Chemical tools for the study of proteolytic activities associated with antigen presentation

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Chapter 5

A cysteine protease probe with an additional amine-reactive group[1]

Introduction

Phagocytosis is, in many cases, the first step towards the presentation of exogenic antigenic proteins on MHC class II molecules of professional antigen presenting cells.[2] The ingested antigens travel to the lysosomes and are degraded by a set of different hydrolases. Some of the peptide fragments generated by these proteases escape further degradation by binding to MHC class II molecules. The loaded MHC class II molecules are transported to the cell surface for presentation to CD4+ T-cells (helper T cells). Depending on other factors, like the conditions of the antigen presentation, the CD4+ T-cells can initiate a B-lymphocyte or CD8+ cytotoxic T-lymphocyte mediated immune response.

The cathepsin family of lysosomal cysteine proteases plays an important role in the process of antigen processing: these proteases are able to cleave the majority of peptide bonds in antigens.[3] The activity of the different proteases in the lysosomal pathway is not constant.[4] Different protease activities are added to the maturing lysosome sequentially and

the activity of all proteases is also dependent on the pH. As a result, the processing of epitopes is governed by gradients of tightly regulated protease activities.

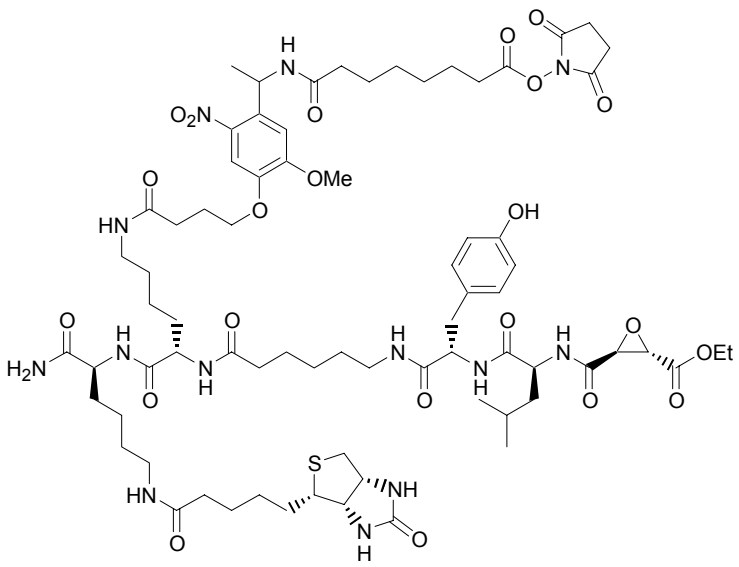

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Figure 1. Target structure

Here, a cysteine protease probe is described for use in the monitoring of the activity of the lysosomal cysteine proteases that a model antigen encounters after internalisation. The new probe contains an amine reactive group to allow conjugation to a model antigen, as well as a photolabile linker to release the probe from the model antigen before analysis of the target proteases. A known cysteine protease activity based probe, DCG-04,[5] was equipped with an activated ester to facilitate conjugation of this probe to a lysine side chain amine of an antigen of choice (**1**, Figure 1). In a typical experiment, probe **1** could be conjugated to ovalbumin (**2**) to furnish construct **3** (Figure 2). This ovalbuminated probe captures an active cathepsin of the cysteine protease family to give **4**. This way, the biotin-bearing cysteine protease probe has become covalently bound to the targeted cysteine protease. An photolabile ortho-nitrobenzyl linker would allow for photorelease of the ovalbumin carrier protein, and biotinylated cathepsin **5** could be visualised by standard avidin based blotting techniques.

