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The Preparation of Fluorescence-Quenched Probes for Use in the Characterization of Human Factor Xa Substrate Binding Domains

Karen M. Bromfield [1], Julia Cianci [2] and Peter J. Duggan [2,3,*]

[1] Centre for Green Chemistry, Monash University, Clayton, Victoria, 3800. Australia.
[2] School of Chemistry, Monash University, Clayton, Victoria, 3800. Australia.
[3] CSIRO Molecular Science, Private Bag 10, Clayton South, Vic 3169, Australia; Phone: +61-3-9545 2560; Fax: +61-3-9545 2446.

* Author to whom correspondence should be addressed; e-mail peter.duggan@csiro.au

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Abstract: The preparation and characterization by LCMS of a library of 55 fluorescence-quenched peptides is described. The peptides bear a terminal anthranilamide fluorophore and a penultimate 2,4-dinitrophenyl-L-lysine quencher, and will be used to probe the substrate binding domain of the human blood coagulation enzyme, factor Xa.

Keywords: Solid phase peptide synthesis, parallel synthesis, fluorescence-quenched substrates, protease, factor Xa.

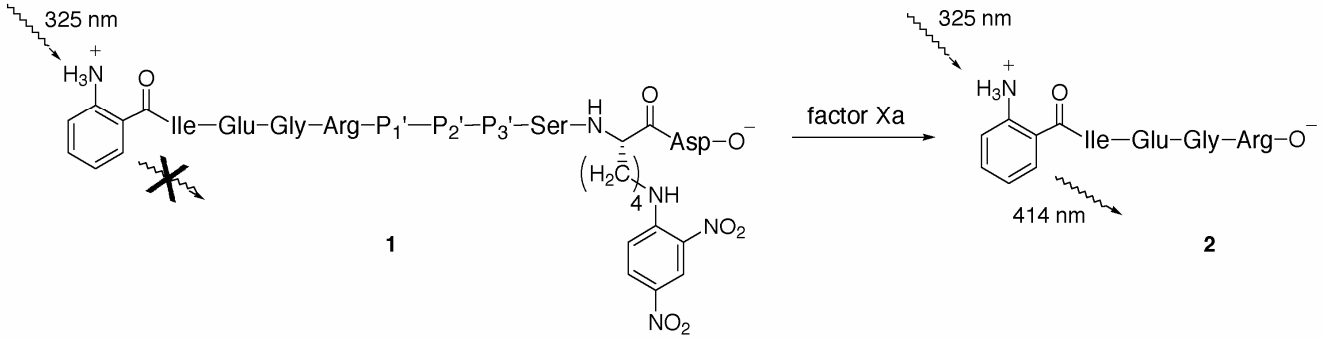
Introduction

Factor Xa is an arginine-specific serine protease that operates at the convergence of the extrinsic and intrinsic blood coagulation pathways [1]. The prothrombinase complex, which consists of factors Xa and Va, phospholipid and calcium, is responsible for limited proteolysis of prothrombin to give thrombin. These facts, along with factor Xa's relatively specific physiological role [2], make it a prime target for the development of new selective anticoagulants. Intimate knowledge of the protein

sequences most preferred by factor Xa provides insight into the enzyme's physiological role, its interaction with natural inhibitors and allows more effective design of selective anticoagulants. Most peptidic inhibitors of serine proteases have been designed to associate with the substrate binding sites on the N-terminal ("non-prime") side of the cleavage site [3]. However, inhibitors that associate with the enzyme on both sides of the catalytic serine [4] have the potential to bind more strongly and be more selective between related proteases.

We have previously probed the selectivity of bovine factor Xa for the first three substrate amino acid residues on the "prime" side of the cleavage site, P_1', P_2' and P_3'[5]. Fluorescence quenched peptide substrates [6] spanning both sides of the scissile bond (P_1-P_1') were used to investigate such P' specificities in a similar way to that used for other proteases [7,8]. These probes contain an N-terminal 2-aminobenzoyl (Abz) group and a penultimate 2,4-dinitrophenyl (Dnp) derivitized lysine (e.g. **1**, Figure 1). The fluorescence of the N-terminal Abz group (λ_{ex} = 325 nm, λ_{em} = 414 nm) is quenched in the intact peptides by the Dnp group through intramolecular resonance energy transfer [6]. Peptide hydrolysis by factor Xa relieves this intramolecular fluorescence quenching and the resulting increase in fluorescence is proportional to the concentration of the released fluorophoric fragment (**2**). This enables determination of kinetic parameters by monitoring the change in fluorescence intensity with time.

