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(54) Title: SUBSTRATES AND INHIBITORS OF PROTEOLYTIC ENZYMES			
(57) Abstract			
<p>The present invention relates to the field of compounds which are substrates or inhibitors of proteolytic enzymes and to apparatus and methods for identifying substrates or inhibitors for proteolytic enzymes. We have devised a combinatorial method for the rapid identification of binding motifs which will greatly expedite the synthesis of inhibitors of a variety of proteolytic enzymes such as aspartyl proteases, serine proteases, metallo proteases and cysteinyl proteases.</p>			

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Substrates And Inhibitors of Proteolytic Enzymes

The present invention relates to the field of compounds which are substrates or inhibitors of proteolytic enzymes and to apparatus and methods for identifying substrates or inhibitors for proteolytic enzymes.

Many therapeutically useful drugs act as enzyme inhibitors. In particular, proteolytic enzyme inhibitors have been the focus of much attention in the pharmaceutical industry, because they play a variety of roles in a multitude of biological systems. Their proteolytic activities are related to processes ranging from cell invasion associated with metastatic cancer to evasion of an immune response, as seen in certain parasitic organisms; from nutrition to intracellular signalling to the site-specific proteolysis of viral proteases and eukaryotic hormone-processing enzymes. However, the traditional random screening methods for the identification of lead molecules as inhibitors of proteolytic enzymes are often laborious and time-consuming. Therefore new and efficient methods which can accelerate the drug discovery process are greatly in demand.

We consider that proteases contain an active catalytic site which tends to become increasingly activated as the recognition pockets¹ (S_1 and S_2 etc) and (S_1' and S_2' etc) become better occupied. Therefore, it is important that those parts (P_1 and P_2 etc) (P_1' and P_2' etc) of the inhibitor that best fit into these pockets are identified as quickly as possible in order to design novel protease

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inhibitors. Therefore, we have devised a combinatorial method for the rapid identification of these binding motifs which will greatly expedite the synthesis of inhibitors of a variety of proteolytic enzymes such as aspartyl proteases, serine proteases, metallo proteases and cysteinyl proteases.

Proteases of interest include (but are not limited to):

1. Aspartyl proteases, such as renin, HIV, cathepsin D and cathepsin E etc.
2. Metalloproteases, such as ECE, gelatinase A and B, collagenases, stromolysins etc.
3. Cysteinyl proteases, such as apopain, ICI, DerPI, cathepsin B, cathepsin K etc.
4. Serine proteases, such as thrombin, factor VIIa, factor Xa, elastase, trypsin.
5. Threonyl proteases, such as proteasome S.

The use of a fluorescence resonance energy transfer (FRET) substrate for the analysis of proteolytic enzyme specificity was first published by Carmel.² Since then many different quenched fluorogenic substrates for measuring enzyme inhibition have been described in the literature.⁴⁻¹¹ These substrates contain a fluorophore, F, in a P position (*vide supra*), which is quenched by another group, Q, present in a P' position (*vide supra*) and separated from F by the scissile bond. The advantage of

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the positioning of these residues, F and Q, is that cleavage of a peptide bond occurs between the two natural residues and, therefore, represents a more natural hydrolytic event rather than the cleavage and release of a C-terminal chromophore.

For example, Bratovanova and Petkov¹² have synthesised fluorogenic substrates from peptide 4-nitroanilides. N-acylation of peptide 4-nitroanilides with the aminobenzoyl (ABz) group yielded substrates that are internally quenched by the presence of the 4-nitroanilide moiety. Upon hydrolysis of the aminoacyl-4-nitroanilide bond, the highly fluorescent N-ABz group is released attached either to an amino acid or peptide.

Immobilised libraries, where substrates are attached to a polymer or biopolymer support, have also been used for mapping protease binding sites.¹³ Singh et al. reported recently that enzymatic substrate activity of 38 selected octapeptides attached via a linker to controlled pore glass is predictive of the same activity of similar peptides in solution. However, these results are preliminary and only for a specific example. Therefore, it is not clear whether immobilised substrates attached to polymers can reliably replace soluble substrates in mapping the hindered protease binding sites, especially since the hydrophilic or lipophilic nature of the polymer and the size of the interstices within the polymer are bound to influence the reaction between the enzyme and its substrates.

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Mixtures of internally quenched, fluorogenic substrates have also recently been described in which the quencher group, Q, is 2,4-dinitrophenyl (Dnp) and is attached to the P side of the scissile bond, while the fluorogenic group, is N-methyl anthranilic acid (Nma) and is attached to the P' side.¹⁴

Examples of other Donor-Acceptor Chromophore Pairs that have been applied to Biological Systems are shown in Table 1.

Table 1 : Donor-Acceptor Chromophore Pairs That Have Been Applied To Biological Samples

	Donor	Acceptor	Donor	Acceptor
	Naphthalene	Dansyl	IAEDANS	TNP-ATP
	IANBD	DDPM	ϵ -A	IANBD
15	IAEDANS	DDPM	NBD	SRH
	DNSM	LY	ISA	TNP
	IAEDANS	IANBD	Dansyl	ODR
	E-A	F ₂ DNB	DANZ	IAF
	Pyrene	Bimane	FNAI	EITC
20	ANAI	IPM	NBD	LRH
	IAANS	IAF	IAF	ELA
	ϵ -A	F ₂ DPS	FITC	ENAI
	ϵ -A	DDPM	Proflavin	ETSC
	IAEDANS	TNP	CPM	TNP-ATP
25	MNA	DACM	IAEDANS	IAF
	PM	NBD	CPM	Fluorescein
	FITC	TNP-ATP	IAEDANS	FITC
	DANZ	DABM	FITC	TMR
	NCP	CPM	IAF	TMR
30	NAA	DNP	CF	TR
	LY	TNP-ATP	CPM	FTS
	IAF	diI-C ₁₈	ϵ -A	TNP-ATP
	IAF	TMR	CPM	FM
	FMA	FMA	LY	EM
35	PM	DMAMS	FITC	EITC
	mBBR	FITC	IAEDANS	DiO-C ₁₄
	mBBR	DABM	IAF	ErITC
	ϵ -A	NBD	FITC	EM
	Pyrene	Coumarin	FITC	ETSC
40	IPM	FNAI	FITC	ErITC
	IAEDANS	DABM	BPE	CY5

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ANAI, 2-anthracene N-acetylimidazole; BPE, B-phycoerythrin; CF, carboxyfluorescein succinimidyl ester; CPM, 7-doethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin; CY5, carboxymethylindocyanine-N-hydroxysuccinimidyl ester; diI-C₁₈, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine; DiO-C₁₄, 3,3'-ditetradecyloxacarbocyanine; DABM, 4-dimethylanilinophenylazo-phenyl-4'-maleimide; DACM, (7-(dimethylamino)coumarin-4-yl)-acetyl; DANZ, dansylaziridine; DDPM, N-(4-dimethylamino-3-5-dinitrophenyl)maleimide; DACM, di-methylamino-4-maleimidostilbene; DMSM, N-(2,5-dimethoxystiben-4-yl)-maleimide; DNP, 2,4-dinitrophenyl; ϵ -A, 1,N⁶-ethenoadenosine; EIA, 5-(iodoacetamidoeosin; EITC, eosin-5-isothiocyanate; ENAI, eosin N-acetylimidazole; EM, eosin maleimide; ErITC, erythrosin-5'-isothiocyanate; ETSC, eosin thiosemicarbazide; F₂DPB, 1,5-difluoro-2,4'-dinitrobenzene; F₂DPS, 4,4'-difluoro-3,3'-dinitrophenylsulphone; FITC, fluorescein N-acetylimidazole; FTS, fluorescein thiosemicarbazide; IAANS, 2-((4'-iodoacetamido)anilino)naphthalene-6-sulphonic acid; IAEDANS, 5-(2-((iodoacetyl)amino)ethylamino)-naphthlene-1-sulphonic acid; IAF, 5-iodoacetamidofluorescein; IANBD, N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa1,3,diazole; IPM, 3(4-isothiocyanatophenyl)7-diethyl-4-methylcoumarin; ISA, 4-(iodoacetamido)salicylic acid; LRH, lissaminerho-2,1,3-benzoxadiazol-4-yl; NCP, N-cyclohexyl-N'-(1-pyrenyl)carbodiimide; ODR, octadecylrhodamine; PM, N-(1-pyrene)-maleimide; SRHsulphurhodamine; TMR, tetramethylrhodamine; TNP, trinitrophenyl; TR, Texas red.

from: Wu, P. and Brand, L. 1994. Anal. Biochem. 218, 1-13.

The specificity of soluble peptide libraries have been determined.^{15,16} Berman et al. described¹⁶ an HPLC mass spectrometry technique in which 6 mixtures of 128 peptides were synthesised which were N-terminally labelled with the Dnp group in order to allow UV monitoring on the HPLC. The disadvantage of this approach is that each assay mixture has to be individually analysed, because no fluorogenic substrate is revealed, and that the effective concentration of each separate component is limited by the size of the mixture because of overall solubility factors.

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Drevin et al.¹⁷ have suggested the use of individually synthesised fluorogenic substrates for the determination of enzyme activity using a chromophore which chelates lanthanide ions. Garmann and Phillips have suggested the use of FRET substrates in which the fluorogenic and quencher moieties are attached via thiol or amino functional groups after the peptide has been synthesised, but this has the disadvantage that they are not in library form and that these functional amino and thiol groups need to be selectively revealed after the peptide has been synthesised. Wang et al. have suggested the use of the EDANS and DABCYL fluoescor and quencher pairing for the individual synthesis of substrates for proteolytic enzymes.

The above methods which have used FRET techniques for the mapping of the active site around a specific protease suffer from one or more of the following disadvantages:

- i. because of general aqueous insolubility they do not produce mixtures of compounds in a form suitable for high throughput screening in aqueous solution.
- ii. the derivatised compounds cannot be prepared in combinatorial library form using solid phase techniques.
- iii. the mixtures which have been used^{8,9} were not self-decoding, and needed time-consuming deconvolutive resynthesis for identification of the active molecules.

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Description of the Drawings

Figures 1 to 14 inclusive exemplify component distributions in the plates of a library matrix;

Figure 15 illustrates a reaction scheme for production of compound number 4: Boc-Val-Ala-Leu-H wherein

- i. isobutylchloroformate, N-methylmorpholine, then N,O-dimethylhydroxylamine HCl, THF.
- ii. HCl, dioxan. iii. isobutylchloroformate, N-methylmorpholine, then Boc-Ala-OH, THF.
- iv. HCl, dioxan. V. Boc-Val-Osuc, N-methylmorpholine, DMF. Vi. LAH;

Figure 16 illustrates a reaction scheme for production of active inhibitors of *Der PI*.

Brief Description of the Invention

The present invention relates to the field of:

- i. Compounds which are substrates or inhibitors of proteolytic enzymes.
- ii. Apparatus and methods which provide the rapid generation of structure-activity relationships using auto-deconvoluting combinatorial libraries, which facilitate the invention of novel inhibitors of proteolytic enzymes.
- iii. Apparatus and methods which provide the detection and measurement of proteolytic enzyme activity using combinatorial FRET (fluorescence resonance energy transfer) libraries of molecules.

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- iv. Apparatus and methods which provide the establishment of biological assays for proteolytic enzymes through the rapid discovery of highly active substrates for proteolytic enzymes.

We describe herein apparatus and methods which can be used for the rapid generation of structure-activity relationship (SAR) data and, therefore, the characterisation of the binding motif of any protease, and which will, therefore, facilitate:

- i. the development of a sensitive enzyme inhibition assay by using the best compound in the library as the fluorogenic substrate for the proteolytic enzyme under scrutiny.
- ii. the invention of novel compounds which are proteolytic enzyme inhibitors by rapid characterisation of the best binding motif.
- iii. computer aided drug design to design potent inhibitors using known methodology, and also in prioritising which pre-synthesised compounds in the in-house and commercially available databases to assay.

In a first aspect the invention provides novel compounds represented by the formula A-B-C-D-nE-F [I] in which; A represents a fluorescor internally quenched by F; B, C, D, and E represent groups such that the scissile bond between any two of these groups is a suitable bond; F represents a quencher capable of internally quenching the fluorescor A; and

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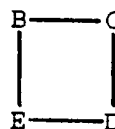
n represents an integer between 1 and 4 inclusive.

In some embodiments the suitable bond is an unsubstituted amide bond (see Example 1); in other embodiments the suitable bond is an ester bond (see Example 2).

- 5 In preferred embodiments B, C, D, E are amino acids or hydroxy acids. That is a molecule with an amine or hydroxy terminus and a carboxylic acid terminus. The amine/hydroxy group may be positioned on the same carbon atom or separated by a number of atoms and atom types.
- 10 The molecules may be cyclic or alicyclic. They may be linear or cyclic on the same structure

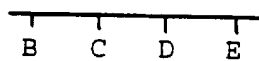
B-C-D-E

or

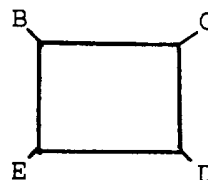


or BCDE can be on a central scaffold (linear or cyclic)

15

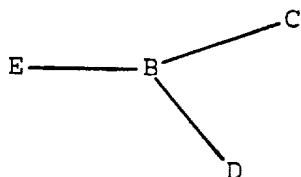


or



or one of the residues can be the backbone

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Where the symbol -B-C-D-E- or -B-C-D-nE- is used herein,
5 it is to be understood to include all of these linear and
cyclic variants within its definition.

At least one but not all the bonds between B, C, D, E need
to be scissile. Non scissile bonds may include
sulphonamide, urea, aminomethylene.

10 Several non-limiting examples of "scaffold" molecules are
shown in Table 2, in which substituents R₁-R₄ correspond to
possible variable groups B, C, D and E.

In a second aspect the invention provides a combinatorial
library of FRET compounds comprising a mixture of
15 compounds of formula [I].

In a third aspect the invention provides for the use of
such a combinatorial FRET library in a method which
provides rapid generation of structure-activity
relationships (SAR) which comprises detection and
20 measurement of proteolytic enzyme activity by carrying out
an assay with a library of combinatorial FRET
(fluorescence resonance energy transfer) molecules to find
a substrate or substrates for the enzyme. According to
this method an identified substrate can be synthesised and
25 used in biological assay for proteolytic enzymes. Novel
substrates are included in the scope of the invention.

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In a forth aspect the invention provides for the use of such a combinatorial FRET library in a method for detection and measurement of proteolytic enzyme activity against compounds of the library.

