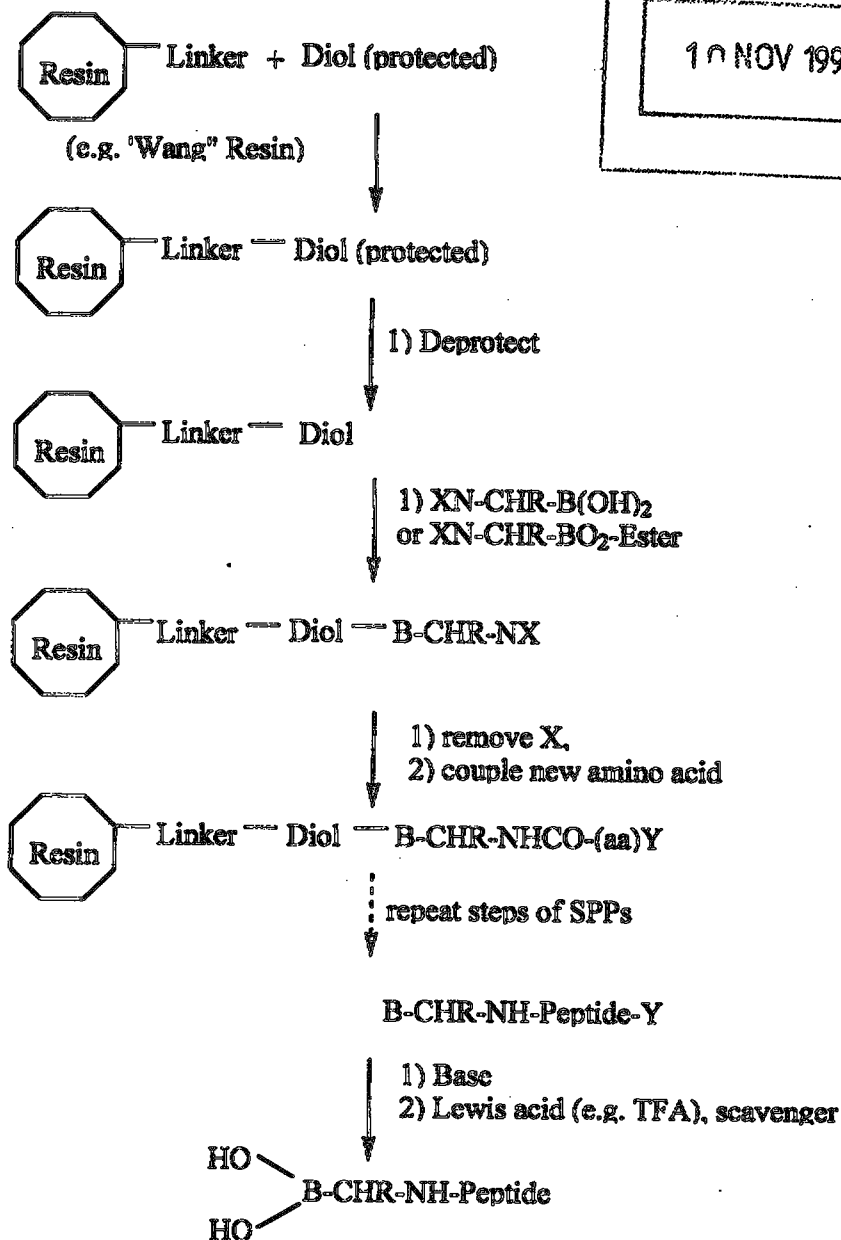
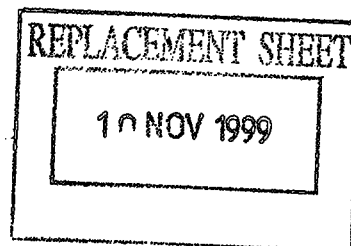
20 Thus, boronic acids can be directly esterified onto diol-containing resins, and then chain extension continued from the N-terminal end by, for example, standard Fmoc-chemistry. Subsequently the boronic acid ester can be cleaved, either by mineral acids, to give the free boronic acids [peptide-B(OH)₂], or by transesterification, *e.g.* by a concentrated solution of a diol, especially a hindered diol such as pinanediol, for example.

25

The literature describes ways of preparing diol-containing solid phase resins, which can be derivatised by aldehydes and are suitable also to be derivatised by boronic acids/esters (*e.g.* Xu, Z.-H., McArthur, C.R and Leznoff, C.C. 'The monoblocking of symmetrical Diketones on Insoluble Polymer Supports', *Can.J.Chem.*, 1993, 61, 1405-1409. and Leznoff, C.C. and Sywanyk, W. 'Use of Polymer Supports in Organic Synthesis. 9. Synthesis of Unsymmetrical Carotenoids on Solid Phase', *J.Org.Chem.*, 1997, 42, 3203-3205).

30

A general procedure is as follows:



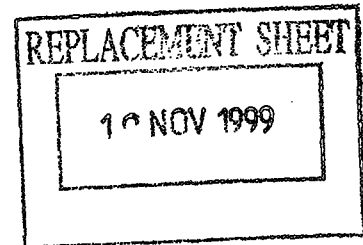
The diol is a compound having two or more alcoholic hydroxy groups.

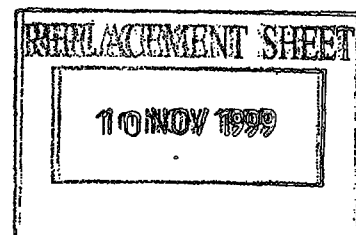
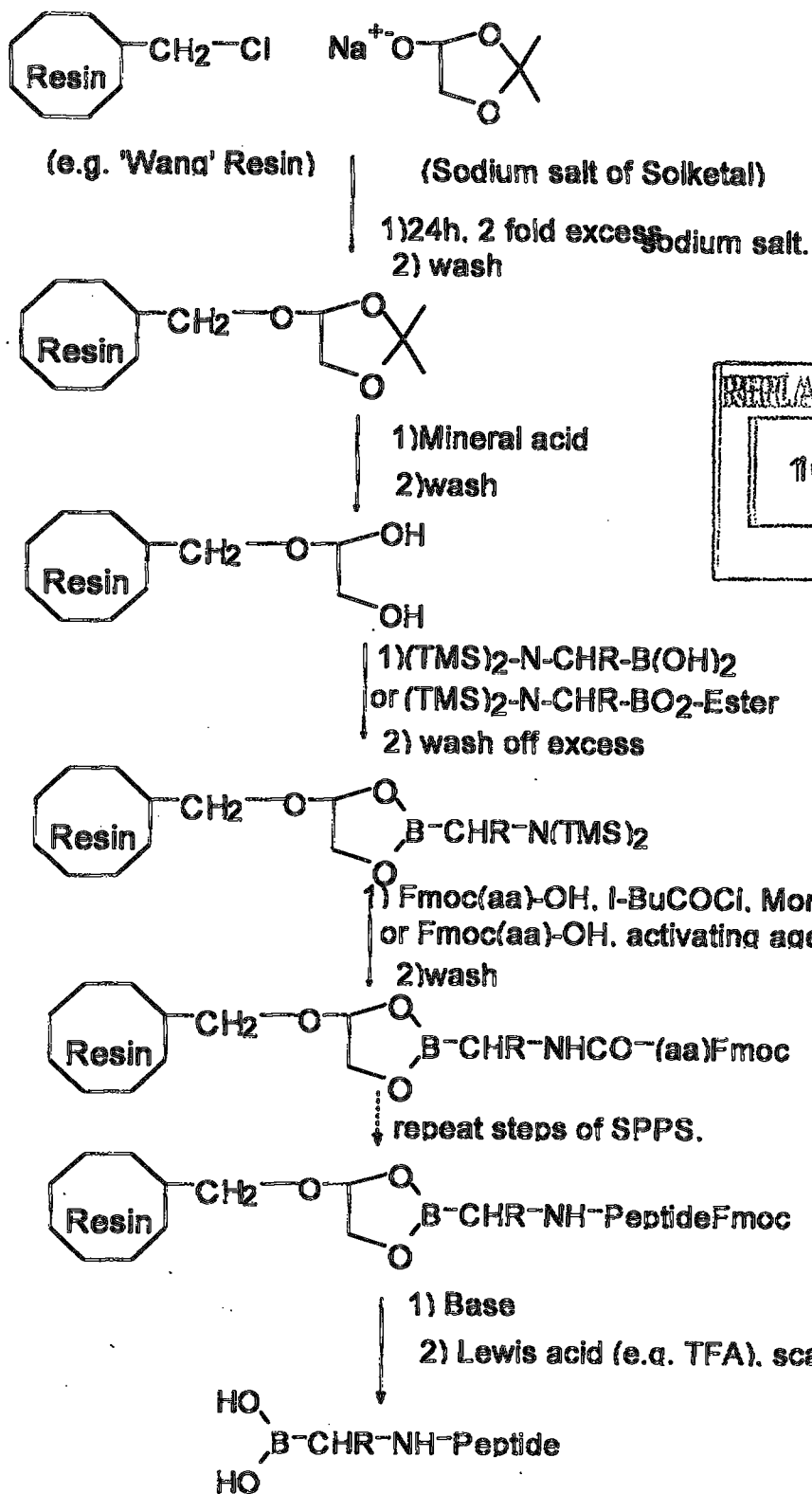
X and Y are protecting groups.

5 R is the side chain of an amino acid boronate/boronic acid.

Typically, the resin is washed after each step. In suitable embodiments the diol is not protected before it is reacted with the resin.

A more specific procedure is set out below:





TMS = Trimethylsilyl

SPPS = solid phase peptide synthesis

The invention therefore opens the way to the preparation of peptide boronates by solid phase chemistry, for example in preparing a library of peptide boronates by a combinatorial method.

