Supporting Information

Fluorous-based Peptide Microarrays for Protease Screening

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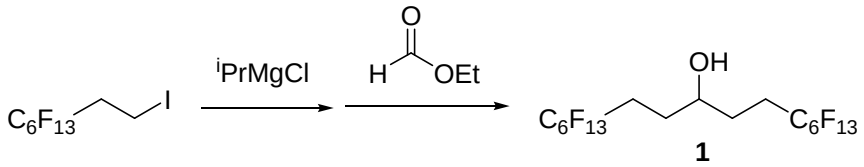
A. Materials and General Methods.

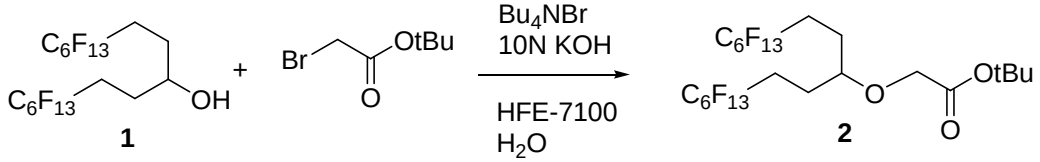
All chemicals were purchased from commercial sources unless indicated otherwise. The non-fluorous peptides were purchased from Sigma or Calbiochem. Their catalog numbers are the following: Bz-Phe-Val-Arg-AMC (**RM1**) (Calbiochem, 605211), Boc-Val-Pro-Arg-AMC (**RM2**) (Sigma, B9385), Cbz-Phe-Arg-AMC (RM3) (Calbiochem, 03-32-1501), Ac-Ile-Glu-Pro-Asp-AMC (DM1) (Calbiochem, 368068), Suc-Ala-Ala-Pro-Phe-AMC (**FM1**) (Calbiochem, 230914), Suc-Ala-Phe-Lys-AMC (**KM3**) (Sigma, S0763). The proteases were purchased from Sigma, Calbiochem, or Haematologic Technologies. The catalog numbers are the following: Human α-thrombin (Haematologic Technologies, HCT-0020), Human plasmin (Haematologic Technologies, HCPM-0140), α-Chymotrypsin from bovine pancreas (Sigma, C3142), Trypsin from bovine pancreas (Sigma, T1426) and Granzyme B (Calbiochem, 368043). ^{1}H NMR spectra were recorded on a Bruker AC-270 spectrometer. Low resolution mass spectra were obtained with an Agilent 1100 LC/MSD system. Purities of the peptides were determined by HPLC. Column, Agilent Prep C-18 (part No. 443905-902, size = 4.6 x 150 mm, 5 μ); Solvent, **A** = 0.025 % TFA in H_2O, **B** = 0.025 % TFA in MeCN; Gradient, **A**:**B** = 90:10 (at 0 min) to 5:95 (at 20 min); Flow rate, 1.0 mL/min; Detection, UV at 220 nm. All other commercial reagents and solvents were used as received without further purification. The fluorous-coated glass slides were obtained from Fluorous Technologies, Inc. (Pittsburgh, PA).

B. Synthesis of fluorous tagged aminocourmarin (H-ACC-[F26]).



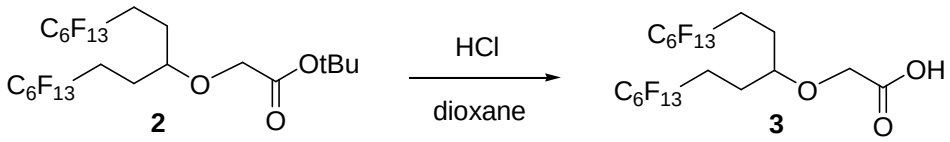
1

1,5-Bis(perfluorohexyl)propan-3-ol (1): 2-(Perfluorohexyl)ethyl-1-iodide (11.5 mL, 48.1 mmol) was added to an ice-cooled solution of iPrMgCl (1.0 M in THF, 40 mL) slowly so that the internal temperature was kept below 13 ^{o}C. After 20 min, ethyl formate (1.61 mL, 19.9 mmol) was added over the period of 5 min. After a minute, the ice-water bath was removed. After half an hour, it was cooled on ice-water, and then ice-cooled 1N HCl (50 mL) was added slowly. It was extracted with Et_2O (1 x 50 mL). The organic layer was washed with aqueous Na_2SO_4 (once) and was dried over $MgSO_4$. After removal of the solvent, white solid formed (13.4 g, 93% yield) and it was used without further purification. ^{1}H NMR (270 MHz , $CDCl_3$): δ 3.75–3.55 (m, 1H), 2.60–2.10 (m, 4H), 1.95–1.60 (m, 4H).

