Electronic Supporting Information

Eliminating Caspase-7 and Cathepsin B Cross-Reactivity on Fluorogenic Caspase-3 Substrates

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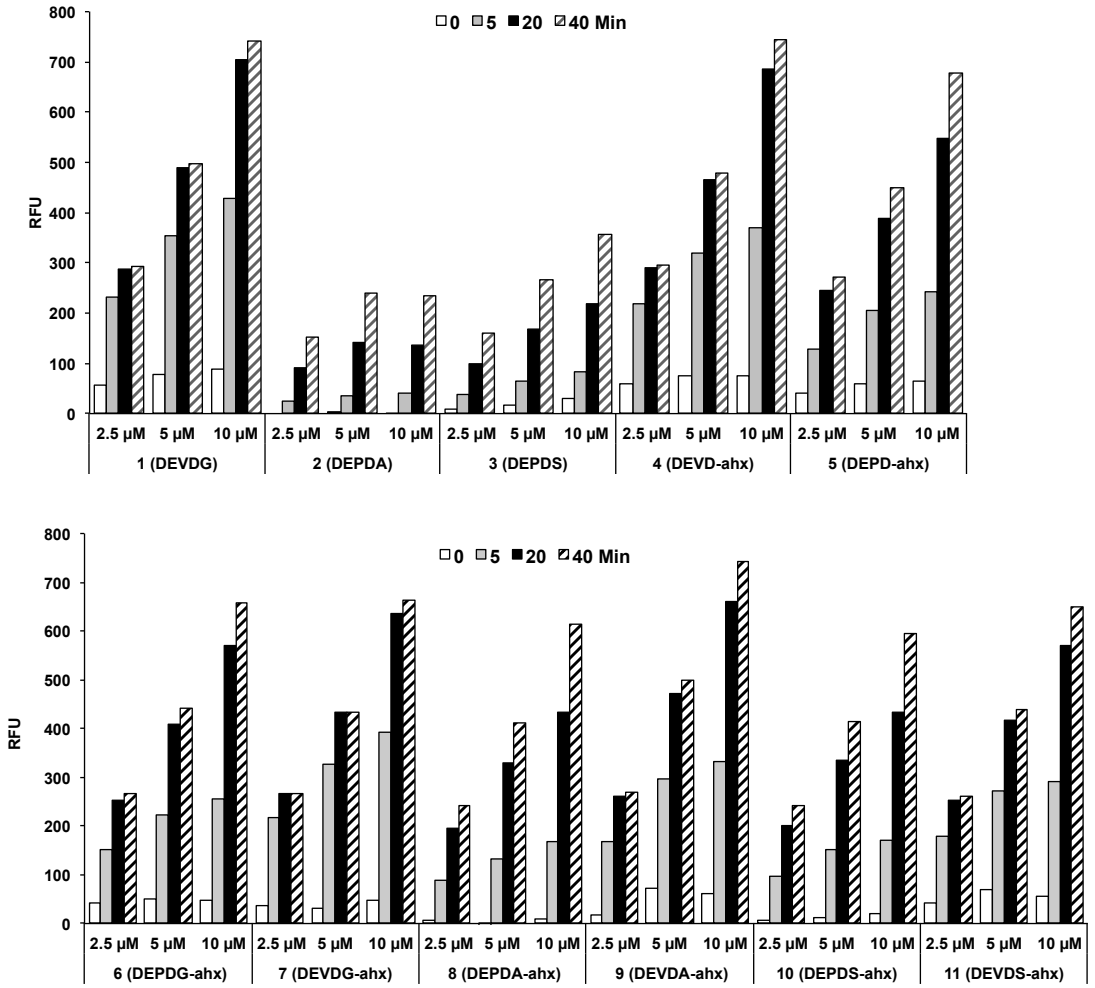


Figure S1. Preliminary screen of time-dependent activation of substrates **1**–**11** (2.5, 5 and 10 µM, n = 3) with caspase-3 (15 nM).

