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

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Chapter 6

A cell penetrating cysteine protease probe[1]

Introduction

Around a decade ago, it was found that specific oligopeptides can traverse lipid bilayers that make up the cell membrane of mammalian cells, and that they can do so when covalently bound to proteins or other relevant biomolecules. This property, which was first identified for peptides derived from the HIV-1 Tat[2] and *Drosophila* Antennapedia[3] (Ant) proteins, has attracted much attention. As a result, a wide variety of natural and synthetic peptides displaying transferring properties have been identified and used to deliver cargo into cells.[4-6] Common to most of these sequences is the presence of numerous basic amino acid residues, as is evident in the Tat (GRKKRRQRRRPPQ) and Ant (RQIKIWFQNRRMKWKK) peptides. It is thought that these cationic residues are responsible for the translocation activity.

To investigate the role of arginine residues further, oligoarginine peptides built from seven to eleven arginine moieties were used in uptake experiments. The group of Futaki showed that oligoarginine translocated both fluoresceine and carbonic anhydrase into RAW cells.[7] The optimal number of arginine residues for translocation was found to lie between six and eight. Wender *et al.* synthesised both all-D and all-L oligoarginine peptides, and

demonstrated that both are capable of translocation of fluoresceine.[8] They found that nine arginine residues were optimal to induce translocation, and that the all-D peptide is a better translocator than the corresponding all-L peptide. In another study,[9] the uptake of fluoresceine conjugates with oligomers of arginine, lysine or histidine was compared, and it was found that only the oligoarginine conjugates effected fluorescence within cells. Octamers and nonamers of arginine exhibited comparable uptake efficiency, and again the all-D peptides residues performed better than the all-L peptides.

The mechanism of translocation mediated by cell penetrating peptides still has to be revealed. From the observation that the all-D peptides are at least as efficient in translocation as the corresponding all-L peptides, it can be concluded that there is no specific receptor involved. There is, however, no consensus whether the translocation is caused by spontaneous permeabilisation of the cell membrane or by active uptake of the cargo *via* the endosomal pathway. Evidence supporting the latter option is accumulating slowly,[10] but the possibility of multiple pathways can not be excluded yet. The endosomal pathway is a feature of the cell that is used to regulate uptake of nutritients and communication signals from the environment of the cell. There are different uptake pathways for molecules to enter the cell.[11] Generally, the first step involves a membrane rearrangement resulting in the formation of a small, membrane enclosed vesicle in the cytosol. These vesicles can travel to the endoplasmatic reticulum or golgi apparatus, and deliver their cargo there, or enter the lysosomal pathway, resulting in the degradation of the contents of the vesicle through the action of lysosomal cysteine proteases.[12]