Figure 1. Intramolecular quenching of the Abz fluorophore by the Dnp-lysine residue in the peptidic probes is relieved on cleavage, adjacent to the arginine residue, by the protease, factor Xa.



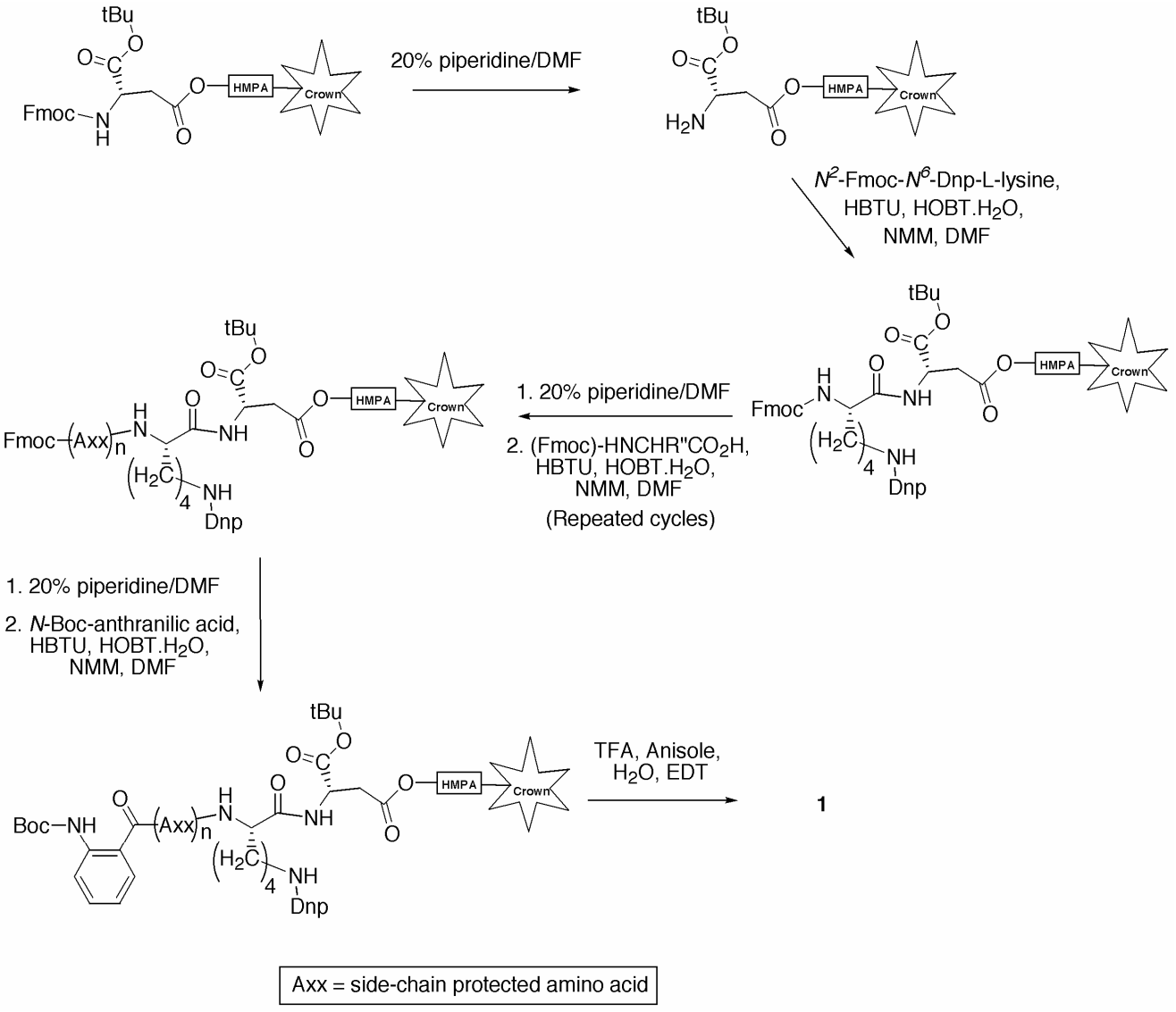
Results and Discussion

While peptidic probes of the type mentioned above have been sited a number of times in the biological literature, a detailed description of the preparation and characterization of such compounds, as well as their precursors, *N*-(Boc)-anthranilic acid and N^2-Fmoc-N^6-dinitrophenyl-L-lysine, has yet to appear. In this article, we provide a comprehensive description of the preparation of a library of peptidic fluorescence-quenched probes that are being used to probe the "prime-side" substrate

specificities of human factor Xa. The biological results obtained with these probes will be published at a later date.