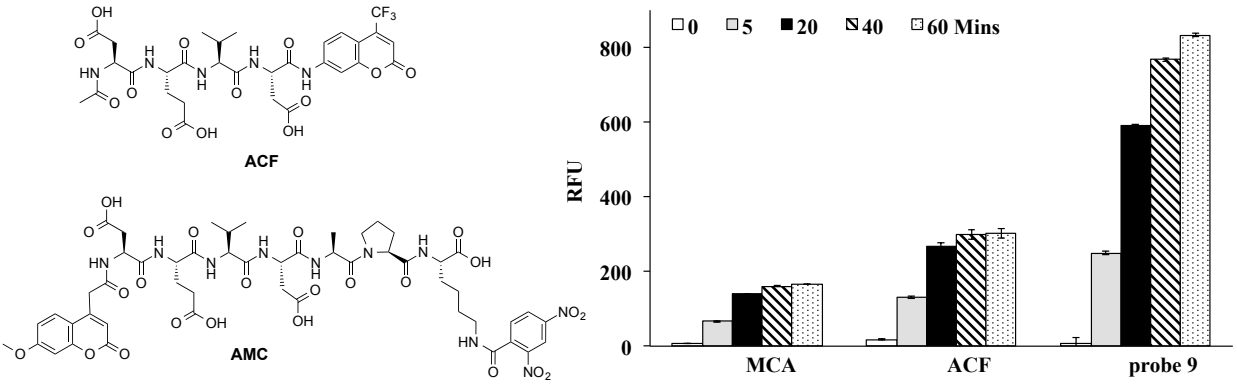


Figure S2. Comparison of the relative increase in fluorescence with substrate **9** ($\lambda_{Ex/Em}$ 485/525 nm) and commercial control substrates **MCA** ($\lambda_{Ex/Em}$ 325/395 nm) and **AFC** ($\lambda_{Ex/Em}$ 400/505 nm), all at 20 µM concentration (n = 3), upon incubation with caspase-3 (15 nM).