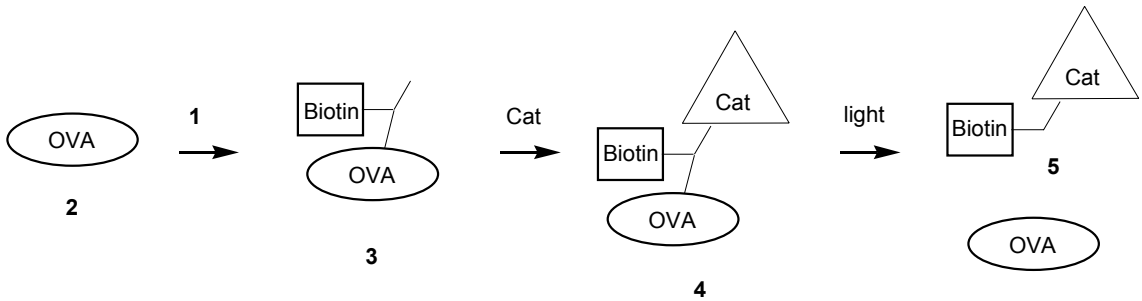
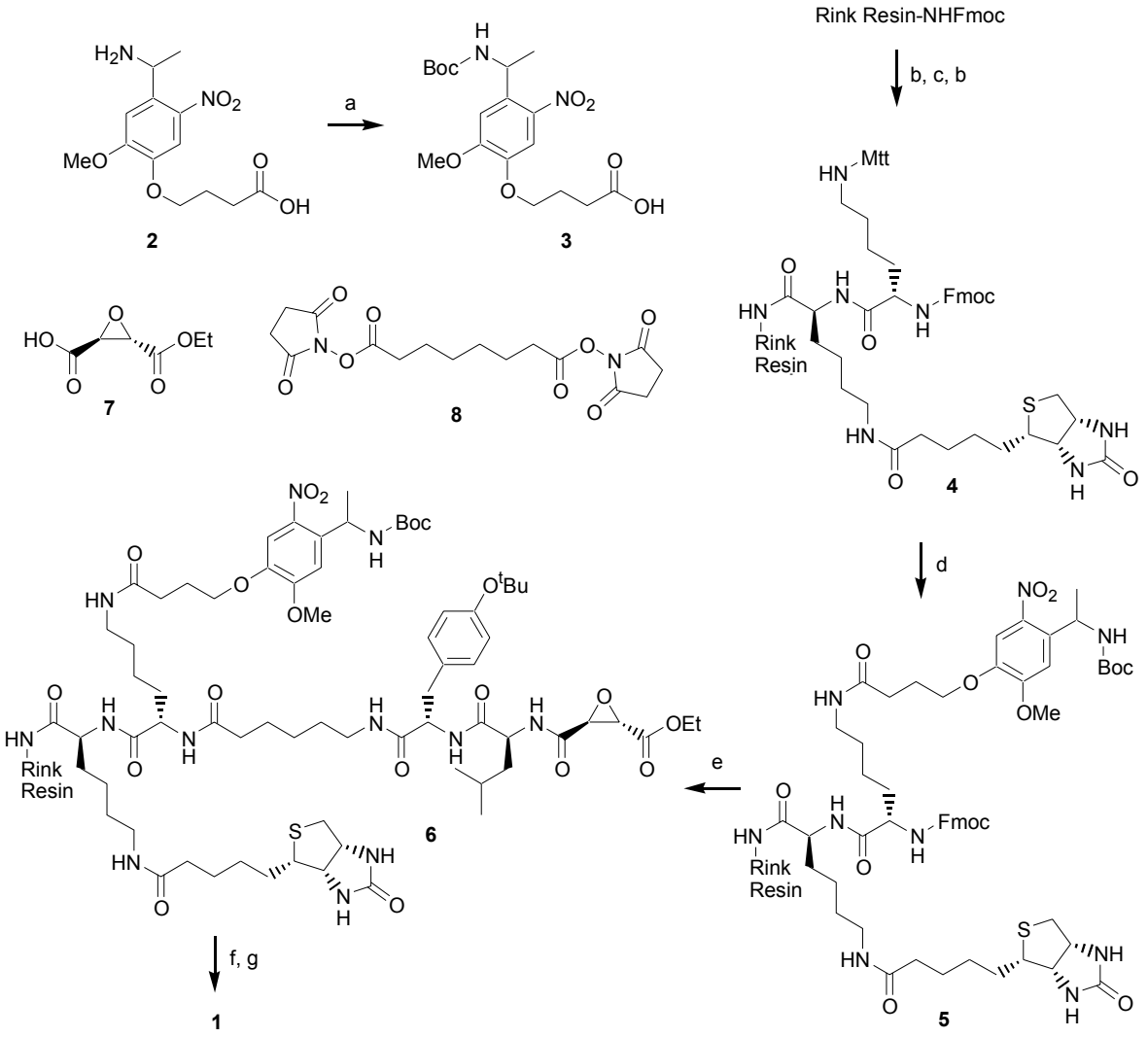