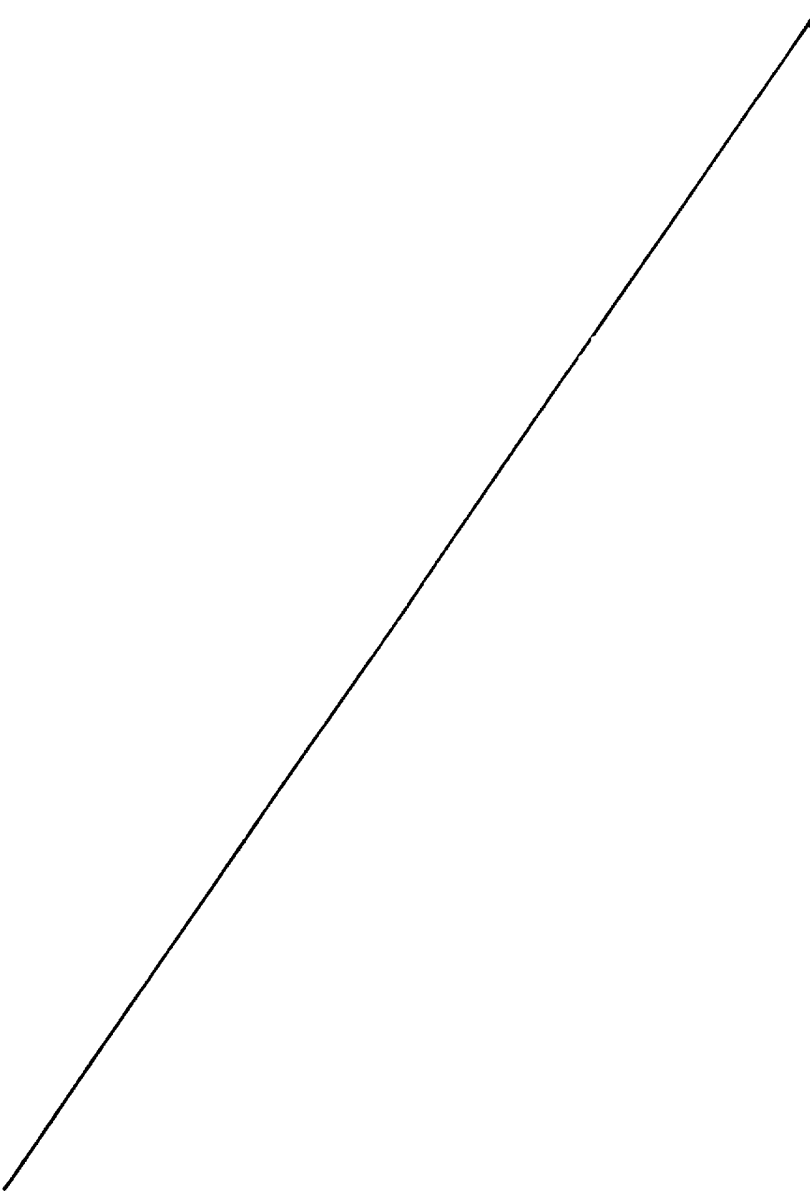
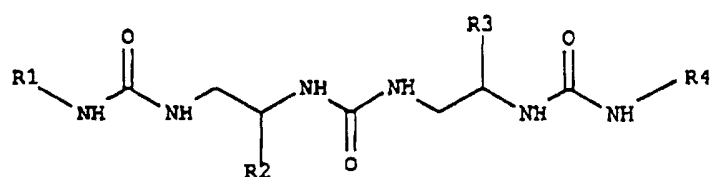
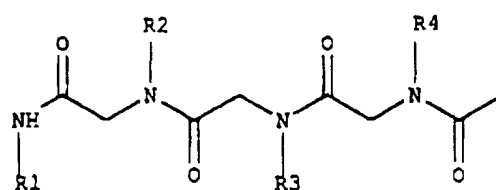
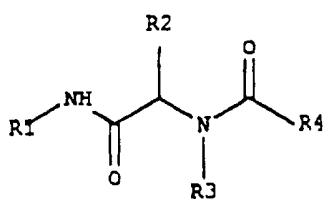
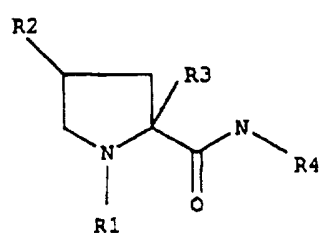
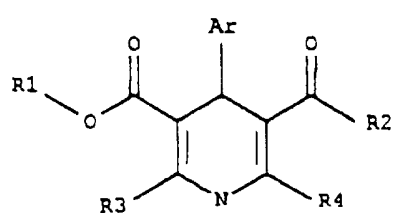
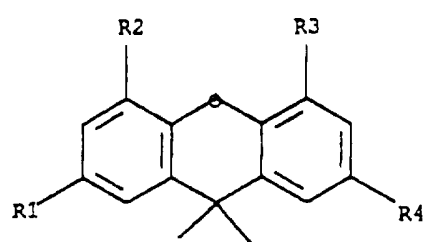
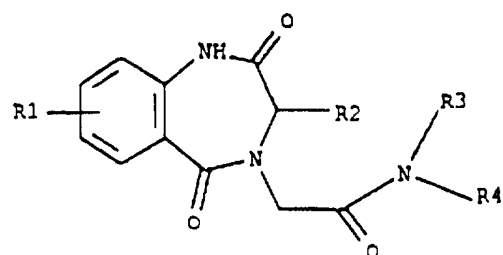
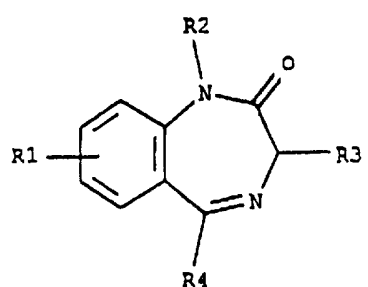
- 5 In a fifth aspect the invention provides a method which comprises the identification of an enzyme inhibitor or inhibitors wherein a FRET compound which has been
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TABLE 2



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identified as a substrate is used in an inhibition assay with the enzyme separately against a panel of possible inhibitors.

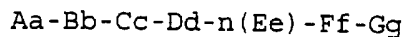
In a sixth aspect the invention provides a set of
5 compounds which comprises two complementary FRET compound libraries. Such a set will be referred to hereafter as "apparatus" because it allows for the screening or assay method for identifying substrates or inhibitors of
10 proteolytic enzymes. This set of compounds constituting an apparatus is capable of providing an auto-deconvoluting combinatorial library as will be described below.

In a seventh aspect the invention provides a method of identifying and synthesising an inhibitor of a proteolytic enzyme which comprises detection and measurement of
15 proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET (fluorescence resonance energy transfer) molecules, deconvoluting the library to find a substrate or substrates for the enzyme and
20 synthesis of an inhibitor based on the substrate or substrates. The direct product of this method is one or more novel proteolytic enzyme inhibitors.

In an eighth aspect the invention provides an inhibition assay which uses a FRET molecule, which has been
25 identified as a substrate for the enzyme, wherein the molecule is assayed with the enzyme separately against a panel of possible inhibitors.

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In a ninth aspect the invention provides a complementary pair of compound libraries L1 and L2 which constitute a set containing compounds of formula:



5 giving $a \times b \times c \times d \times e \times f \times g = M_n$ compounds in each library, there being a predetermined number (P1, P2) of mixtures each consisting of a predetermined number (Q1, Q2) of individual identifiable compounds in each library, wherein both L1 and L2 contain the same M_n compounds, but
 10 wherein any two compounds which are found together in one mixture of Q1 compounds of L1 are not found together in any one of the P2 mixtures of L2.

In a tenth aspect the invention provides a method of screening for enzymic activity using the libraries L1, L2
 15 described above in which the P1 mixtures of L1 and the P2 mixtures of L2 are each placed separately into individual wells of well plates, the well plates having wells arranged in a format adapted to allow deduction of a unique active compound formula from the presence of
 20 activity in one well of L1 and one well of L2.

The apparatus of the invention preferably comprises two complementary compound libraries, L1 and L2, each containing $n \times 1600$ compounds of the invention, of the type $A-B_{1-10}-C_{1-10}-D_{1-8}-n(E_{1-2})-F-G$ [II], in which:

25 A = a fluorescor internally quenched by F, preferably an unsubstituted or substituted anthranilic acid derivative, connected by an amide bond to B

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B, C, D, E, are natural or unnatural amino acid residues connected together by suitable bonds, although B, C, D and E can be any set of groups, provided that the scissile bond between D-E is an unsubstituted bond.

5 F = a quencher capable of internally quenching the fluorescor A, preferably an unsubstituted or substituted 3-nitrotyrosine derivative.

G = optionally present and is a hydrophilic moiety, preferably an aspartyl amide moiety. If present, G
10 advantageously ensures that all compounds in the library are imparted with aqueous solubility. Also, G should not be a substrate for any type of enzyme.

n = any integer between 1 and 4 inclusive.

In an alternative, the scissile bond could be between B-C
15 or C-D.

(Note that A and F herein correspond generally and respectively to moieties F and Q of the prior art referred to above).

The numbers represented in subscript following residues B,
20 C, D and E refer to the number of possibilities from which those residues are selected. Thus, by way of illustrative example, A-B_{1,5}-C-D-E_{1,2}-F-G represents a mixture of the following ten compounds:

A-B₁-C-D-E₁-F-G

25 A-B₂-C-D-E₁-F-G

A-B₃-C-D-E₁-F-G

A-B₄-C-D-E₁-F-G

A-B₅-C-D-E₁-F-G

A-B₁-C-D-E₂-F-G

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A-B₁-C-D-E₂-F-G

A-B₂-C-D-E₂-F-G

A-B₄-C-D-E₂-F-G

A-B₅-C-D-E₂-F-G

- 5 The general combinatorial formula for each library can be expressed as:

$$A_1-B_{10}-C_{10}-D_8-n(E_2)-F_1-G_1 \quad [III]$$

providing $1 \times 10 \times 10 \times 8 \times n \times 2 \times 1 \times 1 = 1600n$ compounds.

- 10 Both compound libraries, L1 and L2, of the above type are synthesized using solid phase techniques using the Multipin approach²⁴ such that each library contains 1600n compounds as 80n mixtures of 20 distinct, identifiable compounds. These 20 component mixtures are then placed
15 separately into each of 80 wells of a 96 well plate (the other two lanes are used for control experiments) and then screened against a known quantity of the protease.

Thus it is an important part of the invention that regardless of the number of compounds contained in the two
20 libraries L1 and L2 (e.g. in the preferred embodiment: 1600n, where n = any integer between 1 and 4) the libraries themselves are complementary and amenable to deconvolution without recourse to resynthesis. It is also an important part of the invention that the library matrix
25 has been especially formatted so that the most important site pairings P₂ and P₁ for proteolytic enzymes can be identified immediately without recourse to resynthesis.

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Those compounds of the type A-B-C-D-E-F-G that are the better substrates for the protease will be cleaved, and can be readily identified because the fluorophore, A, will be cleaved from its nearby quencher F, in a time dependent manner which can be easily quantified. The fluorescent quenching by F of A only occurs when the two are in nearby proximity, normally within 30 angstrom units. Hence cleavage of a scissile bond (e.g. the scissile bond D-E) allows F to move further away from A and thus allow A to fluoresce when excited by light of the correct wavelength.

1. In this manner the most active compound can be rapidly identified without the need for further resynthesis and deconvolution. Moreover, the wells that show the most rapid development of fluorescence can also be analysed by mass spectrometry, since by comparison with the original mixture, the identity of the most efficient substrate can be found by its disappearance into its two component parts, e.g A-B-C-D and E-F-G.

Hence the problem of library deconvolution can be overcome and the most active substrate for the enzyme can be rapidly identified.

In addition, after the initial treatment of the proteolytic enzyme with the library mixtures, L1 and L2, the residual enzymatic activity in each well can be quantified by the addition of the most potent fluorogenic substrate for the enzyme, S1, which is found in the 16xn compound library. Because of the nature of the library

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design this can be quickly prepared and purified. If there is no appearance of increased fluorescence with the known substrate, S1, then the presence of an enzyme inhibitor can be inferred, which again can be quickly identified without the need for resynthesis.

The general description of the library layout will now be described with reference to figures 1 to 14.

For example, when $n=1$ and the library contains 1600 compounds, in the first column of the first row (A1) (Fig.1) in the first plate (P1) of the library L1, (hereinafter designated as location A1,P1,L1) there will be one C component, C_1 , one D component, D_1 , the ten B components and the two E components (E_1 and E_2) (Fig. 2). In the tenth column of the first row (A10) in the first plate (P1) of the library L1, (hereinafter designated as location A10,P1,L1) there will be one C component, C_{10} , one D component, D_1 , the ten B components and the two E components (E_1 and E_2). In the tenth column of the eighth row (H10) in the first plate (P1) of the library L1, (hereinafter designated as location H10,P1,L1) there will be one C component, C_{10} , one D component, D_8 , the ten B components and the two E components (E_1 and E_2). Hence all 1600 components are present in the one plate, because the 80 wells each contain 20 components.

A second complementary library is synthesised as follows (Fig. 3). In the first column of the first row (A1) of the first plate (P1) of the library, L2, (hereinafter designated as location A1,P1,L2), there will be ten C components, two D components (D_1 and D_2), one B component,

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B₁, and one E component, E₁. In the tenth column of the first row (A10) of the first plate (P1) of the library, L2, (hereinafter designated as location A10,P1,L2), there will be ten C components, two D components (D₁ and D₂), one
5 B component, B₁₀, and one E component, E₁. In the first column of the second row (B1) of the first plate (P1) of the library, L2, (hereinafter designated as location B1,P1,L2), there will be ten C components, two D components (D₁ and D₂), one B component, B₁, and one E
10 component, E₂. In the tenth column of the second row (B10) of the first plate (P1) of the library, L2, (B10,P1,L2) there will be ten C components, two D components (D₁ and D₂), one B component, B₁₀, and one E component, E₂. Hence only the first two rows are used to accommodate 400
15 compounds in total.