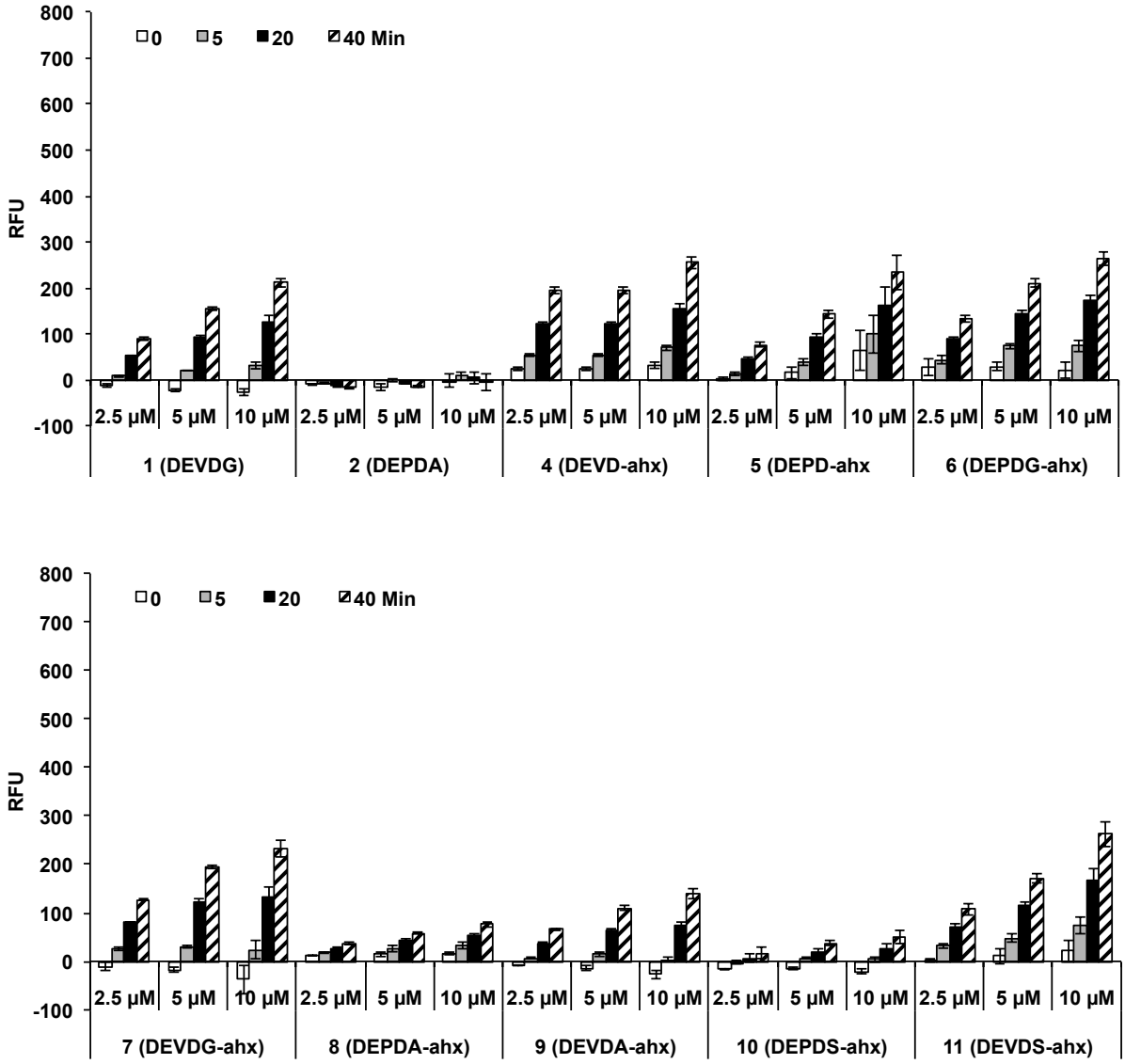


Figure S3. Screen of time-dependent activation of substrates **1**–**2** and **4**–**11** (2.5, 5 and 10 µM, n = 3) with caspase-7 (15 nM).

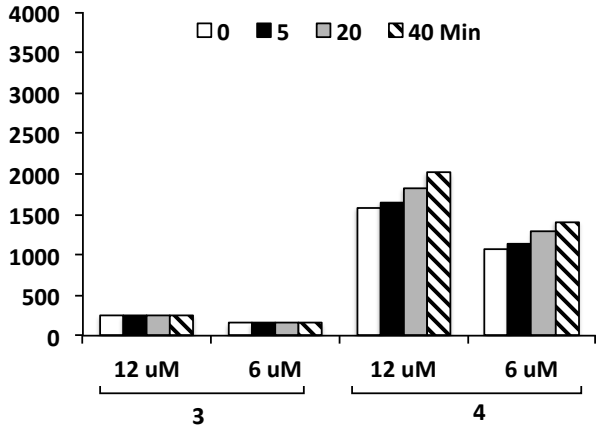


Figure S4. Comparison time-dependent activation of substrates **3** and **4** (n = 3) with caspase-7 (15 nM). Substrate **3** does not show time-dependent activation (y-axis = relative fluorescence intensity, uncorrected).