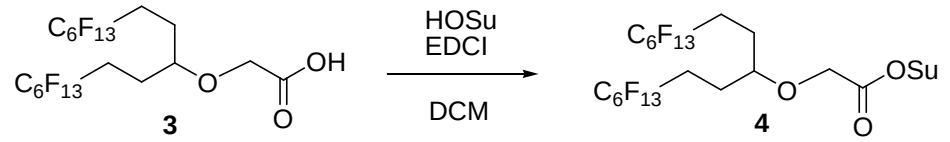


1 **2**

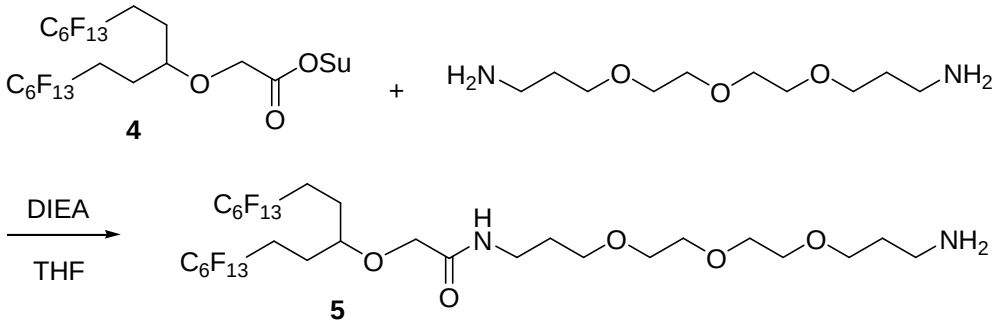
***tert*-Butyl 2-[1,5-bis(perfluorohexyl)pentan-3-yloxy]acetate (2):** To a solution of 1,5-bis(perfluorohexyl)propan-3-ol **1** (5.71 g, 7.89 mmol) and Bu_4NBr (0.52 g, 1.6 mmol) in HFE-7100 (35 mL) was added 10 N KOH (16 mL) at room temperature. It was vigorously stirred for 2 min and then tert-butyl bromoacetate (6.8 mL, 46 mmol) was added. The mixture was vigorously stirred for 17 h. It was then diluted in Et_2O (0.2 L), and was washed with H_2O (1x), 1N HCl (1x) and brine (1x). After drying over $MgSO_4$, the solvent was concentrated under vacuum. The residue was purified by flash chromatography on silica gel (eluent = 50:2 hexane–Et_2O) to give the desired product (5.48 g, 83% yield). ^{1}H NMR (270 MHz, $CDCl_3$): δ 3.98 (s, 2H), 3.60 (t, *J* = 5.4 Hz, 1H), 2.50–2.10 (m, 4H), 1.94–1.75 (m, 4H), 1.48 (s, 9H).



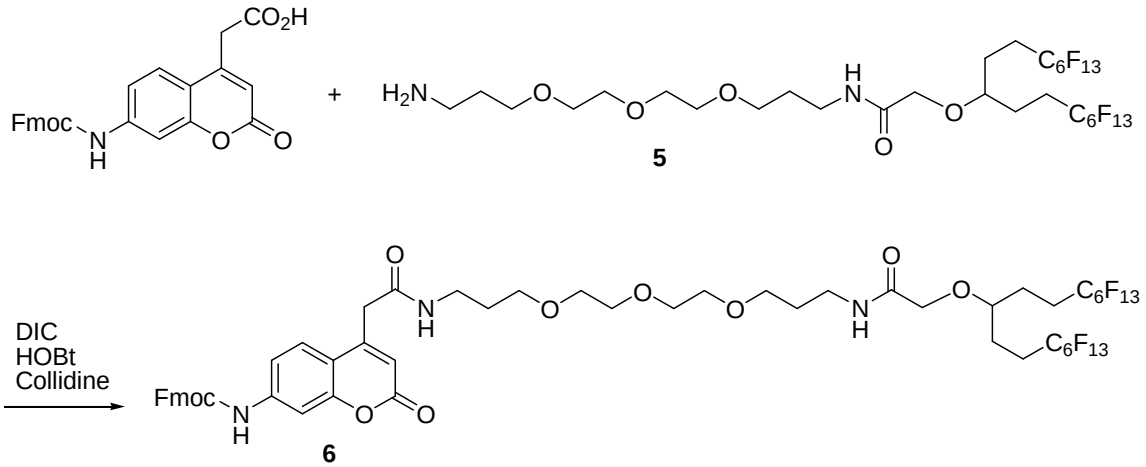
2-[1,5-Bis(perfluorohexyl)pentan-3-yloxy]acetic acid (3): *tert*-Butyl 2-[1,5-bis(perfluoro-hexyl)pentan-3-yloxy]acetate **2** (5.26 g, 6.28 mmol) was dissolved in 4 M hydrochloric acid in dioxane (45 mL) at room temperature. After 3 days, it was diluted in Et_2O (0.2 L). It was washed with H_2O (2x) and aqueous Na_2SO_4 (1x), and dried over $MgSO_4$. The crude product was purified by flash chromatography on silica gel (eluent = 10:1 hexane–Et_2O with 1% v/v AcOH) to give the desired product as white solid (4.60 g, 94% yield). ^{1}H NMR (270 MHz, $CDCl_3$): δ 4.18 (s, 2H), 3.63 (t, *J* = 5.4 Hz, 1H), 2.50–2.10 (m, 4H), 1.95–1.75 (m, 4H).



***N*-{2-[1,5-Bis(perfluorohexyl)pentan-3-yloxy]acetoxy}succimide (4):** EDCI-HCl (0.80 g, 4.2 mmol) was added to a solution of 2-[1,5-bis(perfluorohexyl)pentan-3-yloxy]acetic acid **3** (2.11 g, 2.70 mmol) and HOSu (0.52 g, 4.52 mmol) in 1:1 DCM–MeCN (40 mL) at room temperature under a nitrogen atmosphere. After 7 h, it was diluted with Et_2O (200 mL). It was washed with H_2O (2x) and aqueous Na_2SO_4 (1x), and was dried over $MgSO_4$. Removal of the solvent gave white solid (2.36 g, 99% yield) that was used for the next step without any purification. ^{1}H NMR (270 MHz, $CDCl_3$): δ 4.47 (s, 2H), 3.68 (t, *J* = 5.3 Hz, 1H), 2.88 (s, 4H), 2.50–2.05 (m, 4H), 2.00–1.75 (m, 4H).