Substrates suitable for the present study were prepared using parallel solid phase peptide synthesis techniques incorporating Fmoc/HBTU/TFA chemistry (see General Experimental section for a list of abbreviations used in this article) and is summarised in Scheme 1. Briefly, this involved building the peptides on a solid phase bearing a HMPA linker, with aspartate attached through the side chain carboxylic acid and Fmoc-protected on the nitrogen, and with the α-carboxylic acid protected with a *tert*-butyl group.

Scheme 1.



The Fmoc protecting group was removed with piperidine and the next amino acid, with Fmoc and side chain protection, was attached with HBTU activation. The terminal Fmoc group was then cleaved, again with piperidine, and the two-step process was repeated with the next protected amino acid until the desired sequence was obtained. The peptide was then cleaved from the resin, and the amino acid side chain protecting groups removed, by treatment with TFA in the presence of anisole and EDT. Where coupling steps were found to be low yielding, a "double coupling" was performed, in which the solid phase was treated twice with the activated amino acid prior to Fmoc removal. In some cases, it was also necessary to re-treat the cleaved peptides with TFA to remove the last traces of *tert*-butyl side-chain protecting groups. This overall strategy typically allowed the production of peptides in >60% yield and >90% purity (see Tables 2-4).A library of 55 fluorescence-quenched peptides was prepared with the sequence being; Abz-Ile-Glu-Gly-Arg-P_1'-P_2'-P_3'-Ser-Lys(Dnp)-Asp-OH (**1**). The most common naturally occurring amino acids, excluding cysteine, were substituted at the P_1', P_2' or P_3' positions while the remainder of the sequence was unchanged, with alanine being used as the default residue for the P_1'-P_3' positions.

Conclusions

We have presented a detailed description of the preparation and characterization of a library of 55 fluorescence-quenched peptidic probes for use in determination of the "prime-side" substrate specificities of human factor Xa. More than 87% of these peptides were obtained in >60% yield and all were obtained in >90% purity, as judged by LCMS.

Acknowledgements

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Experimental

General

Abbreviations: Abz: 2-aminobenzoyl; BOP: benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate; Dnp: 2,4-dinitrophenyl; EDT: 1,2-ethanedithiol; HBTU: 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorphosphate; HMPA: hydroxymethylphenoxy-acetic acid; HOBt.H_2O : hydroxybenzotriazole monohydrate; LCMS: Liquid chromatography mass spectrometry; NMM: *N*-methylmorpholine; Pmc: 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Trt: trityl. All *N*-Fmoc-amino acids, HBTU and HOBt.H_2O were obtained from Novabiochem (Merck). All other reagents were obtained from Sigma-Aldrich. Solvents were used as supplied (AnalR grade) unless otherwise stated. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DPX

300MHz spectrometer (^{1}H-, 300 MHz; ^{13}C-, 75 MHz). The spectra were run at ambient temperature using the appropriate deuterated solvent with all chemical shifts referenced to the residual protonated solvent peak. Chemical shifts (δ) are reported in parts per million (ppm), followed by their multiplicities, coupling constants (*J* Hz) where appropriate, intensities and assignments. Infrared spectra were recorded using a Perkin Elmer 1600 Series Fourier Transform Spectrophotometer. The spectra of oils were recorded as thin films (neat) mounted on NaCl plates, whereas solids were recorded as either nujol mulls on NaCl plates or as KBr disks. Principal diagnostic absorptions are reported in wavenumbers (cm^{-1}) followed by assignment were appropriate. All mass spectra used are electrospray ionisation (ESI) unless otherwise stated. High resolution mass spectra were recorded on a Bruker BioApex 47e Fourier Transform mass spectrometer. Samples were dissolved in methanol or 1:4 dichloromethane - methanol, and ionized using an ESI source. Melting points of solid compounds were measured on a Reichert hot stage melting point apparatus and are uncorrected. Flash chromatography refers to wet-packed columns with Merck silica gel 60 (0.040-0.063 mm, 230-400 mesh ASTM) as the stationary phase. Pre-absorption was carried out using the same silica. Air pressure was applied to columns to give an approximate flow rate of 2.5 mL of eluent per minute. Thin layer chromatography was performed on silica coated aluminium-backed sheets (silica gel 60/ F_{254}). Compounds were routinely visualised under short wavelength (254 nm) ultraviolet light. Unless otherwise stated, LCMS analysis was undertaken by Phillip Holt (Centre for Green Chemistry) with the following setup: Equipment; Gilson 215 Liquid handler/autosampler, Gilson 819 injector module, Gilson 811 dynamic mixer 700 μL chamber, Gilson 306 Pumps with 50.SC heads, Agilent 1100 series G1315A Diode array detector, Column Alltima C_{18} 150 x 4.6 mm, 5 μm particle size, Alltech Australia, Mass Spectrometer; Micromass ZMD from Waters Australia, software; Micromass MassLynx version 3.5. LC Method: Dry samples were dissolved in 300 μL of 30% acetonitrile/water, of which 20 or 40 μL was injected into the HPLC instrument. HPLC Solvents: A: 0.5% Formic acid in Milli Q water, B: 0.5% Formic acid in 80% acetonitrile, 20% Milli-Q water. Flow rate of 1 mL/min and gradient 10-100% B over 10 minutes. MS Method: (Electrospray Ionization +ve Mode) capillary 3kV, cone 30V, source temp 80°C, de-solvation temperature 150°C, desolvation gas 200L/hr, cone gas 50L/hr. Scanning; 350 to 800 amu in 0.8 seconds, 0.1 seconds interscan delay.