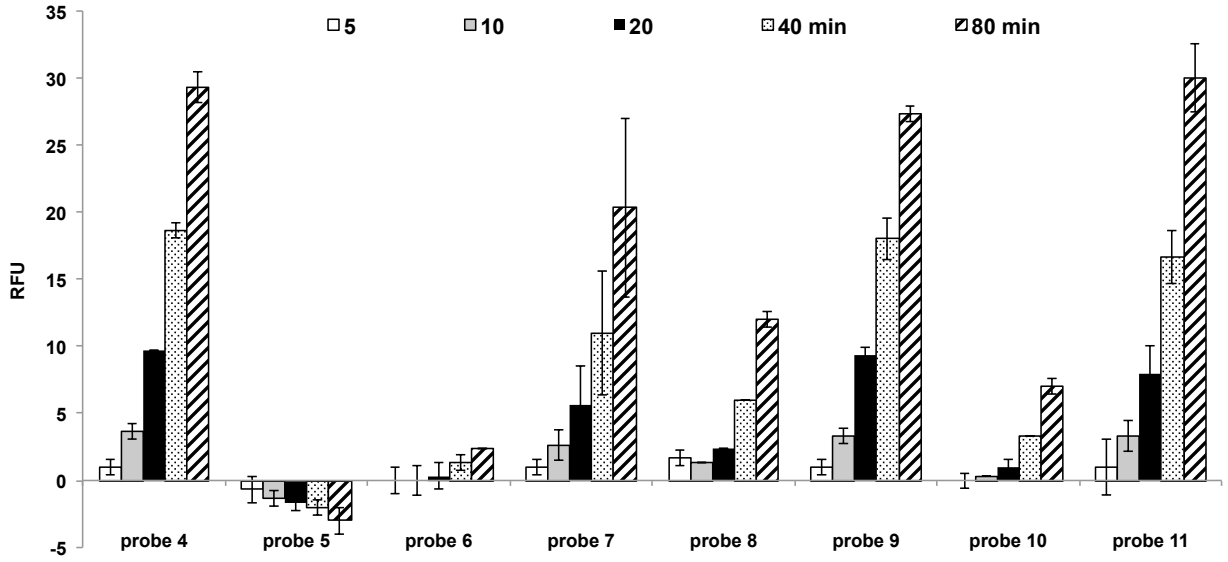
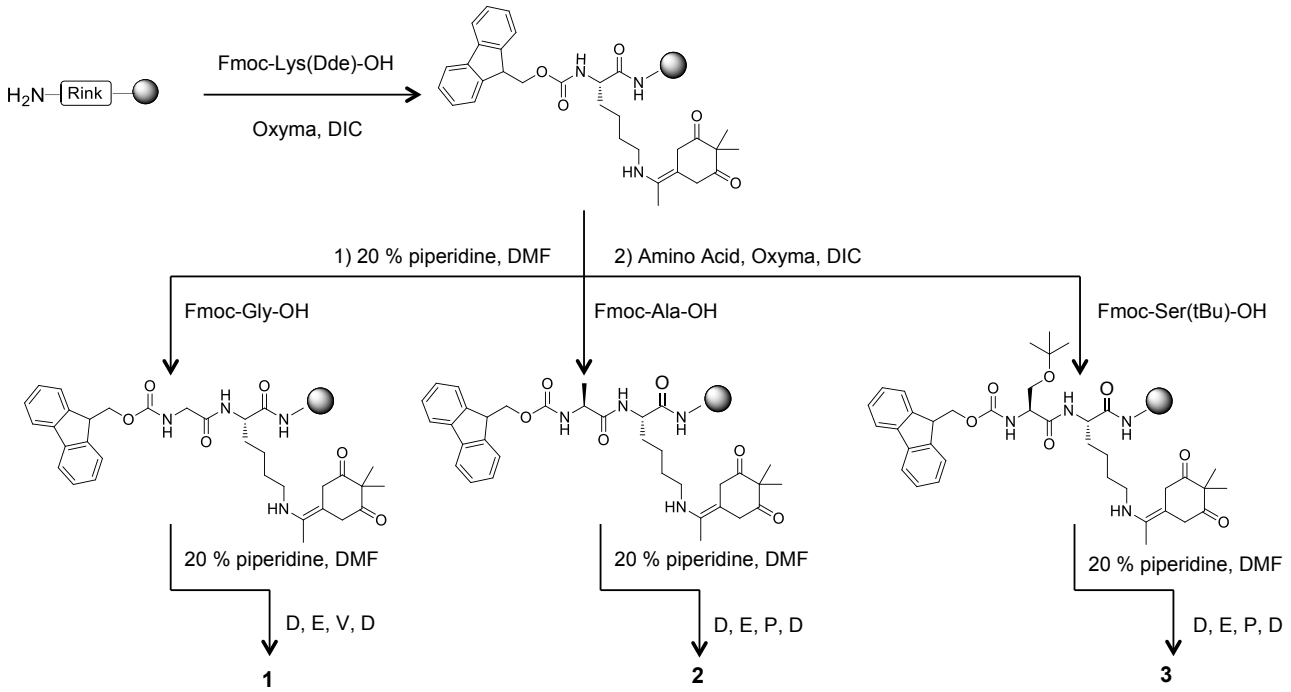


Figure S5. Time-dependent activation of **4–11** (3.75 μM, n = 3) with cathepsin B (25 nM). Note that the apparent low increase in fluorescence can partly be attributed to the low fluorescence intensity of fluorescein in the acidic cathepsin B buffer (pH 5.0).