Figure 2. Schematic representation of the cathepsin labeling approach. Ovalbumin (OVA) is conjugated to probe **1**, a cathepsin (Cat) reacts with the probe and OVA is photoreleased to furnish labeled cathepsin **5**.

Synthesis

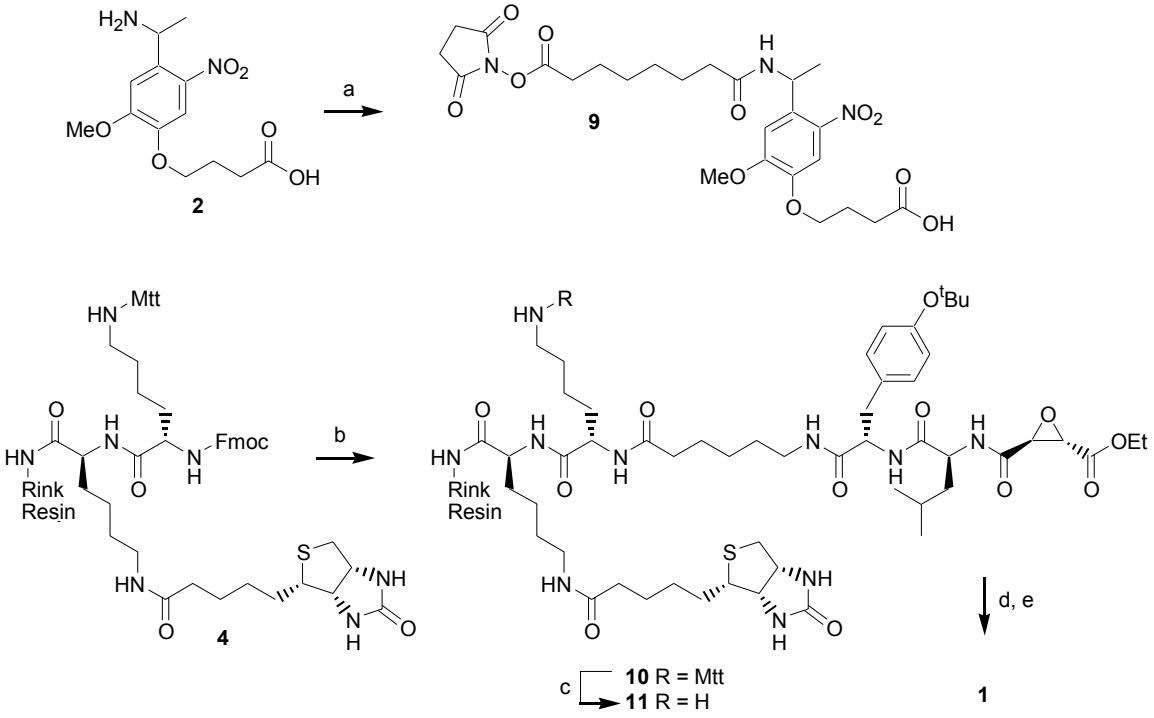
The first route of synthesis that was explored is depicted in Scheme 1. Suitable protected photocleavable linker **3** (Scheme 1) was obtained by Boc-protection of known photolabile amino acid **2**.[6] Immobilised biocytin was created as described in Chapter 3 and elongated with Fmoc-Lys(Mtt)-OH to afford **4**. The lysine side chain amine was liberated by treatment with 1% TFA in CH_2Cl_2, followed by acylation with Boc-protected nitrovaleryl acid **3** to give immobilised **5**. The peptide was elongated by standard Fmoc-based SPPS to