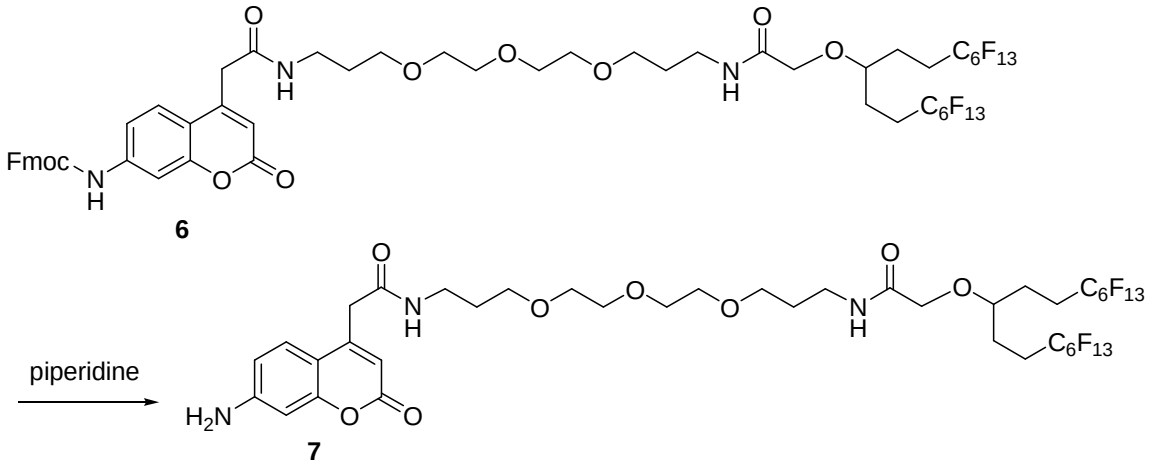


***N*-(13-amino-4,7,9-trioxatridecyl)-2-[1,5-Bis(perfluorohexyl)pentan-3-yloxy]acetamide (5):** A solution of *N*-{2-[1,5-bis(perfluorohexyl)pentan-3-yloxy]acetoxy}succimide **4** (4.91 g, 5.59 mmol) in THF (20 mL) was added to a solution of 1,13-diamino-4,7,9-trioxatridecane (25 mL, 0.11 mol) in THF (200 mL) and DIEA (20 mL) at room temperature under a nitrogen atmosphere. After 2 h, it was poured into a separatory funnel that contained Et_2O (0.5 L) and H_2O (0.6 L). The reaction flask was rinsed with Et_2O (0.1 L), and the Et_2O was added to the separatory funnel. The mixture was swirled gently to prevent emulsion, and the aqueous layer was drained. The ether layer was washed with water (3 times, 0.6 L, 0.4 L and 0.4 L each). (The separation of the two layers was extremely slow due to emulsion.) The organic layer was concentrated under vacuum. The residue was mixed with toluene (20 mL) and was concentrated under vacuum to give the desired product as pale yellow oil (3.62 g, 66% yield). It was used for the next step without further purification. MS (ESI-positive) *m/z* = 985.0 $[M+H]^+$.



Fmoc-ACC-[F26] (6): DIC (0.30 mL, 1.94 mmol) was added to a solution of *N*-(13-amino-4,7,9-trioxatridecyl)-2-[1,5-bis(perfluorohexyl)pentan-3-yloxy]acetamide **5** (0.95 g, 0.97 mmol), *N*-Fmoc-7-amino-4-carboxylmethylcoumarin (0.85 g, 1.93 mmol) (prepared according to Maly *et al*. *J. Org. Chem.* **2002**, *67*, 910–915), HOBt (0.26 g, 1.93 mmol), and 2,4,6-collidine (0.28 mL, 1.93 mmol) in DMF (4.2 mL) at room temperature under a nitrogen atmosphere. After 1 day, DIC (0.10 mL, 0.65 mmol) was added. After another day, DMF (4 mL) was added to dissolve precipitate. The desired product was isolated by F-SPE using FluoroFlash cartridge (size = 20 g) as follows. The cartridge was conditioned with 9:1 DMF-H_2O (80 mL). For the organic wash, 9:1 DMF-H_2O (80 mL)

