Direct visualization of enzyme inhibitors using a portion mixing inhibitor library containing a quenched fluorogenic peptide substrate. Part 1. Inhibitors for subtilisin Carlsberg

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A PEGA-resin was derivatized with a 2:1 mixture of 4-hydroxymethylbenzoic acid and Fmoc-Lys(Boc)-OH and the fluorogenic substrate Ac-Y(NO_2)FQPLAVK(ABz)-PEGA was assembled using the active ester approach. Following esterification of the 4-hydroxymethylbenzoic acid with Fmoc-Val-OH a library $X_1X_2X_3$-x_4-X_5X_6V was assembled by the portion mixing method. The library was subjected to extensive hydrolysis by subtilisin Carlsberg and the rate of hydrolysis was highly dependent on the potential inhibitor contained in each bead. The most persistently dark beads were collected and sequenced to yield repeatedly highly lipophilic peptides. Some of these were synthesized and found to be strong inhibitors of subtilisin Carlsberg in solution.

Many of the drugs used in medicine today are inhibitors of enzymes and in particular proteolytic enzymes have been the target of drug design. Usually lead compounds have been identified by random screening of a vast number of compounds in an enzyme inhibitor assay or they have been obtained through an exhaustive design and synthesis process.[1-8] These screenings are laborious and expensive and often require a large quantity of the appropriate enzyme or the application of functional assays with living organisms.

The search for inhibitors of enzymes involved in the invasion and pathogenesis of microorganisms or in the regulation of *e.g.* blood- and serum-proteins has been a major effort in medicinal research. New and efficient procedures which can reduce the cost of this research may increase the rate of success in our attempts to combat the most serious infectious diseases.

We have recently described a beaded polyethylene glycol cross-linked polyamide (PEGA) resin designed for peptide synthesis[9,10] with an open structure permitting biologically active proteins into the interior.[11] This polymer has been used for quantitative solid phase glycosylation with a glycosyl transferase[12] and a library technique was developed for the complete characterization of the specificity of proteolytic enzymes.[13,14] A portion mixing library[15,16] of substrates containing a C-terminal Lys [2-aminobenzoic acid (ABz)] and an N-terminal 3-nitrotyrosine (YNO_2) as an efficient donor–acceptor pair for intramolecular resonance energy transfer[17-19] was hydrolysed and the most active substrates, contained in fluorescing beads, were identified by amino acid sequencing. It is important for the visual inspection that the energy transfer is efficient and this is secured by the complete integral overlap between the emission of the donor (ABz) and the excitation of the acceptor (YNO_2) at 400–450 nm. In fluorescing beads the YNO_2 has been removed from the resin bound donor by an enzymic cleavage reaction.

The volume of the bead may be regarded as a confined space with pseudo concentrations of resin bound compounds. Thus, a local environment could be envisaged in a single bead in which two different compounds could compete for the same or two separate but interdependent binding sites on the same protein (Fig. 1). This situation is particularly interesting for the determination of inhibitors of proteolytic enzymes but in principle it applies to any enzyme or interaction between ligand and receptor. The use of the confined volume of a resin bead for the enzyme assay carried out with the peptide and the inhibitor