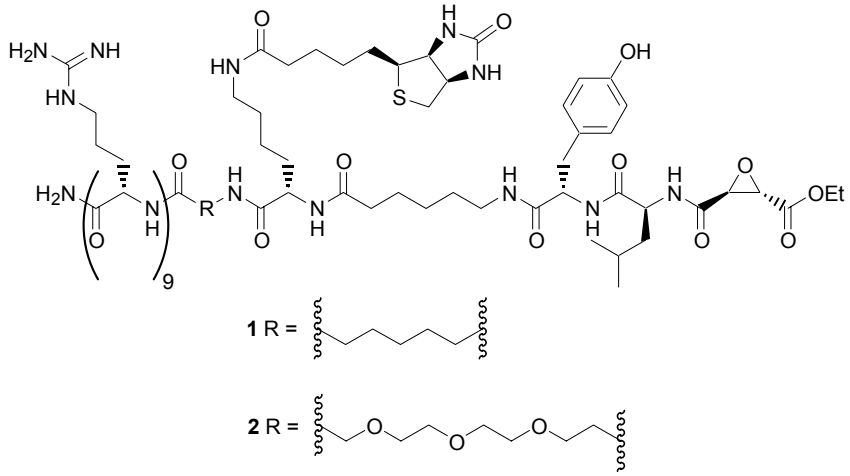


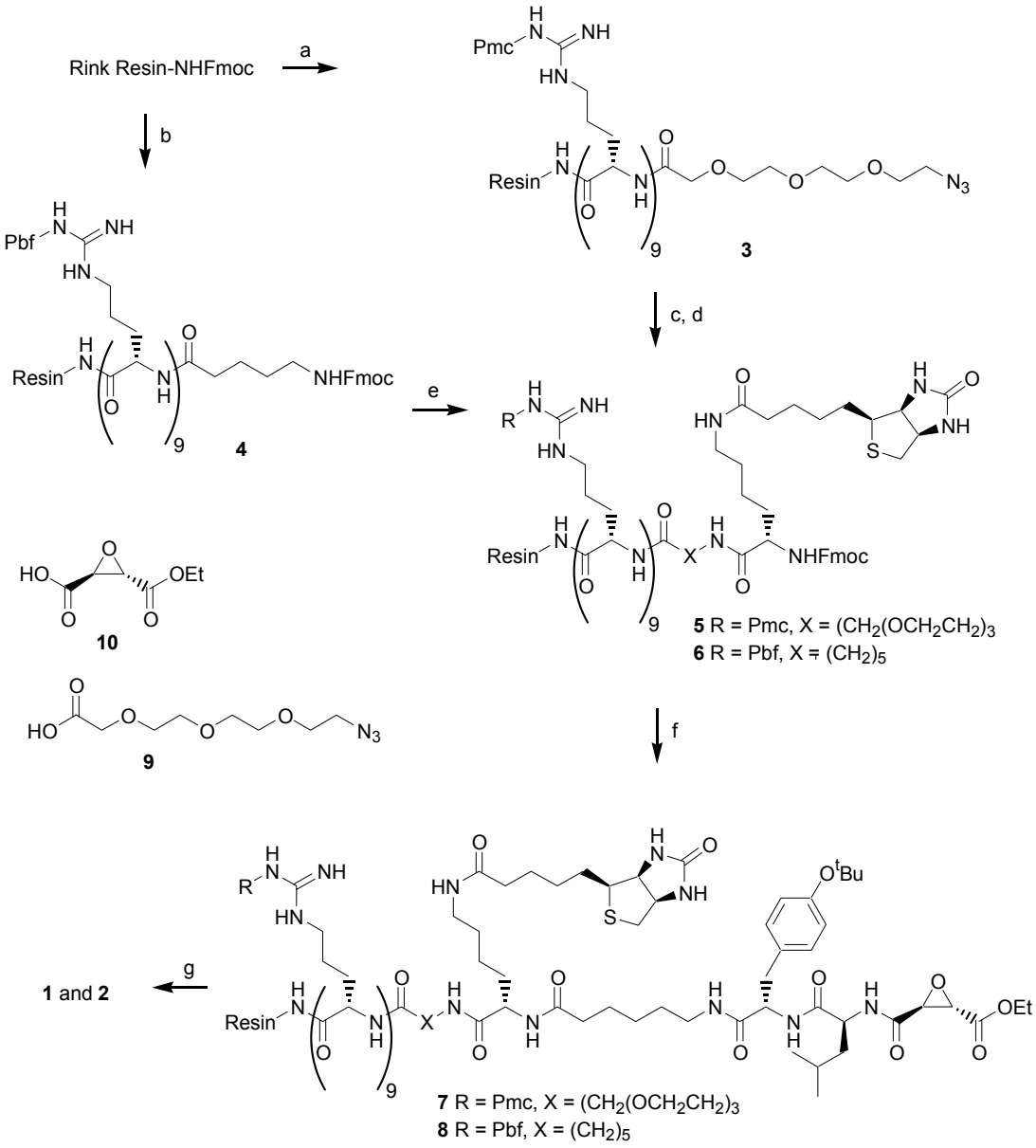
Figure 1. Target structures.

It was envisaged that involvement of the endosomal pathway could be demonstrated by the activity of lysosomal cysteine proteases, which may be visualised *via* a suitable activity based probe. Fusion of an activity based cysteine protease probe to the nonaarginine cell penetrating peptide would yield a probe that enters the cell in the fashion dictated by the nonaarginine sequence. Any cysteine protease that is encountered in the uptake pathway can be visualised, thus generating insight in the actual path that the probe, and thus the nonaarginine peptide, follows. For this purpose, two conjugates were synthesized of nonaarginine with DCG-04,[13] a broad class cysteine protease activity based probe. The cell penetrating peptide is linked to DCG-04 either by a short hydrophobic or by a longer hydrophilic linker (compounds **1** and **2**, figure 1).