N^{2}-*Fmoc*-N^{6}-*dinitrophenyl-L-lysine*

Following the procedure of Anastasi *et al.* [9], diisopropylethylamine (1.5 mL, 8.6 mmol) was added to commercial *N*^{6}-dinitrophenyl-L-lysine (1.50 g, 4.3 mmol) in 1:1 acetonitrile/water (22 mL). The resulting solution was stirred vigorously at 0°C and Fmoc-succinimidyl carbonate (1.50 g, 4.4 mmol) was added over 25 minutes. After a further 30 minutes, the reaction mixture was allowed to warm to room temperature and left to stir for 20 hours. The reaction mixture was then diluted with ethyl acetate (100 mL) and the organic phase washed with 10% citric acid (100 mL), water (3 x 100 mL) and saturated NaCl (100 mL). The organic extract was dried with $MgSO_4$ and the solvent evaporated under vacuum to give a yellow oil. Flash column chromatography eluting with

9.5:0.25:0.25 chloroform/methanol/acetic acid was followed by co-evaporation with toluene and trituration with hexane to give the title compound as a yellow solid, (2.11 g, 92%); mp: 79-82 °C, lit. 81-83 °C [9]; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 9.09 (d, *J* = 2.6 Hz, 1H, $(NO_2)_2$-Ar-H), 8.50 (br s, 1H, $(NO_2)_2$-Ar-NH), 8.22 (dd, *J* = 9.6, 2.5 Hz, 1H, $(NO_2)_2$-Ar-H), 7.75 (d, *J* = 7.3 Hz, 2H, Fluor-H), 7.61-7.54 (m, 2H, Fluor-H), 7.39 (t, *J* = 7.2 Hz, 2H, Fluor-H), 7.30 (t, *J* = 7.4 Hz, 2H, Fluor-H), 6.86 (d, *J* = 9.6 Hz, 1H, $(NO_2)_2$-Ar-H), 5.38 (d, *J* = 7.5 Hz, 1H, OCONH), 4.56-4.43 (m, 1H, H2), 4.42 (d, *J* = 6.7 Hz, 2H, OCH_2CH), 4.20 (t, *J* = 6.8 Hz, 1H, OCH_2CH), 3.42-3.35 (m, 2H, H6), 2.06-1.93 (m, 2H, H3), 1.90-1.73 (m, 2H, H5), 1.65-1.51 (m, 2H, H4); ^{13}C-NMR (75 MHz, d_6-DMSO): δ 173.4, 155.6, 147.6, 143.3, 140.2, 134.1, 129.1, 129.4, 127.1, 126.5, 124.7, 123.1, 119.6, 114.7, 65.1, 53.2, 46.2, 42.1, 29.9, 27.1, 22.5; IR (nujol): 3436 s, 3370 s, 1733 s, 1692 m, 1619 s, 1589 m, 1520 s, 1503 m, 1337 s; MS: *m/z* 557.3 (MNa^+, 100%), 535.3 (MH^+, 81%), 357.3 ($C_{13}H_{17}N_4O_8$, 34%), 313.2 ($C_{12}H_{17}N_4O_6$, 47%); HRMS: *m/z* [MNa^+, $C_{27}H_{26}N_4O_8$.Na] calcd 557.1648; found 557.1643.