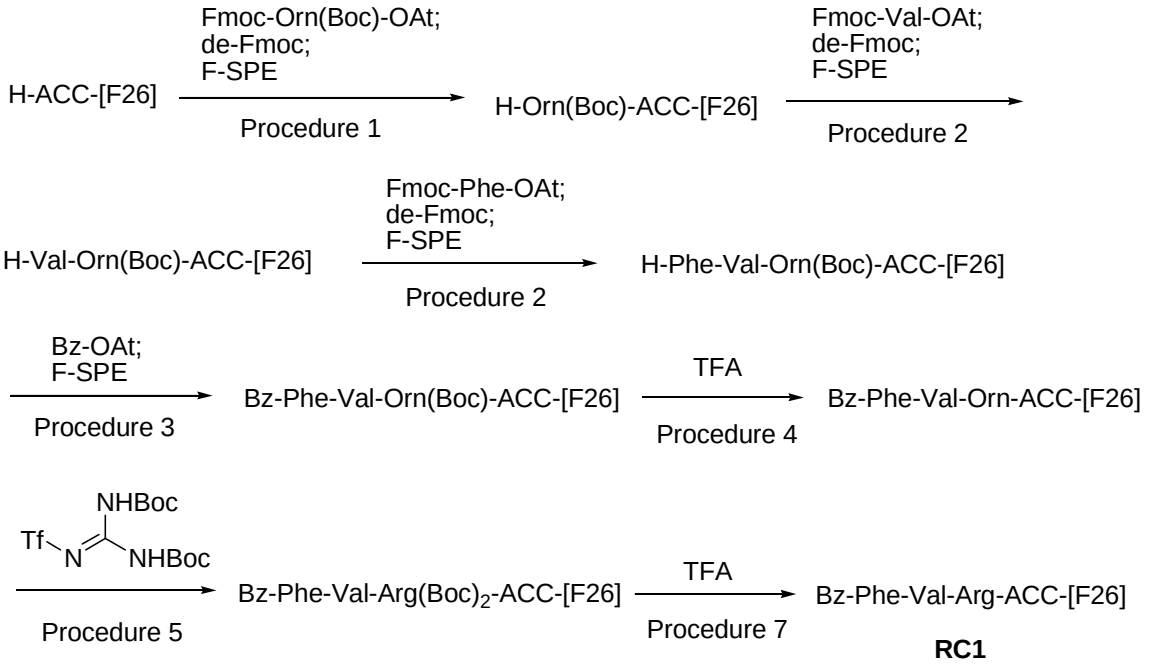
was passed through the cartridge and the solvent was drained. The desired product was eluted with THF (40 mL). The THF fractions were combined and were concentrated to give the desired product (1.26 g, 92% yield) that was used without further purification. ^{1}H NMR (270 MHz, THF-d_8): δ 9.28 (s, 1H), 7.81 (d, J = 7.4 Hz, 2H), 7.78 (d, J = 8.4 Hz, 3H), 7.53 (s, 1H), 7.34–7.18 (m, 7H), 6.20 (s, 1H), 4.60 (d, J = 6.1 Hz, 2H), 4.28 (t, J = 6.0 Hz, 1H), 3.94 (s, 2H), 3.75–3.37 (m, number of protons cannot be determined due to a peak from the solvent), 3.35–3.19 (m, 4H), 2.59–2.15 (m, 4H), 2.00–1.88 (m, 4H), 1.75–1.60 (m, number of protons cannot be determined due to a peak from the solvent). MS (ESI-positive) m/z = 1408.0 $[M+H]^+$.



H-ACC-[F26] (7): To Fmoc-ACC-[F26] **6** (2.99 g, 2.13 mmol), DMF (20 mL) and piperidine (2 mL) were added at room temperature. After 1 h, the desired product was isolated by F-SPE using FluoroFlash cartridges (size = 20 g). The cartridge was conditioned with 9:1 DMF-H_2O (80 mL) with 0.1% DIEA. For the organic wash, 9:1 DMF-H_2O (50 mL) with 0.1% DIEA was passed through the cartridge and then the solvent was drained. The desired product was eluted with THF (46 mL). The THF fractions were combined and were concentrated. The product was further purified by flash chromatography on silica gel using 20:1 DCM-MeOH as the eluent to give pale yellow waxy solid (1.61 g, 64% yield). ^{1}H NMR (270 MHz, THF-d_8): δ 7.45 (d, J = 8.6 Hz, 1H), 7.35–7.15 (m, 2H), 6.48 (dd, J = 2.1, 8.6 Hz, 1H), 6.38 (d, J = 2.1 Hz, 1H), 5.94 (s, 1H), 5.46 (s, 2H), 3.94 (s, 2H), 3.76–3.39 (m, number of protons cannot be determined due to a peak from the solvent), 3.35–3.18 (m, 4H), 2.60–2.15 (m, 4H), 2.05–1.90 (m, 4H), 1.89–1.60 (m, number of protons cannot be determined due to a peak from the solvent). MS (ESI-positive) m/z = 1185.9 $[M+H]^+$.

C. Synthesis of fluorous tagged substrates.

The schemes below show the synthesis of the substrates (RC1, RC2, RC3, DC1, FC1, and KC3). Typical procedures are described after the schemes.



Scheme 1. Synthesis of RC1.

Typical procedure 1: ***Attachment of the first amino acid.***
To a solution of Fmoc-Orn(Boc)-OH (0.32 g, 0.71 mmol) and 2,4,6-collidine (93 μL, 0.71 mmol) in DMF (1.0 mL) was added HATU (245 mg) at room temperature. The mixture was swirled for 1 h. The solution was mixed with H-ACC-[F26] (153 mg, 0.129 mmol). After 16 h, the mixture was loaded to a FluoroFlash SPE cartridge (5 g size) that was conditioned with DMF-H_2O (9:1, 40 mL). The reaction vial was rinsed with DMF (twice, 0.5 and 0.2 mL). The non-fluorous components were washed out with DMF-H_2O (9:1, 16 mL), and then the solvent was drained. The fluorous components were eluted with THF-MeOH (1:1, 34 mL), and it was concentrated under vacuum to give two mixtures of Fmoc-Orn(Boc)-ACC[F26] and H-ACC-[F26] (0.131 g and 0.122 g). Each mixture was treated with a solution of Fmoc-Orn(Boc)-OAt that was prepared by mixing Fmoc-Orn(Boc)-OH (0.16 g, 0.36 mmol), 2,4,6-collidine (47 mL), and HATU (0.12 g, 0.32 mmol) in DMF (0.4 mL) for 1 h. After 16 h, each reaction mixture was loaded to a FluoroFlash SPE cartridge (2 g size) that was conditioned with DMF-H_2O (9:1, 12 mL) prior to use. The non-fluorous components were washed out with DMF-H_2O (9:1, 8 mL), and the solvent was drained. The desired product was eluted with THF-MeOH (1:1, 12 mL). After the removal of the solvent under vacuum, the residue was split into three tubes, and they were further dried under vacuum to give 85, 81, and 78 mg of the product in the three tubes. To one of the tubes were added DMF (0.4 mL) and piperidine (40 mL) at 23 ^oC. After 1 h, the mixture was loaded to a FluoroFlash SPE cartridge (2 g size) that was conditioned with DMF-H_2O (9:1 with 0.1% DIEA, 12 mL). The non-fluorous components were washed out with DMF-H_2O (9:1 with 0.1% DIEA, 8 mL), and the solvent was drained. The desired product was eluted with THF-MeOH (1:1, 12 mL). After removal of the solvent, 70 mg (58% yield) of the product was obtained.