In the first column of the first row (A1) of the second plate (P2) of the library, L2, (hereinafter designated as location A1,P2,L2), there will be ten C components, two D components (D₃ and D₄), one B component, B₁, and one E
20 component, E₁ (Fig. 4). In the tenth column of the first row (A10) of the second plate (P2) of the library, L2, (hereinafter designated as location A10,P2,L2), there will be ten C components, two D components (D₃ and D₄), one B component, B₁₀, and one E component, E₁. In the first
25 column of the second row (B1) of the second plate (P2) of the library, L2, (hereinafter designated as location B1,P2,L2), there will be ten C components, two D components (D₃ and D₄), one B component, B₁, and one E component, E₂. In the tenth column of the second row (B10)
30 of the second plate (P2) of the library, L2, (B10,P2,L2), there will be ten C components, two D components (D₃ and

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D_4), one B component, B_{10} , and one E component, E_2 . Hence only the first two rows are used to accommodate 400 compounds in total.

In the first column of the first row (A1) of the third plate (P3) of the library, L2, (hereinafter designated as location A1,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1 , and one E component, E_1 (Fig 5). In the tenth column of the first row (A10) of the third plate (P3) of the library, L2, (hereinafter designated as location A10,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_{10} , and one E component, E_1 . In the first column of the second row (B1) of the third plate (P3) of the library, L2, (hereinafter designated as location B1,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1 , and one E component, E_2 . In the tenth column of the second row (B10) of the third plate (P3) of the library, L2, (B10,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_{10} , and one E component, E_2 . Hence only the first two rows are used to accommodate 400 compounds in total.

In the first column of the first row (A1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A1,P4,L2), there will be ten C components, two D components (D_7 and D_8), one B component, B_1 , and one E component, E_1 (Fig. 6). In the tenth column of the first row (A10) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A10,P4,L2), there will be ten C components, two D components (D_7 and D_8), one B

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component, B_{10} , and one E component, E_1 . In the first column of the second row (B1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location B1,P4,L2), there will be ten C components, two D components (D₇ and D₈), one B component, B_1 , and one E component, E_2 . In the tenth column of the second row (B10) of the fourth plate (P4) of the library, L2, (B10,P4,L2), there will be ten C components, two D components (D₇ and D₈), one B component, B_{10} , and one E component, E_2 . Hence only the first two rows are used to accommodate 400 compounds in total.

In this fashion two complementary libraries, L1 and L2 are prepared. In library, L1, each of the 80 of wells contains a mixture of 20 components providing 1600 compounds for screening. In library, L2, four plates are used in which only the first two rows are employed, providing 20 wells of 20 components per well per plate, and furnishing the same 1600 compounds as are present in library L1, but in a format in which no two compounds found together in library, L1, will be found together in library, L2.

Thus it is an important part of the invention that the compounds contained in the two libraries L1 and L2 are themselves complementary, in that any two compounds which are found together in a 20 component mixture in the same location (e.g. A1P1L1) in library L1, are not found together in any of the 20 component mixtures in any location of the library L2.

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Thus, for example, with reference to the primary library P1 L1 of figure 2 and the secondary libraries P1 L2, P2 L2, P3 L2 and P4 L2 of figures 3-6 it is possible to deconvolute an exemplary sequence:

5

-B₂-C₃-D₄-E₁-

If this sequence is a substrate fluorescence will occur in P1 L1 at C₃D₄. This gives the information that the substrate is

-?-C₃-D₄-?-

10

If fluorescence occurs in P2 L2 at B₂E₁ it indicates a substrate

-B₂-?-?-E₁-

The confirmation of the substrate as

-B₂-C₃-D₄-E₁-

15

should be provided by non-fluorescence of P1 L2, P3 L2 and P4 L2 which all contain -B₂-C₃-X-E₁- where X is not D₄.

20

In practice it is likely that more than one sequence will result in a substrate. Information as to which positions B-C-D-E- are sensitive to change (i.e. require a specific group) and which are insensitive (i.e. can tolerate more than one choice of group) in the context of the whole sequence gives valuable SAR data which can be used to model and/or synthesise related compounds.

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In analogous examples, where separately $n=2, 3$ or 4 , extra plates are constructed in library L1 format to accommodate the component pairs E_3 and E_4 ($n=2$), E_5 and E_6 ($n=3$), and E_7 and E_8 ($n=4$), respectively. For the respective
5 deconvolution libraries of the type, L2, the respective rows in the plates P1, P2, P3, and P4, are increasingly filled with the paired components D_1 and D_2 , D_3 and D_4 , and D_5 and D_6 , and D_7 and D_8 , respectively.

For example, when $n=3$, and the library contains 4800
10 compounds, in the first column of the first row (A1) in the first plate (P1) of the library L1, (hereinafter designated as location A1,P1,L1) there will be one C component, C_1 , one D component, D_1 , the ten B components and the two E components (E_1 and E_2). In the tenth column
15 of the first row (A10) in the first plate (P1) of the library L1, (hereinafter designated as location A10,P1,L1) there will be one C component, C_{10} , one D component, D_1 , the ten B components and the two E components (E_1 and E_2). In the tenth column of the eighth row (H10) in the first
20 plate (P1) of the library L1, (hereinafter designated as location H10,P1,L1) there will be one C component, C_{10} , one D component, D_8 , the ten B components and the two E components (E_1 and E_2). Hence 1600 components are present in the one plate, because the 80 wells each contain 20
25 components.

In the first column of the first row (A1) in the second plate (P2) of the library L1, (hereinafter designated as location A1,P2,L1) there will be one C component, C_1 , one
D component, D_1 , the ten B components and the two E
30 components (E_3 and E_4). In the tenth column of the first

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row (A10) in the second plate (P2) of the library L1, (hereinafter designated as location A10,P2,L1) there will be one C component, C₁₀, one D component, D₁, the ten B components and the two E components (E₃ and E₄). In the
5 tenth column of the eighth row (H10) in the second plate (P2) of the library L1, (hereinafter designated as location H10,P1,L1) there will be one C component, C₁₀, one D component, D₈, the ten B components and the two E components (E₃ and E₄). Hence 1600 components are present
10 in the one plate, because the 80 wells each contain 20 components.

In the first column of the first row (A1) in the third plate (P3) of the library L1, (hereinafter designated as location A1,P3,L1) there will be one C component, C₁, one
15 D component, D₁, the ten B components and the two E components (E₅ and E₆). In the tenth column of the first row (A10) in the third plate (P3) of the library L1, (hereinafter designated as location A10,P3,L1) there will be one C component, C₁₀, one D component, D₁, the ten B
20 components and the two E components (E₅ and E₆). In the tenth column of the eighth row (H10) in the third plate (P3) of the library L1, (hereinafter designated as location H10,P3,L1) there will be one C component, C₁₀, one C component, C₈, the ten B components and the two E
25 components (E₅ and E₆). Hence 1600 components are present in the one plate, because the 80 wells each contain 20 components. In total the three plate, P1, P2 and P3, contain 1600 compounds/plate 4800 compounds in total.

For example, when n = 4, and the library contains 6400
30 compounds, in the first column of the first row (A1) in

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the first plate (P1) of the library L1, (hereinafter designated as location A1,P1,L1) there will be one C component, C_1 , one D component, D_1 , the ten B components and the two E components (E_1 and E_2) (Fig. 7). In the tenth column of the first row (A10) in the first plate (P1) of the library L1, (hereinafter designated as location A10,P1,L1) there will be one C component, C_{10} , one D component, D_1 , the ten B components and the two E components (E_1 and E_2). In the tenth column of the eighth row (H10) in the first plate (P1) of the library L1, (hereinafter designated as location H10,P1,L1) there will be one C component, C_{10} , one D component, D_8 , the ten B components and the two E components (E_1 and E_2). Hence all 1600 components are present in the one plate, because the 80 wells each contain 20 components.

In the first column of the first row (A1) in the second plate (P2) of the library L1, (hereinafter designated as location A1,P2,L1) there will be one C component, C_1 , one D component, D_1 , the ten B components and the two E components (E_3 and E_4) (Fig. 8). In the tenth column of the first row (A10) in the second plate (P2) of the library L1, (hereinafter designated as location A10,P2,L1) there will be one C component, C_{10} , one D component, D_1 , the ten B components and the two E components (E_3 and E_4). In the tenth column of the eighth row (H10) in the second plate (P2) of the library L1, (hereinafter designated as location H10,P2,L1) there will be one C component, C_{10} , one D component, D_8 , the ten B components and the two E components (E_3 and E_4).

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In the first column of the first row (A1) in the third plate (P3) of the library L1, (hereinafter designated as location A1,P3,L1) there will be one C component, C_1 , one D component, D_1 , the ten B components and the two E components (E_5 and E_6) (Fig. 9). In the tenth column of the first row (A10) in the third plate (P3) of the library L1, (hereinafter designated as location A10,P3,L1) there will be one C component, C_{10} , one D component, D_1 , the ten B components and the two E components (E_5 and E_6). In the tenth column of the eighth row (H10) in the third plate (P3) of the library L1, (hereinafter designated as location H10,P3,L1) there will be one C component, C_{10} , one D component, D_8 , the ten B components and the two E components (E_5 and E_6).

In the first column of the first row (A1) in the fourth plate (P4) of the library L1, (hereinafter designated as location A1,P4,L1) there will be one C component, C_1 , one D component, D_1 , the ten B components and the two E components (E_7 and E_8) (Fig. 10). Likewise, in the tenth column of the first row (A10) in the fourth plate (P4) of the library L1, (hereinafter designated as location A10,P4,L1) there will be one C component, C_{10} , one D component, D_1 , the ten B components and the two E components (E_7 and E_8). In the tenth column of the eighth row (H10) in the fourth plate (P4) of the library L1, (hereinafter designated as location H10,P4,L1) there will be one C component, C_{10} , one D component, D_8 , the ten B components and the two E components (E_7 and E_8).

A second complementary library is synthesised as follows.
In the first column of the first row (A1) of the first

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plate (P1) of the library, L2, (hereinafter designated as location A1,P1,L2), there will be ten C components, two D components (D_1 and D_2), one B component, B_1 , and one E component, E_1 (Fig. 11). In the tenth column of the first row (A10) of the first plate (P1) of the library, L2, (hereinafter designated as location A10,P1,L2), there will be the ten C components, two D components (D_1 and D_2), one B component, B_{10} , and one E component, E_1 . In the first column of the eighth row (H1) of the first plate (P1) of the library, L2, (hereinafter designated as location H1,P1,L2), there will be the ten C components, two D components (D_1 and D_2), one B component, B_1 , and one E component, E_8 . In the tenth column of the eighth row (H10) of the first plate (P1) of the library, L2, (H10,P1,L2) there will be the ten C components, two D components (D_1 and D_2), one B component, B_{10} , and one E component, E_8 . Hence the matrix containing all ten columns and all eight rows are used to accommodate 1600 compounds in total.

In the first column of the first row (A1) of the second plate (P2) of the library, L2, (hereinafter designated as location A1,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_1 , and one E component, E_1 (Fig. 12). In the tenth column of the first row (A10) of the second plate (P2) of the library, L2, (hereinafter designated as location A10,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_{10} , and one E component, E_1 . In the first column of the second row (B1) of the second plate (P2) of the library, L2, (hereinafter designated as location B1,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_1 , and one E

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component, E_2 . In the tenth column of the eighth row (H10) of the second plate (P2) of the library, L2, (H10,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_{10} , and one E component, E_8 .

5 In the first column of the first row (A1) of the third plate (P3) of the library, L2, (hereinafter designated as location A1,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1 , and one E component, E_1 (Fig. 13). In the tenth column of the first
10 row (A10) of the third plate (P3) of the library, L2, (hereinafter designated as location A10,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_{10} , and one E component, E_1 . In the first column of the second row (B1) of the third plate (P3) of
15 the library, L2, (hereinafter designated as location B1,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1 , and one E component, E_2 . In the tenth column of the eighth row (H10) of the third plate (P3) of the library, L2, (H10,P3,L2),
20 there will be ten C components, two D components (D_5 and D_6), one B component, B_{10} , and one E component, E_8 .

In the first column of the first row (A1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A1,P4,L2), there will be ten C components, two D
25 components (D_7 and D_8), one B component, B_1 , and one E component, E_1 (Fig.14). In the tenth column of the first row (A10) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A10,P4,L2), there will be ten C components, two D components (D_7 and D_8), one B
30 component, B_{10} , and one E component, E_1 . In the first

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column of the second row (B1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location B1,P4,L2), there will be ten C components, two D components (D₇ and D₈), one B component, B₁, and one E component, E₂. In the tenth column of the eighth row (H10) of the fourth plate (P4) of the library, L2, (H10,P4,L2), there will be ten C components, two D components (D₇ and D₈), one B component, B₁₀, and one E component, E₈.

It will be apparent to those skilled in the art that the synthesis of two orthogonal sets of mixtures in solution providing two complementary FRET libraries indexed in two dimensions for autodeconvolution does not require the currently preferred arrangement -B₁₋₁₀-C₁₋₁₀-D₁₋₈-E₁₋₈-.

The general concept of two orthogonal sets of mixtures indexed in two dimensions can be applied to various permutations of numbers of wells, plate layout, number of permutations per mixture etc.

A numerical interrelationship can be defined as follows:

General Deconvolution Formulae

$$-Bb-Cc-Dd-n(Ee)- \quad (I)$$

- 1) Primary and Secondary plates preferably have the same number of compounds per well [X]: otherwise there are two values, having X_p and X_s respectively.
- 2) The primary library comprises [np] plates.

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If $R_p.C_p = R_s.C_s$, then the number of plates in the secondary library is also $[np]$. If not, the number of plates in the secondary library $[ns]$ is:

$$ns = \frac{R_p.C_p}{R_s.C_s} . np$$

eg. A primary library of $np=4$, $R_p=8$, $C_p=10$ can be set out in an $R_s=4$, $C_s=5$ secondary library with the number of plates equal to:

$$ns = \frac{8 \times 10}{4 \times 5} . np$$

$$= 16 \text{ plates.}$$

Number of compounds per well

$$-Bb-Cc-Dd-np(Ee) - \quad (1)$$

Number of possible combinations $[k]$ is given by:

$$k = b.c.d.np.e \quad (2)$$

When number of wells on a plate = $[N]$, number of compounds per well = $[X]$ and number of plates = $[np]$:

$$k = X.N.np \quad (3)$$

However, number of wells $[N]$ is also defined by the number of rows $[R_p]$ and number of columns $[C_p]$:

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$$N = R_p \cdot C_p \quad (4)$$

Combining (3) and (4):

$$k = X \cdot R_p \cdot C_p \cdot n_p \quad (5)$$

Combining (2) and (5):

$$5 \quad b \cdot c \cdot d \cdot n_p \cdot e = X \cdot R_p \cdot C_p \cdot n_p \quad (6)$$

Cancelling $[n_p]$ from both sides of the equation:

$$b \cdot c \cdot d \cdot e = X \cdot R_p \cdot C_p \quad (7)$$

Two of the variables (e.g. $b \cdot c$) on the left side of the equation must each be equal in number to the number of
 10 columns $[C_p]$, whilst a remaining variable (e.g. d) on the left side must be equal in number to the number of rows $[R_p]$. So:

$$[C_p]^2 \cdot R_p \cdot e = X \cdot R_p \cdot C_p \quad (8)$$

Cancelling $[C_p]$ and $[R_p]$ from both sides of the equation:

$$15 \quad C_p \cdot e = X \quad (9)$$

where $[e]$ is the number of variants along a fixed row; and

if $R_p = C_p$, then $R_p \cdot e = X$.