5 The invention includes a method for making a compound comprising a peptide boronic acid or peptide boronate ester, the method comprising:

(i) providing a solid phase having coupled thereto alcoholic hydroxy groups;

10

(ii) causing an amino acid boronic acid or peptide boronic acid to react with the hydroxy groups whereby the boronic acid residue becomes esterified to the solid phase;

15

(iii) causing the carboxyl group of the amino acid sequentially following, in the end product peptide boronic acid or boronate ester, selectively to react with the amino group of the sequentially preceding amino acid coupled to the solid phase;

20

(iv) repeating step (iii) as often as necessary;

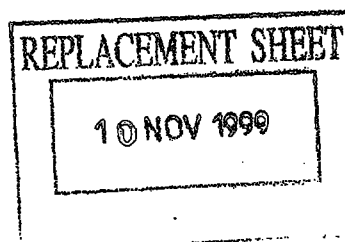
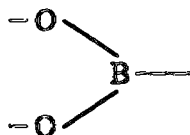
(v) cleaving the resultant peptide boronate from the resin,

the method optionally comprising one or more further steps to make said compound.

25

The alcoholic hydroxy groups coupled to the solid phase are preferably arranged such that pairs of the groups can be bonded to a boron atom, i.e. such that a boron atom can be di-esterified by them:

30



In some embodiments the hydroxy groups are in a 1,2-arrangement (i.e. on adjacent carbons); in other embodiments they are spaced apart on chains (e.g. a residue of $\text{NH}(\text{CH}_2\text{CH}_2\text{OH})_2$).

5 Preferably each amino acid coupled to the solid phase has a protected amino group and step (iii) comprises deprotecting the amino group of the sequentially preceding amino acid. Preferably the cleavage of step (v) is performed with acid or by transesterification.

10 More generally, there is provided the use in solid phase synthesis of a boronic acid residue attached to the solid phase through hydroxy group residues.

Also provided is a method for making a compound comprising a boron atom, the method comprising:

15

(i) providing a solid phase having coupled thereto alcoholic hydroxy groups;

20

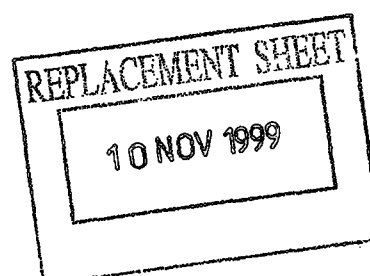
(ii) causing a boronic acid or boronate ester to react with the hydroxy groups whereby the boronic acid residue becomes esterified to the solid phase; and

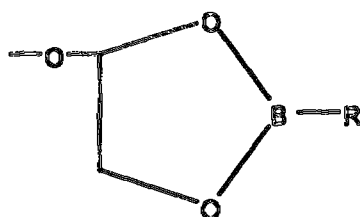
(iii) performing one or more further steps to make said compound.

25 The alcoholic hydroxy groups are preferably arranged as described above.

In other aspects, there are provided a solid phase material having coupled thereto boronic acid residues through hydroxy groups, as well as a solid phase material having coupled thereto a moiety of the Formula IV:

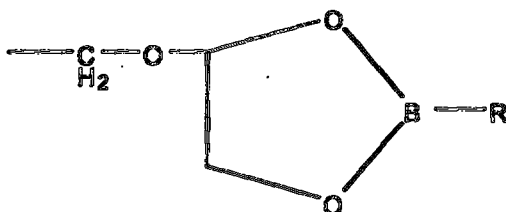
30





(IV)

wherein R is a residue bonded to the boron atom, and is usually an organic moiety. Residue R is in one class of material free of functional groups reactive with alcoholic hydroxy groups (but the material may contain such functional groups in protected form, *e.g.* prior to deprotection). In another class of material, such functional groups are unprotected, protecting groups having previously been removed. R is typically an organic moiety having one or more functional groups to enable R to undergo a chemical reaction; any protectable functional groups may be protected. In one class of materials, the solid phase has coupled thereto a moiety of Formula V:



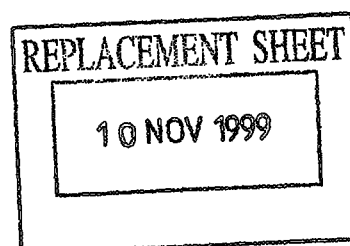
(V)

One or both of the hydrogen atoms of the $-\text{CH}_2-$ group may be replaced by other groups compatible with the use of the material, *e.g.* alkyl groups (for example methyl or butyl).

In a yet further class of materials, the left hand oxygen of Formula IV is part of an ester.

A first class of solid phase synthetic methods comprises performing the following steps to make a target amino acid sequence:

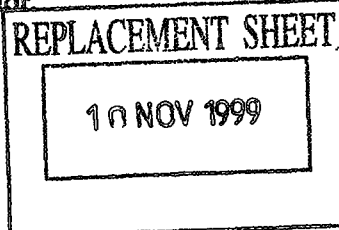
- (i) providing a solid phase having coupled thereto functional groups capable of reacting with a carboxyl group;



- (ii) causing a compound reactive with the functional groups and comprising an amino group protected by a base-labile protecting group to react with the functional groups to form an acid labile bond;
- (iii) deprotecting the amino group with a base;
- 5 (iv) causing the carboxyl group of an amino acid whose amino group is protected by a base-labile protecting group to react with the deprotected amino group resulting from step (iii);
- (v) deprotecting the protected amino acid with a base;
- (vi) causing the amino acid sequentially following, in the target sequence, 10 the sequentially preceding amino acid coupled to the solid phase to react with the deprotected amino group of the sequentially preceding amino acid, the sequentially following amino acid having its amino group protected by a base-labile protecting group;
- (vii) deprotecting the protected amino acid group with base;
- 15 (viii) repeating steps (vi) and (viii) as often as necessary; and
- (ix) cleaving the acid labile bond with acid or by transesterification,

characterised in that a compound comprising a boronic acid group $[-B(OH)_2]$ or a derivative thereof, especially an ester, is incorporated in the solid phase-linked 20 compound prior to cleavage of the acid labile bond.

As described above in relation to the method of making a peptide or peptide-containing compound, step (ii) of the method may comprise reacting an optionally derivatised carboxy group of an amino acid with a functional group coupled directly 25 or indirectly to a solid phase, for example by a method known *per se* in solid phase chemistry. The amino acid may be the compound comprising a boronic acid or ester group, i.e. an amino acid boronic acid or ester. Alternatively, step (ii) may comprise reacting the compound comprising a boronic acid or ester group in the form of an amino acid or peptide boronic acid or ester with a diol coupled to the solid phase. In 30 either case that an amino acid (or peptide) boronic acid or ester is used, the process is suitable for making a peptide boronate inhibitor of a serine protease catalytic site, optionally in the synthesis of a bifunctional serine protease inhibitor.



The other variants described above of the method of making a peptide or peptide-containing compound are applicable to said first class of solid phase synthetic methods.

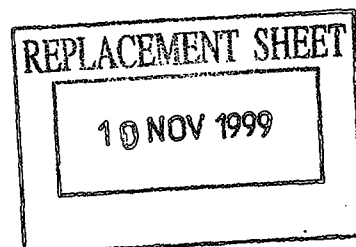
5 A second class of solid phase synthetic methods comprises performing the following steps to make a target amino acid sequence:

- (i) providing a solid phase having coupled thereto functional groups capable of reacting with a carboxyl group;
- 10 (ii) causing a compound reactive with the functional groups and comprising an amino group protected by an acid-labile protecting group to react with the functional groups, to form a base labile bond;
- (iii) deprotecting the amino group with an acid;
- (iv) causing the carboxyl group of an amino acid whose amino group is
15 protected by an acid-labile protecting group to react with the deprotected amino group resulting from step (iii);
- (v) deprotecting the protected amino acid with an acid;
- (vi) causing the amino acid sequentially following, in the target sequence, the sequentially preceding amino acid coupled to the solid phase to react with
20 the deprotected amino group of the sequentially preceding amino acid, the sequentially following amino acid having its amino group protected by an acid-labile protecting group;
- (vii) deprotecting the protected amino acid group with acid;
- (viii) repeating steps (vi) and (viii) as often as necessary; and
- 25 (ix) cleaving the base labile bond with base or by transesterification,

characterised in that a compound comprising a boronic acid group $[-B(OH)_2]$ or a derivative thereof, especially ester, is incorporated in the solid phase-linked compound prior to cleavage of the acid labile bond.

30

The above-described variants of the first class of solid phase synthetic methods are applicable also to the second class.



As described above in relation to the method of making a peptide or peptide-containing compound, methods of the first and second classes of solid phase synthetic methods may comprise a step of coupling to a solid phase-linked compound prepared by previous steps of the process a compound other than an amino acid or peptide.

5

The invention may be applied to the synthesis of bifunctional serine protease inhibitors, as described below.

BI-FUNCTIONAL SERINE PROTEASE INHIBITORS

10

The bifunctional serine protease inhibitors comprise:

(a) a catalytic site-directed moiety (CSDM) which binds to and inhibits the active site of a serine protease;

15

(b) an exosite associating moiety (EAM); and, optionally,

(c) a connector moiety bonded between the EAM and the CSDM,

the CSDM and the EAM being capable of binding simultaneously to a molecule of the serine protease.

20

The Catalytic Site-Directed Moiety (CSDM)

The catalytic site-directed moiety (CSDM) binds to and inactivates the catalytic site of a serine protease enzyme. The structure of the CSDM is not critical to the invention. It may comprise the amino acid sequence of any known inhibitor of a serine protease catalytic site, for example.

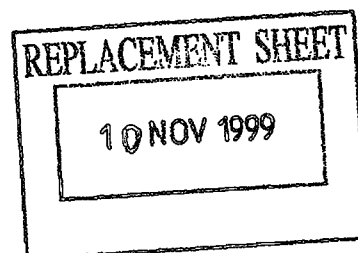
25

One class of CSDMs is included in the following Formula I:



30

wherein aa^1 , aa^2 , and aa^3 represent natural or unnatural acid residues and $(aa^4)_m$ one or more optional amino acid residues linked to the amino group of aa^3 . Alternatively any one or more aa groups may be analogues of amino acid residues in which the α -



hydrogen is replaced by a substituent. The sequence of amino acids and/or amino acid analogues binds to the serine protease active site. Suitable sequences are described later in this specification. X represents H or a substituent on the N-terminal amino group. Z is -COOH or a C-terminal extension group (carboxy replacement group), for example as
 5 known in the art. In preferred compounds Z is a heteroatom acid group, e.g. -B(OH)₂, -P(OH)₂ or PO(OH)₂, or a derivative thereof, for example a carboxylic acid ester, a dioxo-boronate [-B(Osubstituent)₂] or a phosphate [-PO(Osubstituent)₂] or BF₂. Preferred heteroatom analogue groups are -B(OH)₂ and -P(O)(OH)₂; a less preferred heteroatom analogue group is S(O)₂OH. Amongst other possible Z groups there may be
 10 mentioned -CN, -COCH₂Cl and -COCH₂F. In preferred embodiments m is from 0 to 7 and more usually 0 to 5, e.g. 0, 1 or 2, especially 0. Normally, n=1.