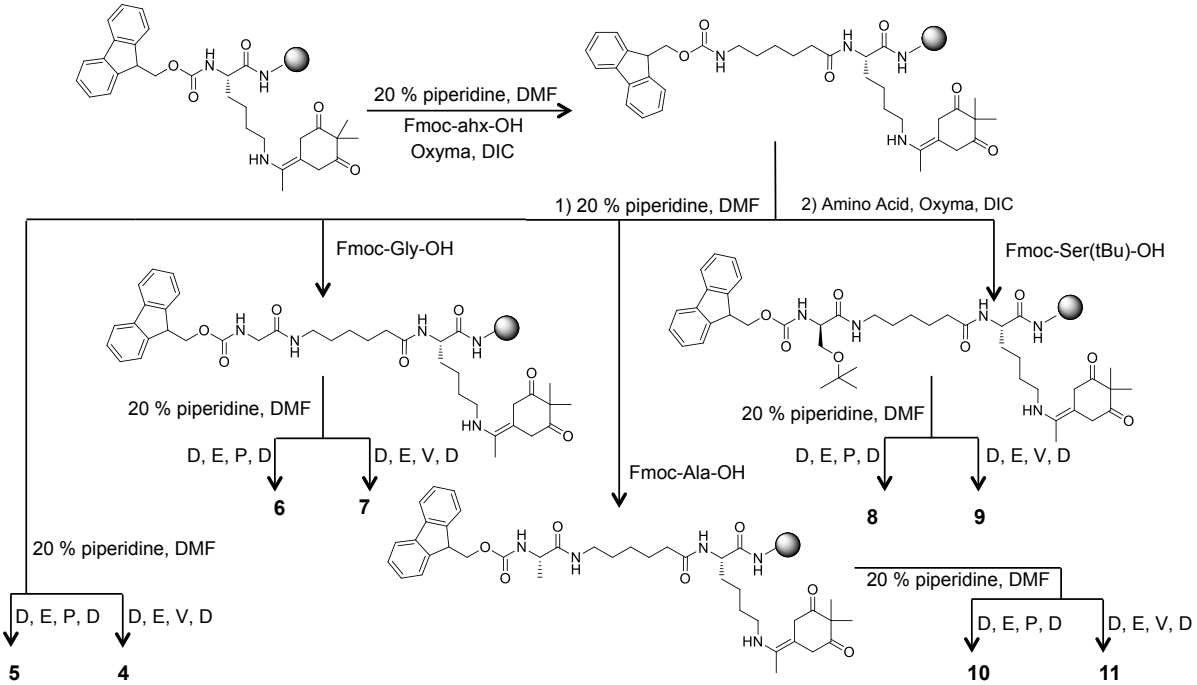
2. Experimental

2.1 Solid-phase synthesis of 1–11

The fluorogenic substrates **1–11** were synthesised (Scheme S1 and S2) on aminomethyl polystyrene resin (1 % DVB, 100–200 mesh, loading 1.2 mmol/g). Fmoc Rink-linker (*p*-{*(R,S)*-*α*-[1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl}-phenoxyacetic acid) was coupled to the resin, using the general coupling procedure below. The bifunctional spacer Fmoc-Lys(Dde)-OH (synthesised as reported by Portal[1]) was then coupled, followed by Fmoc deprotection and coupling of the relevant amino acid sequence (including εAhx-spacers where present). Once the sequences were complete, the Dde protecting group was removed with $NH_2OH \cdot HCl$/imidazole in NMP–DCM (see below),[2] and methyl red coupled to the lysine side chain. The *N*-terminus Fmoc group was then deprotected and 5(6)-carboxyfluorescein coupled. Ninhydrin and acetaldehyde/chloranil tests were used to confirm the presence of amines after each deprotection and coupling step. After cleavage from the resin, the compounds were purified by preparative HPLC, and analysed by MALDI-ToF MS and HPLC.



Scheme S1. Solid-phase synthesis of substrates **1**–**3**.



Scheme S2. Solid-phase synthesis of substrates **4**–**11**.