*2-Amino(*tert-*butoxycarbonyl)benzoic acid*

Following the method of Mougenot and Marchand-Brynaert [10] NaOH (0.65 g, 16.5 mmol), in water (16 mL) and *tert*-butanol (22 mL), was added to anthranilic acid (2.05 g, 14.9 mmol). Di-*tert*-butyl dicarbonate (3.23 g, 14.8 mmol) was then added with vigorous stirring over 15 minutes. The reaction was left to stir at room temperature for 17 hours after which time it was cooled to 0°C and acidified to pH 2-3 with citric acid (10% w/v). The reaction mixture was then extracted with ether (2 x 100 mL then 2 x 50 mL) and the combined organic extracts were dried over $MgSO_4$ and the solvent evaporated under vacuum. The residue was recrystallised from dichloromethane and methanol to give Boc protected anthranilic acid as white needles (2.16 g, 61%); mp: 150-152 °C, lit. 155-157 °C [11]; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 10.03 (s, 1H, NH), 8.47 (dd, *J* = 8.6, 0.8 Hz, 1H, H6), 8.09 (dd, *J* = 8.0, 1.6, Hz, 1H, H3), 7.57 (dt, *J* = 7.9, 1.6 Hz, 1H, H4), 7.04 (dt, *J* = 7.6, 1.1 Hz, 1H, H5), 1.55 (s, 9H, *t*-Bu); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.0, 152.9, 143.1, 135.7, 132.1, 121.5, 119.2, 113.5, 81.0, 28.5; IR (nujol): 3318 s, 1728 s, 1670 s; MS: *m/z* 260.3 (MNa^+, 38%), 204.2 (MNa^+-*t*Bu, 16%), 182.2 (MH^+-*t*Bu, 9%), 138.2 (MH^+-Boc, 100%); HRMS: *m/z* [MNa^+, $C_{12}H_{15}NO_4$.Na] calcd 260.0899; found 260.0894.

General Procedure for the Synthesis of Individual Fluorescence Quenched Peptides

Peptides were prepared following the Mimotopes procedures [12] with some modifications. These modifications allowed the use of HBTU as the activating agent, in place of BOP. All solvents/reagents were dried and purified as specified [12]. Mimotopes Series I crowns bearing HMPA linkers and an Fmoc protected Asp residue (which formed the *C*-terminus of the completed peptides) were the starting point of the synthesis of all peptides. These crowns conveniently fit into 1.6 mL Eppendorf tubes, which were used as reaction vessels for the Fmoc-deprotection, coupling and washing steps.

Fmoc Deprotection

Each crown was immersed in 20% piperidine/DMF (750 μL) for 20 minutes. The crown was then washed several times, firstly with DMF (750 μL) for 2 minutes, then with methanol (3 x 750 μL) also for 2 minutes each. The crowns were then allowed to dry at room temperature on a watchglass for 30 minutes.

Amino Acid and Activation Solutions

The desired L-amino acid with *N*-Fmoc protection (0.116 mmol, see Table 1 for mass) was dissolved in DMF (320 μL). A second 'activating' solution containing HBTU (44.2 mg, 0.116 mmol), HOBt.H_2O (17.8 mg, 0.116 mmol) and NMM (19.2 μL, 0.175 mmol) in DMF (445 μL to give final volume of 510 μL) was also prepared.

Amino Acid Coupling

The Fmoc-L amino acid solution (310 μL of the 320 μL) was added to the activating solution (490 μL of the 510 μL) to give a coupling solution (800 μL) containing Fmoc-L-amino acid (140 mM), HBTU (140 mM), HOBt.H_2O (140 mM) and NMM (210 mM). After 2 minutes, the dry deprotected crown was immersed in the coupling solution and left to stand for at least 2 hours. The crowns were then removed and washed with methanol (750 μL) for 5 minutes, dried at room temperature for 2 minutes and then washed with DMF (750 μL) for 5 minutes. The crown was then subjected to repeated cycles of the above deprotection and coupling steps to give the amino acid sequence required. An alternative post-coupling washing procedure was used when the next cycle could not be started immediately. This involved the crown being washed with DMF (750 μL) for 5 minutes, dried for 2 minutes and then washed with methanol (750 μL) for 5 minutes. After drying, the crown was stored at room temperature in a covered petri dish until needed.