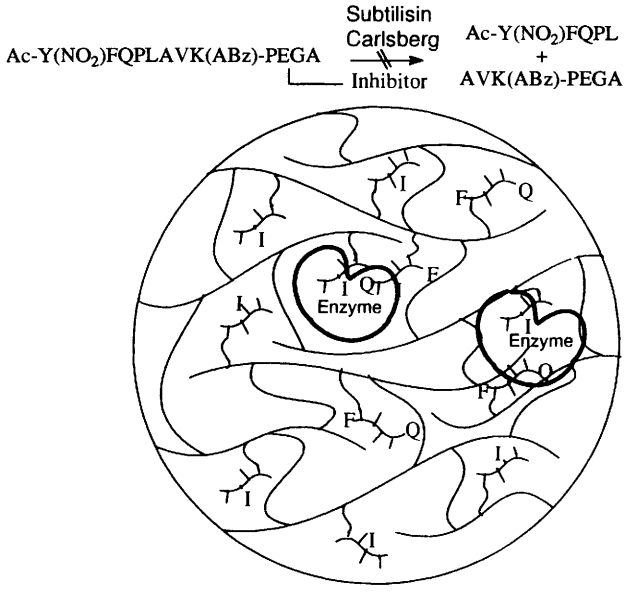

Fig. 1 The confined volume of a PEGA-bead used for an enzyme-inhibitor assay

linked to the solid phase will allow the assay to be performed with a very large number of potential inhibitors simultaneously. The present report describes the use of a library of potential protease inhibitors in which the beads also contain an optimized substrate for subtilisin Carlsberg. Subtilisin Carlsberg was selected as the enzyme due to its broad specificity, its ready availability in a pure form and because the properties of this enzyme in the interior of the PEGA-resin have been described. The technique greatly facilitates the search for lead compounds for inhibitor design.

Results and discussion

The principle of obtaining two different peptides within the confined volume of a bead (1.5×10^{-3} mm^3) was realized by temporary shielding of 66% of the functional groups with 4-hydroxymethylbenzoic acid and utilizing the selectivity of active esters for amino nucleophiles. After assembly of the first peptide and esterification of the hydroxy function the second