Synthesis

The synthesis of probe **1** (Scheme 1) starts with the conjugation of nine Pbf-protected arginine residues to the acid labile rink resin *via* standard Fmoc-based SPPS. Elongation of the growing peptide chain with an aminohexanoic acid spacer yields protected and immobilised **4**. The biotin moiety was introduced by direct coupling of a side chain biotinylated lysine building block and the synthesis was continued by Fmoc-based SPPS furnishing peptide **8** on the solid support. Cleavage of the peptide from the solid phase with concomitant removal of the arginine en tyrosine side chain protecting groups gave endocytosis probe **1** in 11% overall yield after RP-HPLC purification.

The synthesis of probe **2** was performed in a slightly different way. Rink resin was elongated with nine Pmc-protected arginine residues and spacer **9**.[14] The azide group in compound **3** was transformed to an amine by a Staudinger reduction. Elongation with Fmoc-Lys(Mtt)-OH and removal of the hyper acid-labile Mtt protecting group was followed by biotinylation of the lysine side chain to give immobilised **5**. From that point, the synthesis was continued by Fmoc-based SPPS furnishing peptide **7** on the solid support. Cleavage of the peptide from the solid phase and RP-HPLC purification afforded probe **2** in 13% yield.



Scheme 1. Synthesis of polyargininated cysteine protease probes **1** and **2**. Reagents and conditions: General reagents for SPPS: Fmoc cleavage: 20% (v/v) piperidine in NMP; amino acid condensation: Fmoc protected amino acid, HCTU, DiPEA, NMP. a) Repeated cycles of SPPS: Building blocks employed: Fmoc-Arg(Pmc)-OH (9×), **9**; b) Repeated cycles of SPPS: Building blocks employed: Fmoc-Arg(Pbf)-OH (9×), Fmoc-Ahx-OH; c) Me_3P, 20% water in dioxane, then Fmoc-Lys(Mtt)-OH, HCTU, DiPEA, NMP; d) 1% TFA in CH_2Cl_2, then biotin, HCTU, DiPEA, NMP; e) 20% piperidine in NMP, then Fmoc-Lys(Biotin)-OH; f) Repeated cycles of SPPS: Building blocks employed: Fmoc-Ahx-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Leu-OH, epoxirane **10**; g) TFA/TIS/H_2O (95/2.5/2.5 v/v/v), 3 h. Overall yield of **1** and **2**: 11% and 13%, respectively.

Biological activity of the probes

Next, the labeling efficiency of the newly synthesised probes was investigated. Cell lysates and endocytic extracts[15] from different origin were incubated with **1**, **2** or a broad class cysteine protease probe developed in Chapter 5 (**11**; Figure 2). The labeling with **1** can be abolished by pre-treatment of the lysate with E-64,[16] a broad-class cysteine protease inhibitor (Figure 3, lane 1), indicating that **1** is a label for active cysteine proteases. For the other lanes, it is of interest to note that cathepsins labeled with either **1** or **2** are modified with a probe that is 1.5 kD heavier than **11**, and thus all cathepsin band are shifted 1.5 kD upwards. In U937 extracts (lane 2 and 3), probe **1** and **2** label only Cat S and Cat B, whereas **11** also hits Cat X (lane 4). In DC cell lysates, the new probes clearly target Cat B, which may appear as a double band (lane 5 and 6). Cathepsin probe **11** (lane 7) also hits Cat X and Cat S. Monocyte extract labeling with probe **1** and **2** (lane 8 and 9) shows Cat B, Cat H and Cat S. Again, **11** also labels Cat X. Overall, this experiment demonstrates that Cat B, Cat H and Cat S are targeted by **1** and **2** with an efficiency comparable to broad class cysteine protease inhibitor **11**.

Encouraged by these results, the labeling efficiency in living cells was addressed. In a pulse experiment, immature dendritic cells (DC) were incubated with control **11** or probe **1**. Immature DC are known for their high endocytic activity, whereas in mature DC endocytosis is greatly reduced.[17] The cells were washed and lysed in the presence of JPM-565[18] to block remaining cysteine protease activity. The labeling of cysteine proteases was visualised *via* strept-HRP blotting (Figure 4). As a control, immature and mature dendritic cell lysates were labeled with **11** (lane 1 and 2), and it shows that maturity of the dendritic cells does not affect cathepsin labeling. In lane 3, 4 and 5, it is seen that labeling of Cat X, Cat B and Cat S with **11** is almost completely abolished at 4°C, and that it is independent of