Side Chain Deprotection and Cleavage

After coupling of the final residue, Abz in this case, the crown was washed with DMF (750 μL) for 2 minutes, then with methanol (2 x 750 μL) for 2 minutes each and allowed to air dry for 30 minutes. The crown was then placed in a 10 mL plastic centrifuge tube containing the cleavage solution, which consisted of TFA (1.24 mL), EDT (37.5 μL), anisole (75 μL), thioanisole (75 μL) and water (75 μL). After 2.5 hours, the crown was then removed and washed with TFA (0.5 ml). The TFA wash was then added to the peptide solution that remained in the centrifuge tube and the mixture was concentrated to ~10% volume under a flow of nitrogen gas (~2 hours).

Trituration

To the concentrated peptide solution was added a solution of 1:2:0.1% ether/hexane/ mercaptoethanol (8 mL) and the mixture was shaken. The suspension was placed in a freezer (approx. -20°C) for 30 minutes, centrifuged and the supernatant decanted from the solid. Additional 1:2 ether/hexane (4 mL) solution was added and again shaken, cooled, centrifuged and the supernatant removed. The resulting pellet was dried under nitrogen, suspended in H_2O and freeze-dried to give the desired peptide typically in quantitative yield. The purity of the product was determined using LCMS.

Table 1: Mass Required of Fmoc and Side-chain Protected Amino Acids

Amino Acid Code	**Protecting Group**	**Mass (mg)**[A]
Ala	–	36.0
Arg	Pmc	76.6
Asn	Trt	69.0
Asp	OtBu	47.6
Cys	Trt	67.7
Gln	Trt	70.6
Glu	OtBu	49.2
Gly	–	34.4
His	Trt	71.7
Ile	–	40.9
Leu	–	40.9
Lys	Boc	54.2
Met	–	42.9
Phe	–	44.8
Pro	–	39.0
Ser	tBu	44.3
Thr	tBu	46.0
Trp	Boc	60.9
Tyr	tBu	53.1
Val	–	39.2
Lys(Dnp)	–	61.8
Abz [B]	–	27.4

[A] 0.116 mmol, [B]Nitrogen protected with Boc rather than Fmoc.

Synthesis of P_1'-P_3' Fluorescence Quenched Substrate Library

The factor Xa P_1', P_2' and P_3' library consisted of 55 peptides. All had the sequence Abz-Ile-Glu-Gly-Arg-P_1'-P_2'-P_3'-Ser-Lys(Dnp)-AspOH were P_1', P_2' and P_3' were varied individually using alanine as the default residue. The procedure for the synthesis of these probes followed the "General Procedure for the Synthesis of Individual Fluorescence Quenched Peptides" (above) with the following exceptions: Following the coupling of the P_1' amino acid, and before Fmoc removal, the crowns were subjected to a second immersion in fresh coupling solution (containing the activated P_1' amino acid) followed by the appropriate washes, before the next deprotection and coupling cycles were undertaken. In addition, instead of individual eppendorf tubes, a reaction tray containing 96 wells was utilized in conjunction with a pin holder on which crowns attached to stems were mounted. All Fmoc-deprotection, coupling, washes and drying was undertaken with the crowns attached to the pin holder. This allowed efficient synthesis of the large number of peptides in parallel. Side chain deprotection and cleavage was still performed in individual centrifuge tubes. To allow for the smaller reaction vessels in the 96-well plate, quantities used differed as follows: The deprotection and washing steps all used 700 μL. The amino acid solution contained 0.102 mmol (rather than the 0.116 mmol quantities listed in Table 1) of the required protected amino acid in DMF (280 μL). The activating solution contained HBTU (38.9 mg, 0.102 mmol), HOBt.H_2O (15.7 mg, 0.102 mmol) and NMM (17 μL, 0.155 mmol) in DMF (405 μL resulting in a final volume of 450 μL). The amino acid solution (270 μL of the 280 μL) was added to the activating solution (430 μL of the 450 μL) to give the coupling solution (700 μL) containing Fmoc-L-amino acid (140 mM), HBTU (140 mM), HOBt.H_2O (140 mM) and NMM (210 mM), to which the crown was added. The peptidic products were individually cleaved from the crowns and triturated in 10 mL plastic centrifuge tubes in the same way as that described above for the preparation of individual peptides. The yields obtained are shown in Tables 2-4. Characterisation by LCMS in most cases identified the major constituent to be the desired peptide, as indicated by the retention time and *m/z* reported in the Tables. For some peptides, however, a second peak, which was identified as the desired peptide with a *t*-butyl group still attached was also observed in the LCMS chromatogram. In these cases, a second treatment with the TFA cleavage solution, followed by trituration and freeze-drying, completed the deprotection of the peptides ensuring that they were obtained in greater than 90% purity. In some cases, initial LCMS analysis also revealed the presence of another ion, which was apparently due to fragmentation of the peptide in the mass spectrometer. These latter ions corresponded to the doubly protonated peptide having lost the 2 *N*-terminal residues (Abz-Ile).