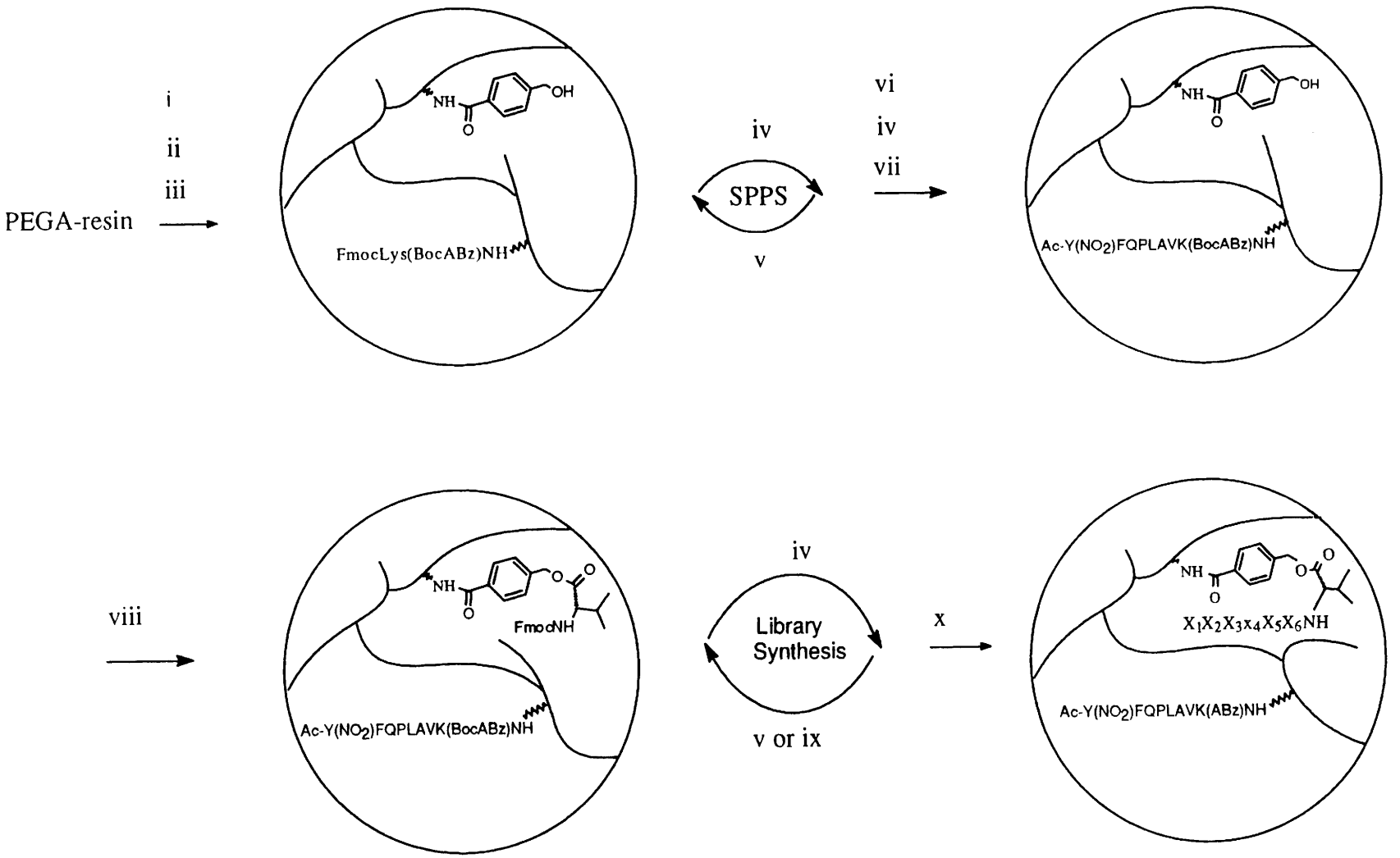

Fig. 2 Synthesis of the putative inhibitor library and a quenched fluorogenic peptide substrate **1** in the same resin. *Reagents:* i, Fmoc-Lys(Boc)-OH (0.5 equiv.), 4-hydroxymethylbenzoic acid (1.5 equiv.), TBTU (2 equiv.), NEM (2 equiv.); ii, TFA; iii, Boc-ABz-ODhbt; iv, Fmoc-amino acid-OPfp, Dhbt-OH; v, 20% piperidine–DMF, vi; Fmoc-Tyr(NO_2)-OH, TBTU, NEM; vii, Ac-ODhbt; viii, Fmoc-Val-OH, MSNT, *N*-methylimidazole; ix, Fmoc-D-amino acid, TBTU, NEM used for residue x_4; x, 95% TFA–H_2O.

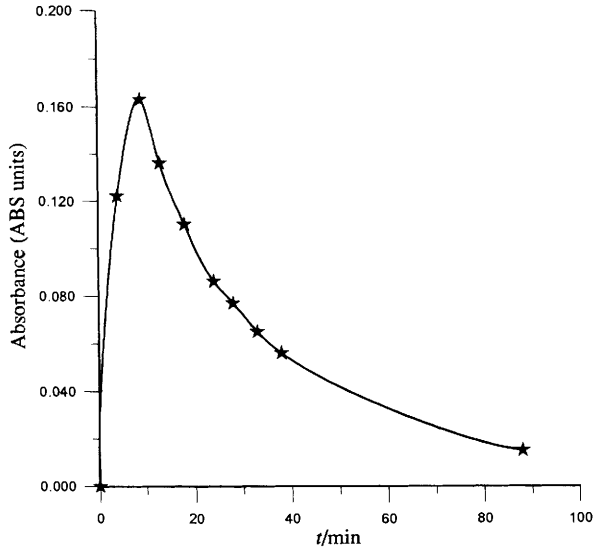

Fig. 3 Monitoring the UV-absorption of nitrotyrosine at 425 nm in the eluate from a column with the library resin using a 5×10^{-8} mol dm^{-3} solution of subtilisin Carlsberg (E. C. no. 3.4.21.14.a) in bicine buffer

Table 1 Potential inhibitors found as darker beads[a]

Compound	Sequence	% Cleavage of substrate
2	VFNiVWV	24
3	I I I xNYV[b]	24
4	MYWfFFV	60
5	WYAxWVV	64
6	WMVfLHV	50
7	PVVnIFV	24
8	AMMxMIV	22
9	KMLwFVV	70
10	VMIxFNV	64
11	PFYiQIV	45
12	GWMkLWV	90
13	YFLx-FV	33
14	VMRxLIV	80

[a] Small letters indicate D-amino acids. [b] The letter x indicates an amino acid eluting as the acid stable cys (Bu^t), which was used in the synthesis.

peptide could be synthesized and due to the base labile and acid stable character of the ester bond it could be retained during the deprotection affording the deprotected library (Fig. 2).