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Example

for a 10 x 10 x 8 x 8 format over 4 plates:

np.e = 8 => e = 2

10 x 2 = X

5 X = 20.

From an understanding of the general deconvolution formulae shown above, those skilled in the art will readily appreciate that the advantageous results of self-deconvolution according to the invention are obtainable
10 utilising a number of different arrangements of wells, plate layouts, mixtures etc and that such variants on the preferred embodiment illustrated herein are intended to be within the scope of the present invention.

The FRET strategy is based on the synthesis of two
15 orthogonal sets of mixtures in solution. These solutions are each indexed in two dimensions. Thus the data from, for example, a protease scan identifies the most active compounds without the need for decoding or resynthesis. The positional preferences of sub-units (in this case
20 amino acids) are optimised with respect to all other variant positions simultaneously. The synergistic relationship between all four positions is realised and both positive, beneficial and negative, deactivating data are generated. This leads to families (sub-populations)
25 of substrates and their sub-unit preferences. The data can be fed into molecular modelling programs to generate

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pharmacophoric descriptors that encompass both the desirable features (from the positive data) and indicate undesirable interactions (from the negative data sets). Note that a one dimensional scan only indicates one position at a time as 'most active' and does not explore the synergistic relationship between positions.

The invention will now be described by reference to the following examples.

Example 1:

In this Example the proteolytic enzyme of interest is Der P1, which is found in house dust mite faeces. The example illustrates the synthesis of a number of FRET compounds in which the suitable bond is an unsubstituted amide bond, their use as a library for screening for potential substrates of Der P1, and subsequent identification and synthesis of active inhibitors of the enzyme.

Purification of Der pI.

Crude mite extract (~100mg, SmithKline-Beecham, U.K) was dissolved in 5mL Phosphate Buffered Saline (PBS; 50 mM potassium phosphate; pH 7.4 containing 150 mM NaCl Der pI was purified by affinity column chromatography using 4C1 antibody (indoor Biotechnology, Deeside, U.K.) The crude preparation was mixed with ~2 mL of affinity resin for 2 h at 4°C and then washed with 2-3 volumes of PBS. Elution of bound protein was carried out using 5mM glycine containing 50%(v/v) ethylene glycol. Fractions(2.2 mL) were collected and neutralised with 0.8 mL of 0.2 M sodium phosphate

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buffer, pH 7.0. The fractions were pooled and dialysed overnight against 4 L PBS followed by a second dialysis against 2 L PBS for 2-3h. The total protein was concentrated as required by ultrafiltration (MacroSep; Flowgen, U.K.)

Synthesis of compounds

The compounds were synthesised using the Multipin approach^{25,26} using Fmoc-Rink amide Macro crowns (Chiron Mimotypes Pty., Ltd., 11 Duerdin Street, Clayton Victoria 3168, Australia) with a loading of 7 μ Moles.

The amino acid residues of each of the compounds were linked using amide bonds in a suitable form. The coupling chemistry employed is similar to that reported in the literature²⁷ for fluorenylmethoxycarbonyl protected amino acids and activated pentafluorophenyl esters, in which the side-chains are protected using acid labile protecting groups known to those skilled in the art, such as Boc- (for the -NH₂ of Lysine, -NH₂ of anthranilic acid and guanidino of arginine), tBu- (for the -OH groups of serine, threonine and tyrosine), t-Bu for the -COOH group of Aspartic acid and Glutamic acid, Trityl- (for the Amide of Asparagine and Glutamine, and the amine functionality of the Histidine ring.

The N- α -fluorenylmethoxycarbonyl protecting group of the coupled residues were cleaved using 20% piperidine in dimethylformamide (DMF) for 30 minutes at 20° C. The coupling reactions for the free acids such as Boc-ABz-OH (Boc-2-aminobenzoic acid), and Fmoc-(3-nitro)tyrosine-OH

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were accomplished using 10 equivalents of a mixture of the free acid (1 eq.) :TBTU (0.98 eq.): HOBt (0.98 eq.) : N-methylmorpholine (1.96 eq.) in dimethylformamide (500 μ L) as solvent for 5 hours at 20° C. The other amino acids were
5 coupled as their pentafluorophenyl esters²⁶ for 2-6 hours.

Hence, in order to couple approximately equal ratios of each component in the mixture of the derivatised amino acids as their pentafluorophenyl esters, a solution of a total of 0.98 equivalents (relative to the amino group
10 loading on the crown) of the mixture of amino acid pentafluorophenyl esters : HOBt (1 eq.) in DMF (500 μ L) were coupled for 16 hours at 20° C. The pins were then washed well with DMF and then recoupled using the same mixture under the same conditions. A third coupling of 10
15 equivalents (relative to the amino group loading of the crown) for 2 hours in DMF was performed using this coupling protocol with equimolar mixtures of the derivatised pentafluorophenyl esters of the amino acids in slightly less than 1 equivalent, it is possible to obtain
20 approximately equal amounts of the coupled products to the crown. In this fashion the libraries are constructed with 20 compounds present on each crown. The compounds were cleaved from the crowns directly into the 80 designated wells of the desired 96 well plate. In the cleavage
25 protocol each crown was treated with a mixture (600 μ L) containing trifluoroacetic acid (95%), triethylsilane (5%) for 2 hours at 20° C. The crowns were then washed with trifluoroacetic acid (500 μ L) and this was then combined with the cleavage solution.

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The Fmoc-Rink amide Macro crowns (Chiron Mimotypes Pty., Ltd., 11 Duerdin Street, Clayton Victoria 3168, Australia) at 7 μ Mol loading per crown, were coupled with a 10 fold excess of a mixture containing L-Fmoc-Asp(O-t-Bu)-OH (1eq) using TBTU (0.98 eq) and N-methylmorpholine (1.96 eq.) in the presence of HOBt (0.98 eq.) in DMF at 0.14M concentration. After deblocking of the Fmoc group with 20% piperidine in DMF for 30 minutes and subsequent washing with DMF and then methanol, coupling of the Fmoc-(3-nitro)tyrosine-OH was accomplished using 10 equivalents of a mixture of the Fmoc-(3-nitro)tyrosine-OH (1 eq.) :TBTU (0.98 eq.) : HOBt (0.98 eq.) : N-methylmorpholine (1.96 eq.) in dimethylformamide as solvent at 0.14 M concentration for 5 hours at 20° C. Removal of the Fmoc group (*vide infra*) was followed by coupling of the mixtures of amino acids in the ratios outlined and under the conditions described (*vide infra*).

In a particular example the amino acids comprising group B include Ala, Val, Leu, Ser, Asn, Gln, Glu, Lys, Phe, Pro. The amino acids comprising group C include Ala, Val, Leu, Ser, Asn, Gln, Glu, Lys, Phe, Pro. The amino acids comprising group D include Ala, Val, Ile, Leu, Nle, Ser, Glu, Phe. For n=4, the amino acids comprising group E include Ala, Val, Ile, Leu, Nle, Ser, Glu, Phe. Otherwise any selection from the amino acids can be made for n=1, 2, or 3.

The plates containing the combined cleavage solutions and were then evaporated to dryness to yield the component mixtures using a rotary centrifuge ("SPEEDVAC", Savant Instruments Inc., Farmingdale, NY) at 800 rpm for 1 hour

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at 20° C under a reduced pressure of 10⁻² mmHg. Each component was then transferred to the final mother plate using a (50%: 45%: 5%) mixture of acetonitrile: water: acetic acid. The plates were then lyophilised to dryness
5 using at 20° C under a reduced pressure of 10⁻² mmHg, and then stored at -20° C. In this fashion libraries of the type shown in Figures 2-14 were prepared.

In further detail, the Multipin approach which was employed is described below:

10 **Multipin Synthesis Of Potential Substrates of Der pI**

The 'Chiron' multipin kit consists of a standard 8 x 12 pin holder containing 96 'pin stems' to which are reversibly attached 'crowns'. The 'crowns' provide a reactive polymer surface upon which a growing peptide is
15 anchored during solid phase peptide synthesis. Each crown (the equivalent of the peptide-resin in standard solid phase synthesis) can be considered to be an independent reactor by performing simultaneous synthesis in individual 1mL wells of industry standard 96 well plates. Each well,
20 and thus each crown, can be charged with a unique set of reagents providing unique sequences to each crown. Common steps such as washing or removal of N α protection can be performed concomitantly.

Synthesis is based upon the use of N α -
25 fluorenylmethyloxycarbonyl (Fmoc) protected amino acids. Side-chains of tri-functional amino acids are protected with acid labile groups such as trityl or tert-butyl. The addition of amino-acid residues to the growing peptide

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chain, a process termed 'coupling' proceeds through the utilisation of pre-formed pentafluorophenyl (pfp) esters or activation of the free acid, using the reagents HBTU or BOP in the presence of tertiary base (NMM) and HOBT as catalyst.

The experimental techniques used are fully documented (Maeji, N. J. Bray, A. M. Valerio, R. M. and Wang, W., *Peptide Research*, 8(1), 33-38, 1995 and Valerio, R. M. Bray, A. M. and Maeji, N. J. *Int. J. Pept. Prot. Res*, 44, 158-165, 1994) and the main steps are briefly as follows.

General Methods

Preparation of Multipin Assembly

Whilst wearing standard plastic gloves, Fmoc-Rink Amide derivitized macrocrowns are assembled (simply clipped) onto stems and slotted into the 8 x 12 stem holder in the desired pattern for synthesis.

Removal of N α -Fmoc Protection

A 250mL solvent resistant bath is charged with 200 ml of a 20% piperidine/DMF solution. The multipin assembly is added and deprotection allowed to proceed for 30 minutes. The assembly is then removed and excess solvent removed by brief shaking. The assembly is then washed consecutively with (200mL each), DMF (5mins) and MeOH (5mins, 2mins, 2mins) and left to air dry for 15mins.

Quantitative UV Measurement of Fmoc Chromophore Release

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A 1cm path length UV cell is charged with 1.2mL of a 20% piperidine/DMF solution and used to zero the absorbance of the UV spectrometer at a wavelength of 290nm. A UV standard is then prepared consisting of 5.0mg Fmoc-
5 Asp(OBut)-Pepsyn KA (0.08mmol/g) in 3.2mL of a 20% piperidine/DMF solution. This standard gives $Abs_{290} = 0.55-0.65$ (at RT). An aliquot of the multipin deprotection solution is then diluted as appropriate to give a theoretical $Abs_{290} = 0.6$, and this value compared with the
10 actual experimentally measured absorbance showing the efficiency of previous coupling reaction.

Coupling of amino-acid residues

Whilst the multipin assembly is drying, the appropriate $N\alpha$ -Fmoc amino acid pfp esters (10 equivalents calculated
15 from the loading of each crown) and HOBT (10 equivalents) required for the particular round of coupling are accurately weighed into suitable containers.
Alternatively, the appropriate $N\alpha$ -Fmoc amino acids (10 equivalents calculated from the loading of each crown),
20 desired coupling agent e.g. HBTU (9.9 equivalents calculated from the loading of each crown) and activation eg HOBT (9.9 equivalents calculated from the loading of each crown), NMM (19.9 equivalents calculated from the loading of each crown) are accurately weighed into
25 suitable containers.

The protected and activated Fmoc amino acid derivatives are then dissolved in DMF (500 μ l for each macrocrown, e.g. for 20 macrocrowns, 20 x 10eq x 7mmoles of derivative would be dissolved in 10 000 μ L DMF). The appropriate

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derivatives are then dispensed to the appropriate wells ready for commencement of the 'coupling cycle'. As a standard, coupling reactions are allowed to proceed for 2-6 hours (depending upon nature of coupling e.g. Ala to Ala
5 2 hours Val to Leu 6 hours.

When coupling Fmoc amino-acid pentafluorophenyl esters, 10eq of derivative in DMF (400 μ l) with bromophenol blue stock solution (100 μ l) is used for each macrocrown. This allows monitoring of the progress of the acylation
10 reaction through the disappearance of the deep blue coloration of bromophenol blue in the presence of unreacted amine to a pale yellow upon completion of acylation.

Preparation of Bromophenol Blue Stock Solution

15 Bromophenol blue (20mg) is dissolved in DMF (50mL) and HOBt (10mg) added.

Washing Following Coupling

If a 20% piperidine/DMF deprotection is to immediately follow the coupling cycle, then the multipin assembly is
20 briefly shaken to remove excess solvent washed consecutively with (200mL each), MeOH (5mins) and DMF (5mins) and deprotected (see above). If the multipin assembly is to be stored, then a full washing cycle consisting brief shaking then consecutive washes with
25 (200mL each), DMF (5mins) and MeOH (5mins, 2mins, 2mins) is performed.

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Acidolytic Mediated Cleavage of Peptide-Pin Assembly

Acid mediated cleavage protocols are strictly performed in a fume hood. A polystyrene 96 well plate (1mL / well) is labelled, then the tare weight measured to the nearest mg.

5 Appropriate wells are then charged with a trifluoroacetic acid / triethylsilane (95:5, v/v, 600 μ l) cleavage solution, in a pattern corresponding to that of the multipin assembly to be cleaved.

The multipin assembly is added, the entire construct

10 covered in tin foil and left for 2hrs. The multipin assembly is then added to another polystyrene 96 well plate (1mL /well) containing trifluoroacetic acid / triethylsilane (95:5, v/v, 600 μ l) (as above) for 5 mins.

The cleaved assembly is washed with DMF (200 μ L, 5mins),

15 MeOH (200 μ L, 5mins), the spent crowns removed and discarded, the stems removed and washed by sonication in methanol (1hr, RT).

Work up of Cleaved Peptides

The primary polystyrene cleavage plate (2hr cleavage) and

20 the secondary polystyrene plate (5min wash) (see above) are then placed in the SpeedVac and the solvents removed (minimum drying rate) for 90mins.

The contents of the secondary polystyrene plate (see above) are transferred to their corresponding wells on the

25 primary plate using an acetonitrile / water / acetic acid

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(50:45:5, v/v/v) solution (3 x 150 μ l) and the spent secondary plate discarded.