Typical procedure 2: ***Attachment of N-protected amino acid followed by F-SPE.***
To a solution of Fmoc-Val-OH (37 mg, 0.11 mmol) and 2,4,6-collidine (14 μL) in DMF (0.40 mL) was added HATU (35 mg, 0.09 mmol) at room temperature. After 1 h, this solution was added to H-Orn(Boc)-ACC-[F26] (36 mg, 0.026 mmol) and the mixture was swirled well for 15 h. Octyl-1-amine (14 μL, 0.085 mmol) was added and after 20 min, piperidine (45 μL) was added. (In case the amino acid is protected with Cbz, addition of octylamine and piperidine was skipped.) After 1.5 h, the mixture was loaded to a FluoroFlash F-SPE cartridge (2 g size, conditioned with 9:1 DMF-H_2O with 0.1 % DIEA). The reaction tube was rinsed with DMF (2 x 0.1 mL). The undesired organic components were washed out with 9:1 DMF-H_2O with 0.1 % DIEA (8 mL), and the solvent was drained. The desired product was eluted with 1:1 THF-MeOH (12 mL). It was concentrated to give H-Val-Orn(Boc)-ACC-[F26] (28 mg) that was used for the next coupling reaction without further purification.

Typical procedure 3: ***Capping of N-terminus with benzoyl or acetyl group followed by F-SPE.***
To a solution of HOAt (0.105 mmol) and DIEA (0.109 mmol) in DMF (0.40 mL) was added BzCl (10 mL, 0.086 mmol) at room temperature. (In case of acetyl capping, AcOAt was prepared by mixing HATU, AcOH, and 2.4.6-collidine in DMF at 1:1:1:1 ratio in 0.18 M). After 1 h, this solution was added to H-Phe-Val-Orn(Boc)-ACC-[F26] (38.5 mg), and the mixture was swirled for 1 day. The mixture was loaded to a FluoroFlash F-SPE cartridge (2 g size, conditioned with 9:1 DMF-H_2O with 0.1 % DIEA). The reaction tube was rinsed with DMF (2 x 0.1 mL). The undesired organic components were washed out with 9:1 DMF-H_2O with 0.1 % DIEA (8 mL) and the solvent was drained. The desired product was eluted with 1:1 THF-MeOH (12 mL). It was concentrated to give Bz-Phe-Val-Orn(Boc)-ACC-[F26] that was used for the next coupling reaction without further purification.

Typical Procedure 4: ***Deprotection of Orn(Boc).***
Cbz-Phe-Orn(Boc)-ACC-[F26] (18 mg, 0.011 mmol) was dissolved in TFA (0.5 mL) on ice bath. After 20 min, the TFA was removed under vacuum without heat and the residue was dissolved in EtOH (0.5 mL). EtOH was removed under vacuum to give Cbz-Phe-Orn-ACC-[F26] (20 mg). The product was used for the next step without further purification.

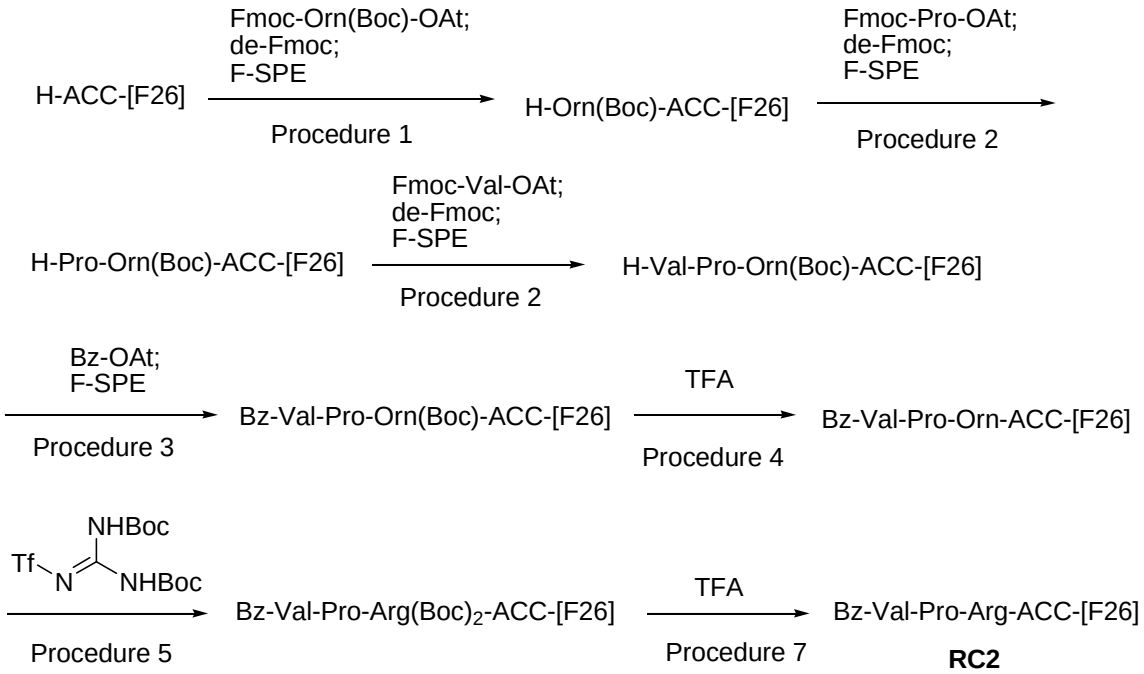
Typical Procedure 5: ***Guanidine formation.***
To a solution of Bz-Phe-Val-Orn-ACC-[F26] (approx 0.021 mmol) in 2:1 DCM-THF (0.8 mL) was added DIEA (36 μL, 0.21 mmol) and *N*,*N*'-bis(tert-butoxycarbonyl)-*N*"-trifluoromethanesulfonyl guanidine (22 mg, 0.056 mmol) at room temperature. After 19 h, DIEA (36 μL, 0.21 mmol) and *N*,*N*'-bis(tert-butoxycarbonyl)-*N*"-trifluoromethanesulfonyl guanidine (22 mg, 0.056 mmol) were added again. It was swirled for 3 days and the solvent was removed under vacuum. The residue was dissolved in DMF (0.2 mL) and was loaded to a FluoroFlash F-SPE cartridge (2 g size, conditioned with 9:1 DMF-H_2O with 0.1 % DIEA). The reaction tube was rinsed with DMF (2 x 0.1 mL). The undesired organic components were washed out with 9:1 DMF-H_2O with 0.1 % DIEA (8 mL) and the solvent was drained. The desired product was