The derivatization of the PEGA resin was performed assuming a similar reactivity of 4-hydroxymethylbenzoic acid (HMBA) and N^{α}-fluoren-9-ylmethoxycarbonyl-N^{ε}-*tert*-butyloxycarbonyllysine [Fmoc-Lys(Boc)-OH] but the amino acid analysis showed that the former was slightly less reactive yielding a 2:1 ratio of incorporation with a 3:1 ratio of reactants. Each of the beads contained ~200 pmol of peptide which is sufficient for MALDI-TOF analysis or for amino acid sequencing. The Boc-group could be cleaved and the side-chain derivatized with Boc-ABz-ODhbt (Dhbt = 4-oxo-3,4-dihydro-1,2,3-benzotriazin-3-yl). The use of active esters effectively led to synthesis of the substrate, Y(NO_2)-FQPLAVK(ABz) **1** without acylation of the hydroxy group reserved for the inhibitor synthesis. The amino group of the N-terminal substrate amino acid [Tyr(NO_2)] was *N*-acetylated with Ac-ODhbt to block the substrate in the sequence analysis. The esterification with Fmoc-Val-OH, *N*-methylimidazole and 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole[20] (MSNT) was efficient as determined by amino acid analysis and the library was readily assembled in a custom made library generator[14] allowing easy wet-mixing of the resin and parallel dispensing of solvents and deprotection reagents. The cleavage of the putative inhibitors from single beads afforded single products according to MALDI-TOF mass spectrometry.

The library was subjected to a continuous flow of a solution of the pure crystalline enzyme, subtilisin Carlsberg (in total 1.5 mg in 1000 cm^3) (Fig. 3). The continuous flow process was selected in order to exclude the possibility of acyl transfer reactions occurring. However, similar inhibitor sequences were isolated when larger enzyme concentrations were used in a batch reaction with the library. Immediately the first beads began to fluoresce and a large difference in rate was observed from bead to bead (Fig. 4). After 24 h almost all of the library fluoresced, but there were still a few non-fluorescent beads left. Some of these were collected and sequenced by Edman degradation–gas phase sequencing to afford putative inhibitors represented by the peptides listed in Table 1.

The most inhibitory peptides found as determined from the suppression of substrate cleavage were synthesized. These as well as non inhibitory peptides and constructed putative inhibitors were tested as inhibitors in solution. The inhibitors, all containing a D-amino acid, were completely stable to a 10^{-6} mol dm^{-3} solution of enzyme as determined by HPLC. The IC_{50}s were determined in the presence of the quenched fluorogenic subtilisin substrate ABz-FQPLDEY(NO_2)D-OH.[13] An IC_{50} ~3 μmol dm^{-3} was measured for the best inhibitor (Table 2).

In the present study a fluorogenic substrate containing ABz and Tyr(NO_2) was used, but in principle any fluorogenic donor–acceptor pair or just a dye linked to the N-terminal of the substrate could have been applied. However, the use of the fluorogenic donor–acceptor allowed the initial stage of hydrolysis to be monitored and it was found that in most of the beads the potential inhibitors had a major influence on the rate and large differences in the fluorescence of individual beads could be observed. After 24 h only few beads were still darker than the bulk. These were assumed to contain the inhibitory peptides and some were collected for sequencing. In all of the beads collected some of the product AVK(ABz) of cleavage of the substrate could be found indicating that the enzyme reaction was not completely inhibited in the interior of the PEGA-resin. However, all of the inhibitors were highly lipophilic compounds and none of them showed any sign of proteolytic cleavage. No immediate consensus sequence could be determined and all of the hydrophobic amino acids were represented. This is in agreement with the known specificity of the important sub-sites S_1 and S_4[21,22] of subtilisin Carlsberg. Ile, Phe and in particular Cys(Bu^t) were found as the D-amino acid at position 4. The Cys(Bu^t) was detected as a late eluting peak in the HPLC from the sequence analysis and was independently confirmed by analysis of the inhibitors synthesized below. The D-Cys(Bu^t) is stable to the presence of trifluoroacetic acid (TFA) during the synthesis.