Analysis of Products

1.0 μ l of each well (see above) is diluted to 400 μ l with
5 0.1%aq TFA and analysed by HPLC-MS. Column Vydac C4
(214TP52, narrowbore, 21 x 250mm). Eluents :- Solvent A =
0.1%aq trifluoroacetic acid, Solvent B = acetonitrile /
10%A. Gradient :- 10-90% B in A over 27mins, 250ml / min,
215nm UV detection. The individual substrates described
10 below were prepared by the above methods and shown by
HPLC-MS to be >95% with the correct mass.

Final Lyophilisation of Peptides

The primary polystyrene plate (plus the washings from the
secondary plate) is covered with tin foil, held to the
15 plate with an elastic band. A pin prick is placed in the
foil directly above each well and the plate placed at
-80°C for 30mins. The plate is then lyophilised on the
'Heto freeze drier' overnight. Where appropriate
individual peptides were then weighed and dissolved to
20 10mM stock solutions in DMSO prior to biological
screening. Alternatively the 20 component mixture is
weighed and the peptide/20 component ratio is calculated.

Further coupling of amino acid residues was carried out
according to the multipin approach described above. Whilst
25 the multipin assembly was drying, the appropriate N α -Fmoc
amino acids (10 equivalents calculated from the loading of
each crown), HATU coupling agent (9.9 equivalents

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calculated from the loading of each crown), HOAt catalyst
 (9.9 equivalents calculated from the loading of each
 crown) and DIPEA (19.9 equivalents calculated from the
 loading of each crown) were accurately weighed into
 5 suitable containers.

The protected N α -Fmoc amino acids and coupling agents were
 then dissolved in DMF (500 μ l for each macrocrown) and
 activated by the addition of DIPEA. The appropriate
 derivatives were then dispensed to their appropriate wells
 10 and as standard coupling to each macro crown was allowed
 to proceed for 2 hours.

When coupling particularly hindered amino acid residues
 such as N-Methyl, C α -Methyl or unusual amino acids (whose
 coupling efficiency is unknown) the coupling reaction was
 15 repeated, as standard, for a further 2 hours.

Substrates for Der pI

Using the general techniques described above, the
 following compounds were prepared and assayed as potential
 substrates against Der pI purified as described above.

20	Peptide [SEQ ID Nos. 1-76]	Measured K _m (μ M)
	Abz-Val-Ala-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	12
	H-Val-Ala-Nle-Ser-TyrNO ₂ -Asp-NH ₂	NS
	H-Ala-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
	Ac-Val-Ala-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
25	H-Val-Ala-Nle-Ser-Tyr(NO ₂)-NH ₂	NS
	H-Ala-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NS

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	Ac-Val-Ala-Nle-Ser-Tyr(NO ₂)-NH ₂	NS
	Abz-Val-Ala-Nle-Ser-Tyr(NO ₂)-NH ₂	NM
	Abz-Val-Ala-Nle-Ser-NH ₂	NM
	Abz-Val-Ala-Nle-Ser-Phe-Asp-NH ₂	NM
5	Abz-Val-Ala-Nle-Ser-Tyr-Asp-NH ₂	NM
	Abz-Val-Ala-Nle-Ser-Ala-Asp-NH ₂	NM
	Abz-Val-Ala-Nle-Ser-Lys-Asp-NH ₂	NM
	Abz-Val-Ala-Nle-Ser-eAHA-Asp-NH ₂	NM
	Abz-Ala-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NM
10	Abz-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
	Bz-Val-Ala-Nle-Ser-Tyr(NO ₂)-NH ₂	NM ^a
	Bz(2-carboxy)-Val-Ala-Nle-Ser-Tyr(NO ₂)-NH ₂	NM ^a
	Chex-Val-Ala-Nle-Ser-Tyr(NO ₂)-NH ₂	NM ^a
	n-Bu-Val-Ala-Nle-Ser-Tyr(NO ₂)-NH ₂	NM ^a
15	Piv-Val-Ala-Nle-Ser-Tyr(NO ₂)-NH ₂	NM ^a
	Bz-Val-Ala-Nle-Ser-Tyr(NO ₂)-NH ₂	NM ^a
	Abz-Val-Ala-Lys-Ser-Tyr(NO ₂)-Asp-NH ₂	14
	Abz-Val-Ala-Gln-Ser-Tyr(NO ₂)-Asp-NH ₂	6
	Abz-Val-Ala-Thr-Ser-Tyr(NO ₂)-Asp-NH ₂	6
20	Abz-Val-Ala-hLeu-Ser-Tyr(NO ₂)-Asp-NH ₂	4
	Abz-Val-Ala-Cha-Ser-Tyr(NO ₂)-Asp-NH ₂	5
	Abz-Val-Ala-His-Ser-Tyr(NO ₂)-Asp-NH ₂	> 20
	Abz-Val-Ala-ACH-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
	Abz-Val-Ala-DNle-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
25	Abz-Val-Ala-3pyr-Ser-Tyr(NO ₂)-Asp-NH ₂	10
	Abz-Val-Ala-Hyp-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
	Abz-Val-Ala-ACP-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
	Abz-Val-Lys-hLeu-Ser-Tyr(NO ₂)-Asp-NH ₂	35
	Abz-Val-DAla-hLeu-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
30	Abz-Val-Tic-hLeu-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
	Abz-Val-ACH-hLeu-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
	Abz-Val-Met(O)-hLeu-Ser-Tyr(NO ₂)-Asp-NH ₂	35

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	Abz-Val-2Nal-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	NS
	Abz-Val-ACP-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	NS
	Abz-Val-DLys-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	NS
	Abz-Val-DGln-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	NS
5	Abz-Val-3pyr-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	NS
	Abz-Val-Cha-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	NS
	Abz-DVal-Ala-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	NS
	Abz-Gln-Ala-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	12
	Abz-Lys-Ala-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	>15
10	Abz-Tic-Ala-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	NS
	Abz-ACH-Ala-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	NS
	Abz-Met (O) -Ala-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	20
	Abz-3pyr-Ala-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	>10
	Abz-2Nal-Ala-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	15
15	Abz-Leu-Ala-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	18
	Abz-Cha-Ala-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	9
	Abz-Bip-Ala-hLeu-Ser-Tyr (NO ₂) -Asp-NH ₂	2.5
	Abz-Bip-Ala-hLeu-Tyr-Tyr (NO ₂) -Asp-NH ₂	3
	Abz-Bip-Ala-hLeu-Leu-Tyr (NO ₂) -Asp-NH ₂	3.7
20	Abz-Bip-Ala-hLeu-Lys-Tyr (NO ₂) -Asp-NH ₂	2
	Abz-Bip-Ala-hLeu-Asp-Tyr (NO ₂) -Asp-NH ₂	5.0
	Abz-Bip-Ala-hLeu-Abu-Tyr (NO ₂) -Asp-NH ₂	1.7
	Abz-Bip-Ala-hLeu-Cha-Tyr (NO ₂) -Asp-NH ₂	2.5
	Abz-Bip-Ala-hLeu-Met (O) -Tyr (NO ₂) -Asp-NH ₂	5
25	Abz-Bip-Ala-hLeu-Thr-Tyr (NO ₂) -Asp-NH ₂	2.5
	Abz-Bip-Ala-hLeu-3pyr-Tyr (NO ₂) -Asp-NH ₂	4
	Abz-Bip-Ala-hLeu-Bu ^t Gly-Tyr (NO ₂) -Asp-NH ₂	4
	Abz-Bip-Ala-hLeu-Hyp-Tyr (NO ₂) -Asp-NH ₂	4
	Abz-Phe-Val-Ala-Nle-Ser-Tyr (NO ₂) -Asp-NH ₂	NM
30	Abz-3.Pyr-Val-Ala-Nle-Ser-Tyr (NO ₂) -Asp-NH ₂	NM
	Abz-1.Naph-Val-Ala-Nle-Ser-Tyr (NO ₂) -Asp-NH ₂	17
	Abz-2.Naph-Val-Ala-Nle-Ser-Tyr (NO ₂) -Asp-NH ₂	NM

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	Abz-Tyr-Val-Ala-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NM
	Abz-Bip-Val-Ala-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	10
	Abz-Lys-Val-Ala-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	15
	Abz-Glu-Val-Ala-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	20
5	Abz-Leu-Val-Ala-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NM
	Abz-Hyp-Val-Ala-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NS

NS indicates that the peptide was not hydrolysed by *Der* pI.

NM indicates that the peptide was a substrate for *Der* pI, but the K_m was not measured.

10 NM^a indicates that the peptide was a substrate for *Der* pI, and its cleavage was followed by HPLC-MS showing hydrolysis to occur between-Nle-Ser-.

Ranked in order of cleavage rate :-Bz > n-But > Piv >
15 Bz(2-carboxy) > Abz.

Coupling of amino acid residues was carried out according to the multipin approach described above. Whilst the multipin assembly was drying, the appropriate N α -Fmoc amino acids (10 equivalents calculated from the loading of
20 each crown), HATU coupling agent (9.9 equivalents calculated from the loading of each crown), HOAt catalyst (9.9 equivalents calculated from the loading of each crown) and DIPEA (19.9 equivalents calculated from the loading of each crown) were accurately weighed into
25 suitable containers.

The protected N α -Fmoc amino acids and coupling agents were then dissolved in DMF (500 μ l for each macrocrown) and activated by the addition of DIPEA. The appropriate

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derivatives were then dispensed to their appropriate wells and standard coupling to each macrocrown was allowed to proceed for 2 hours.

When coupling particularly hindered amino acid residues such as N-Methyl, C α -Methyl or unusual amino acids (whose coupling efficiency is unknown) the coupling reaction was repeated, as standard, for a further 2 hours.

The following sequences were synthesised in this way:

Peptide Sequence [SEQ ID Nos. 77-85]		Measured Km (μ M)
10	Abz-Val-Ala-(NMe)Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
	Abz-Val-(NMe)Ala-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
	Abz-Val-Ala-Aib-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
	Abz-Val-Aib-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
	Abz-Deg-Ala-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
15	nBu-D.Ser-D.Nle-D.Ala-D.Val-p.Aba-NH ₂	NS
	Bz-Val-Ala-Statine-Ser-eAha-NH ₂	NS
	Abz-p.Aba-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NS
	Abz-Cmpi-Nle-Ser-Tyr(NO ₂)-Asp-NH ₂	NS

NS-Not Substrate: Not hydrolysed by Der pI

20 NM-Substrate but not measured: Substrate for Der pI but not measured.

Assay procedure

Each mixture of 20 compounds in the libraries of the apparatus described herein was screened at a concentration of 1.0 μ M per compound in an assay against the cysteinyl

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protease *Der* pI. The most active wells were identified by the rate of emission of fluorescence at 420 nm when the samples were irradiated at 320 nm. An analysis of the two complementary libraries showed that the best substrates
5 for the enzyme were:

Abz-B-C-D-E-Tyr(NO₂)-Asp-NH₂

Where

B= Valine>Alanine, Glutamine, Leucine, Phenylalanine

C= Alanine>>Glutamine, or Lysine.

10 D= Leucine, Norleucine or Alanine>Serine

E=Serine

The best substrate was:

[SEQ ID:1] Abz-Val-Ala-Nle-Ser-Tyr(NO₂)-Asp-NH₂

This compound was then resynthesised as a single component
15 using the peptide synthesis methodology described herein. The k_{cat}/K_m value for the pure substrate in the *Der* pI assay was measured as $3.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, and was considered to be suitably high for use in a high throughput assay for the general screening of inhibitors of *Der* pI.

20 High throughput assay development

Plate assays were carried out in 96 well plate format, using 0.1 ug of *Der* pI per 100 μL assay volume in each well and using 20 μM of the substrate. All assays were performed in Assay Buffer (AB; 50 mM potassium phosphate,
25 pH 8.25 containing 1mM ethylenediaminetetraacetic acid (EDTA) and 1mM dithiothreitol (DTT). The *Der* pI enzyme is

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pre-activated by addition of DTT and this is incubated at room temperature for 5 min. prior to initiation of the assay. As an example for the screening methodology, each well contains a 5 μ L of a 20 μ M solution of the test compound in DMSO, 10 μ L of a 200 μ M aqueous solution of the substrate, **2**, and 85 μ L of Der pI in AB is added to initiate the reaction. Enzyme activity is monitored by fluorescence using 320 nm for excitation and 420nm for the emission wavelengths using a Labsystems Fluroskan Ascent machine. Kinetic measurements were carried out using a Hitachi F-4500 Fluorescence Spectrophotometer.

Synthesis of inhibitors of Der pI

The best substrate described above was shown by HPLC-mass spectroscopic analysis of the enzyme/ assay solution, to be cleaved between the Norleucine-Serine amide bond. Replacement of the terminal Abz group by a series of derivatives (e.g. Boc-, Pivaloyl, Benzoyl, and 2- carboxy-Benzoyl) affected substrate activity and specificity for the Der pI enzyme. With this knowledge of the P₁-P_{1'} cleavage site and for the P₄-P₃-P₂-P₁ motif, the compound Boc-Val-Ala-Leu-H, **4**, was synthesised as shown in Scheme 1a, figure 15.

Attachment of a suitable Michael acceptor such as CH=CH-CO₂Et, and -CH=CH-SO₂ Ph to the motif (Scheme 2, figure 16), provided active inhibitors of the enzyme with apparent IC₅₀ values of 50nM, 1000 nM and 100nM respectively.

Preparation of an Acyloxymethylketone Series

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A series of acyloxymethylketone compounds having active *Der PI* inhibitor activity was prepared by the following procedures:

N-Benzoyl-L-valyl-L-alanyl-L-norleucine

- 5 N-Benzoyl-L-valyl-L-alanyl-L-norleucine was prepared by solid phase benzoylated peptide synthesis as follows:

Resin loading (step 1)

- 2-Chlorotritylchloride resin (4.9g, 1.05mmol/g, Novabiochem) was swelled in dichloromethane (40ml) and a
10 suspension of Fmoc-L-norleucine added and stirred for 5 minutes. A solution of diisopropylethylamine in DCM(10ml, 57mmol in 30ml) was added over 5minutes and the resulting mixture stirred at room temperature for 2 hours. Methanol (5ml) added and reaction mixture stirred for a further
15 10minutes before resin filtered and washed with 3x DCM, 2x DMF, 2x 2-propanol, 2x DMF, 2x 2-propanol, methanol, 2x ether and dried under vacuum for 24 hours.