In one class of compounds, the (aa²)-(aa¹) natural peptide linkage is replaced by another linkage (ψ). Additionally or alternatively other natural peptide linkages may
 15 be replaced by another linkage.

Catalytic site inhibitors of serine proteases are well known in the art. A short review of serine protease inhibitors, i.e. inhibitors of the serine protease catalytic site, is to be found in EP-B-145441, which patent discloses a class of serine proteases having a C-
 20 terminal boron group. Other patent specifications describing serine protease inhibitors include EP 293881, EP 471651 (equivalent to US 5288707), EP 235692, US 4963655 and WO 89/09612 (concerned particularly with inhibitors of Factor VII/VIIa in the [TF:VII/VIIa] complex).

25 For inhibitors of trypsin-like enzymes, preferred classes of P1 residues of CSDMs are (i) Arg, Lys and their analogues, and (ii) hydrophobic residues; further description of preferred P1 groups for thrombin which are also for other trypsin-like enzymes may be found in the aforesaid specification entitled "Thrombin Inhibitors" and in PCT/GB96/00352. Chymotrypsin-like serine proteases bind preferentially to CSDMs
 30 having phenylalanine-like and alanine-like side chains on the P1 residue. The following table A indicates the most preferred (P4)P3P2 residues for eight particular serine proteases:

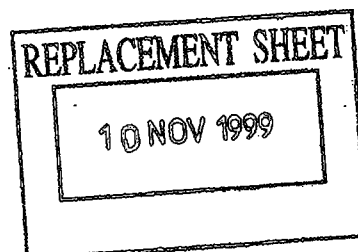


Table A

Enzyme	Residue Sequence
Thrombin	D-Phe-/substituted D-Phe-/D-Dpa-/Dba-/Pms-/α-Nal-/β-Nal-/TMSal-/Chg-/Phg-/D-Tiq-/para-ether of D-Tyr-/NaSO ₂ -Pro
Factor Xa	IleuGluGly, PyroGluGly, ArgGly, ChaGly, LeuArg
Factor VIIa	L-PhePhe, NalPhe, D-TiqPhe, NalThr, NalPhg
Factor IXa	ValVal
Factor XIa	K-BzlGluGly, Glu(OBzl)Ala, GlyArg, GlyLys
Factor XIIa	GlnGly
Urokinase	PhePro, GluGly
Protein Ca	LeuSerThr

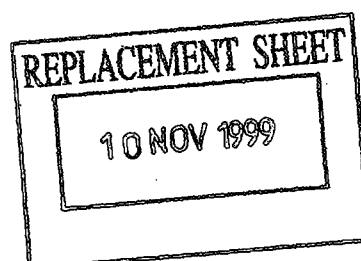
In each case, a preferred amino acid may be replaced by an analogue thereof.

5

The Exosite Associating Moiety (EAM)

The exosite associating moiety (EAM) is a moiety which binds to an exosite (ABE) of a serine protease. Thrombin has a well defined exosite to which there binds, in addition to a fibrinogen amino acid sequence C-terminal to the thrombin cleavage site, non-substrate ligands of thrombin such as hirudin. Hirudin sequences such as Hir⁵³⁻⁶⁴ have been used in bifunctional peptides named "hirulogs". The hirulogs are described in US 5196404; a further description of thrombin EAMs may be found in the aforesaid UK patent application entitled "Thrombin Inhibitors". EAMs for thrombin are often termed "anion binding exosite associating moieties" (ABEAMs).

The implications from the crystal structure of FXa (Padmanabhan K et al, "Structure of Human Des (1-45) Factor Xa at 2.2Å Resolution" *J Mol. Biol* 1993, 232, 947-966) are that the sequences 35-41 and 70-81, containing 8 acidic residues constitute a cation binding exosite. The natural polypeptide inhibitors antistasin and ghilanten contain the electropositive cation exosite associating sequences ¹⁰⁸CRPKRKLIPR¹¹⁷ and ¹⁰⁸CKPKRKLVPV¹¹⁷ respectively.



It is clear that serine protease interactions in P' sites (C-terminal to the cleavage point) can be as important to specificity as those at P sites (for example Ding, L.; Coombs, G.S.; Strandberg, L.; Navre, M.; Coreyu, D.R. and Madison, E.L. Origins of the
 5 Specificity of Tissue-type Plasminogen Activator, Proc.Natl.Acad.Sci. 1995, 92, 7627-7631), and larger ligands need to be compared spanning both sites, as are available rapidly from library screening. Recently (Lawson 1992) has shown that screening of peptide sequences, containing significantly P' as well as P binding units, allows detection of FVIIa/TF activity where existing substrates were too insensitive.
 10 Peptide libraries are showing great efficacy for screening for biological activity in a variety of applications (e.g Eichler, 1994) and are useful for the present invention.

The invention contemplates that sequences C-terminal to the cleavage point of other serine proteinase substrates form an EAM, as follows:

15

Table B

Enzyme	Natural Substrate	Cleavage Site (—)
Factor Xa	Prothrombin	YIDGR--IVEGSDAEIGMSPWQ
Factor Xa	Prothrombin	AIEGR--TATSEYQTFFNPRTFGS
Factor Xa	Factor VII	SKPQGR--IVGGKVC
Factor VIIa	Factor X	NLTRR--IVGGQECKDGEC
Factor IXa	Factor X	NLTRR--IVGGQECKDGEC
Factor XIa	Factor IX	SKLTR--AEAVFPDVDYVN
Factor XIIa	Factor IX	FNDFTR--VVGGEDAKPGQF
Factor XIIIa	Factor XI	KIKPR--IVGGTASVRGE
Factor XIIIa	Plasma Kallikrein	KTSTR--IVGGTNSSWGE
Protein Ca	Factor VIII	ELR--MKNNEEAEDYDDDLTDSEMD
t-PA/UK	Plasminogen	PKKCPGR--VVGCCVAHPHSWPWQVSLRT

The Connector Moiety

REPLACEMENT SHEET

10 NOV 1999

The compounds of the present invention may contain a connector moiety which interconnects the CSDM and the EAM, the connector moiety being capable of permitting the CSDM and the EAM to bind simultaneously to a molecule of the
 5 respective serine proteinase inhibitor. In the case of thrombin, the connector moiety is bonded to the CSDM as an N-terminal extension or as or through a side chain thereof.

The connector moiety may be bonded to the CSDM either as a C-terminal extension or, alternatively, as an N-terminal extension or as or through a side chain. However, if
 10 the compound is a thrombin inhibitor, the connector moiety may not be a C-terminal extension of the CSDM.

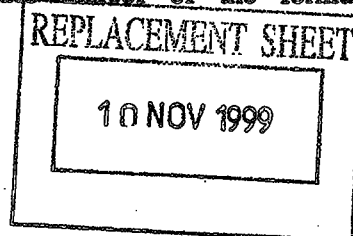
Especially if the connector moiety is an N-terminal extension of the CSDM, or if it is comprised in a side chain thereof, it desirably comprises an amino acid sequence
 15 containing at least two adjacent Gly residues, *e.g.* at its N-terminal end. In one class of compounds the connector preferably comprises a peptide "spacer" and a non-peptide "linker". A representative connector structure is:



20

wherein λ represents a non-peptide linker and σ a spacer comprising a sequence of amino acids, λ and σ suitably being joined by a peptide bond. The spacer σ is preferably linked to the EAM and the linker λ to the CSDM, although compounds in which σ is linked to the CSDM and λ to the EAM form a less preferred embodiment
 25 included in the invention.

The linker is typically a residue of a compound having functional groups to react with the N-terminal amino group of the spacer and a functional group of the CSDM, such as the N-terminal group, for example. A preferred linker, therefore, has two carboxylate
 30 groups, *e.g.* is a dicarboxylic acid which can form amide bonds with the N-terminal amino groups of the CSDM and the spacer. Particularly preferred linkers are a residue of glutaric acid ($\text{HO}_2\text{C}(\text{CH}_2)_3\text{CO}_2\text{H}$) and homologues thereof of the formula



(HO₂C(CH₂)_hCO₂H) wherein h is an integer of 2 or from 4 to 6. The alkylene residue [- (CH₂)₂₋₆-] may be substituted by one or more substituents which do not sterically hinder the linker, whereby the desirable flexibility of the linker is maintained.

- 5 Less preferably, the linker may comprise for example the residue of another compound having two carboxyl groups whose carbon atoms are separated by from 2 to 6 atoms.

The amino acid sequence of the spacer is not critical to the invention but it preferably comprises at least two adjacent Gly residues, normally at its N-terminal end. The length
10 of the spacer is dependent upon *inter alia* the position on the CSDM to which the linker is attached.

A further description of connector moieties suitable for peptide thrombin inhibitors can be found in the aforesaid UK patent application entitled "Thrombin Inhibitors" filed on
15 the same day as the present application.

The connector moiety may have one or more natural amide bonds replaced by other linkages.

20

SYNTHESIS

N-terminal coupling methods may be used in making the bifunctional inhibitors described above. In one embodiment the CSDM, including any directly attached
25 amino acid(s), is synthesised by N-terminal coupling. This technique is especially useful if the CSDM has C-terminal heteroatom group; in this method the resin bound peptide chain made using N-terminal coupling is derivatized to activate its carboxy termini, then a free α -aminoboronate ester or acid is coupled to the resin bound sequence. Finally the peptide boronate (comprising the CSDM) is cleaved from the
30 resin by strong acid (e.g. HF or TFA) prior to being joined to the remainder of the final product.