In the present assay the beads were treated with a high concentration of subtilisin Carlsberg for an extended period of time. It has previously been demonstrated that PEGA resins preserve the activity of enzymes for extended periods.[12] This feature of the PEGA-resin and the fact that it is completely permeable to proteins up to ~60 kD, yet mechanically robust, makes it particularly suited for enzyme assays even with unstable proteases which would denature and lose activity in contact with, *e.g.*, polystyrene or silica. The permeability and polarity of the PEGA-resins may furthermore be varied by the composition of the monomers used.

A conceptually new method for the identification of enzyme inhibitors has been presented. Inhibitors were defined for subtilisin Carlsberg, which is a protease known to have a broad specificity indicating the versatility of the method. The best

Table 2 Synthetic inhibitors and their IC_{50}-values with subtilisin Carlsberg (2.5×10^{-8} mol dm^{-3}) and the substrate ABz-FQPLDEY(NO_2)D-OH (7×10^{-7} mol dm^{-3})[a]

Compound	Structure	m/z (M + H$^+$) Found (required)	IC_{50}-values (mmol dm^{-3})	Comments
15	KSLpNVVF	904.4 (904.1)	NI[b]	Enhancing the initial rate to a small extent at mmol dm^{-3} concentration
16	KALpNVVF	888.0 (888.1)	NI[b]	Designed as a non-substrate/non-inhibitor compound
17[c]	IIIc(Bu^t)NYVF	1041.6 (1041.4)	weak	
18	KMMpISVF	954.0 (953.2)	2 ± 0.3	As **19** but containing a hydrophilic amino acid towards the C-terminal
19	KMMpMVVF	982.5 (983.3)	0.4 ± 0.04	Combination of **8** and **9**. Proline was selected for x_4
20[d]	PVVnIFVF	934.9 (934.5)	0.095 ± 0.005	Moderate solubility
21[e]	VFNiVWV	875.7 (876.1)	0.091 ± 0.005	Low solubility in aqueous buffers
22	MMMpMMMF	1049.5 (1050.4)	0.055 ± 0.003	Designed (Table 1) soluble inhibitor
23[f]	AMMc(Bu^t)MIVF	1001.6 (1001.4)	0.0031 ± 0.0002	Most active in library

[a] Small letters indicate D-amino acids. [b] NI = no inhibition. [c] Corresponding to compound **3**. [d] Corresponding to compound **7**. [e] Corresponding to compound **2**. [f] Corresponding to compound **8**. The variation was estimated from dual determinations.