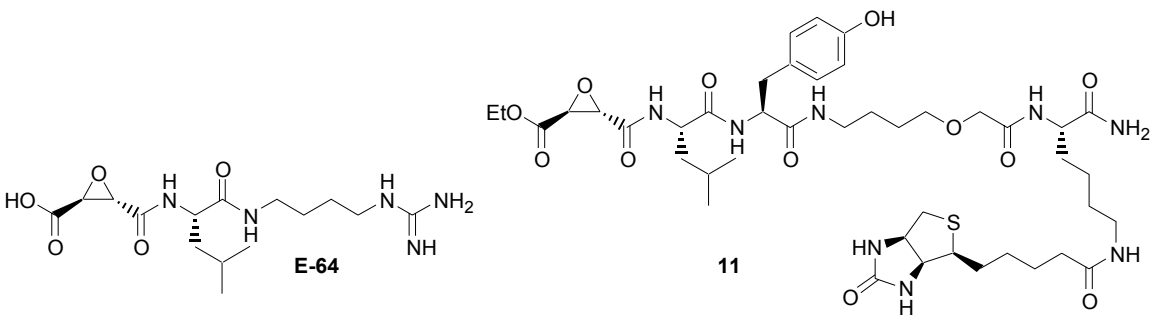


Figure 2. Broad class epoxide based cysteine protease active site alkylating agents used as reference compounds.

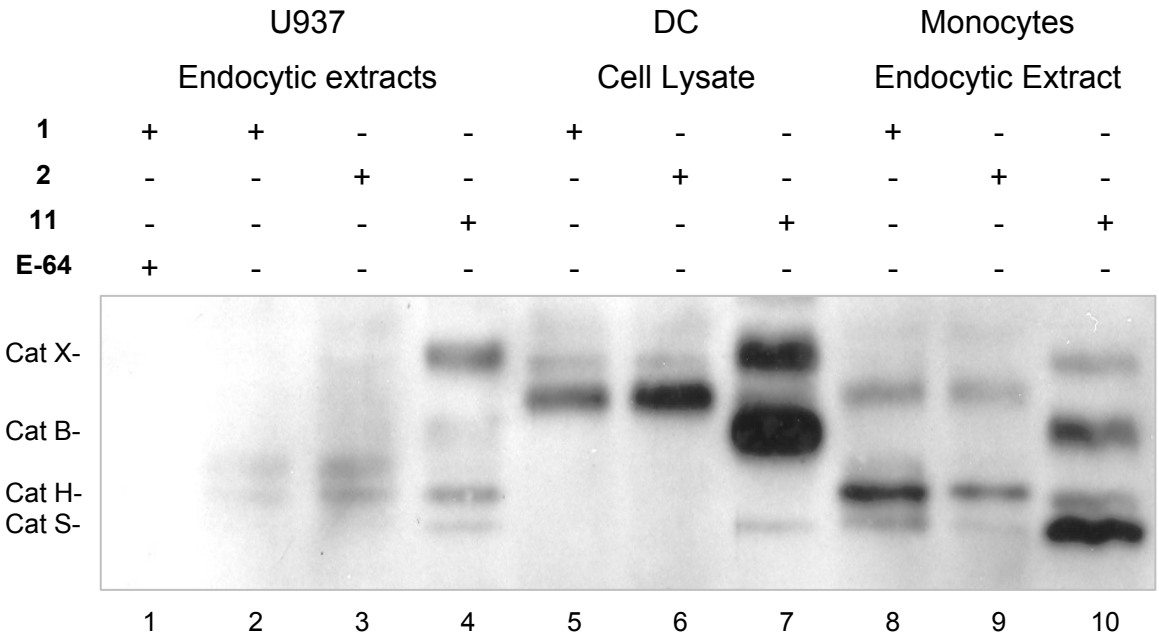


Figure 3. Labeling behaviour of **1** and **2** in cell lysates and endocytic extracts. Lysates and extracts were incubated with **1**, **2** and **11** at 10 μM and ambient temperature for 30 minutes. In lane 1, the extract was pretreated with 40 μM E-64 for 1 hour. Samples were separated by SDS-PAGE and transferred to polyvinyliden difluoride membrane. Incubation with strept-HRP conjugate allowed the visualisation of active cathepsins.