Scheme 1. Synthesis of photocleavable activated probe **1**. Reagents and conditions: a) Boc_2O, dioxane, H_2O, NaOH; b) 20% piperidine in NMP, then Fmoc-Lys(Mtt)-OH, HCTU, DiPEA, NMP; c) 1% TFA in CH_2Cl_2, then biotin, HCTU, DiPEA, NMP; d) 1% TFA in CH_2Cl_2 then **3**, HCTU, DiPEA, NMP; e) Repeated cycles of SPPS: Fmoc cleavage: 20% piperidine in NMP; amino acid condensation: Fmoc-protected amino acid, HCTU, DiPEA, NMP; Fmoc-protected building blocks were used in the following order: Fmoc-Ahx-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Leu-OH, **7**; f) TFA/H_2O (95/5 v/v); g) **8**, DiPEA, DMF.

give protected and immobilized **6**. The peptide was cleaved from the resin by treatment with TFA/H_2O/TIS, removing also the acid labile side chain protecting groups. The crude intermediate was purified to homogeneity by RP-HPLC and reacted with excess bisactivated ester **8**. LC/MS analysis confirmed the formation of product **1** but HPLC purifications did not effect separation of the target compound from the excess of **8**.

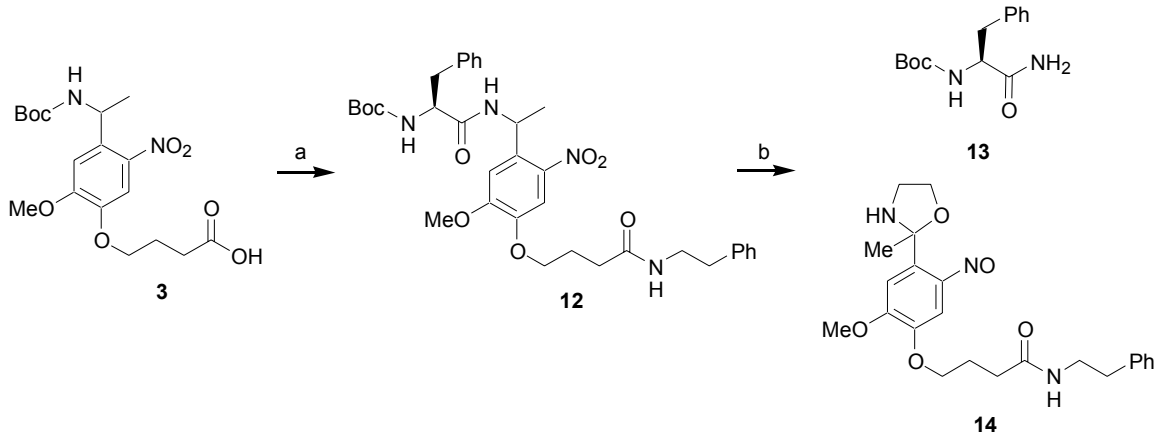
To circumvent this purification problem, it was decided to perform all coupling steps on the solid support. For this purpose, activated ester **9** (Scheme 2) was prepared by reaction of photolabile linker **5** with disuccinimidyl suberate **8**. Elongation of **4** by standard Fmoc-based SPPS afforded Mtt-protected immobilised peptide **10**. After Mtt cleavage, the lysine side chain was elaborated using acid **9** at low temperature. Nucleophilic attack on the less reactive OSu ester was suppressed by the reaction conditions. Liberation of the peptide from the solid support followed by RP-HPLC purification afforded activated ester **1** in 6% overall yield.