eluted with 1:1 THF-MeOH (12 mL) to give Bz-Phe-Val-Arg(Boc)$_2$-ACC-[F26] (42 mg) that was used for the next step without further purification.

Typical Procedure 7: ***Final deprotection.***
Bz-Phe-Val-Arg(Boc)2-ACC-[F26] (42 mgl) was dissolved in 95:2.5:2.5 TFA-TIS-H_2O (1.0 mL) at room temperature. After 2 h, the solvents were removed under vacuum. The residues were co-evaporated successively with EtOH (0.5 mL), MeCN (0.25 mL) and H_2O (0.25 mL each). The total weight of the product (**RC1**) was 34 mg (0.019 mmol).

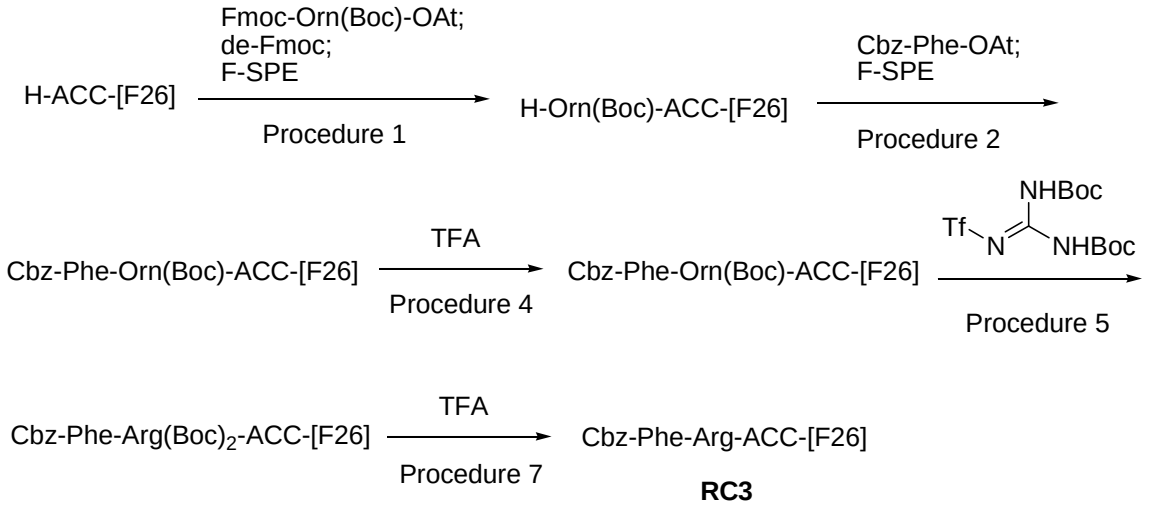
Purities of the peptides were determined by HPLC. Column, Agilent Prep C-18 (part No. 443905-902, size = 4.6 x 150 mm, 5 μ); Solvent, **A** = 0.025 % TFA in H_2O, **B** = 0.025 % TFA in MeCN; Gradient, **A**:**B** = 90:10 (at 0 min) to 5:95 (at 20 min); Flow rate, 1.0 mL/min; Detection, UV at 220 nm.

RC1: 87% yield from **H-ACC-[F26]**. Purity 92%. MS (ESI positive) m/z = 846.6 $[M+2H]^{2+}$.



