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

Swieten, Paul Franciscus van

Chapter 4

Development of an isotope-coded activity based probe for the quantitative profiling of cysteine proteases[1]

Introduction

Proteomics research aims at the study of the expression levels and functioning of (subsets of) the proteins present in a biological sample. Proteomics research presents a number of challenges to the researchers. The protein content in a cell is dynamic and cannot be amplified easily. Further, there is a difference of several orders of magnitude between the most and the least abundant proteins. Traditionally, 2D-gel electrophoresis has been used to separate mixtures of proteins, allowing identification of the proteins, originally using Edman degradation. Nowadays, protein sequencing is normally performed using mass spectrometry techniques, as introduced by Watanabe and coworkers in their groundbreaking report.[2] This mass spectrometry based approach also opened the way to quantitative analyses on protein fragments.

The use of isotope labeled amino acids for relative quantification of protein was introduced by the group of Chait.[3] Isotopic labeling was accomplished by growing cells on ^{15}N enriched growth media. Two cell pools, one isotope labeled and one unlabeled, are mixed, lysed and digested with trypsin. The two isotope forms of each peptide have very similar chromatographic properties. In practical applications, they coelute and therefore can serve as mutual internal standards in the mass spectrometer. The relative intensity of both peptides reflects the relative abundance of the protein in the two cell pools. The amino acid sequence of the peptides, and thereby the identity of the proteins they originated from, was established by tandem ms.

Aebersold and coworkers used an isotope coded affinity tag (ICAT) to introduce an affinity tag, biotin, and an isotope label.[4] The ICAT strategy is based on the presence of eight D (heavy) or H (light) atoms in a biotin-containing cysteine-reactive probe. The light and heavy probe share almost all physical and chemical properties, and only in a mass spectrometer the mass difference is revealed. After labeling a denatured protein sample from two different sources with either the light or heavy ICAT probe, the combined fragments are digested using trypsin. The biotinylated fragments are enriched for, and both the relative quantity and sequence identity of the proteins from which the biotinylated peptides originated are determined by automated multistage mass spectrometry.[5] By selecting only cysteine containing tryptic peptides the sample complexity is reduced while protein quantification and identification are still achieved. ICAT experiments have been used by others to study the influence of perturbations on protein expression levels, to gain new insights in specific cellular processes, and to study protein complexes and post-translational modifications.[6]

Quantitative activity based profiling

In chemical proteomics approaches, a complex biological mixture of proteins is simplified before analysis by labeling a specific set of related proteins with an affinity- or fluorescence tag.[7] For instance, broad-spectrum, irreversible protease inhibitors have been used in the profiling of serine proteases,[8] cysteine proteases,[9] and the catalytically active subunits of the proteasome.[10, 11] A relevant example of a chemical proteomics probe is represented by **1a** (DCG-04, Figure 1), developed by Bogyo and coworkers as an irreversible cysteine protease inhibitor, and applied by Lennon-Duménil *et al.* to monitor the proteolytic activity of maturing phagosomes in live antigen-presenting cells.[12,13]

Compound **1a** consists of three functionalities: 1) an electrophilic epoxysuccinate, that alkylates the active site cysteine residue, 2) a short peptide sequence that allows recognition of the probe by the cathepsin family of cysteine proteases and 3) biotin, for the detection and isolation of the modified enzymes (Figure 1). The biotin is connected to the peptide epoxysuccinate by an aminohexanoic acid residue. It was reasoned that incorporation of an isotopic encoded entity, in analogy to the ICAT reagent,[4] would allow for both quantitative and functional assessment of the cathepsin family of cysteine proteases from complex biological samples.

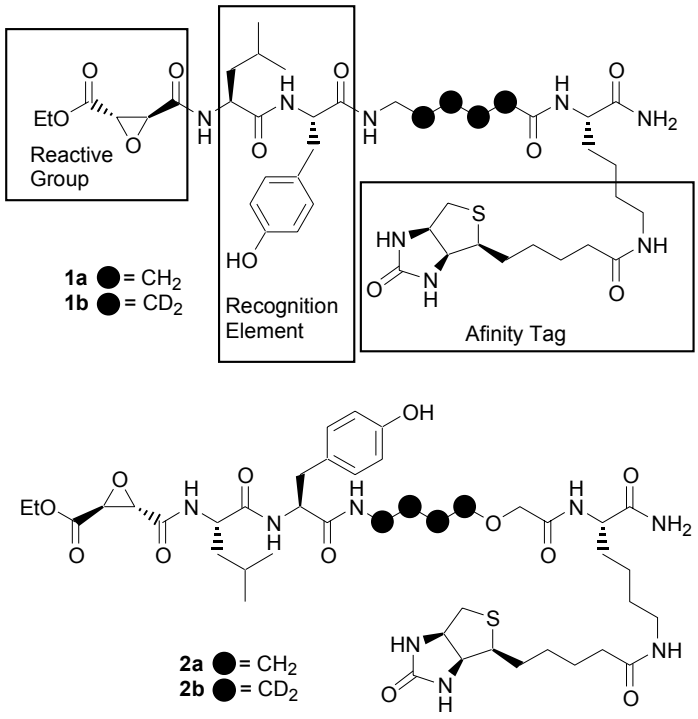
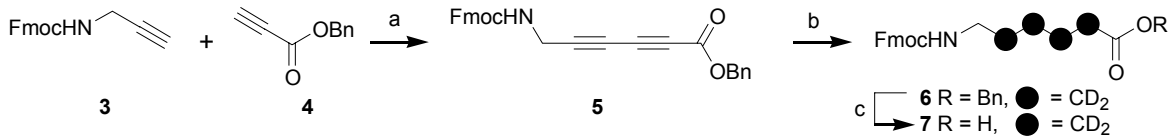