Scheme 2. Synthesis of photocleavable activated probe **1.** Reagents and conditions: a) **8**, Et_3N, DMF; b) Repeated cycles of SPPS: Fmoc cleavage: 20% piperidine in NMP; amino acid condensation: Fmoc-protected amino acid, HCTU, DiPEA, NMP; Fmoc-protected building blocks were used in the following order: Fmoc-Ahx-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Leu-OH, **7**; c) 1% TFA in CH_2Cl_2; d) **9**, HATU, DiPEA, NMP, -20°C; e) TIS/H_2O/TFA (2.5/2.5/95 v/v/v).

Inhibitory results and photocleavage characteristics

Next, the photocleavage characteristics of the linker were investigated. Model compound **12**, conveniently synthesised from **3** in three steps (Scheme 3), was chosen for initial studies. Photocleavage proceeded smooth, and after 4 h all starting material had been converted to amide **12** and aminal **13** as judged from LC/MS analysis of the reaction mixture.



Scheme 3. Initial photocleavage studies. Reagents and conditions: a) 2-phenylethylamine, DIC, DMAP, CH_2Cl_2, then TFA/CH_2Cl_2 (1/1 v/v) then Boc-Phe-OH, DIC, DMAP, CH_2Cl_2; b) **12** was dissolved in DMSO/MeOH/MeCN (10/45/45 v/v/v) and two drops of ethanolamine were added. The reaction mixture was irradiated in a quartz cuvet using a Rayonet reactor equipped with 7 × 3500 Å (15 W) lamps. Samples were taken at t=0, 1 h, 2 h, 4 h, 8 h and analyzed by LC/MS.

With the photocleavage conditions in hand, an ovalbumin-**1** conjugate was synthesized. Ovalbumin was incubated with 3 equivalent of OSu ester **1** for 2 h at 4°C, and unreacted and hydrolyzed **1** were removed by dialysis. The resulting construct was exposed to the action of Cat B at 37°C for 30 min. Western blot analysis indicated the presence of biotinylated Cat B, but no heavy band for the Cat B-**1**-Ova was observed. This observation forced us to exclude the presence of **1** after dialysis.

As a model for the dialysis of **1**, ovalbumin was incubated with DCG-04 (compound **1a,** Chapter **4**) under the same conditions as **1**. Unexpectedly, not only cathepsin labeling was found upon incubation with Cat B, but also labeling of ovalbumin was detected by Western blotting with strept-HRP. This would indicate that ovalbumin could react with the epoxide moiety in DCG-04. To test which nucleophile was responsible for this labeling, ovalbumin was treated with maleimide to block all cysteine residues. DCG-04 was added

and western blotting indicated no alkylation with DCG-04. However, labeling returned if a dialysis step was included after maleimide treatment, albeit with lower efficiency as seen before. As a third control, DCG-04 and ovalbumin were mixed in blotting buffer, boiled briefly to denaturate and separated on SDS-PAGE. Again, biotinylation was confirmed with western blotting. From these experiments, it was concluded that 1) the dialysis protocol is insufficient for removal of DCG-04 and possibly also for **1** and 2) that boiling in Laemmli sample buffer is sufficient to effect alkylation of cysteine residues within ovalbumin. These results demand a proper purification protocol for ovalbumin after the reaction with **1** and a method to prove that **1** is attached to ovalbumin *via* the photocleavable side chain and not through the electrophilic site of the epoxide moiety.

Conclusions

The synthesis and purification of probe **1** required a strategy that allowed all coupling steps to be perfomed on a solid support. The target compound was obtained in 6% overall yield. The photocleavage characteristics of the nitrovaleryl linker were established. The labeling of ovalbumin with **1**, in order to study the inhibition characteristics of the construct, needs optimization. The purity and homogeneity of ovalbumin labeled with probe **1** could not be confirmed, due to possible alkylation of ovalbumin *via* a cysteine thiol moiety. This would indicate that the labeling reaction of lysine with hydroxysuccimidyl ester is unsuitable to be performed in the presence of an epoxide moiety, and possibly an bioorthogonal labeling approach using the Staudinger ligation or Huisgen cyclization could produce efficient labeling of ovalbumin.