Table 2: Characterization of P_1' Probe Library

Peptide Code[A]	MW	Yield (%)[B]	Retention Time (min)[C]	Relative Peak Area (%)[D]	*m/z* [E]	Assignment
KBL1D	1346.32	84	9.17	100	674.0	$(M+2H^+)/2$
KBL1E	1360.35	76	9.08	>90	681.0	$(M+2H^+)/2$
KBL1F	1378.41	96	9.39	100	690.1	$(M+2H^+)/2$
KBL1G	1288.28	42[F]	11.6[G]	100	644.9	$(M+2H^+)/2$
KBL1H	1368.37	49[F]	10.70[G]	100	457.2	$(M+3H^+)/3$
					685.0 (80 %)	$(M+2H^+)/2$
					568.8 (35 %)	$(M-232+2H^+)/2$
KBL1I	1344.39	72[F]	12.2[G]	100	673.1	$(M+2H^+)/2$
KBL1K	1359.40	78	8.73	100	454.3	$(M+3H^+)/3$
					680.6 (45 %)	$(M+2H^+)/2$
					564.5 (20 %)	$(M-232+2H^+)/2$
KBL1L	1344.39	46[F]	12.18[G]	100	673.0	$(M+2H^+)/2$
KBL1M	1360.43	73	9.21	100	682.3	$(M+2H^+)/2$
KBL1N	1345.33	78	8.84	>90	673.7	$(M+2H^+)/2$
KBL1P	1328.35	69	9.01	>90	665.2	$(M+2H^+)/2$
KBL1Q	1359.36	55[F]	11.64[G]	>90	680.5	$(M+2H^+)/2$
KBL1R	1387.42	92	8.66	>90	463.7	$(M+3H^+)/3$
					694.6 (60 %)	$(M+2H^+)/2$
					578.6 (40 %)	$(M-232+2H^+)/2$
KBL1S	1318.31	54[F]	11.75[G]	100	660.0	$(M+2H^+)/2$
KBL1T	1332.34	62[F]	11.70[G]	100	667.1	$(M+2H^+)/2$
KBL1V	1330.36	64	9.16	100	666.2	$(M+2H^+)/2$
KBL1W	1417.44	91	9.38	100	709.8	$(M+2H^+)/2$
KBL1Y	1394.41	79	9.27	>90	698.0	$(M+2H^+)/2$

[A] For example: KBL1E contains a Glu (E) residue at P_1', Ala at P_2' and P_3'; [B] Isolated yield based on approximate crown loading of 6.1 μmol. Q = quantitative; [C] See General Experimental section for LCMS conditions; [D] Derived from total ion current chromatogram; [E] Relative Abundance of 100% unless otherwise stated; [F] After second TFA treatment, yield calculated from the original crown loading of 6.1 μmol; [G] LCMS gradient run was extended from the typical 10-100% solvent B over10 minutes to 10-100% solvent B over 15 minutes.