REPLACEMENT SHEET

10 NOV 1999

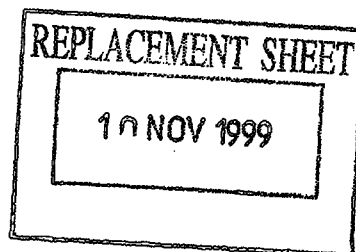
When synthesising compounds whose CSDM contains a P1-P2 non-natural amide bond, it is convenient to premake as intermediates the binding subsite affinity moiety [X-(aa⁴)_m-(aa³)_n-(aa²) of Formula I] and the specificity pocket affinity moiety with its attached C-terminal group [(aa¹)-Z of Formula I]. The two intermediates contain
 5 suitable functional groups to react together to form the target non-natural amide bond [ψ] and are caused or allowed to react together to form the compound (or a precursor thereof to undergo one or more further functional group transformations).

Suitable synthetic techniques for making peptides containing a non-amide bond ψ are
 10 described in PCT/GB96/00352.

In an exemplary process for synthesising a serine proteinase inhibitor in which the connector moiety and its attached EAM form an N-terminal extension of the CSDM, the EAM is prepared by Fmoc solid phase peptide chemistry, *e.g.* using an Fmoc-polyamide
 15 continuous flow method. A suitable solid phase for this purpose is the pre-derivatised solid support Fmoc-Leu-PEG-PS. The peptide-conjugated resin is subsequently treated with, for example, glutaric anhydride, one carboxyl group of which reacts with the N-terminal amino group of the EAM. A pre-synthesised peptide boronate CSDM is reacted with the resin/peptide/glutaric acid conjugate to form the final compound, which
 20 is cleaved from the resin, for example by treatment with 100% TFA.

Another method of using peptide boronate esters in making the inhibitors is a completely novel technique in which boronic acids [-B(OH)₂] are directly esterified onto diols coupled to a resin. Chain extension is continued from the amino group of the
 25 amino acid by, for example, standard Fmoc chemistry. The boronic acid ester is cleaved from the resin by acid (*e.g.* TFA) to give the peptide boronate [peptide-B(OH)₂], or by transesterification, for example, by concentrated solution of a hindered diol, such as pinanediol, for example.

30 The invention therefore includes a method of making a said bifunctional inhibitor comprising performing the following steps to make a target amino acid sequence:



- (i) providing a solid phase having coupled thereto functional groups capable of reacting with an amino group or, preferably, with a carboxyl group or a reactive derivative thereof;
- 5 (ii) causing the amino or carboxyl group (which may be in the form of a reactive derivative thereof) of a terminal amino acid of an amino acid sequence of a compound of the invention selectively to react with said functional groups;
- 10 (iii) coupling the amino acid sequentially following, in the target sequence, the sequentially preceding amino acid coupled to the solid phase to said preceding amino acid; and
- (iv) repeating step (iii) as often as necessary.

15 In step (i), the functional groups coupled to a solid phase may be on a moiety which is incorporated in the end product compound, e.g. may be an amino group (which may be derivatised) of an amino acid coupled directly or indirectly to the solid phase.

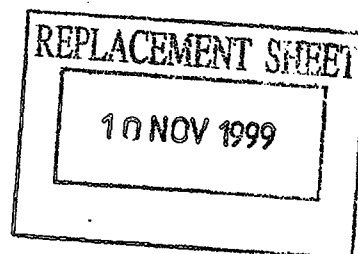
20 One or more additional steps may be, and often are, included in the method to obtain the compound of the invention. Thus, preferred methods include, when desired, a step (v) of coupling a said sequentially following amino acid of a step (iii) to said preceding amino acid of the step through a compound having two functional groups capable of reacting with an amino group, whereby one of said functional groups becomes bonded to the amino group of said preceding amino acid and the other to the amino group of

25 said following amino acid.

The sequentially following amino acid of a step (iii) may be part of a larger moiety, e.g. of an amino acid sequence optionally containing a replacement for a natural peptide bond.

30

In the method, any one or more carboxylate groups reacted with an amino group may be in the form of a reactive carbonyl-containing derivative thereof, such as an activated carboxyl group, for example an acid anhydride.



Before use, the final compound of the solid phase synthesis is cleaved from the solid phase, for example in a manner known *per se*. The cleaved compound may be subjected to one or more further chemical reactions before the end product compound is obtained.

5

In a preferred embodiment, the terminal amino acid reacted with the functional groups attached to the solid phase is the C-terminal amino acid of the EAM and step (iii) is repeated to couple successive amino acids of the EAM sequence and successive amino acids of any contiguous connector peptide, to form an uninterrupted amino acid sequence.

10

The final amino acid of the uninterrupted amino acid sequence coupled to the solid phase may be reacted with a compound having two carboxylate groups or reactive derivatives thereof, for example the anhydride of a dicarboxylic acid, to bond one of the two carboxylates to the amino group of the final amino acid. The unreacted carboxylate or carboxylate derivative is typically reacted with the amino group of an amino acid, which is normally the N-terminal amino acid of the CSDM. In this latter case, the amino acid may already be bonded to the remainder of the CSDM, i.e. the CSDM may be separately made in whole (or in part) for joining to the unreacted carboxylate (derivative). The compound having two carboxylate groups is preferably a linker as described above.

15

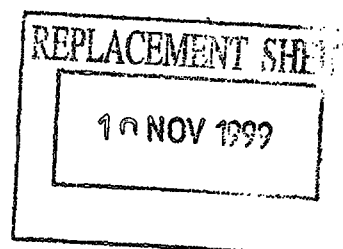
20

In some preferred methods, there is used a preformed CSDM having a heteroatom group in place of a C-terminal carboxy group. The heteroatom group is preferably a boronate or boronate derivative as described above.

25

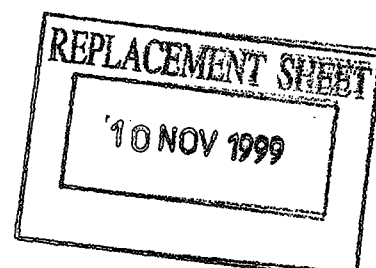
An amino acid or other moiety reacted with the solid phase material (the solid phase and any attached molecules) desirably has all its reactive functional groups which could interfere with the synthesis protected, other of course than the group to be reacted with the solid phase material. Any protected functional group of the reacted amino acid or moiety which is subsequently itself to be reacted is deprotected before it is subjected to reaction.

30



A first preferred method, therefore, comprises:

- 5
- (i) providing a solid phase having coupled thereto functional groups capable of reacting with a carboxyl group or a reactive derivative thereof;
- (ii) contacting the solid phase with the C-terminal amino acid of an EAM, the amino acid having a protected amino group and optionally a derivatised carboxy group, and causing or allowing the carboxy groups of the amino acid molecules to react with the functional groups of the solid phase;
- 10
- (iii) deprotecting the amino groups of the reacted amino acid, whereby the solid phase becomes provided with free amino groups;
- (iv) repeating steps (ii) and (iii) with successive amino acids of the EAM and optionally of a contiguous spacer peptide to form on the solid phase an amino acid sequence from the C-terminal of the EAM to, at the free end of the sequence, the N-terminal of the spacer;
- 15
- (v) contacting the solid phase with a linker compound having two carboxyl groups or reactive residues thereof, and causing or allowing linker carboxy groups or reactive carboxy residues to react with the N-terminal amino groups of the spacer sequence;
- 20
- (vi) contacting the solid phase having the linker compound coupled thereto with the N-terminal amino acid of a CSDM sequence and causing or allowing the amino groups of the amino acid molecules to react with the carboxy groups or reactive carboxy residues of the linker compound, the N-terminal amino acid of the CSDM sequence optionally being part of a complete CSDM;
- 25
- (vii) if necessary, repeating steps (ii) and (iii) with successive amino acids of the CSDM to complete the CSDM sequence; and
- 30



(viii) cleaving the resultant compound from the functional groups of the solid phase.

A second preferred method comprises:

5

(i) providing a solid phase having coupled thereto functional groups capable of reacting with a carboxyl group or a reactive derivative thereof;

10

(ii) contacting the solid phase with the C-terminal amino acid of an EAM, the amino acid having a protected amino group and optionally a derivatised carboxy group, and causing or allowing the carboxy groups of the amino acid molecules to react with the functional groups of the solid phase;

15

(iii) deprotecting the amino group of the reacted amino acid, whereby the solid phase becomes provided with free amino groups;

20

(iv) repeating steps (ii) and (iii) with successive amino acids of the EAM to form on the solid phase an amino acid sequence from the C-terminal of the EAM to, at the free end of the sequence, the N-terminal of the EAM;

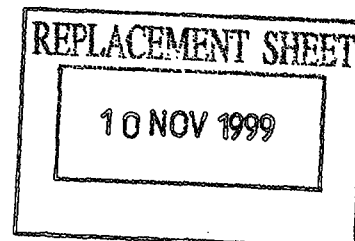
25

(v) contacting the solid phase with a linker compound having two carboxyl groups or reactive residues thereof, and causing or allowing linker carboxy residues to react with the N-terminal amino groups of the EAM;

30

(vi) optionally contacting the solid phase having the linker compound coupled thereto with the N-terminal amino acid of a peptide spacer sequence and causing or allowing the amino groups of the amino acid molecules to react with the carboxy groups or reactive carboxy residues of the linker compound;

(vii) optionally repeating steps (ii) and (iii) with successive amino acids of the spacer and then repeating step (ii) with the N-terminal amino acid of a CSDM sequence, the N-terminal amino acid of the CSDM sequence optionally being part of a complete CSDM;



(viii) if necessary, repeating steps (ii) and (iii) with successive amino acids of the CSDM to complete the CSDM sequence; and

5 (ix) cleaving the resultant compound from the functional groups of the solid phase.

In either of the preceding methods the synthesised compound is preferably cleaved from the solid phase by acid.

10

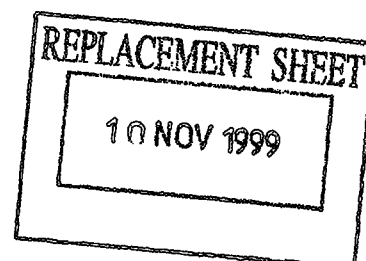
The preceding methods preferably involve the use of a CSDM amino acid or amino acid sequence (e.g. a complete CSDM) having a C-terminal boron group.

15 In the first and second preferred methods the functional groups coupled to a solid phase may be part of a moiety which is incorporated in the end product compound, e.g. may be an amino group (which may be derivatised) of an amino acid coupled directly or indirectly to the solid phase.