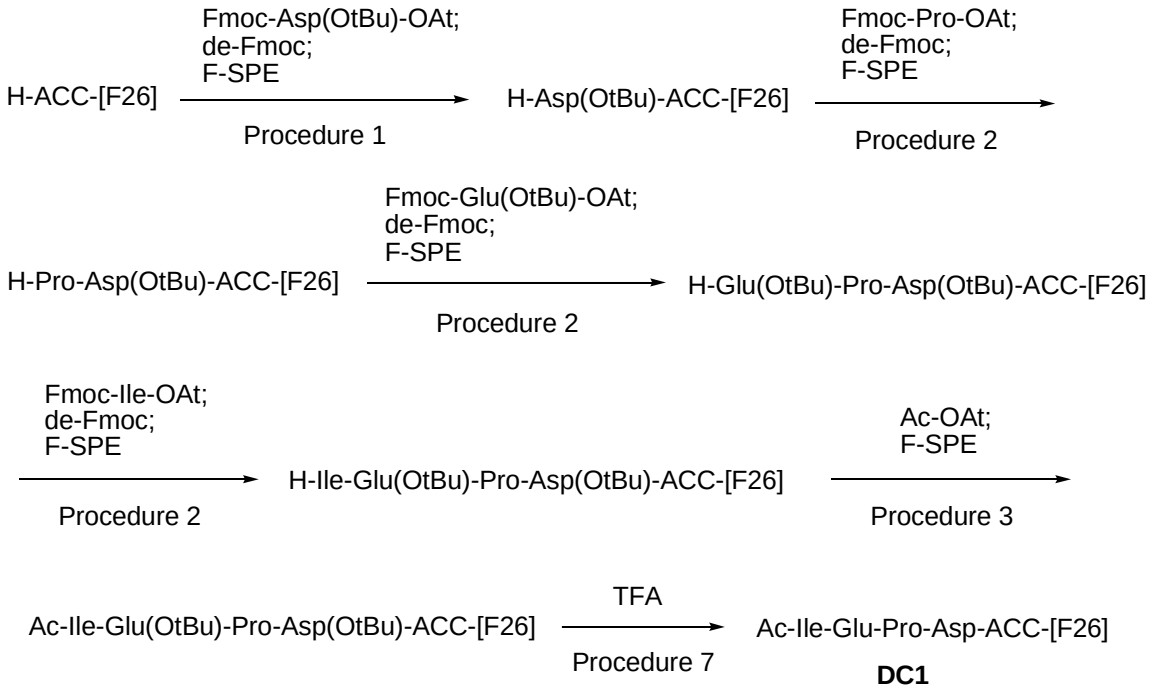
Scheme 2. Synthesis of RC2.

RC2: 93% yield from **H-ACC-[F26]**. Purity 84%. MS (ESI positive) m/z = 821.6 $[M+2H]^{2+}$.



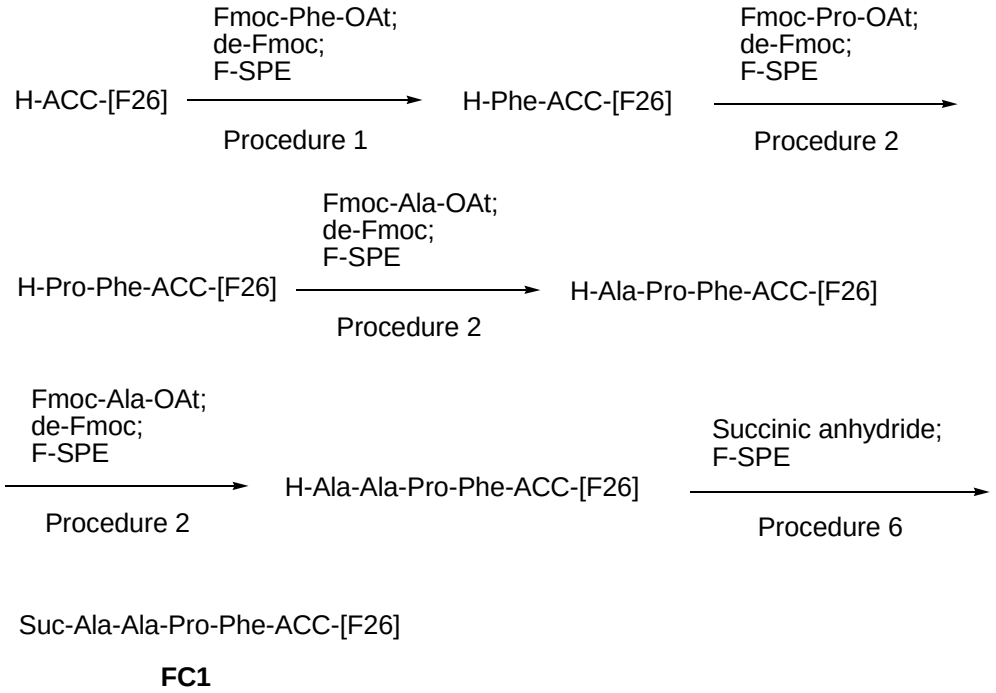
Scheme 3. Synthesis of RC3.

RC3: 45% yield from **H-ACC-[F26]**. Purity 80%. MS (ESI positive) m/z = 812.1 $[M+2H]^{2+}$.



Scheme 4. Synthesis of DC1.

DC1: 76% yield from **H-ACC-[F26]**. Purity 80%. MS (ESI positive) m/z = 841.6 $[M+2H]^{2+}$.

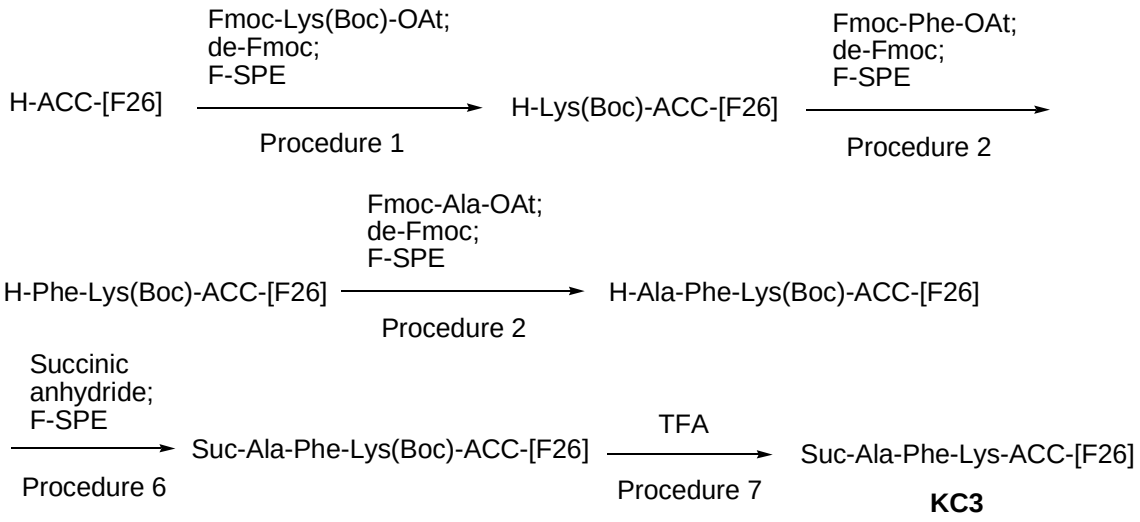


Scheme 5. Synthesis of FC1.