Experimental part

General methods and materials: Solvents used in the solid phase peptide synthesis, DiPEA and TFA were all peptide synthesis grade (Biosolve) and used as received. The protected amino acids, Rink amide MBHA resin (0.78 mmol g^{-1}) and HCTU were obtained from NovaBiochem. Ethyl (2S,3S)oxirane-2,3-dicarboxylate was prepared as described in Chapter 4. Fmoc-Lys(Mtt)-OH was from Senn Chemicals, SPPS was carried out using a 180° Variable Rate Flask Shaker (St. John Associates, Inc.). LC/MS analysis was performed on a Jasco HPLC system (detection simultaneously at 214 and 254 nm) coupled to a Perkin Elmer Sciex API 165 mass spectrometer equipped with a custom-made Electrospray Interface (ESI). An analytical Alltima C_{18} column (Alltech, 4.6 × 250 mm, 5μm particle size) was used. Buffers: A: H_2O; B: CH_3CN; C: 0.5% aq. TFA. For RP HPLC-purifications a Biocad "Vision" automated HPLC system (PerSeptive

Biosystems, Inc.) was used. The applied buffers were A, B and C. ^{1}H-NMR spectra were recorded with a Bruker DMX 600 instrument at 600 MHz with chemical shifts (δ) relative to tetramethylsilane. When appropriate, reactions were performed under the exclusion of light.

Synthesis of 4-(4-(1-(succinimidylsuberoylamino)ethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (9): A solution of known 4-(4-(1-(amino)ethyl)-2-methoxy-5-nitrophenoxy)butanoic acid[6] (298 mg, 1 mmol) and Et_3N (0.14 ml, 1 mmol) in DMF (5 ml) was added dropwise to a solution of disuccinimidyl suberate (Alldrich, 386 mg, 1.3 mmol) in DMF (5 ml) and the mixture was stirred for 1.5 h until TLC-analysis (ethyl acetate) indicated a completed reaction. The mixture was concentrated *in vacuo* and the residue was taken up in ethyl acetate. The white precipitate was filtered off and the remainder solution was purified by silica gel chromatography (0-1% acetic acid in ethyl acetate) to yield 250 mg (0.45 mmol, 45%) of a faint yellow solid. ^{1}H NMR ($CDCl_3$): δ, 7.56 (s, 1H), 7.19 (s, 1H), 6.52 (d, *J*=7.3 Hz, 1H), 5.6-5.4 (m, 1H), 4.2-4.0 (m, 2H), 3.92 (s, 3H), 2.84 (s, 4H), 2.59 (t, 4H, *J*=7.3 Hz), 2.3-2.0 (m, 4H), 1.8-1.2 (m, 11H). ESI-MS $C_{25}H_{33}N_3O_{11}$ + H^+ requires 552.2, found 551.9.