Table 3: Characterization of P_2' Probe Library

Peptide Code[A]	MW	Yield (%)[B]	Retention Time (min)[C]	Relative Peak Area (%)[D]	*m/z* [E]	Assignment
KBL2A	1302.31	92	9.28	>90	652.0	$(M+2H^+)/2$
KBL2D	1346.32	Q	9.16	>90	674.2	$(M+2H^+)/2$
KBL2E	1360.35	92	9.23	>90	681.2	$(M+2H^+)/2$
KBL2F	1378.41	93	9.50	>90	690.2	$(M+2H^+)/2$
KBL2G	1288.28	62	9.30	>90	645.2	$(M+2H^+)/2$
KBL2H	1368.37	78	8.77	100	457.3	$(M+3H^+)/3$
					685.2 (55 %)	$(M+2H^+)/2$
					569.0 (20 %)	$(M-232+2H^+)/2$
KBL2I	1344.39	73	9.34	100	673.2	$(M+2H^+)/2$
KBL2K	1359.40	Q	8.77	100	453.7	$(M+3H^+)/3$
					680.7 (95 %)	$(M+2H^+)/2$
					564.6 (25 %)	$(M-232+2H^+)/2$
KBL2L	1344.39	79	9.43	>90	673.3	$(M+2H^+)/2$
KBL2M	1360.43	Q	9.36	100	681.9	$(M+2H^+)/2$
KBL2N	1345.33	67	9.17	100	673.7	$(M+2H^+)/2$
KBL2P	1328.35	Q	9.19	>90	665.2	$(M+2H^+)/2$
KBL2Q	1359.36	93	9.17	100	680.4	$(M+2H^+)/2$
KBL2R	1387.42	98	8.92	100	694.6	$(M+2H^+)/2$
					578.4 (63 %)	$(M-232+2H^+)/2$
KBL2S	1318.31	89[F]	9.12	100	660.0	$(M+2H^+)/2$
KBL2T	1332.34	87	9.16	>90	667.1	$(M+2H^+)/2$
KBL2V	1330.36	86	9.23	>90	665.9	$(M+2H^+)/2$
KBL2W	1417.44	95[F]	9.47	100	709.7	$(M+2H^+)/2$
KBL2Y	1394.41	92[F]	9.19	100	698.1	$(M+2H^+)/2$

[A] For example: KBL2E contains a Glu (E) residue at P_2', Ala at P_1' and P_3'; [B] Isolated yield based on approximate crown loading of 6.1 μmol. Q = quantitative; [C] See General Experimental section for LCMS conditions; [D] Derived from total ion current chromatogram; [E] Relative Abundance of 100% unless otherwise stated; [F] After second TFA treatment, yield calculated from the original crown loading of 6.1 μmol.

Table 4: Characterization of P_3' Probe Library

Peptide Code[A]	MW	Yield (%)[B]	Retention Time (min)[C]	Relative Peak Area (%)[D]	*m/z* [E]	Assignment
KBL3D	1346.32	91	9.30	>90	674.1	$(M+2H^+)/2$
KBL3E	1360.35	86	9.17	100	681.0	$(M+2H^+)/2$
KBL3F	1378.41	86	9.47	100	690.1	$(M+2H^+)/2$
KBL3G	1288.28	82	8.94	100	654.1	$(M+2H^+)/2$
KBL3H	1368.37	91	8.83	100	457.3	$(M+3H^+)/3$
					685.1 (40 %)	$(M+2H^+)/2$
					569.0 (30 %)	$(M-232+2H^+)/2$
KBL3I	1344.39	68	9.36	100	673.0	$(M+2H^+)/2$
KBL3K	1359.40	95	8.84	>90	454.3	$(M+3H^+)/3$
					680.7 (55 %)	$(M+2H^+)/2$
					564.6 (40 %)	$(M-232+2H^+)/2$
KBL3L	1344.39	61	9.41	>90	673.0	$(M+2H^+)/2$
KBL3M	1360.43	61	9.34	100	682.0	$(M+2H^+)/2$
KBL3N	1345.33	96	9.01	100	673.5	$(M+2H^+)/2$
KBL3P	1328.35	89	9.12	100	665.2	$(M+2H^+)/2$
KBL3Q	1359.36	94	9.06	100	680.7	$(M+2H^+)/2$
KBL3R	1387.42	93	8.75	>90	463.7	$(M+3H^+)/3$
					694.8 (50 %)	$(M+2H^+)/2$
					578.6 (25 %)	$(M-232+2H^+)/2$
KBL3S	1318.31	59[F]	11.55[G]	100	660.0	$(M+2H^+)/2$
KBL3T	1332.34	54[F]	11.81[G]	100	667.1	$(M+2H^+)/2$
KBL3V	1330.36	74	9.21	100	666.2	$(M+2H^+)/2$
KBL3W	1417.44	89	9.39	100	709.8	$(M+2H^+)/2$
KBL3Y	1394.41	75	9.17	100	698.2	$(M+2H^+)/2$

[A] For example: KBL3E contains a Glu (E) residue at P_3', Ala at P_1' and P_2'; [B] Isolated yield based on approximate crown loading of 6.1 μmol. Q = quantitative; [C] See General Experimental section for LCMS conditions; [D] Derived from total ion current chromatogram; [E] Relative Abundance of 100% unless otherwise stated; [F] After second TFA treatment, yield calculated from the original crown loading of 6.1 μmol; [G] LCMS gradient run was extended from the typical 10-100% solvent B over10 minutes to 10-100% solvent B over 15 minutes.

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