Typical Procedure 6: ***Capping of N-terminus with succinic anhydride followed by F-SPE***.

To a solution of H-Ala-Ala-Pro-Phe-ACC-[F26] (approx. 0.021 mmol) in DMF (0.40 mL) was added 2,4,6-collidine (14 μL, 0.11 mmol) and succinic anhydride (11 mg, 0.11 mmol) at room temperature. After 1 day, 2,4,6-collidine (14 μL, 0.11 mmol) and succinic anhydride (11 mg, 0.11 mmol) were added again. After another day, TFA (16 μL) was added and the mixture was loaded to a FluoroFlash F-SPE cartridge (2 g size, conditioned with 9:1 DMF-H_2O with 0.1 % DIEA). The reaction tube was rinsed with DMF (2 x 0.1 mL). The undesired organic components were washed out with 9:1 DMF-H_2O with 0.1 % DIEA (8 mL) and the solvent was drained. The desired product was eluted with 1:1 THF-MeOH (12 mL) to give Suc-Ala-Ala-Pro-Phe-ACC-[F26] (29 mg).

FC1: 67% yield from **H-ACC-[F26]**. Purity 93%. MS (ESI positive) m/z = 836.6 $[M+2H]^{2+}$.

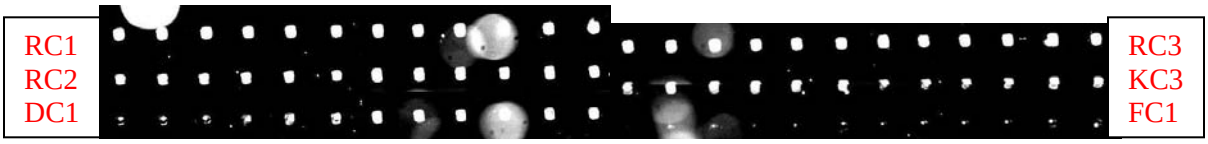


Scheme 6. Synthesis of KC3.

KC3: 79% yield from **H-ACC-[F26]**. Purity 87%. MS (ESI positive) m/z = 816.6 $[M+2H]^{2+}$.

D. Microarray Pictures.

Thrombin:



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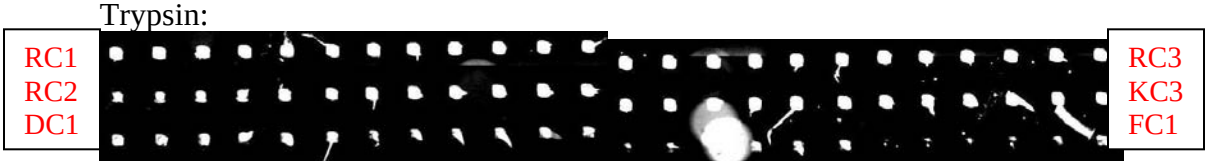

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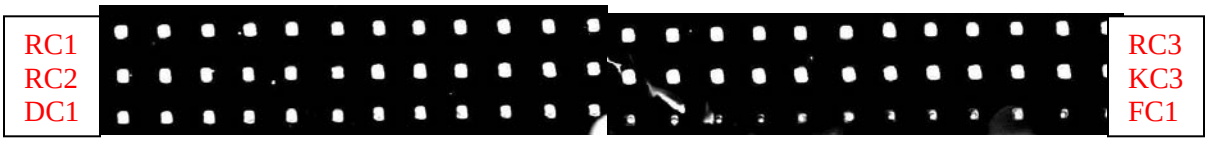

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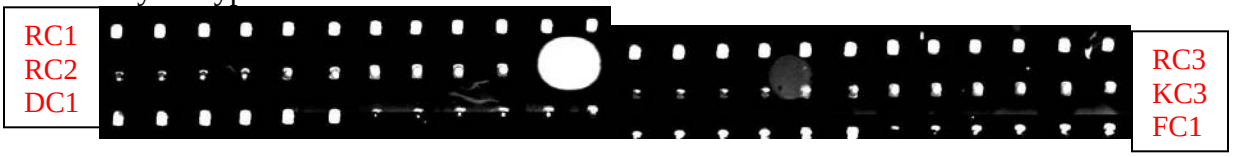
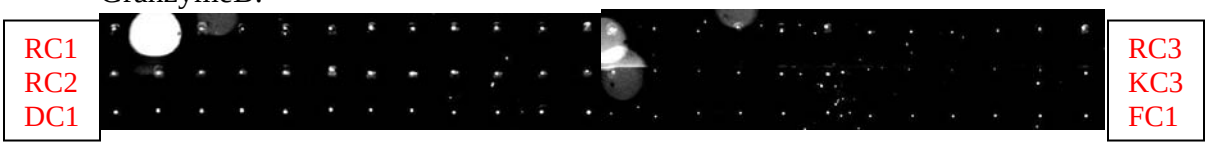


GranzymeB:



E. References.

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