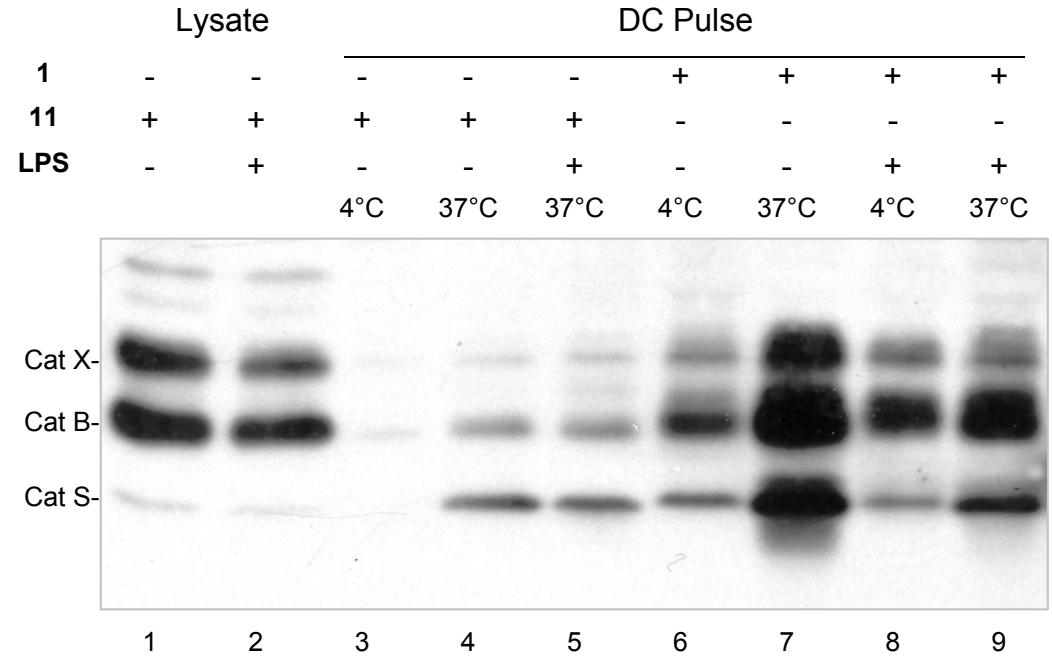


Figure 4. Labeling of dendritic cells with probe **1** or **11**. Cells were incubated for 1 hour with 10 μM of either **11** or **1**. Cells were lysed in the precence of 100 μM JPM-565 and the labeling was addressed by developing a blot.

maturity. However, in comparable experiments (data not shown), labeling generally decreased upon maturation. This indicates that labeling with **11** is at least partly independent of endocytosis. In lane 6-9, cathepsin labeling is greatly reduced at 4°C relative to 37°C, but not blocked. This indicates contributions both of energy dependent and energy independent pathways. In mature cells (lane 9), the uptake is lower than in immature dendritic cells (lane 7), which indicates that endocytosis is a large component of the uptake in immature cells at 37°C. Interestingly, in mature cells the difference in uptake between 4°C and 37°C (lane 8 and 9) is not so pronounced, which suggests that in mature cells no energy dependent pathway is employed for the uptake of the probe. This suggests that the energy-dependent component of uptake is abolished both at 4°C and by maturation, and endocytosis could be this component in both cases.

Conclusion

In this chapter, it is shown that a broad class cysteine protease probe that is hardly cell permeable can be made cell penetrating *via* the attachment of a nonaargine cell penetrating vector. The labeling of cysteine proteases with the new probes in living cells seems to be largely energy-dependent, because it is greatly reduced at 4°C.

These probes may play a role in the elucidation of the pathway that is employed to take up nonaarginine vectors. Further experiments addressing the role of individual steps of the uptake pathway could help to gain insight in details of the uptake. Furthermore, it will be interesting to investigate the uptake efficiency and the targeting of cysteine proteases in other cell types or tissues.

Experimental section

General methods and materials: Solvents used in the solid phase peptide synthesis, DiPEA and TFA were all of peptide synthesis grade (Biosolve) and used as received. The protected amino acids, Rink amide MBHA resin (0.78 mmol/g) 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.) or on a 443A Peptide Synthesiser (Applied Biosystems). 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). High resolution mass spectra were recorded on a Finnigan LTQ-FT (Thermo electron). An analytical Alltima C_{18} column (Alltech, 4.6 × 250 mm, 5 μm 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. U937 cells were cultured on complete medium (RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal calf serum, and antibiotics).