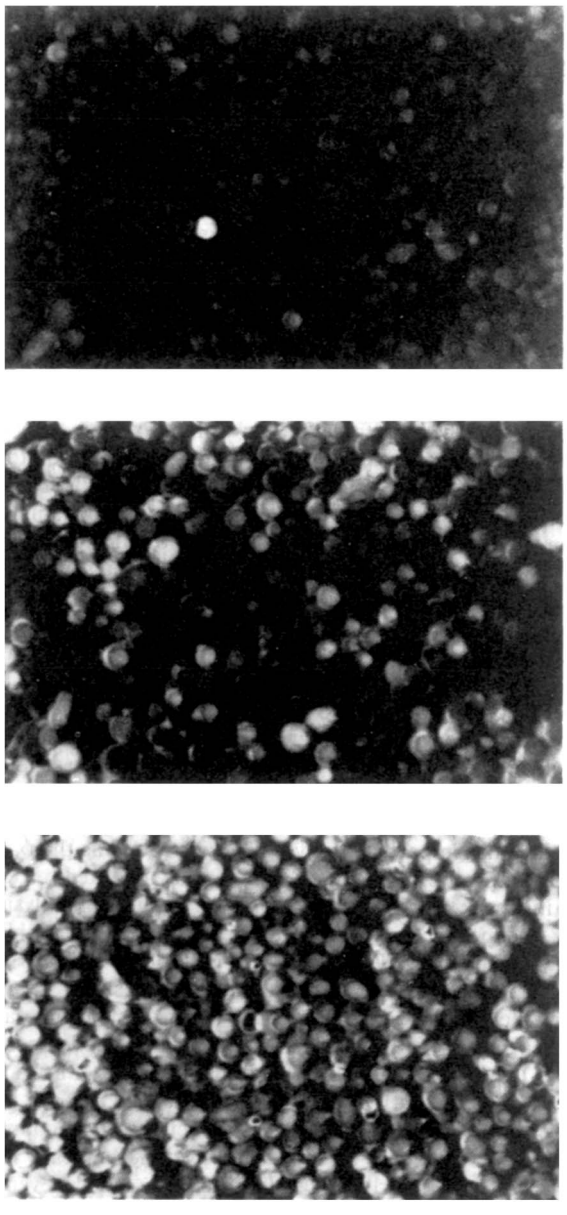

Fig. 4 Pictures illustrating the progress of substrate cleavage. (*a*) The library before enzyme addition with one fully fluorescent bead added. (*b*) After 30 min of enzyme reaction large differences in rate of substrate cleavage in individual beads are observed. (*c*) After 24 h only few dark beads remain for collection and sequence analysis.

inhibitory activity of the first generation found with this method was in the 3 μmol dm^{-3} range. The selectivity for even better inhibitors may be achieved by using the information obtained in the present work and by varying the reactivity of the substrate and the concentration of the inhibitor in the resin beads.

Experimental

N,*N*-Dimethylformamide (DMF) was DNA-grade from Labscan and was used without purification. Piperidine and *N*-methylimidazole were from Sigma. The *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), Fmoc-amino acids, MSNT, *N*-ethylmorpholine (NEM) and pentafluorophenyl esters were from Bachem (Switzerland) or NovaBiochem (Switzerland) and subtilisin Carlsberg was from NOVO Industries (Denmark). Bis-aminopropylethylene glycol 1900 and 300 were from Fluka. Macrosorb SPR-250 was from Phase Separations (UK). PEGA-beads were collected from a glass plate by adherence to a closed glass capillary under an Optical Star Fluorescence Microscope with a 320 nm band pass filter for the excitation and a 410 nm low pass filter for the detection of emitted light. Collected beads were placed directly on sequencer cartridge filters (400379). Peptides were sequenced on an Applied Biosystems Sequencer, model 477A equipped with an on-line phenylthiohydantoin-(PTH)–amino acid HPLC, model 120A using the program 'Micro' supplied by the manufacturer. Peptides were hydrolysed in evacuated tubes with 6 mol dm^{-3} HCl for 24 h and analysed by amino acid analysis on an LKB Alpha Plus instrument. Enzyme assays in solution were carried out with a Perkin-Elmer LS50 fluorescence spectrophotometer in 1 cm^3 quartz cells. All washing procedures in the peptide synthesis were with six volumes of solvent when not otherwise indicated. Fmoc cleavage was performed with two volumes of 20% piperidine–DMF. A Waters Delta Prep 3000 with a 991 Photodiode Array Detector and a Waters 1000 Prep Pak module were used for preparative purifications and an analytical Waters HPLC system with a RCM 25 × 10 module containing a 25 NV C_{18} column was used for analysis. Buffers were A: 0.1% aqueous TFA and B: 0.1% TFA in 90% aqueous acetonitrile. All automatic peptide syntheses were performed on an ABI-432A instrument using the standard TBTU-procedure. ES-MS was performed on a VG Quatro instrument from Fission and MALDI-TOF MS on an instrument from Bruker-Fransen or a Finnigan Lasermat 2000. All buffers were sterile filtered (Millipore) and equipment used for enzyme reactions was rinsed with ethanol and dried.