Synthesis of 1: Fmoc-protected biocytinylated Rink amide resin (132 mg, 50 μmol) was elongated by standard Fmoc-based SPPS to give resin-bound Lys(Bio)Lys(Mtt)AhxTyr(tBu)Leu-Epoxirane. In brief, where appropriate removal of the Fmoc protecting group was accomplished by treatment of the resin-bound peptide with 20% (v/v) piperidine in NMP for 20 min. Peptide coupling steps were performed by treatment of the resin with a premixed (5 min) solution of the of the appropriate acid (5 eq.), HCTU (5 eq.) and DiPEA (6 eq.) in NMP (0.5 ml) for 1 hour unless stated otherwise. Coupling efficiencies were monitored with the Kaiser test and couplings were repeated if necessary. After coupling and deprotecting steps the resin was washed with NMP (5×). After the last coupling step, the resin was washed with CH_2Cl_2 (3×). Of 25 μmol of resin-bound Lys(Bio)Lys(Mtt)AhxTyr(tBu)LeuEpoxirane, the lysine side chain Mtt protecting group was removed by treatment with 1% TFA in CH_2Cl_2 (4×10 min). The resin was washed (CH_2Cl_2 5×, then NMP 2×) and neutralised with 10% DiPEA in NMP. To cooled (-20°C) resin was added a premixed (5 min) and cooled (-20°C) solution of active ester **8** (55 mg, 0.10 mmol), HATU (38 mg, 0.10 mmol) and DiPEA (34 μl, 0.20 mmol) in NMP and the resin was agitated for 1 h, and allowed to warm to ambient temperature. The resin was washed extensively (alternating CH_2Cl_2-MeOH 3×, alternating CH_2Cl_2-Et_2O 3×), transferred into a clean vial and treated with TFA/H_2O/TIS (1 ml, 95/2.5/2.5 v/v/v) for 1 h. The mixture was filtered and the resin washed with TFA (2× 1 ml). The filtrate was diluted (toluene, 10 ml) and concentrated in vacuo. The crude product was coevaporated (toluene, 3×) and purified to homogeneity by RP-HPLC, applying a linear gradient (20-55% B in 3 column volumes) to yield 2.38 mg (1.5 μmol, 6.1%) of product. ^{1}H NMR (DMSO, 300 K, DMX 600): δ, 9.12 (s, 1H), 8.50 (d, 1H, *J* = 7.6 Hz), 8.44 (d, 1H, *J* 7.6 Hz), 8.07 (d, 1H, *J* = 8.2 Hz), 7.89

(d, 2H, *J* = 7.7 Hz), 7.83-7.77 (m, 2H), 7.77-7.65 (m, 6H), 7.46 (s, 2H), 7.14 (s, 1H), 6.99-6.91 (m, 5H), 6.60 (d, 4H, *J* = 8.3 Hz), 6.41 (s, 1H), 6.34 (s, 1H), 5.38-5.31 (m, 1H), 4.37-4.26 (m, 5H), 4.21-4.08 (m, 8H), 4.04-3.94 (m, 3H), 3.86 (s, 3H), 3.69 (d, 1H, *J* = 1.7 Hz), 3.57 (d, 1H, *J* = 1.7 Hz), 3.11-3.04 (m, 3H), 2.83-2.74 (m, 6H), 2.73-2.50 (m, 8H), 2.23-1.88 (m, 12H), 1.67-1.09 (m, 28H), 0.87-0.74 (m, 7H). ESI-MS: $C_{74}H_{109}N_{13}O_{22}S + H^+$ requires 1564.8, found 1565.0;

Conjugation of 1 to ovalbumin: Ovalbumin (22 nmol) was incubated with **1** (66 nmol, 5 µl of a 13 mM stock in DMSO) in phosphate buffer (0.5 ml, 50 mM, pH 7.8) for 2 h at 4°C. To purify the labeled ovalbumin, the reaction mixture was filtered over a 10 kD cutoff filter and the resulting solution was lyophilized. A sample of the ovalbumin conjugate was boiled for 3 min in 4× Laemmli sample buffer (5% SDS, 46% glycerol, 0.156 mM Tris-HCl pH 6.8, 0.25% bromophenol blue, 1.4 M β-mercaptoethanol in H_2O) and resolved by 12.5% SDS-PAGE. Proteins were visualized by silver staining (Amersham). Western blotting and incubation with streptavidin-horseradish peroxidase (strept-HRP) conjugates allowed the visualization of biotinylated ovalbumin by chemiluminescence.

Cathepsin activity profiling: 2 pmol Cat B (R&D Systems) was pre-incubated at 10 µg/ml in 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 5 mM dithiothreitiol, pH 5.0 for 15 min at room temperature. Ovalbumin-**1** conjungate (2 pmol) was incubated with the activated Cat B for 1 h at ambient temperature. The mixture was neutralized to pH 7 by addition of aq. NaOH (0.4 µl of a 1M solution) and resolved by 10% SDS-PAGE followed by Western blotting. Biotinylated proteins were visualized by chemiluminescence after incubation with strept-HRP.

References and notes

1. The work presented in this chapter was performed in close collaboration with Bobby Florea and Micha Slegt.
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