Solid phase peptide synthesis: 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 appropriate acid (5 eq.), HCTU (5 eq.) and DiPEA (6 eq.) in NMP for 1 h. 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×).

Synthesis of 1: Fmoc Rink amide resin (78 mg, 50 μmol) was elongated using automated standard Fmoc-based SPPS to give resin-bound $(Arg(Pbf))_9$. The synthesis was continued by manual Fmoc-based SPPS to give protected and resin-bound **8**. The resin was washed extensively (alternating CH_2Cl_2-MeOH 3×, alternating CH_2Cl_2-Et_2O 3×. An aliquot of resin (100 mg, 16 μmol) was transferred into a clean vial, washed with CH_2Cl_2 and treated with TFA/H_2O/TIS (0.7 ml, 95/2.5/2.5 v/v/v) for 2 h. The mixture was filtered into cold Et_2O and the white precipitate was collected by centrifugation and decantation. The precipitate was washed (Et_2O), followed by RP-HPLC purification of the crude product (linear gradient in B: 20-30% B in 3 column volumes) to yield 4.3 mg (1.8 μmol, 11%) of product. ^{1}H NMR (D_2O, 295 K, DMX 600): δ, 7.08 (d, 2H, J = 7.9 Hz), 6.80 (d, 2H, J = 8.2 Hz), 4.58-4.55 (m, 1H), 4.45-4.41 (m, 1H), 4.38-4.35 (m, 1H), 4.34-4.18 (m, 12H), 4.13-4.09 (m, 1H), 3.68 (br s, 1H), 3.48-3.45 (m, 1H), 3.30-3.25 (m, 1H), 3.24-3.09 (m, 24H), 3.03-2.86 (m, 4H), 2.79 (s, 1H), 2.69 (s, 1H), 2.28-2.18 (m, 6H), 1.80-1.20 (m, 61H), 0.89-0.80 (m, 6H). ESI-MS: $C_{105}H_{189}N_{45}O_{24}S$ + 4 H^+ requires 625.5, found 625.4; $C_{105}H_{189}N_{45}O_{24}S$ + 3 H^+ requires 833.7, found 833.6.

Synthesis of 2: Fmoc Rink amide resin (78 mg, 50 μmol) was elongated using automated standard Fmoc-based SPPS to give resin-bound **3**. The azide moiety was reduced to an amine on solid phase by treatment of the resin, after washing (dioxane 5×), with Me_3P (0.3 ml 1M in toluene, 0.3 mmol, 6 eq.) in dioxane/H_2O (4/1 v/v, 1 ml) for 40 min, followed by extensive washing of the resin (dioxane 3×, alternating CH_2Cl_2-MeOH 3×, and NMP 3×). The peptide chain was elongated manually with Fmoc-Lys(Mtt)-OH and the lysine side chain Mtt protecting groups 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 neutralized with 10% DiPEA in NMP. The lysine side chain amine was acylated with biotin to give resin-bound **5**. The synthesis was continued by manual Fmoc-based SPPS to give protected and resin-bound **7**.

The resin was washed extensively (alternating CH_2Cl_2-MeOH 3×, alternating CH_2Cl_2-Et_2O 3×. An aliquot of resin (55 mg, 10 µmol) was transferred into a clean vial, washed with CH_2Cl_2 and treated with TFA/H_2O/TIS (0.7 ml, 95/2.5/2.5 v/v/v) for 3 h. The mixture was filtered into cold Et_2O and the white precipitate was collected by centrifugation and decantation. The precipitate was washed (Et_2O), followed by RP-HPLC purification of the crude product (linear gradient in B: 20-30% B in 3 column volumes) to yield 3.3 mg (1.3 µmol, 13%) of product. ^{1}H NMR (D_2O, 295 K, DMX 600) δ, 7.09 (d, 2H, *J* = 8.3 Hz), 6.80 (d, 2H, *J* = 8.2 Hz), 4.58-4.55 (m, 1H), 4.47-4.43 (m, 1H), 4.38-4.23 (m, 13H), 4.20-4.16 (m, 1H), 4.11 (s, 2H), 3.76-3.61 (m 20H), 3.59-3.55 (m, 2H), 3.54-3.50 (m, 1H), 3.40-3.36 (m, 1H), 3.35-3.31 (m, 2H), 3.28-3.24 (m, 1H), 3.22-3.12 (m, 24H), 2.99-2.90 (m, 5H), 2.69 (s, 2H), 2.24-2.20 (m, 4H), 1.82-1.20 (m, 60H), 0.88-0.80 (m, 6H). ESI-MS: $C_{103}H_{185}N_{45}O_{21}S$ + 4 H^+ requires 606.5, found 606.5; $C_{103}H_{185}N_{45}O_{21}S$ + 3 H^+ requires 808.3, found 808.3.