Synthesis of the inhibitor library 1

A beaded $PEGA_{1900/300}$ resin was prepared in a polymerization flask with a stirrer as previously described[10] by inverse suspension polymerization of a mixture of bis-acrylamido-PEG_{1900} (25 g), acrylamido-PEG_{300} (0.77 equiv. acryloylation,

5 g) and *N*,*N*-dimethylacrylamide (5 cm^3) in water (75 cm^3) at 0 and 70 °C using a mixture of heptane and tetrachloromethane. Immediately before the aqueous mixture was added to the polymerization flask sorbitan monolaurate (0.6 g) and ammonium peroxodisulfate (0.6 g) were added and the mixture was purged for 3 min with argon. It was then added to the polymerization flask while stirring at 1000 rpm and after 2 min *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (2 cm^3) was added. The polymerization was allowed to continue for a period of 3 h at 70 °C and then cooled to room temperature and filtered, washed thoroughly with water and ethanol and then lyophilized. The loading was determined to be 0.23 mmol g^{-1}, the mol% cross-linking was 25% and the average swelled bead diameter was 0.18 mm. The dried resin (1.17 g, 0.27 mmol) was packed in a syringe synthesizer. It was then swelled in DMF, washed with 20% piperidine–DMF (2 volumes) and with DMF and then the excess of solvent was removed. A solution of 4-hydroxymethylbenzoic acid (61 mg, 1.5 equiv.) and Fmoc-Lys(Boc)-OH (63 mg, 0.5 equiv.) in DMF (11 cm^3) was activated for 5 min with TBTU (173 mg, 2 equiv.) and NEM (68 mm^3, 2 equiv.) and was then added to the resin. After 24 h the resin was washed with DMF and dichloromethane and then was treated with 50% TFA–dichloromethane (2 volumes, 1 and 20 min). It was washed with dichloromethane and DMF and the free amino groups of the lysine side-chains were treated with Boc-ABz-ODhbt (154 mg, ~3 equiv.) in DMF (10 cm^3). The Fmoc-group was cleaved and peptide synthesis was continued using Fmoc-amino acid OPfp esters (3 equiv.) with addition of a catalytic amount of Dhbt-OH (~5 mg) and 20% piperidine–DMF for N^{α}-deprotections. After all the acylations and deprotections had been performed the resin was washed with DMF. Finally, the Fmoc-Tyr(NO_2) (3 equiv.) was activated with TBTU (3 equiv.) as described above and coupled to the resin-bound peptide. Removal of Fmoc, washing, acetylation with Ac-ODhbt (3 equiv.) and then washing afforded the peptide Ac-Y(NO_2)FQPLAVK(ABz)-PEGA. The resin was washed with dichloromethane and sucked dry. It was treated with 0.1 mol dm^{-3} aq. NaOH, washed with water and freeze dried.

Fmoc-Val-OH (570 mg, 4 equiv.) dissolved in 12 cm^3 dichloromethane was activated with MSNT (500 mg, 4 equiv.) and *N*-methylimidazole (102 mm^3, 3 equiv.) and then added to the resin **1**. After 2.7 h it was washed with dichloromethane and DMF and the Fmoc-group was cleaved. Amino acid analysis of a sample showed the ratio between Val and the amino acids of the substrate to be 2:1. The resin was transferred to a 20 column library generator[14] with a mixing chamber above the columns and vacuum and pressure regulation of reagent flow. A hexapeptide library containing D-amino acids at position 4 was synthesized by standard procedures as previously described[13,14] using Pfp esters (3 equiv.) and Dhbt-OH catalyst as above, except for the Fmoc-D-amino acids (3 equiv.) which were activated with TBTU (3 equiv.) and NEM (3 equiv.). In each cycle after the Fmoc cleavage the synthesizer was turned upside-down and the resin was mixed by vigorous agitation on a shaking table. When turned to the upright position the resin was distributed uniformly into the 20 columns of the synthesizer. The resin was washed with DMF and dichloromethane and treated with three portions of 95% aqueous TFA (10 min, 10 min and 5 h). It was washed with DMF, 20% piperidine–DMF (1 volume), DMF and dichloromethane and was freeze dried. A few beads were collected and each bead was cleaved with base and the filtrate neutralized and analysed by MALDI-TOF mass spectrometry. Single peaks were detected in the mass range 700–1100.