Coupling procedure. The appropriate Fmoc-protected amino acid (4 eq, 0.1 M) and Oxyma (4 eq) in DMF was stirred for 10 min. DIC (4 eq) was added and the mixture stirred for a further 5 min. The coupling solution was added to the corresponding resin (1 eq, ~ 250 mg resin), preswollen in DCM, and shaken for 1 hr. After filtration, the resin was washed with DMF (3 × 10 mL), DCM (3 × 10 mL), and MeOH (3 × 10 mL).

Fmoc deprotection. The resin was preswollen in DCM, and treated with 20 % piperidine in DMF (v/v) (3 mL, 2 × 5 min). The resin was isolated by filtration, and washed with DMF (3 × 10 mL), DCM (3 × 10 mL), and MeOH (3 × 10 mL).

Dde deprotection. $NH_2OH{\cdot}HCl$ (1.25 g, 1.80 mmol) and imidazole (0.92 g, 1.35 mmol) were suspended in 5 mL of NMP and the mixture was sonicated until complete dissolution. Before the reaction, 5 volumes of the deprotection solution were diluted with 1 volume of DCM. The solution was added to the corresponding resin (100–125 mg, 20 mg/mL), stirred for 2 h, the deprotected resin was isolated by filtration, and washed with DMF (3 × 10 mL), DCM (3 × 10 mL), and MeOH (3 × 10 mL).

Cleavage from the resin. Prior to cleavage, the resin was additionally washed with 20% piperidine in DMF. The resin (100–125 mg), preswollen in DCM, was treated with 2–3 mL of TFA–TIS–H_2O (95:2.5:2.5) for 3 h. The cleavage solution was isolated from the resin by filtration, and the compounds precipitated by addition of cold Et_2O and isolated as dark red solids by centrifugation.

2.2 Purification and characterisation of 1–11

Preparative HPLC was performed using Agilent Technologies 1100 instrument with UV detection (collection at 495 nm), fitted with an Agilent Eclipse XDB-C18 (7 μm, 21.1 × 250 mm) column. Samples were eluted with a gradient of H_2O–ACN with 0.1% formic acid (30 to 95 % ACN in 25 min; isocratic 95 % ACN for 5 min), flow rate 4 mL/min. Analytical HPLC was performed using Agilent Technologies 1100 instrument with UV (495 nm) and Evaporative Light Scattering (ELSD) detectors (Polymer Lab 100 ES), fitted with a Supelco Discovery® C18 (5 μm, 5 cm) column. Samples were eluted with a gradient of H_2O-MeOH with 0.1% formic acid (5 to 95 % MeOH in 10 min; isocratic 95 % for 4 min, 5 % 1 min, 1 min isocratic), flow rate 1 mL/min. MALDI-ToF MS were recorded on Voyager-DE™ STR using Biospectrometry™ workstation, and calibrated using an internal standard reference peak of [Glu 1]-Fibrinopeptide, sequence EGVDNEEGFFSAR ($C_{66}H_{95}N_{19}O_{26}$) ($[M+H]^+$ 1570.67678 for positive ionisation and $[M–H]^–$ 1568.66222 for negative ionisation). Sinapic acid was used as a matrix.

Table S1. Characterisation of substrates **1**–**11**.

Substrate	Maldi-ToF MS		HPLC			
	Calculated	**Found**	t_r (min)	**Purity** (%)[a] ELSD	**Purity** (%)[b] λ 495 nm	**Yield** (mg)[c]
1	$[C_{68}H_{77}N_{12}O_{20}]^{1-}$ 1381.5382	1381.6293	6.95	100	92	45 (80%)
2	$[C_{69}H_{77}N_{12}O_{20}]^{1-}$ 1393.5382	1393.7742	6.70	100	100	34 (62%)
3	$[C_{69}H_{79}N_{12}O_{21}]^{1-}$ 1411.5487	1411.5534	6.63	100	100	48 (85%)
4	$[C_{72}H_{85}N_{12}O_{20}]^{1-}$ 1437.6008	1437.8162	6.68	100	100	57 (98%)
5	$[C_{72}H_{83}N_{12}O_{20}]^{1-}$ 1435.5852	1435.4716	6.86	100	90	18 (76%)
6	$[C_{74}H_{86}N_{13}O_{21}]^{1-}$ 1492.6066	1492.7576	6.78	100	100	63 (93%)
7	$[C_{74}H_{88}N_{13}O_{21}]^{1-}$ 1494.6223	1494.8761	6.82	100	100	47 (80%)
8	$[C_{75}H_{88}N_{13}O_{21}]^{1-}$ 1506.6223	1506.9024	6.67	100	100	41 (69%)
9	$[C_{75}H_{90}N_{13}O_{21}]^{1-}$ 1508.6379	1508.8114	6.83	84	100	45 (76%)
10	$[C_{75}H_{88}N_{13}O_{22}]^{1-}$ 1522.6172	1522.8054	6.72	100	100	50 (89%)
11	$[C_{75}H_{90}N_{13}O_{22}]^{1-}$ 1524.6328	1524.6401	6.45	100	100	60 (90%)