20 In one class of processes of the invention, the functional groups coupled to the solid phase are part of an amino acid boronate which is incorporated in the end product compound, i.e. the solid phase has coupled thereto a diol to which is bound an amino acid boronate.

25 In another class of methods, an amino acid whose side chain has an amino or carboxyl group is coupled to a solid phase through the carboxyl group or amino group to the side chain. Chain extension is carried out from one of the functional groups of the amino acid, for example Fmoc synthesis from the amino group. The other functional group is then reacted with some other constituent part of the end product, for example an amino acid boronate (to form the P1 residue of the CSDM). Solid phase synthesis
30 of boron-containing peptides is, however, of applicability to any such peptides, and not only to the serine protease inhibitors of the invention.

Use



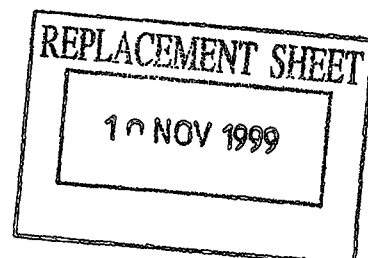
The bifunctional inhibitors made according to the present invention are useful as inhibitors or substrates of serine proteases, e.g. thrombin, and may be used *in vitro* or *in vivo* for diagnostic and mechanistic studies of such enzymes. More generally, the novel peptides may be useful for research or synthetic purposes. Furthermore, because of their inhibitory action, the inhibitors are useful in the prevention or treatment of diseases caused by an excess of thrombin or another serine proteases in a regulatory system particularly a mammalian system, e.g. the human or animal body, for example control of the coagulation system. The pharmaceutically useful compounds have a pharmaceutically acceptable group as any N-terminal substituent (X).

The anti-thrombotic compounds may be employed when an anti-thrombogenic agent is needed. Generally, these compounds may be administered orally or parenterally to a host in an effective amount to obtain an anti-thrombogenic effect. In the case of larger mammals such as humans, the compounds may be administered alone or in combination with one or more pharmaceutical carriers or diluents at a dose of from 0.02 to 10mg/Kg of body weight and preferably 1-100mg/Kg, to obtain the anti-thrombogenic effect, and may be given as a single dose or in divided doses or as a sustained release formulation. When an extracorporeal blood loop is to be established for a patient, 0.1-10mg/Kg may be administered intravenously. For use with whole blood, from 1-100 mg per litre may be provided to prevent coagulation.

Pharmaceutical diluents or carriers for human or veterinary use are well known and include sugars, starches and water, and may be used to make acceptable formulations of pharmaceutical compositions (human or veterinary) containing one or more of the subject peptides in the required pharmaceutically appropriate or effective amount or concentration. The pharmaceutical formulations may be in unit dosage form. Formulations of the compounds include tablets, capsules, injectable solutions and the like.

30

The anti-coagulant compounds may also be added to blood for the purpose of preventing coagulation of the blood in blood collecting or distribution containers, tubing or implantable apparatus which comes in contact with blood.



The methods of the invention are useful for the synthesis of serine protease inhibitors and other compounds. They are useful in combinatorial chemistry.

5 The invention will be further described and illustrated by the Examples which now follow.

EXAMPLES

10

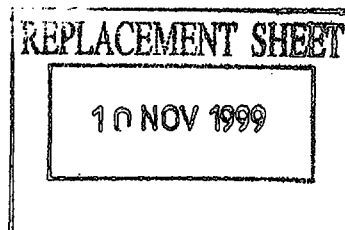
In the examples, amino acid residues are of L-configuration unless otherwise stated.

1. $[-D\text{-Phe-Pro-BoroBpgOPin}] \text{CO}(\text{CH}_2)_3\text{COGly}_2\text{Gln}(\text{Tyr}^{63})\text{Hir}^{51-64}$

15 a. $\text{GlyGlyGln}(\text{Tyr}^{63})\text{Hir}^{51-64}$

$\text{GlyGlyGln}(\text{Tyr}^{63})\text{Hir}^{51-64}$ which has the amino acid formula: H-Gly-Gly-Gln-His-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Tyr-Leu-OH, was prepared by solid phase peptide chemistry on a Milligen® 9050 PepSynthesizer using an Fmoc-polyamide continuous
20 flow method and proprietary 9050 Plus on column monitoring software. Pre-derivatised solid support, Fmoc-Leu-PEG-PS (1.6g, 0.22meq/g) was used throughout; Fmoc-Leu-PEG-PS comprises polyethylene glycol derivatised polystyrene with HMBA linker. Fmoc groups were removed using 20% piperidine in DMF. Fmoc-amino acids (4 equiv.) as their pentafluorophenyl esters with side chain protection where appropriate
25 (e.g. Fmoc-L-Tyr(tBu)OPfp, Fmoc-L-Glu(tBu)OPfp, Fmoc-L-Asp(tBu)OPfp, Fmoc-L-Asn(Trt)OPfp and Fmoc-His(boc)OPfp, were coupled sequentially. Once the required peptide sequence was complete the N-terminal Fmoc group was removed using 20% piperidine in DMF. A positive ninhydrin test indicated that the Fmoc group had been removed. The peptide-conjugated resin was subsequently decanted on a filter and
30 washed 'off line' with dichloromethane, methanol and dichloromethane before being dried *in-vacuo* for a few hours.

b. $\text{HO}_2\text{C}(\text{CH}_2)_3\text{COGlyGlyGln}(\text{Tyr}^{63})\text{Hir}^{51-64}$



The peptide obtained in Example 1a was suspended in DMF (5ml) and treated with glutaric anhydride (300mg) and 4-methyl-morpholine (200mg) in a round bottomed flask (25ml). The reaction mixture was swirled overnight. The resin was washed with
 5 DMF, DCM and MeOH, and then dried *in-vacuo* overnight to obtain the target compound.

c. H-D-Phe-ProBoroBpgOPin

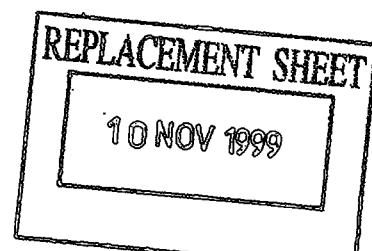
10 H-D-Phe-ProBoroBpgOPin was prepared by adding a 40% solution of HBr in acetic acid (20ml) to Cbz-D-Phe-Pro-BoroBpgOPin (2g) in a round bottomed flask (100ml) fitted with a septum and flushed with nitrogen. The flask was swirled to effect complete dissolution of the protected tripeptide. When the gas evolution ceased after approximately 30 minutes, anhydrous ether (200ml) was added and the reaction mixture
 15 was stored in a refrigerator for 4 hours. The reaction mixture was filtered, the residue was dissolved in EtOH (1ml) and dry ether was added to precipitate the produce (800mg) as a white solid (M+H), 516; Tlc (C/M/A, 95/5/3), Rf=0.05.

d. [-D-PheProBoroBpgOPin]CO(CH₂)₃COGly₂Gln(Tyr⁶³)Hir⁵¹⁻⁶⁴

20

To synthesise [-D-PheProBoroBpgOPin]CO(CH₂)₃COGly₂Gln(Tyr⁶³)Hir⁵¹⁻⁶⁴, the dry resin HOCO(CH₂)₃COGly₂Gln(Tyr⁶³)Hir⁵¹⁻⁶⁴ was suspended in DMF (10ml), before TBTU (129mg, 0.4mmol) and H-D-Phe-ProBoroBpgOPin (230mg, 0.4mmol) were added to the reaction mixture. After 5 minutes stirring, triethylamine (40mg, 0.04mmol)
 25 was added and the flask left stirring overnight.

The fully protected peptide resin was washed with dichloromethane, methanol and dichloromethane and then dried under vacuum. Cleavage from the resin with simultaneous deprotection of side chain protecting groups was achieved by treating the
 30 resin with 100% TFA for two hours. TFA was removed and the free peptide with a C-terminal carboxylic acid was generated by precipitation with cold dry ether. The crude peptide was collected by filtration and washed with further portions of ether.



Purification of the crude peptide was carried out by reversed-phase HPLC using a Vydac™ C-18 preparative column (TP silica, 10µm, 25mm x 300mm). The column was eluted with a 30-90% linear gradient of solvent A (0.1% TFA in water) and solvent B (0.1 TFA in acetonitrile). The column eluants were monitored at 230nm, and fractions
5 were collected appropriately. The purity of the products were determined by analytical RP-HPLC and mass spectrometry.

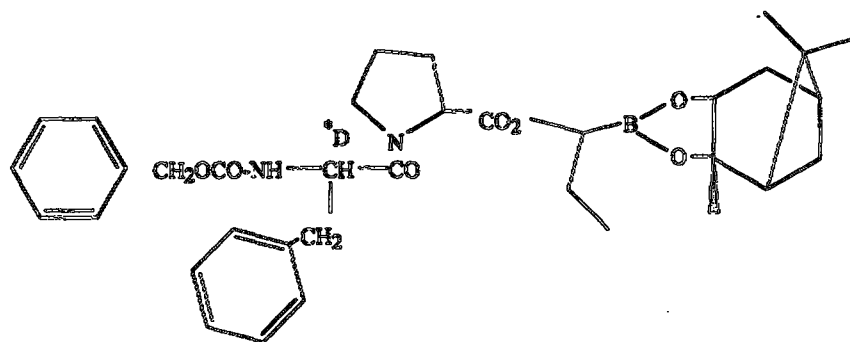
2. Cbz-D-Phe-Pro-ψ(CO₂)-boroethylglycine pinanediol