DC: Isolation and cultures: PBMC (peripheral blood mononuclear cells) were isolated by Ficoll/Paque (Life Technologies, Inc., Grand Island, NY) density gradient centrifugation of heparinized blood obtained from buffy coat of healthy volunteers from the blood bank of the University of Tübingen. Isolated PBMC were plated (1×10^8 cells/8 ml per tissue culture flask) into tissue culture flasks (cellstar 75 cm^2, Greiner Bio-One GmbH, Frickenhausen, Germany) in complete medium. After 1.5 h of incubation at 37°C, nonadherent cells were removed, and the adherent cells (12–19% of the incubated cells) were cultured in complete medium supplemented with GM-CSF and IL-4 for 6 days.[19, 20] For maturation, medium was supplemented with LPS (Sigma) at day 6 for additional 24 h.

Isolation of monocytes: Human PBMC were freshly isolated from buffy coats of donor blood by density gradient centrifugation (Ficoll), followed by several washing steps with PBS and a Percoll gradient (Percoll™, Amersham Biosciences, Uppsala, Sweden) consisting of 12 ml 2× PBS and 14 ml Percoll. After centrifugation four distinct layers become discernable, where (from top to bottom) the first contains debris and few thrombocytes, the second enriched monocytes with little lymphocytes and the remaining thrombocytes, the next layer is enriched for lymphocytes with little thrombocytes while the bottom phase contains remaining granulocytes and red blood cells. The second phase (containing the bulk of monocytes) was carefully collected. The majority of the remaining contaminating cells were T cells (15-20%) with small amounts of B cells present (<10%). Cells were kept on ice for all successive procedures to prevent non-specific stimulation. Generation of postnuclear supernatants immediately followed monocyte enrichment.

Active site labeling of cysteine proteases and detection by streptavidin-blotting: Endocytic extracts of at least 1×10^8 U937 cells or at least 5×10^7 monocytes were prepared following the method described.[15] DC lysates were prepared as follows: 5×10^5 immature DC were washed with

PBS, centrifuged and resolved with 100 µl 2× lysis buffer (100 mM citrate/phosphate, 2 mM EDTA, 1% NP40, pH 5) and kept to lysis for 30 min at 4°C. After pelleting membrane fragments by centrifugation for 10 min at 13.000 rpm in an eppendorf-centrifuge, the supernatant contained crude cell lysate. 3 µg total endocytic protein of U937, 1.5 µg endocytic protein of primary monocytes or 3 µg DC cell lysate protein was incubated with 10 µM of **1**, **2** or **11** at ambient temperature for 30 min. Reactions were terminated by addition of SDS reducing sample buffer and immediate boiling. Samples were resolved by 12.5% SDS-PAGE gel, and then blotted on a PVDF-membrane and visualised using strept-HRP and the ECL-detection kit. In lane 1, E-64 was competed against **1**. The same amount of total endocytic protein was incubated with 40 µM of E-64 for one hour at ambient temperature, and **1** was added to a concentration of 10 µM. After 30 min, the reaction was terminated and samples were treated as described above.

Uptake and labeling in living cells *via* a pulse experiment: At least 1×10^6 DC per time point were pulsed in complete culture medium (300 µl per time point) for 1 hour with 10 µM **1** or **11** at 37°C. Additionally, at least 1×10^6 DC were incubated for the same time with 10 µM of either **1** or **11** at 4°C. After the pulse cells were washed to remove excess **1** or **11** at 4°C for four times by centrifugation, removing of supernatant and resolving the pellet in PBS. Cells were lysed with 50 µl of 2× lysis buffer (100 mM citrate/phosphate, 2 mM EDTA, 1% NP40, pH 7), supplemented with 100 µM free JPM-565. SDS reducing sample buffer was added to 50 µg total protein of each time point and immediately boiled.

References and notes

1 The work presented in this chapter was performed in close collaboration with M. Reich, C. Driessen and R. Brock at the University of Tübingen.

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