Amino acid deprotection (step 2)

- Fmoc-L-norleucine loaded resin was deprotected by
20 treatment with 20% piperidine in DMF over 4 hours. The swollen resin was filtered, washed with 5x DMF, 2x ether and dried under vacuum for 24 hours.

Peptide chain extension (step 3)

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L-Norleucine loaded resin (5mmol) was added to a solution of Fmoc-L-alanine (6.23g, 20mmol), hydroxybenzotriazole (3.0g, 20mmol), 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (7.59g, 20mmol) and diisopropylethylamine (6.97ml, 40mmol) in DMF (20ml) and allowed to swell over 4 hours with mild agitation. Resin was filtered and washed with 4x DMF, 2x ether and dried under vacuum overnight.

Steps (2) and (3) were carried out repetitively with Fmoc-L-alanine and Fmoc-L-valine to afford resin bound tripeptide H-L-valyl-L-alanyl-L-norleucine.

Peptide chain benzylation (step 4)

L-Valyl-L-alanyl-L-norleucine loaded resin (1g, approx. 1mmol) was added to a solution of benzoic acid (0.488g, 4mmol), hydroxybenzotriazole (0.6g, 4mmol), 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (1.52g, 4mmol) and diisopropylethylamine (1.40ml, 8mmol) in DMF (5ml) and allowed to swell over 6 hours with mild agitation. Resin was filtered and washed with 4x DMF, 2x ether and dried under vacuum overnight.

Resin cleavage (step 5)

N-Benzoyl-L-valyl-L-alanyl-L-norleucine loaded resin (1.0g, approx. 1mmol) was treated with a 1% solution of trifluoroacetic acid in dichloromethane (20ml) containing triethylsilane (320ml, 2mmol) for 1 hour. Resin was removed by filtration and washed with dichloromethane

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(3x10ml). Organic layer was collected, evaporated and titrated with ether to afford N-benzoyl-L-valyl-L-alanyl-L-norleucine. (285mg). Electrospray-MS m/z 407 [MH⁺].

Bromomethylketone formation (step 6)

5 **N-Benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone**
N-Benzoyl-L-valyl-L-alanyl-L-norleucine (140mg, 0.34mmol) was suspended in dry THF (3ml) and dry DMF was added dropwise to afford homogeneity. The reaction mixture was cooled to -10°C and isobutylchloroformate (129ml, 1.0mmol)
10 and N-methylmorpholine (109ml, 1.0mmol) added with stirring under Argon. The mixture was stirred for 30 minutes before a solution of diazomethane in ether (5ml, approx. 2mmol) was added. The reaction mixture was allowed to warm to room temperature over 1 hour before a 1:1 solution of
15 acetic acid and 50% HBr (1ml, 3.0mmol HBr) was added dropwise and stirred for 15 minutes. The organic phase was diluted with ethylacetate (40ml), washed with water (10ml), brine (10ml) and sodium bicarbonate (2x10ml), dried over MgSO₄, solvent removed under vacuum. This
20 afforded an off white solid (152mg) which could be further purified as required by prep. HPLC. Electrospray-MS m/z 482 [MH⁺] and 484 [MH⁺].

Acyloxymethylketone formation (step 7)

25 **N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2, 6-bis(trifluoromethyl) benzoyloxymethyl ketone**

A mixture of potassium fluoride (0.1mmol, 6mg) and 2,6-bis(trifluoromethyl)benzoic acid (0.066mmol, 17mg) in dry DMF (500ml) was stirred over molecular sieves at room

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temperature for 5 minutes. A solution of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone (0.033mmol, 16mg) in dry DMF (500ml) was added and the reaction mixture stirred for 1 hour. The reaction mixture was
5 passed through a short silica plug and washed with 5% methanol in dichloromethane. Solvent was removed under vacuum and the residue purified using prep. HPLC. Freeze drying afforded (6.4mg) as a white lyophilisate. Electrospray-MS m/z 660 [MH⁺].

10 Similarly the following compounds were prepared:

N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2, 6-dimethylbenzoyloxymethyl ketone (Electrospray-MS m/z 552 [MH⁺]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2,6-dimethylbenzoic acid.

15 N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2-hydroxybenzoyloxymethyl ketone (Electrospray-MS m/z 540 [MH⁺]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2-hydroxybenzoic acid.

20 N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2,6-dichlorobenzoyloxymethyl ketone (Electrospray-MS m/z 592 [MH⁺] and 594 [MH⁺]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2,6-dichlorobenzoic acid.

25 N-Benzoyl-L-valyl-L-alanyl-L-norleucine benzoyloxymethyl ketone (Electrospray-MS m/z 524 [MH⁺]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and benzoic acid.

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N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2,3,4,5,6-pentafluorobenzoyloxymethyl ketone (Electrospray-MS m/z 614 [MH⁺]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2,3,4,5,6-pentafluorobenzoic acid.

5 N-Benzoyl-L-valyl-L-alanyl-L-norleucine 1,1-dimethylpropyloxymethyl ketone (Electrospray-MS m/z 504 [MH⁺]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 1,1-dimethylpropanoic acid.

10 N-Benzoyl-L-valyl-L-alanyl-L-norleucine N(-benzyloxycarbonyl)-D-serinyl-(O-tert-butyl)oxymethyl ketone (Electrospray-MS m/z 697 [MH⁺]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and N-benzyloxycarbonyl-D-serine-O-tert-butylether.

15 N-Benzoyl-L-valyl-L-alanyl-L-norleucine N(-benzyloxycarbonyl)-D-serineoxy methyl ketone (Electrospray-MS m/z 641 [MH⁺]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and N-benzyloxycarbonyl-D-serine.

20 N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2-furanoxy methyl ketone (Electrospray-MS m/z 514 [MH⁺]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2-furan carboxylic acid.

25 N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2,6-dichlorophenylacyloxy methyl ketone (Electrospray-MS m/z 606 [MH⁺], 608 [MH⁺]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2,6-dichlorophenylacetic acid.

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Standard Prep. HPLC conditions were as follows: C4 preparative HPLC system (Vydac, 22x 250mm) eluting at 10ml per minute a gradient of 5-95% (90% acetonitrile (0.1% TFA)) over 30 minutes.

- 5 The following compounds were prepared by the techniques and procedures described beneath each named compound.

Preparation of Ethyl-(S)-(E)-3-((tert-butoxy carbonyl amino valyl alanyl) amino-6-methyl-hept-2-enoate

- 10 To a suspension of sodium hydride (46mg, 1.9mmol) in anhydrous THF (4ml) cooled to 0°C was added a solution of triethylphosphonoacetate (420mg, 1.9 mmol) in THF (2 ml) dropwise over 5 minutes and the mixture stirred until gas evolution ceased. The solution was added dropwise to a solution of BocVAL-CHO (600mg, 1.56mmol) in dry THF cooled to -10°C. The reaction mixture was stirred for 1 hour and saturated ammonium chloride (10ml) was added. A white solid precipitated which was removed by filtration and the filtrate was partitioned between ethyl acetate and water. The organic layer was dried with magnesium sulphate and
20 evaporated to give an oil which was crystallised from acetonitrile water to yield the title compound, 640mg, 91%.

MS (EI +ve) required ($M^+(C_{23}H_{41}N_3O_6)+1$)=456: found(M^+H) =456, ($M^+-tBOC+1$) =356 (100%).

- 25 **Preparation of (S)-(E)-3-((tert-butoxy carbonyl amino valyl alanyl) amino-6-methyl-hept-2-enoic acid**

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The ethyl ester (455mg, 1mmol) was dissolved in dioxane (10ml) and water added followed by lithium hydroxide (126mg, 3mmol). The solution was stirred for 3 hours and 1M HCl aq was added until the pH reached neutrality. The dioxane was removed by rotary evaporation and the pH adjusted to 4 with 1M HCl aq. The title compound precipitated, filtered and washed with water to yield 420mg, 98%.

MS (EI +ve) required $(M^+(C_{21}H_{37}N_3O_6)+1)=428$: found $(M^++H) =428$ (100%).

Preparation of 1,1,1-Trifluoroethyl-(S)-(E)-3-((tert-butoxy carbonyl amino valyl alanyl) amino-6-methyl-hept-2-enoate

The acid (BocVAL-CO₂H) (50mg, 0.117mmol) and dimethylaminopyridine (29mg, 0.24mmol) was dissolved in dry dichloromethane (1ml) and cooled to 0°C. Water soluble carbodiimide hydrochloride salt (26mg, 0.13mmol) in 0.5ml dichloromethane was added and the solution stirred for 5 minutes. 1,1,1-Trifluoroethanol (0.017ml, 0.23mmol) in 0.5ml dichloromethane was added and the reaction was allowed to warm to room temperature after 1 hour and the reaction mixture stirred overnight. The reaction mixture was washed 2x2ml 0.5M citric acid solution, 1x2ml water, 1x2ml saturated sodium bicarbonate solution, 1x2ml water, dried with magnesium sulphate and evaporated to dryness to give the title compound.

MS (EI +ve) required $(M^+(C_{23}H_{38}N_3O_6F_3)+1)=510$: found $(M^++H) =510$, $((M^+-tBOC)+1) =410$, $((M^+-tBu)+1) =454$ (100%).

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**Preparation of Ethyl-(S)-(E)-3-(N-benzoyl valyl alanyl)
amino-6-methyl-hept-2-enoate**

The tert-butoxy carbonyl protected ethyl ester (16.6mg, 0.036mmol) was dissolved in 4.0M HCl in dioxane (2ml) stirred at room temperature for 30 minutes and evaporated to dryness. The residue was dissolved in DMF (0.5ml) and N-methylmorpholine (7.36mg, 0.073mmol) added followed by benzoyl chloride (5.4mg, 0.038mmol) in DMF 0.5ml. The reaction stirred for 2 hours, diluted with 0.1% trifluoroacetic acid solution (4ml) and acetonitrile (2ml) and injected onto a C4 preparative HPLC system (22x250mm) eluting at 10ml per minute, monitoring at 215nm and a gradient of 10-90% system B over 25 minutes and holding at 90% for 15 minutes. System A = 0.1% TFA in water, system B = 90% acetonitrile, 10% system A. The peak eluting at 26-28 minutes was collected and lyophilised to a white solid, yield 4.5mg, 27%.

Analysis by MS (EI +ve) required $(M^+(C_{25}H_{37}N_3O_5)+1)=460$:
found $(M^+H) = 460$.

Preparation of Diethyl Phenylsulfonylmethylphosphonate

The Diethyl Phenyl sulfonylmethylphosphonate was prepared using a method adapted from I. Shahak, and J. Almog. (Synthesis, 145,1970).

The commercially available diethyl phenylthiomethylphosphonate (1.0 ml, 4.1 mmol) was dissolved in dichloromethane (10 ml). Sulphuric acid (10 ml, 25 %) was added and the mixture cooled on ice. Solid

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potassium permanganate was then added in three aliquots of 0.5g with stirring. After the additions the reaction appeared to be complete. Solid sodium metabisulfite was added slowly until the mixture turned colourless. This was then extracted with ethyl acetate (x3) and the combined organic washings washed with saturated sodium bicarbonate solution followed by brine before drying over sodium sulphate. The volatiles were removed in vacuo. The residue was purified by flash chromatography on silica eluting initially with ethyl acetate/hexane 8/2 followed by pure ethyl acetate.

In this way the desired product, diethyl phenylsulfonylmethylphosphonate (1.0 g, quant) was obtained as a colourless solid.

The product was analysed by mass spectrometry (MS) (MALDI-TOF): required (M^+ ($C_{11}H_{17}O_5PS$) +1) = 292; obtained (M^+ +1) = 292

Preparation of (S)-(E)-3-((tert-butoxycarbonylamino-valyl)alanyl)amino-1-phenylsulfonyl-5-methyl-1-hexene

Diethyl phenylsulfonylmethylphosphonate (38 mg, 129 mmol) was dissolved in dry THF (10 ml) and then cooled to 0°C under an atmosphere of nitrogen. Sodium hydride (8 mg of 60% dispersion in oil, 200 mmol) was added and the mixture stirred for 15 mins (effervescence). The aldehyde ^tBoc-Val-Ala-Leu-CHO (50 mg, 129 mmol) was then added to the resulting solution and the mixture was stirred for 60 mins. The reaction was quenched by the addition of dilute hydrochloric acid (0.1 M), followed by extraction with

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ethyl acetate (x3). The separated organic phase was sequentially washed with saturated sodium bicarbonate solution and brine before drying over sodium sulphate. The volatiles were removed *in vacuo*. The residue was purified
5 by flash chromatography on silica eluting with ethyl acetate/hexane 4/6. An unidentified by-product was eluted first (12 mg) followed by the desired product (S)-(E)-3-((*tert*-butoxycarbonylamino-valyl)alanyl)amino-phenylsulfonyl-5-methyl-1-hexene (22 mg, 32%) as a solid.

10 MS (electrospray) required ($M^+(C_{26}H_{41}O_6N_3S)+1$) = 523: found (M^+Na) = 546, ($(M-t\text{ Boc})+1$) = 424 (100%).

Preparation of Diethyl methylsulfonylmethylphosphonate

The commercially available Diethyl
methylthiomethylphosphonate was converted to the title
15 compound using the method of I. Shahak and J. Almog (*Synthesis*, 171, 1969).

Preparation of (S)-(E)-3-((*tert*-butoxycarbonylamino-valyl)alanyl)amino-1-methylsulfonyl-5-methyl-1-hexene

Diethyl methylsulfonylmethylphosphonate (30 mg, 130 mmol)
20 was dissolved in dry THF (5 ml) and then cooled to 0°C under an atmosphere of nitrogen. Sodium hydride (7 mg of 60% dispersion in oil, 175 mmol) was added and the mixture stirred for 15 mins (effervescence). The aldehyde 'Boc-Val-Ala-Leu-CHO (50 mg, 129 mmol) was then added to the
25 resulting solution and the mixture then stirred for 60 mins. The reaction was quenched by addition of dilute hydrochloric acid (0.1 M), followed by extraction with

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ethyl acetate(x3). The separated organic phase was sequentially washed with saturated sodium bicarbonate solution and brine before drying over sodium sulphate. The volatiles were then removed in vacuo. The residue was
5 purified by flash chromatography on silica eluting with ethyl acetate/hexane 8/2. An unidentified by-product was eluted first (4 mg), followed by the desired product (S)-(E)-3-((tert-butoxycarbonylamino-valyl)alanyl)amino-methylsulfonyl-5-methyl-1-hexene (24 mg, 40%) as a solid.