10

a. Cbz-D-Phe-Pro-ψ(CO₂)-BoroEtg pinanediol

1-Chloroethane-pinanediol boronate ester (0.321g, 1.25x10⁻³mol) added with stirring to Cbz-D-Phe-Pro-OH (0.6g, 1.52x10⁻³mol). When the addition had been completed,
15 DBU (0.23g, 1.52mmol) in CH₂Cl₂ was added to the mixture and allowed to stir at room temperature, before being left to stir for an extended period at 4°C before workup. The opaque liquid was washed with HCl (0.1M, 2x50 ml), NaHCO₃ (1%, 50ml). The organic layer was dried by vigorous stirring over anhydrous MgSO₄, and filtered off, to remove the desiccant. The filtrate was concentrated under reduced pressure on a rotary
20 evaporator, to afford a thick, viscous residue. Preliminary examination by ¹H N.M.R. showed the required crude product. The crude sample was dissolved in a small amount of MeOH, applied to the sephadex® LH20 column, and then eluted with a pump using the same solvents. The elution profile was followed with the aid of a U.V. lamp (226nm) and recorder. The void volume, fraction 1-6, and a further bulk volume were
25 collected. From the shape of the chromatogram, it was deemed that fractions 1-6 would be the most likely fractions in which the tripeptide may be found. The fractions were concentrated individually to afford clear slightly coloured viscous residues. One fraction containing the bulk of the material when placed under high vacuum was later afforded as a slightly crystalline product (0.269 yield of 35%). N.M.R., FABMS (Fast Atom
30 Bombardment Mass Spectrometry) and C, H, N were very strong (good) indicators that the compound has been formed.





b. H-Phe-Pro-ψ(CO₂)-BoroEtg pinanediol

5 Cbz-D-Phe-Pro-ψ(CO₂)-BoroEtg pinanediol (from Example 2a) was dissolved in MeOH (30ml) and treated with 10% Pd/C, and purged with argon with stirring, the flask evacuated and pumped with H₂ with stirring for 5H. Ninhydrin staining indicated deprotected product on TLC. The solution was purged with argon for 10 min, filtered and concentrated under reduced pressure to afford a thick black oil, which was dissolved
 10 in CHCl₃, filtered and concentrated. ¹H N.M.R. of the crude product indicated no protected product. The residue from above was chromatographed on a Sephadex LH20 chromatography column. ¹H (60 MHz) N.M.R. showed that the isolated compound displayed many of the characteristics expected on the basis of the putative structure. 122mg of the free amino boronate ester was isolated.

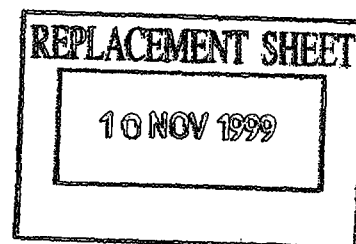
15

3. H-Phe-L-Glu-BoroBpgOPin

a. Fmoc-L-Glu(PEG-PS)OH

20

Tetrakis(triphenylphosphine) palladium(0) [PdP(Ph₃)₄] (1g) was dissolved under Ar in a solution of CH₃Cl containing 5% acetic acid and 2.5% N-methylmorpholine (30ml). This mixture was transferred under Ar to a flask containing Fmoc-L-Glu(PEG-PS)OAl (1.6g). The resin was left to stand for 2 hours with occasional gentle agitation.
 25 The resin was filtered on a sintered glass funnel and washed with 0.5% diisopropylethylamine and sodium diethyldithiocarbamate (0.5%w/w) in DMF (300ml) to remove the catalyst.



b. **Fmoc-L-Glu(PEG-PS)NHBoroBpgOPin**

The dry resin Fmoc-L-Glu(PEG-PS)OH (1.5g) was suspended in DMF(10ml) under
5 Ar. TBTU (129mg, 0.4mmol) and NH₂BoroBpgOPin (165mg, 0.5mmol)w were
added to the reaction mixture. After 5 minutes stirring, triethylamine (40mg,
0.4mmol) was added and the flask left stirring overnight. The resin was washed with
dichloromethane, methanol and dichloromethane and then dried under vacuum.

10 c. **H-Phe-L-Glu(PEG-PS)NHBoroBpgOPin**

H-Phe-L-Glu(PEG-PS)NHBoroBpgOPin was prepared by solid phase chemistry on a
Milligen 9050 peptide synthesiser.

Fmoc group was removed from the solid support Fmoc-L-Glu(PEG-
15 PS)NHBoroBpgOPin using 20% piperidine in DMF. Fmoc-Phe-OPfp was coupled to
the free N-terminus.

The protected peptide resin was washed with dichloromethane, methanol and
dichloromethane and then dried under vacuum.

20

d. **H-Phe-L-Glu-BoroBpgOPin**

Cleavage of the peptide from the resin was achieved by treating the resin with 100%
TFA for two hours. TFA was removed and the free peptide H-Phe-Glu-NH-
25 BoroBpgOPin was generated by precipitation with cold dry ether. The crude peptide
was collected by filtration and washed with further portions of ether.

Purification of the crude peptide was carried out by reversed-phase HPLC using a
Vydac C-18 preparative column (TP silica:particle size 10mm; 25mmX300mm). The
30 column was eluted with a 30-90% linear gradient of solvent A(0.1%TFA in water)
and solvent B(0.1%TFA in acetonitrile). The column eluants were monitored at
230nm, and fractions were appropriately collected. The purity of the products were
determined by analytical RP-HPLC and mass spectrometry. Product H-Phe-Glu-NH-

REPLACEMENT SHEET

10 NOV 1999

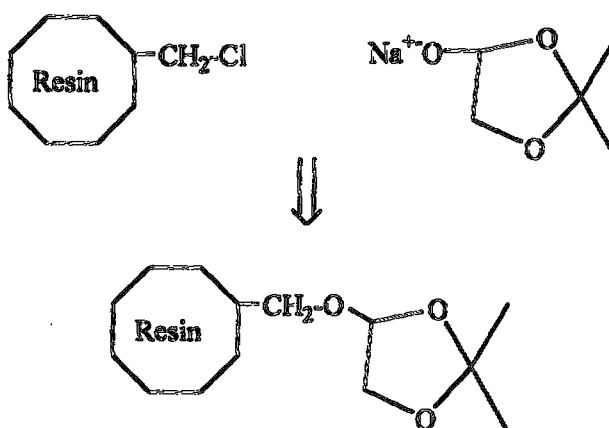
BoroBpgOPin, was obtained in 17% yield, 34mg, ES-MS: 626 $[M+Na]$; retention time analytical HPLC (4x250mm, Vydac, C-18 techsphere), eluted by 10-60% MeCN with 0.1% TFA in water with 0.1% TFA over 25 minutes, gave Rt 23.1 minutes.

5

4. Attachment of Boronic Acid to Merrifield Resin

a. Derivatisation of Resin with protected Diol (2,2-dimethyl-1,3-dioxolane-4-methanol

10



15

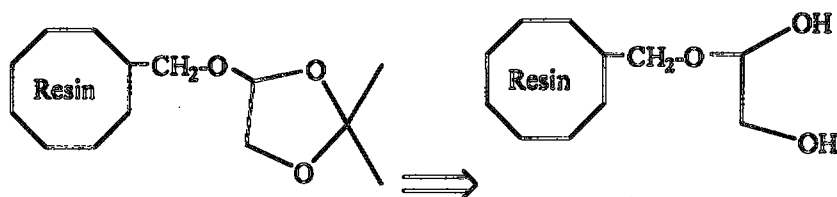
Na (solid, 8g) is added to 2,2-dimethyl-1,3-dioxolane-4-methanol (240ml), under argon gas, and the mixture stirred until it gives a clear solution. Merrifield Resin (Sigma, 1.1 MeQ. Cl per gram, 20g) is added and the mixture stirred overnight, then heated at 80°C for 24h.

Derivatised resin was collected by filtration, washed by 1,4-dioxane (1L), water (3x500ml), and MeOH:water (1:1, 3x500ml), MeOH (3x500ml) and dry ether (3x500ml). An infra red spectrum was obtained by powdering of 1.5-2mg of resin with KBr (dry, 300mg) and compacting into a disc, then scanning on a Perkin® 1600 Fourier Transform I.R. The derivatised resin (Fig. 2) compared to Merrifield resin (Fig. 1) shows distinct stretching signals 1050 to 1150 cm^{-1} (s) for ether stretching frequencies characteristic of a five membered ring; and dialkyl ether stretching at 1060 to 1150 cm^{-1} (s) for alkyl-alkyl stretching.

30



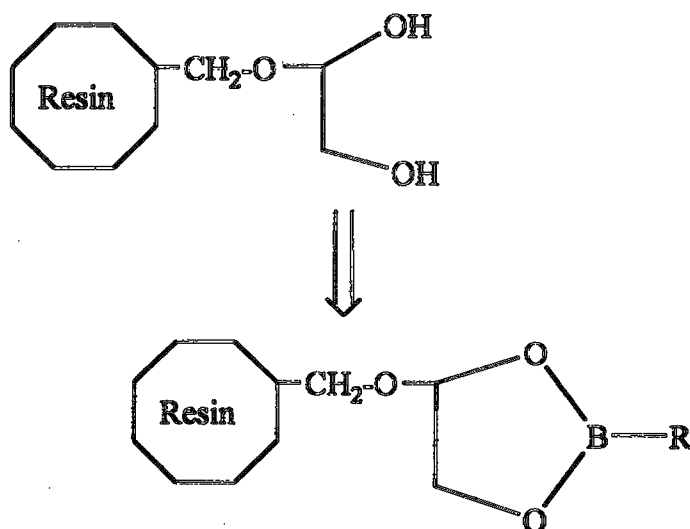
b. Deprotection



The derivatised resin was mixed with HCl (1.5M, 250ml) and 1,4-dioxane (250ml) and the suspension stirred and heated at 80°C. After 72h the resin was washed by water (500ml), MeOH (500ml), DCM (500ml) and Et₂O (500ml), then dried in the air. F.t.-I.R. spectrum of the resin shows distinct O-H stretching frequencies at 3400-3550cm⁻¹ (s), and a main peak at 3413.6 (Fig. 3); this peak is substantially larger than the signal at 2917.6cm⁻¹. In comparison the ether (Fig. 2) and Merrifield resin show only a weak $\cong$ 3400cm⁻¹ signal for background moisture.

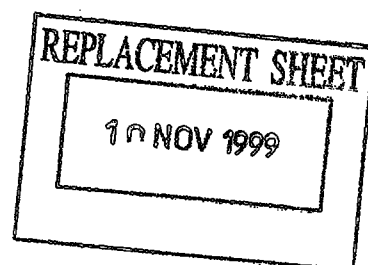
10

c. Reaction of the Derivatised Resin with a boronic acid



The diol resin (5g, 5.5mmol of diol) was suspended in THF (dry, 500ml) and phenylboronic acid (3.35g, 27.5mmol, 5 equivalents), and 4Å sieves (dried at 150°C). After stirring under argon overnight, the resin was filtered under argon in a closed system, washed by THF (500ml) and dried under vacuum. Ft-L.R. (Fig. 4) shows a

15



strong signal at 1026cm^{-1} (aryl-alkyl stretching frequency) for the phenyl ring and a weak signal at 3417cm^{-1} (compared to Fig 3, for the starting diol).