The enzyme inhibitor library assay

The library resin **1** (200 mg) was packed into a sterile disposable syringe column and eluted at 0.66 cm^3 min^{-1} with a solution of pure crystalline subtilisin Carlsberg (5×10^{-8} mol dm^{-3}) in 50 mmol dm^{-3} bicine and 2 mmol dm^{-3} $CaCl_2$ at pH 6; the reaction was followed by the UV absorption of the effluent at 425 nm (Fig. 3) and by inspection of resin aliquots under a fluorescence microscope (Fig. 4). After 24 h the enzyme retained more than 80% of its initial activity. The reaction was terminated by filtering and washing with water, 2% aqueous TFA, water, 2% aq. $NaHCO_3$ and water. The resin was freeze dried and aliquots (25 mg) were plated as a slurry in water on a small petri disk for collection of beads under the fluorescence microscope. Dark beads were transported to the dry glass in the periphery of the slurry and collected with the dry end of a closed capillary. They were then placed on a filter and sequenced. The putative resin-bound inhibitors **2**–**14** were identified and the results are presented in Table 1.

Multiple column peptide synthesis of putative inhibitors 15–23

Peptides were synthesized on a Macrosorb resin 60 mg $column^{-1}$ derivatized with ethylenediamine and 4-hydroxymethylbenzamide. The first amino acid was attached by the MSNT-procedure[20] and peptide assembly was carried out as a multiple column peptide synthesis (MCPS).[23,24] A standard Fmoc amino acid Pfp ester (3 equiv.)–Dhbt-OH protocol with 20% piperidine in DMF for deprotection was used. The D-amino acids were coupled as the free acids (3 equiv.) by *in situ* activation with TBTU.[25] The protecting groups were cleaved off the resin by treatment for 2 h with 95% aqueous TFA. The resin was filtered, washed with 95% aqueous TFA, with dichloromethane, with 20% piperidine, with DMF and with dichloromethane. The resin was dried and the peptides were cleaved off the resin in a 2 h reaction with aq. sodium hydroxide (0.1 mol dm^{-3}). The released peptides were filtered off and after washing the resin the solutions were neutralized to pH 7 on pH paper with HCl (0.1 mol dm^{-3}). The crude products were extracted with DMF and analysed by analytical HPLC (40 min gradient in the range 20–100% B). All the compounds except **23** eluted as a single major peak with the correct mass by ES-MS and MALDI-TOF MS as presented in Table 2. The synthesis of **23** was repeated on an automatic ABI-432A synthesizer. Two of the crude products **21** and **23** were purified to homogeneity by preparative HPLC (yield 61 and 70%, respectively). The resulting peptides had the right composition according to amino acid analysis and MALDI-TOF MS.

Solution assay of the enzyme inhibitors

A solution of buffer A (50 mmol dm^{-3} bicine and 2 mmol dm^{-3} $CaCl_2$ at pH 6), subtilisin Carlsberg solution (10^{-6} mol dm^{-3}) in buffer A, substrate (ABz-FQPLDEY(NO_2)D-OH, 7×10^{-6} mol dm^{-3}) in buffer A and inhibitor (1 mg cm^{-3}) in DMF were prepared. Buffer solution (825–870 mm^3), the substrate solution (100 mm^3) and enzyme solution (25 mm^3) were mixed and the hydrolysis was followed at 25 °C. The initial fluorescence background of the mixture was recorded and found to be 10% of the fluorescence at complete hydrolysis. The influence of the inhibitor on the initial rate of hydrolysis was determined by addition of increasing amounts of inhibitor solutions (0, 5, 20 and 50 mm^3) and the IC_{50} was determined. All determinations were carried out in duplicate. The results are presented in Table 2. A solution of the inhibitor **21** was treated with enzyme (10^{-7} mol dm^{-3}) for 24 h and no degradation was observed by HPLC.

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