10 MS (electrospray) required $(M^+(C_{21}H_{39}O_6N_3S)+1) = 462$: found $(M^+Na) = 484$, $((M^+ - \text{Boc}) + 1) = 362$ (100%).

Preparation of Ethyl diethylphosphorylmethylsulfonate

Prepared in accordance with procedure B described in L. Ghosez et. al. (Tetrahedron, 43, 5125, 1987).

15 The product was analysed on MS (electrospray) required $(M^+(C_7H_{17}O_6PS)+1) = 261$: Found $(M^+H) = 261$, $(M^+Na) = 283$.

Preparation of Diethyl(S)-(E)-3-((tert-butoxycarbonylamino-valyl)alanyl)amino-5-methylhexenylsulfonate.

20 Ethyl diethylphosphorylmethanesulfonate (36 ml, ~138mmol) was dissolved in dry THF (5 ml) and then cooled to 0°C under an atmosphere of nitrogen. Sodium hydride (8 mg of 60% dispersion in oil, 200 mmol) was added and the mixture stirred for 15 mins (effervescence). The aldehyde ^tBoc-
25 Val-Ala-Leu-CHO (50 mg, 129 mmol) was added to the resulting solution and the mixture stirred for 30 mins.

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The reaction was quenched by addition of dilute hydrochloric acid (0.1 M), followed by extraction with ethyl acetate (x3). The separated organic phase was sequentially washed with sodium bicarbonate solution and
 5 brine before drying over sodium sulphate. The volatiles were then removed *in vacuo*. The residue was purified by flash chromatography on silica eluting with ethyl acetate/hexane 1/1. The desired product Diethyl(S)-(E)-3-((*tert*-butoxycarbonylamino-valyl)alanyl)amino-5-
 10 methylhexenylsulfonate, (22 mg, 35%) was obtained as a solid.

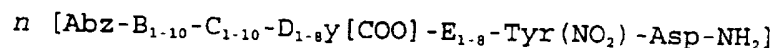
MS(electrospray) required ($M^+(C_{22}H_{41}O_7N_3S)+1$)=492: found (M^++1)=492, ($M^+-t\text{Boc}+1$)=392 (100%)

Example 2:

15 Design of Depsipeptides

Another suitable bond in a compound of general formula (I), (II) or (III) according to the invention is an ester bond to form a depsipeptide. The incorporation of
 20 depsipeptide substrates aided the identification of substrates for low reactivity viral proteases, such as viral serine proteases.

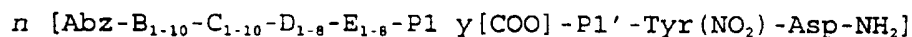
For example, substrates of the general formula



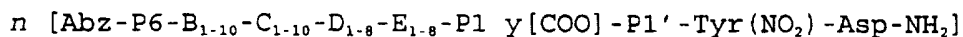
were produced.

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However, a significant proportion of viral proteases only recognise substrate sequences larger than those represented by the general structure above. It is well acknowledged that by the very nature of action of a viral protease (function is to cleave immature viral proteins into the mature viral package) one automatically receives data concerning the natural substrate sites. Thus, the general structure above can be extended by introducing extra fixed amino-acids at appropriate sites. A logical extension would be to introduce the known P1-P1' cleavage site as the depsipeptide bond, then subsequently introduce the four variant positions following the standard format thus;



Furthermore, if these substrates again proved to be too small, one may use the known substrate sequences to introduce additional fixed positions. For instance, with Hepatitis NS3 protease it is known that the natural P6 position is a conserved acidic residue (aspartic or glutamic acid). Thus one could extend the above structure as detailed below.



The novel methodology described herein greatly facilitates the invention of therapeutically useful proteolytic enzyme inhibitors and is commercially exploitable. This is because the best substrate motif for the proteolytic enzyme can be rapidly identified, and, since there exist in the literature a variety of ways for attaching motifs

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which react with the active site of a proteolytic enzyme, especially for aspartyl, metallo, serine and cysteinyl proteases, an enzyme inhibitor can be readily synthesised. Moreover, amide bond replacements or transition state
5 mimetics can be incorporated into the molecule, which would be especially useful for the inhibition of aspartyl or metallo proteases.

The method described also facilitates the rapid development of a screening assay for novel protease
10 inhibitors. The most potent fluorogenic substrate discovered by library screening can subsequently be used for the detection of inhibitors of the particular proteolytic enzyme under scrutiny.

The presence of an inhibitor within the compound libraries
15 described is readily detected by retreatment of the assay mixture with the most active fluorogenic substrate, which will allow the immediate measurement of the remaining proteolytic enzyme activity.

The invention provides self-decoding, combinatorial
20 fluorogenic libraries, and it will greatly facilitate the design and invention of novel protease inhibitors because:

- i. The peptides of the library may have increased aqueous solubility in comparison to peptides containing similar and other
25 fluorogenic and quencher groups.
- ii. The peptides are stable to contaminating exopeptidases.

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- iii. The self deconvolution method described,
coupled with the continuous analysis of the
rate of substrate cleavage data, allows the
immediate identification of the most active
5 binding motif contained within the
substrate library.
- iv. The method allows for the rapid assessment
of the enzyme assay mixture for any
compounds in the library that are acting as
10 enzyme inhibitors.

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Abbreviations

Abbreviations used herein are as follows:

- Abbreviations for amino acids and nomenclature of peptide structures follow the recommendations given in: IUPAC-IUB Commission on Biochemical Nomenclature, (*J. Biol. Chem.*, 247, 997, 1971). All chiral amino acids are of the L configuration unless otherwise stated. Other abbreviations used are :
- Abu , b-amino butyric acid, : Abz, 2-amino benzoyl : ACH,
1-amino-1-carboxy-cyclohexane : ACP, 1-amino-1-carboxy-
cyclopropane : Bip, Biphenylalanine : n-Bu, n-
butoxycarbonyl : Bz, Benzoyl : Bz(2-carboxy), 2-
carboxybenzoyl : Bu^tGly, tert-Butylglycyl : BOP,
benzotriazolyl-oxy-tris-(dimethylamino)-phosphonium
hexafluorophosphate: Cha, cyclohexylalanine : Chex, 1-
carboxycyclohexyl : eAHA, gamma aminohexanoyl : HBTU, O-
benzotriazolyl-N,N,N',N'-tetramethyluronium
hexafluorophosphate : HOBt, 1-hydroxybenzotriazole : Hyp,
trans-4-hydroxyprolinyl : hLeu, homoleucyl : 2Nal, 2-
naphthylalanine : NMM, N-methylmorpholine : Piv, pivoyl :
3pyr, 3-pyridylalanine : Tic, 2-carboxytetrahydroquinolyl
: Tyr(NO₂), 3-nitrotyrosine.
- DMF, dimethylformamide; Fmoc, fluorenylmethoxycarbonyl;
HPLC, high performance liquid chromatography; Pfp,
pentafluorophenyl, tBoc, tert-butoxycarbonyl; tBu, tert-
butyl; TFA, trifluoroacetic acid; Pmc, pentamethyl
chroman, Pbf, pentamethylbenzofuran, TBTU, 2-(1H-

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Benzotriazole-1-yl)-1,1,1,3,3-tetramethyluronium
tetrafluoroborate; Trt, Trityl.

- p.Aba, 4-aminobenzoyl; Aib, Aminoisobutyric acid; Bip,
Biphenylalanine; nBu, n-Butyl; Bz, Benzoyl; Cmpi,
5 Carboxymethylpiperazine; Deg, Diethylglycine; DIPEA, N,N-
Diisopropyl-ethylamine; HATU, O-(7-azabenzotriazol-1-yl)-
1,1,3,3-tetramethyluronium hexafluorophosphate; HOAT, 1-
hydroxy-7-azabenzotriazole; Naph, Naphthylalanine; 3.Pyr,
3-pyridiylalanine; Tyr(NO₂), 3-nitro-tyrosine.

10

References

1. I. Schlechter and A. Berger, Biochem. Biophys. Res. Commun., 1967, 27, 157-162)
2. A. Carmel et al., FEBS Lett., 1973, 30, 11.
- 5 3 M. M. Meldal and I. Svendsen, J. Chem. Soc. Perkin Trans.1, 1995, 1591-1596,
4. M. Meldal and K. Breddam, Anal. Biochem., 1991, 195, 141-147,
5. T. Forster, Ann. Phys., 1948, 6, 55.
- 10 6. A. Yaron, A. Carmel, and E. Katchalski-Katzir, Anal. Biochem. 1979, 95, 228 and references therein.
7. S.A. Latt et al., Anal. Biochem., 1972, 50, 56.
8. A. Persson et al., Anal. Biochem., 1977, 83, 2,96.
9. I. Yu Filppova et al., Bioorg. Khim., 1986, 12, 1172.
- 15 10. J. Pohl et al., Anal. Biochem., 1987, 165, 96.
11. S.J. Pollack et al., J. Am. Chem. Soc., 1989, 111, 5961.
12. E. K. Bratovanova and D.D. Petkov, Analytical Biochem., 1987, 162, 213.
- 20 13. J. Singh et al., J. Med. Chem., 1995, 38, 217-219 and references therein.
14. M. Green et al. in 'Innovation and Perspectives in Solid Phase Synthesis' (R. Epton Ed.) Mayflower Worldwide Ltd., Birmingham, UK. 1994, 239-244.
- 25 15. J. R. Petithory et al., Proc. Natl. Acad. Sci. USA, 1991, 88, 11510-11514
16. J. Berman et al., J. Biol. Chem., 1992, 267, 1434-1437.
- 30 17. H. Drevin, A. -T. Martin, J. Carlsson, S. Oscarsson, T. Lovgren, I. Hemmila and M. Kwiatkowski, WO89/10975, (5-5-1988)

- 68 -

18. A. J. Garman and N. G. Phillips, WO 94/28166 (27-5-1993)
19. G.T. Wang and E.D. Matayoshi, E.P. 428000 (3-11-1989)
- 5 20. G. A. Krafft, G. T. Wang and E.D. Matayoshi, EP 428000, (3-11-1989).
21. G. R. Marshall and M. V. Toth, USP 5,164,300, (11-12-1990).
22. G. R. Marshall and M. V. Toth, USP 5,011,910, (28-10 12-1989).
23. K. T. Chapman, N. A. Thornberry, M. Maccoss, J. R. Weidner, R. A. Mumford, W. K. Hagmann, EP 528487A (16-8-1991)
24. R. P. Haugland, WO 93/04077, (23-8-1991)
- 15 25. R. M. Valerio, A. M. Bray N. J. Maeji, Int. J. Peptide Protein Res., 1994, 44, 158-165
26. M. Bastos, N. J. Maeji and R. H. Abeles, Proc. natl. Acad. Sci., 1995, 92, 6738-6742.
27. 'Solid Phase Peptide Synthesis', E. Atherton and R. 20 C. Sheppard, IRL Press 1989.

- 69 -

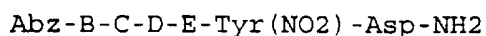
Claims

1. A compound represented by the formula A-B-C-D-nE-F
[I] in which;
A represents a fluorescor internally quenched by F;
5 B, C, D, and E represent groups such that the scissile
bond between any two of these groups is a suitable bond;
F represents a quencher capable of internally quenching
the fluorescor A; and
n represents an interger between 1 and 4 inclusive.
- 10 2. A compound according to claim 1 wherein A represents
an unsubstituted or substituted anthranilic acid
derivative.
3. A compound according to claim 1 or 2 wherein B, C, D
and E independently represent natural or unnatural amino
15 acid residues.
4. A compound according to any preceding claim wherein F
represents an unsubstituted or substituted 3-nitrotyrosine
derivative.
5. A compound according to any preceding claim wherein
20 the scissile bond is a suitable bond selected from the
group consisting of an unsubstituted amide bond, and an
ester bond.
6. A compound according to any preceding claim wherein
the formula further comprises G which ensures the compound
25 is imparted with aqueous solubility.

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7. A compound according to claim 6 which is represented by the formula A-B-C-D-n(E)-F-G in which A, B, C, D, E, F and n are as defined in any one of claims 1 to 6; and
- 5 G represents a hydrophilic moiety which is not an enzyme substrate.

8. A compound according to claim 6 or 7 of general formula:



- 10 Where

B= Valine>Alanine, Glutamine, Leucine, Phenylalanine

C= Alanine>>Glutamine, or Lysine.

D= Leucine, Norleucine or Alanine>Serine

E=Serine

- 15 9. A combinatorial library of FRET compounds comprising a mixture of compounds of formula [I] of claim 1.

10. A set of compounds which comprises two complementary FRET compound libraries.

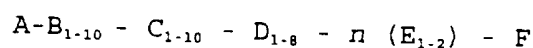
- 20 11. Apparatus which comprises an auto-deconvoluting set of compounds according to claim 10 that facilitates the invention of novel inhibitors of proteolytic enzymes and the rapid generation of structure-activity relationships (SAR) by the detection and measurement of proteolytic enzyme activity.

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12. Apparatus according to claim 11 wherein the set comprises a plurality of mixtures of FRET compounds according to claim 1.

13. Apparatus according to claim 11 or 12 which comprises
5 complementary compound libraries wherein any two FRET molecules in a single mixture of the first library are not found in a single mixture of the second library.

14. Apparatus according to any of claims 11 to 13 wherein the library comprises compounds of represented by the
10 formula:



in which;

A represents a fluorescor internally quenched by F;
B, C, D and E represent groups such that the scissile bond
15 between any two of these groups is a suitable bond;
F represents a quencher capable of internally quenching the fluorescor A; and
n represents an integer between 1 and 4 inclusive.

15. Apparatus according to claim 14 wherein A represents
20 an unsubstituted or substituted anthranilic acid derivative.

16. Apparatus according to claim 14 or 15 wherein B, C, D and E independently represent natural or unnatural amino acid residues.

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17. Apparatus according to any one of claims 14 to 16 wherein F represents an unsubstituted or substituted 3-nitrotyrosine derivative.

18. Apparatus according to any one of claims 14 to 17
5 wherein the scissile bond is a suitable bond selected from the group consisting of an unsubstituted amide bond, and an ester bond.

19. Apparatus according to any one of claims 14 to 18 wherein the formula further comprises G which ensures that
10 compounds in the library are imparted with aqueous solubility.

20. Apparatus according to any one of claims 14 to 19 wherein the library comprises mixtures of compounds represented by the formula:

15
$$A-B_{1-10} - C_{1-10} - D_{1-8} - n (E_{1-2}) - F-G$$

in which;

A, B, C, D, E, F and n are as defined in any one of claims 12 to 16 and

G represents n a hydrophilic moiety which is not an enzyme
20 substrate.