Ref. Leznoff, C.C. and Wong, J.Y., The use of Polymer Supports in Organic Synthesis.

- 5 III. Selective Chemical Reactions on One Aldehyde Group of Symmetrical Dialdehydes., Can.J.Chem., 1973, 51, 3756-3764.

*

*

*

10

Analytical and Activity Data

The following Table 1 contains activity data relating to the invention. In the Table, the designation "Z" denotes benzoyloxycarbonyl and "NHir" refers to normal hirudin.

- 15 "NHir49-64(des-S) refers to the amino acid sequence from amino acid 49 to amino acid 64 of normal hirudin in which the native Tyr(OSO₃H)⁶³ is replaced by Tyr.

- 20 The compounds listed in Table 1 were prepared by the same or analogous methods to the compounds of the preparation Examples 1 and 2 above or, in the case of intermediates, were obtained from sources.

The following techniques were employed for activity measurement:

25 Plasma thrombin time (TT)

A volume of 150 μ l of citrated normal human plasma and 20 μ l of buffer or sample were warmed at 37°C for 1 min. Coagulation was started by adding 150 μ l of freshly prepared bovine thrombin (5NIHu/ml saline) and the coagulation time was recorded on a coagulometer.

30

A phosphate buffer, pH7.8, containing 0.1% bovine serum albumine and 0.02% sodium azide was used. The samples were dissolved in DMSO and diluted with the buffer.



When no inhibitor was used DMSO was added to the buffer to the same concentration as that used in the samples. The inhibitor concentrations were plotted against the thrombin times in a semilogarithmic graph from which the inhibitor concentration that caused a doubling (40 sec) of the thrombin time was determined.

5

Determination of K_i

The inhibition of human α -thrombin was determined by the inhibition of the enzyme catalysed hydrolysis of three different concentrations of the chromogenic substrate S-2238.

10

200 μ l of sample or buffer and 50 μ l of S-2238 were incubated at 37°C for 1 min and 50 μ l of human α -thrombin (0.25 NIH μ /ml) was added. The initial rate of inhibited and uninhibited reactions were recorded at 4.5nm. The increase in optical density was plotted according to the method of Lineweaver and Burke. The K_m and apparent K_m were determined and K_i was calculated using the relationship.

15

$$V = \frac{V_{max}}{1 + \frac{K_m}{[S]} + \frac{(1 + [I])}{K_i}}$$

20

The buffer used contained 0.1M sodium phosphate, 0.2M NaCl, 0.5% PEG and 0.02% sodium azide, adjusted to pH 7.5 with orthophosphoric acid.

The samples consist of the compound disclosed in DMSO.

25

The reader is referred to Dixon, M and Webb, E. C., "Enzymes", third edition, 1979, Academic Press, the disclosure of which is incorporated herein by reference, for a further description of the measurement of K_i .

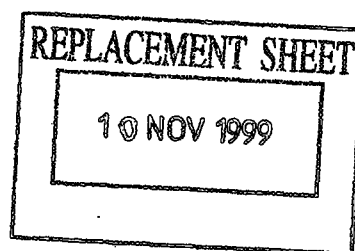


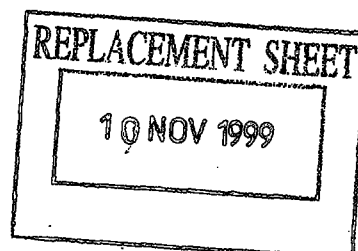
Table 1

	KI μ M	TT μ M
98 GGGDFEPIPL	n/e	100
99 [-BoroBrOPin]CO(CH ₂) ₃ COGGGDFEPIPL	n/e	58
105 GGGGDFEPIPL	n/e	94.9
106 GGGGGDFEPIPL	n/e	15
107 [-BoroBrOPin]OC(CH ₂) ₃ COGGGGDFEPIPL	n/e	58.4
108 [-BoroBrOPin]OC(CH ₂) ₃ COGGGGGDFEPIPL	n/e	63.4
114 GGNSHNDGDFEIPPEYL Hir ⁴⁹⁻⁶⁴	0.613	2
121 HO ₂ C(CH ₂) ₃ COGGGGGDFEPIPL	0.738	30.9
128 [-L-PheProBoroValOPin]OC(CH ₂) ₃ COG ₃ DFEPIPL	n/e 11.7	27.1
129 [-D-PheProBoroValOPin]OC(CH ₂) ₃ COG ₃ DFEPIPL	16.4	33.5
137 [-D-Phe-ProBoroEtgOPin]OC(CH ₂) ₃ COG ₃ DFEPIPL	1.23	10.2
166 [-D-Phe-ProBoroBpgOPin]OC(CH ₂) ₃ COG ₂ NHir ⁴⁹⁻⁶⁴ (des-S)	0.000649	0.029
167 Hir ⁴⁹⁻⁶⁴ (des-S)	n/e 0.155	N.T.
175 [-D-Phe-ProBoroBpgOPin]OC(CH ₂) ₃ COG ₄ NHir ⁴⁹⁻⁶⁴ (des-S)	0.00211	0.065
176 Z-D-Phe-Pro-BoroBpgOPin + Hir ⁴⁹⁻⁶⁴	0.0218	0.636
182 [-D-Phe-ProBoroCegOPin]OC(CH ₂) ₃ COGPGGNHir ⁴⁹⁻⁶⁴ (des-S)	0.00271	N.T.
184 HO ₂ C(CH ₂) ₃ COGPGGNHir ⁴⁹⁻⁶⁴ (des-S)	12.7	56.7
185 [-D-Phe-ProBoroCegOPin]OC(CH ₂) ₃ COGPG ₃ NHir ⁴⁹⁻⁶⁴ (des-S)	n/e 0.75	7.36
186 [-D-Phe-Pro-BoroCegOPin]OC(CH ₂) ₃ COGPG ₃ NHir ⁴⁹⁻⁶⁴ (des-S)	n/e 0.9	4.61
267 [-PheProBoroCegPin]OC(CH ₂) ₃ COG ₂ (EDFEPIPL)	0.762	4.9
268 [-Pgl ² (OEt) ₂]OC(CH ₂) ₃ COG ₂ (EDFEPIPL)	n/e 88.8	66.5
[-D-PheProBoroIrgOPin]OC(CH ₂) ₃ COG ₂ NHir ⁴⁹⁻⁶⁴ (des-S)	N.T.	N.T.

n/e = no effect

S n/e 11.7 = no effect up to a concentration of 11.7 μ M

N.T. = not tested



CLAIMS

1. A method of making a peptide or a peptide-containing compound, comprising performing the following steps to make a target amino acid sequence:

5

(i) providing a solid phase having coupled thereto functional groups;

10

(ii) causing a compound reactive with the functional groups selectively to react therewith, the reacted compound having a functional group capable of reacting with an amino group or with a carboxyl group or a reactive derivative thereof;

15

(iii) causing the amino or carboxyl group (which may be in the form of a reactive derivative thereof) of a terminal amino acid of a target amino acid sequence selectively to react with said functional groups of the reacted compound;

20

(iv) coupling the amino acid sequentially following, in the target sequence, the sequentially preceding amino acid coupled to the solid phase to said preceding amino acid;

(v) repeating step (iv) as often as necessary; and

25

(vi) cleaving a solid phase-linked compound prepared using steps (i)-(iv) from the solid phase by the action of an acid or base,

30

characterised in that a compound comprising a boronic acid group $[-B(OH_2)]$ or an ester or other derivative thereof is incorporated in the solid phase-linked compound prior to its cleavage from the solid phase.

2. A method of claim 1 in which step (ii) comprises reacting an amino group or an optionally derivatised carboxy group of an amino acid with a functional group

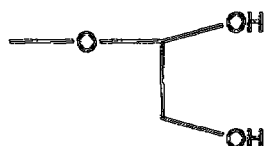
REPLACEMENT SHEET

10 NOV 1999

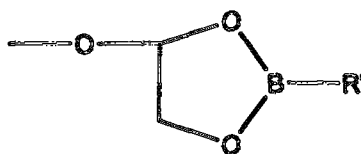
coupled directly or indirectly to a solid phase, for example as part of a conventional solid phase peptide synthesis.

3. A method of claim 1 in which the compound comprising a boronic acid group is an amino acid boronic acid, a peptide boronic acid or a boronate ester of either and step (ii) comprises reacting the amino acid or peptide boronic acid or ester with a diol coupled to the solid phase.

4. A method of claim 3 in which the diol comprises a residue of the formula



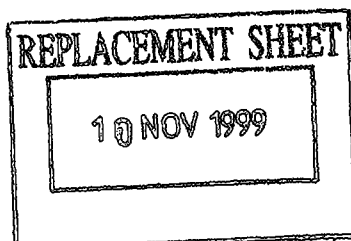
and step (v) results in formation of a solid phase having coupled thereto, directly or via a linker, a moiety of the formula:



- wherein R' is selected from the group consisting of residues of natural and unnatural amino acid and analogues thereof.

5. A method of claim 4 in which the analogues are amino acids having their amino groups replaced by an alternative functional group capable of forming a linkage other than a natural peptide linkage.

6. A method of claim 1 in which step (iii) comprises causing the carboxyl group of said terminal amino acid to react with said functional groups, or causing a reactive derivative of the carboxyl group to do so.





7. A method of claim 6 wherein said compound comprising a boronic acid group or an ester or other derivative thereof comprises an amino acid sequence having a C-terminal boronic acid group or ester or other derivative thereof, and the method further comprises the following additional steps between steps (v) and (vi):

5

(vii) contacting the solid-phase linked compound prepared using steps (i)-(iv) with a linker compound having two carboxyl groups or reactive residues thereof, and causing or allowing the linker carboxy groups or reactive carboxy residues to react with the N-terminal amino group of said compound; and

10

(viii) contacting the solid phase having the linker compound coupled thereto with the N-terminal amino acid of said amino acid sequence having a C-terminal boronic acid group or ester or other derivative thereof, and causing or allowing the amino group of the N-terminal amino acid to react with the carboxy groups or reactive carboxy residues of the linker compound.