[a] Based on ELSD detection; [b] Based on UV detection at 495 nm; [c] Crude yield calculated based on the amount and loading value of the resin used.

3. Enzymatic assays

E. coli-derived recombinant human caspase-3 (cat # 707-C3/CF) and caspase-7 (cat # 823-C7/CF), and murine myeloma cell line N20-derived cathepsin B (cat # 953-CY), were purchased from R & D Systems®. CHAPS, DTT, HEPES, MES, and the control substrates **ACF** (cat # A0466) and **MCA** (cat # M1169) were from Sigma–Aldrich. Fluorescence measurements were performed on a Biotek Synergy HT plate-reader (K_m and k_{cat} determination) or a Tecan Infinite M100 plate-reader operating the Magellan 6 software (initial screening).

3.1 Kinetic assays with Caspase-3/7

Each substrate was dissolved in DMSO to give 10–100 mM stock solutions (stored at −20 °C). Substrate concentrations were calculated by UV/Vis absorption at 490 nm (ε = 72000 M^{-1} cm^{-1}) in caspase buffer (pH = 7.5). One unit of caspase-3 or caspase-7 was added to 100 µL of caspase assay buffer (25 mM HEPES, 0.1 % (w/v) CHAPS, 10 mM dithiotheritol (DTT), pH 7.5) with the substrates at 0.2, 0.4, 0.8, 1.6, 3.1, 6.2, 12.5, 25 µM concentrations (n = 3), to give a final enzyme concentration of 15 nM. The increase in fluorescence ($\lambda_{Ex/Em}$ 485/528 nm) was measured on a plate reader (Biotek Synergy HT) every 1 min for 1 h (61 time points). The rate (µM/min) was calibrated using a 5(6)-carboxyfluorescein conversion factor (0.0045 µM/RFU) and the data plotted against time (min). For initial cleavage rate (0–5 min), plots were fitted using linear regression analysis and the Michaelis-Menten data generated using GraphPad Prism 5. For AFC and MCA ($\lambda_{Ex/Em}$ 355/460 nm) 0.4–25 µM substrate concentrations were used (conversion factor 0.0003 µM/RFU for AFC). The fluorescence from the controls (no enzyme) was removed from the total fluorescence increase of the samples to determine background corrected RFU.

3.2 Cathepsin B assay

Cathepsin B was activated by diluting to 10 μg/mL from a stock solution (50 mg/mL) in activation buffer (25 mM MES, 5 mM DTT, pH 5.0), and incubated for 15 min at rt. 50 μL of substrates **4**–**11** in cathepsin buffer (25 mM MES, pH 5.0) were incubated with 50 μL of activated cathepsin B to give a final substrate concentration of 3.75 μM and enzyme concentration of 25 nM. Increase in fluorescence was measured at 0, 5, 20, 40 and 80 min (Tecan Infinite M100). For direct comparison with caspase-3, caspase-3 (15 nM) and cathepsin B (25 nM) were incubated with 10 μM of 6, 7, 10 and 11 (substrate banks were used to determine RFU-fold increase in fluorescence).

4. References

1. Portal, C., Launay, D., Merritt, A. and Bradley, M. High throughput physical organic chemistry: analytical constructs for monomer reactivity profiling. *J. Comb. Chem*. **2005**, *7*, 554–560

2. Díaz-Mochón, J. J., Bialy, L. and Bradley, M. Full orthogonality between Dde and Fmoc: the direct synthesis of PNA-peptide conjugates. *Org. Lett*. **2004**, *6*, 1127–1129