21. Apparatus according to claim 19 or 20 in which G represents an aspartyl amide moiety.

22. Apparatus according to any one of claims 11 to 21 wherein the library comprises 1600n compounds as 80n
25 mixtures of 20 distinct, identifiable compounds.

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23. Apparatus according to claim 22 wherein the mixtures of 20 distinct, identifiable compounds are placed separately into each of 80 wells of a microtitre plate.
24. Use of a combinatorial FRET library in a method which provides rapid generation of structure-activity relationships (SAR) which comprises detection and measurement of proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET (fluorescence resonance energy transfer) molecules to find a substrate or substrates for the enzyme.
25. Use of a combinatorial FRET library in a method for detection and measurement of proteolytic enzyme activity against compounds of the library.
26. A method of identifying and synthesising an inhibitor of a proteolytic enzyme which comprises detection and measurement of proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET (fluorescence resonance energy transfer) molecules, deconvoluting the library to find a substrate or substrates for the enzyme and synthesis of an inhibitor based on the substrate or substrates.
27. An inhibition assay which uses a FRET molecule, which has been identified as a substrate for the enzyme, wherein the molecule is assayed with the enzyme separately against a panel of possible inhibitors.
28. A method which provides the rapid generation of structure-activity relationships (SAR) which comprises

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detection and measurement of proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET (fluorescence resonance energy transfer) molecules to find a substrate or substrates for the enzyme.

5 29. A method which comprises the identification of an enzyme inhibitor or inhibitors wherein a FRET compound which has been identified as a substrate is used in an inhibition assay with the enzyme separately against a panel of possible inhibitors.

10 30. A method according to claim 29 wherein the assay is performed by using the apparatus according to any of claims 11 to 23.

15 31. A method according to any preceding method claim wherein the compounds of the library are synthesized using a solid phase technique.

32. A complementary pair of compound libraries L1 and L2 which constitute a set containing compounds of formula:

Aa-Bb-Cc-Dd-n(Ee)-Ff-Gg

giving $a \times b \times c \times d \times e \times f \times g = M_n$ compounds in each
20 library, there being a predetermined number (P1, P2) of mixtures each consisting of a predetermined number (Q1, Q2) of individual identifiable compounds in each library, wherein both L1 and L2 contain the same M_n compounds, but wherein any two compounds which are found together in one
25 mixture of Q1 compounds of L1 are not found together in any one of the P2 mixtures of L2.

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33. A method of screening for enzymic activity, wherein the activity is the interaction of proteolytic enzyme applied to a well with one or more compounds of a mixture in the well, using the libraries L1, L2 according to claim 5 32, in which the P1 mixtures of L1 and the P2 mixtures of L2 are each placed separately into individual wells of well plates, the well plates having wells arranged in a format adapted to allow deduction of a unique active compound formula from the presence of activity in one well 10 of L1 and one well of L2.

34. A method according to claim 33 wherein the format complies with general deconvolution formulae in which:

$$(i) \quad ns = \frac{Rp.Cp}{Rs.Cs} Np$$

$$15 \quad (ii) \quad k = b.c.d.np.e$$

$$(iii) \quad k = x.N.np$$

$$(iv) \quad N = Rp.Cp$$

$$(\bar{v}) \quad K = X.Rp.Cp.np$$

$$(vi) \quad b.c.d.e = X.Rp.Cp$$

$$20 \quad (vii) \quad Cp.e = X$$

$$(viii) \quad Rp.e = X, \text{ if } Rp=Cp$$

and wherein

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np = number of primary plates
ns = number of secondary plates
Rp = number of primary rows
Rs = number of secondary rows
5 Cp = number of primary columns
Cs = number of secondary columns
K = number of combinations of compounds
N = number of wells on a plate, and
X = number of compounds per well.

10 35. A method according to claim 34 wherein

np = 4
ns = 16
Rp = 8
Rs = 4
15 Cp = 10
Cs = 5
K = 6400
N = 80
X = 20

20 36. A compound, library or apparatus according to claim 1, 9 or 11 wherein the scissile bond is between D and E.

37. A compound, library, apparatus, method or assay substantially as described herein with reference to example 1.

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	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10		
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10		
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10		
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10		
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10		
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10		
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10		
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10		

5
10 **Figure 1**

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First Example of Library Matrix where $n=1$ Component Distribution in Plate 1, Library 1 ($n=1$)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂
D2	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂
D3	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂
D4	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂
D5	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂
D6	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂
D7	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂
D8	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂	B ₁₋₁₀ E ₁₋₂

Figure 2

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Component Location in Plate 1, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀
	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂
E2	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀
	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂	D ₁₋₂

5 Figure 3

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Component Location in Plate 2, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀
	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄
E2	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀
	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄	D ₃₋₄

5 Figure 4

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Component Location in Plate 3, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀
	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆
E2	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀
	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆	D ₅₋₆

5 Figure 5

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Component Location in Plate 4, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀
	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈
E2	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀	C ₁₋₁₀
	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈	D ₇₋₈

5 Figure 6

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Example Library where $n=4$ Component Distribution in Plate 1, Library 1 ($n=4$)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀
	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂
D2	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀
	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂
D3	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀
	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂
D4	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀
	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂
D5	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀
	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂
D6	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀
	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂
D7	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀
	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂
D8	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀	B ₁₋₁₀
	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂	E ₁₋₂

Figure 7

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Component Location in Plate 2, Library 1 (n=4)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄
D2	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄
D3	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄
D4	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄
D5	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄
D6	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄
D7	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄
D8	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄	B ₁₋₁₀ E ₃₋₄

Figure 8

9/16

Component Location in Plate 3, Library 1 (n=4)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆
D2	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆
D3	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆
D4	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆
D5	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆
D6	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆
D7	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆
D8	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆	B ₁₋₁₀ E ₅₋₆

Figure 9

10/16

Component Location in Plate 4, Library 1 (n=4).

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈
D2	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈
D3	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈
D4	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈
D5	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈
D6	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈
D7	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈
D8	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈	B ₁₋₁₀ E ₇₋₈

Figure 10

11/16

Component Location in Plate 1, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂
E2	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂
E3	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂
E4	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂
E5	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂
E6	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂
E7	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂
E8	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂	C ₁₋₁₀ D ₁₋₂

Figure 11.

12/16

Component Location in Plate 2, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄
E2	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄
E3	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄
E4	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄
E5	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄
E6	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄
E7	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄
E8	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄	C ₁₋₁₀ D ₃₋₄

Figure 12

13/16

Component Location in Plate 3, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆
E2	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆
E3	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆
E4	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆
E5	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆
E6	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆
E7	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆
E8	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆	C ₁₋₁₀ D ₅₋₆

Figure 13

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Component Location in Plate 4, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈
E2	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈
E3	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈
E4	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈
E5	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈
E6	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈
E7	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈
E8	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈	C ₁₋₁₀ D ₇₋₈

Figure 14.

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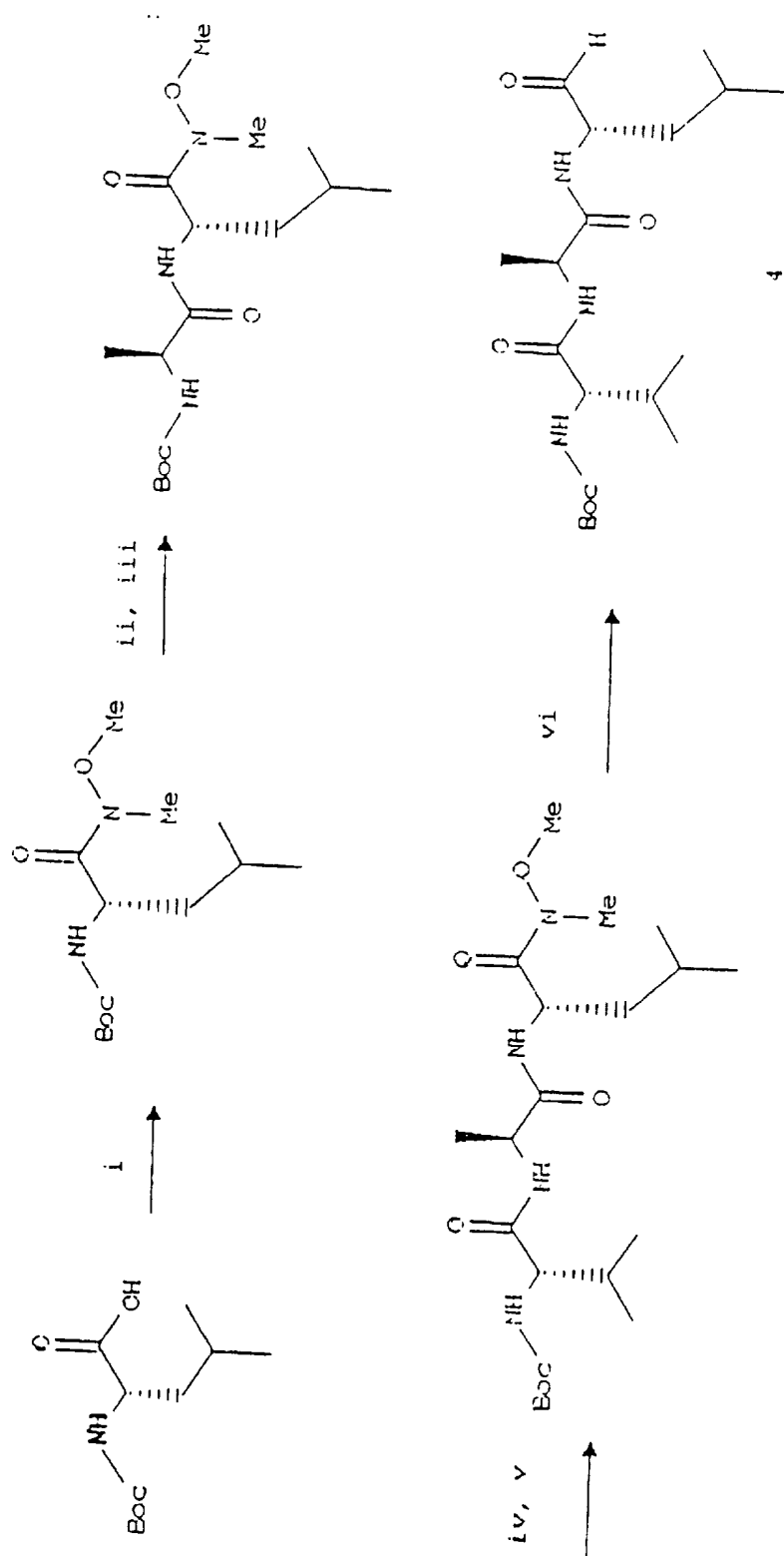


Figure 15

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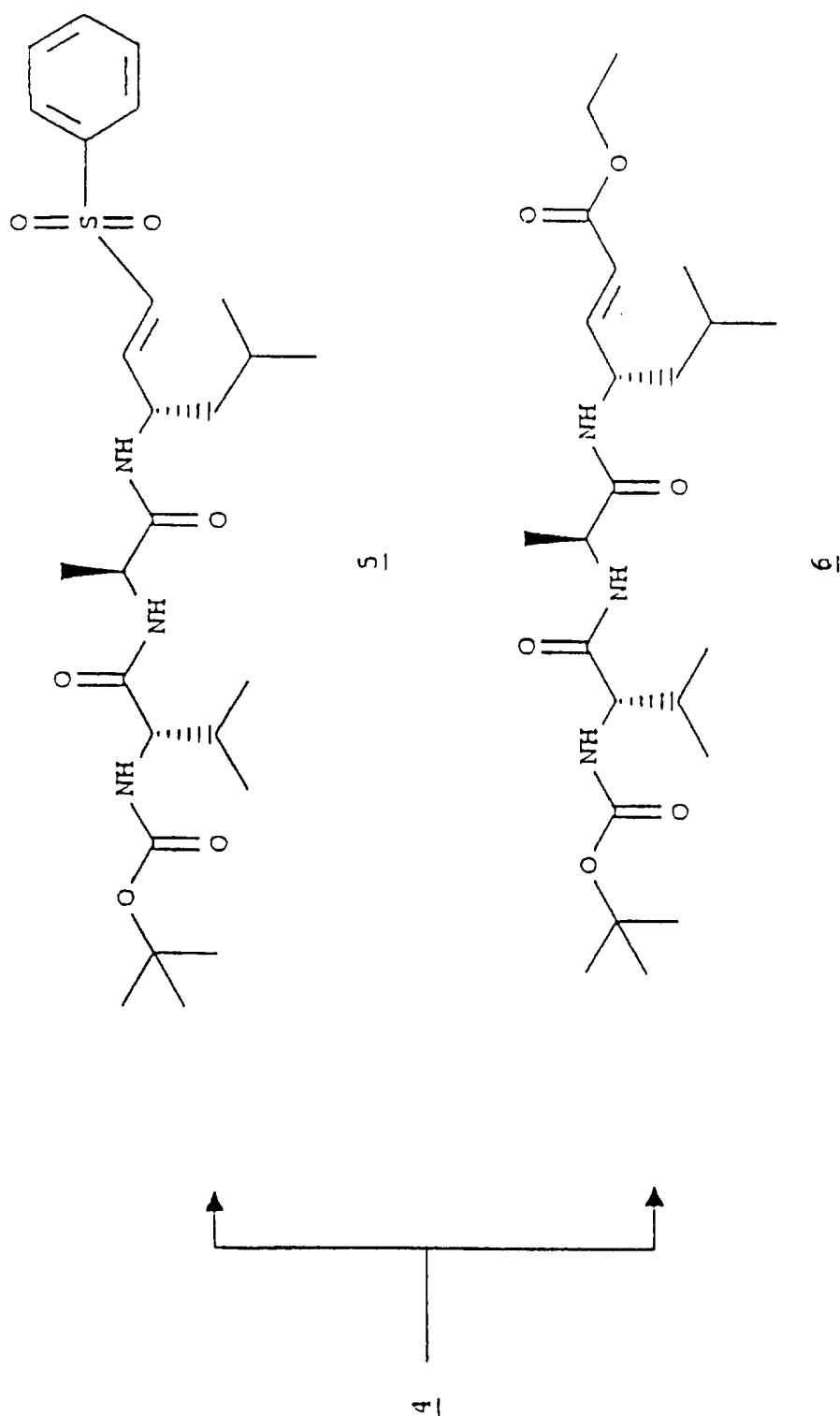


Figure 16