15

8. A method of claim 1 in which step (iii) comprises causing the amino group of said terminal amino acid to react with said functional groups.

20

9. A method of claim 8 in which, between steps (v) and (vi), the carboxy terminus of a resin bound peptide is coupled to a free α -aminoboronate acid or ester and step (vi) comprises cleavage by the action of an acid.

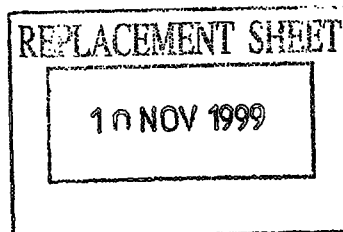
25

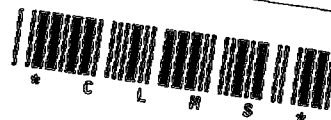
10. A method for making a compound comprising a peptide boronic acid or peptide boronate ester, the method comprising:

(i) providing a solid phase having coupled thereto alcoholic hydroxy groups;

30

(ii) causing an amino acid boronic acid or peptide boronic acid to react with the hydroxy groups whereby the boronic acid residue becomes esterified to the solid phase;



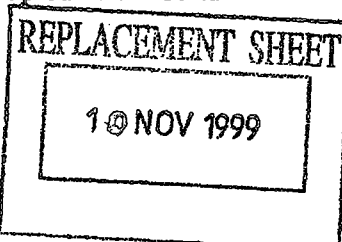


(iii) causing the carboxyl group of the amino acid sequentially following, in the end product peptide boronic acid or boronate ester, selectively to react with the amino group of the sequentially preceding amino acid coupled to the solid phase;

5

(iv) repeating step (iii) as often as necessary;

(v) cleaving the resultant peptide boronate from the resin;



10 the method optionally comprising one or more further steps to make said compound.

11. A method of claim 10, wherein each amino acid coupled to the solid phase has a protected amino group and step (iii) comprises deprotecting the amino group of the sequentially preceding amino acid and/or the cleavage of step (v) is performed with
15 acid or by transesterification.

12. The use of amino acid or peptide boronic acids or boronate esters in the solid phase synthesis of peptide-containing compounds.

20 13. The use in solid phase synthesis of a boronic acid residue attached to the solid phase through hydroxy residues.

14. A method for making a compound comprising a boron atom, the method comprising:

25

(i) providing a solid phase having coupled thereto alcoholic hydroxy groups;

30

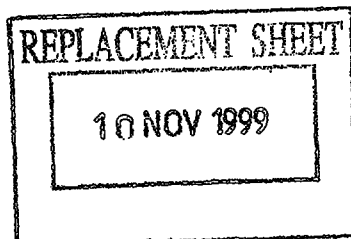
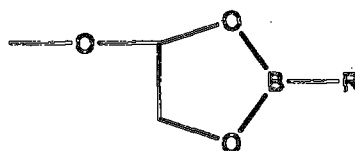
(ii) causing a boronic acid or boronate ester to react with the hydroxy groups whereby the boronic acid residue becomes esterified to the solid phase; and

(iii) performing one or more further steps to make said compound.



15. A solid phase material having coupled thereto boronic acid residues through hydroxy group residues.

5 16. A solid phase material having coupled thereto a moiety of the formula:



10 wherein R is a residue bonded to the boron atom, and the oxygen through which the moiety is bonded to the solid phase is optionally bonded directly to an alkylene residue, which may optionally be inertly substituted, or to a carbonyl group.

17. A product comprising a boronate peptide identified using a library of peptide boronates prepared using a method of any one of claims 1 to 11 or the use of claim 12.

15 18. A method of making a bifunctional serine protease inhibitor comprising:

- (a) a catalytic site-directed moiety (CSDM) which binds to and inhibits the active site of a serine protease;
- (b) an exosite associating moiety (EAM); and, optionally,
- 20 (c) a connector moiety bonded between the EAM and CSDM,

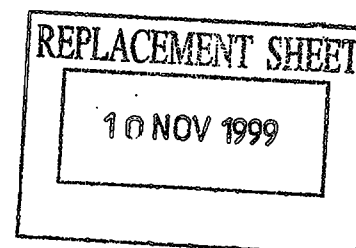
the CSDM and the EAM being capable of binding simultaneously to a molecule of the serine protease, the method comprising performing the following steps to make a target amino acid sequence:

25

- (i) providing a solid phase having coupled thereto functional groups capable of reacting with an amino group or, preferably, with a carboxyl group or a reactive derivative thereof;



- (ii) causing the amino or carboxyl group of a terminal amino acid of the target amino acid sequence selectively to react with said functional groups, the carboxyl group optionally being in the form of a reactive derivative thereof;
- 5 (iii) coupling the amino acid sequentially following, in the target sequence, the sequentially preceding amino acid coupled to the solid phase to said preceding amino acid;
- (iv) repeating step (iii) as often as necessary to form an uninterrupted amino acid sequence coupled to the solid phase;
- 10 (v) reacting the final amino acid of the uninterrupted amino acid sequence coupled to the solid phase with a compound having two carboxylate groups or reactive derivatives thereof; and
- (vi) reacting the unreacted carboxylate or carboxylate derivative with the amino group of an amino acid which is the N-terminal amino acid of the CSDM and is already bonded to the remainder of the CSDM and the CSDM has a boronic acid group or an ester or other derivative thereof in place of a C-terminal carboxy group.
- 15
19. A method of claim 18 in which the terminal amino acid reacted with the functional groups attached to the solid phase is the C-terminal amino acid of the EAM and step (iii) is repeated to couple successive amino acids of the EAM sequence and successive amino acids of any contiguous connector peptide.
- 20
20. A bifunctional serine protease inhibitor whenever made using a method of claim 18 or claim 19.
- 25
21. A pharmaceutical formulation comprising an inhibitor of claim 19 formulated for use as a human or veterinary pharmaceutical.





22. A formulation of claim 21 which includes a pharmaceutically acceptable diluent, excipient or carrier.

5 23. A method a defined in claim 1 of making a peptide or a peptide containing compound substantially as herein described with reference to any example hereof.

24. A solid phase material as defined in claim 17 substantially as herein described with reference to any example hereof.

10

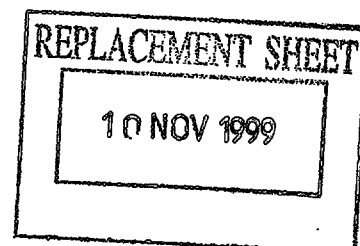
25. A product as defined in claim 17 substantially as herein described with reference to any example hereof.

15 26. A method as defined in claim 18 of making a bifunctional serine protease inhibitor substantially as herein described with reference to any example hereof.

27. A bifunctional serine protease inhibitor as defined in claim 20 substantially as herein described with reference to any example hereof.

20 28. A pharmaceutical formulation as defined in claim 21 substantially as herein described with reference to any example hereof.

25





PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/815, 5/065, A61K 38/55, 38/58		A1	(11) International Publication Number: WO 98/00442															
			(43) International Publication Date: 8 January 1998 (08.01.98)															
(21) International Application Number: PCT/GB97/01574		(74) Agents: BIZLEY, Richard, Edward et al.; Hopworth Lawrence Bryer & Bizley, Merlin House, Falconry Court, Bakons Lane, Bipping, Essex CM16 5DQ (GB).																
(22) International Filing Date: 11 June 1997 (11.06.97)																		
(30) Priority Data: 9613719.5 29 June 1996 (29.06.96) GB		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GR, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TO).																
(71) Applicant (for all designated States except US): THROMBOSIS RESEARCH INSTITUTE [GB/GB]; Emmanuel Kaya Building, Marissa Road, Chelsea, London SW3 6LR (GB).																		
(72) Inventors; and (75) Inventors/Applicants (for US only): DEADMAN, John, Joseph [GB/GB]; Flat 1, 78 Belsize Park Gardens, London NW3 4NG (GB). ELGENDY, Said [EG/GB]; 69 Dyanham Road, West Hampstead, London NW6 2NT (GB). GREEN, Donovan [GB/GB]; 50 Gloucester Road, Walthamstow, London E17 6AH (GB). SKORDALAKES, Emmanuel [GR/GB]; 166 Waller Road, Telegraph Hill, London SE14 5LU (GB). SCULLY, Michael, Finbarr [GB/GB]; Douglas House, Crays Hill, Essex CM11 2KN (GB). GOODWIN, Christopher, Andrew [GB/GB]; 67 Old Fosse Road, Odd Down, Bath, Avon BA2 2SP (GB). KAKKAR, Vijay, Vir [IN/GB]; Hambles Edge, Brook Avenue, Warsash, Hants SO3 6HN (GB).		Published With international search report. Before the expiration of the time limits for amending the claims and to be republished in the event of the receipt of amendments.																
(54) Title: SERINE PROTEASE INHIBITORS																		
(57) Abstract Bifunctional serine protease inhibitors and methods of preparing boron-containing peptides are provided. The serine protease inhibitors comprise a catalytic site-directed moiety, which binds to and inhibits the active site of a serine protease, and an exosite associating moiety, which are joined by a connector moiety. The catalytic site directed moiety and the exosite associating moiety are capable of binding simultaneously to a molecule of the serine protease.																		
<table border="1"><tr><th colspan="2">REPLACED SHEETS</th><th>SG</th></tr><tr><td>☒</td><td>Description</td><td>: paged 1 to paged 40</td></tr><tr><td>☒</td><td>Claim(s)</td><td>: paged 1 to paged 31</td></tr><tr><td>☐</td><td>Drawing(s)</td><td>: paged to paged</td></tr><tr><td>☐</td><td>Abstract</td><td>: paged to paged</td></tr></table>				REPLACED SHEETS		SG	☒	Description	: paged 1 to paged 40	☒	Claim(s)	: paged 1 to paged 31	☐	Drawing(s)	: paged to paged	☐	Abstract	: paged to paged
REPLACED SHEETS		SG																
☒	Description	: paged 1 to paged 40																
☒	Claim(s)	: paged 1 to paged 31																
☐	Drawing(s)	: paged to paged																
☐	Abstract	: paged to paged																