Figure 1. Target Structures

Synthesis

Here, the syntheses of a new azide-protected amino acid spacer, 7-azido-3-oxaheptanoic acid (N_3-Aoh-OH, **14a**) and two d8-enriched amino acids, 2,2,3,3,4,4,5,5 octadeutero-6-(Fmoc-amino)hexanoic acid (Fmoc-Ahx-OH-D8, **7**) and 4,4,5,5,6,6,7,7-octadeutero-7-azido-3-oxaheptanoic acid (N_3-Aoh-OH-D8, **14b**) are described. Their use in the synthesis of two sets of DCG-04-based isotopic encoded activity based probes, namely compound **1b** (in combination with DCG-04 **1a**) and compounds **2a** and **2b** is demonstrated. It is also shown here that neither incorporation of an isotopic label (compounds **1b** and **2b**, Figure 1) nor substitution of the aminohexanoic acid moiety by an 7-amino-3-oxa-heptanoic acid residue (compounds **2ab**) affects the broad spectrum affinity for cysteine proteases of the cathepsin family.



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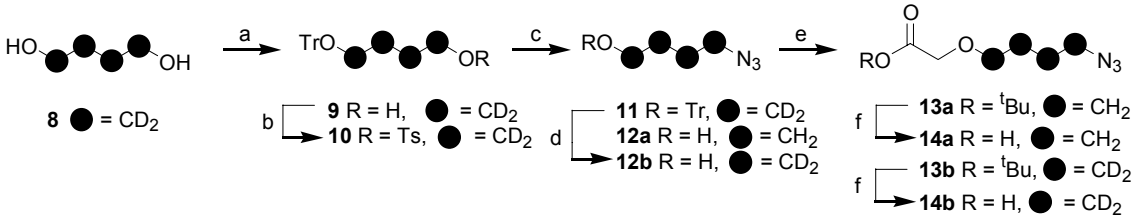
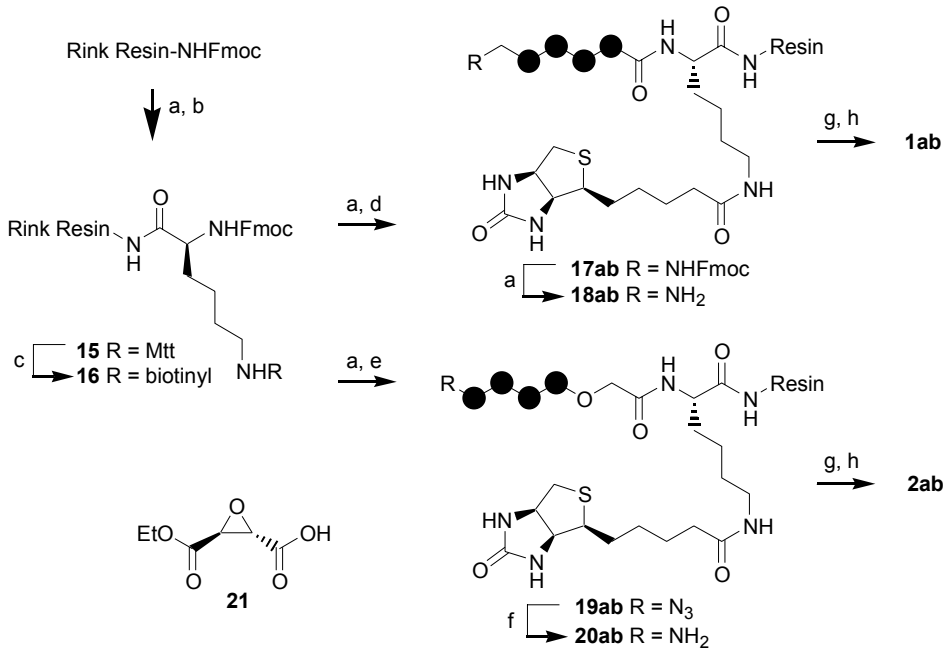
Scheme 1. Synthesis of deuterated Fmoc-Ahx-OH **7**. Reagents and conditions: a) CuCl, *N,N,N′,N′*-tetramethylene ethylenediamine (TMEDA), O_2, THF, 45°C, 36% yield; b) D_2, EtOAc, Pd/C, 84% yield; c) DCl in D_2O, dioxane, 4 h, 90°C, 85% yield.

The synthesis of Fmoc-Ahx-OH-D8 (**7**; Scheme 1) starts with a copper(I) catalyzed oxidative Glaser coupling[14] of Fmoc-protected propargylamine[15] (**3**) and benzyl propiolate[16] (**4**) to furnish diyne **5**. Upon reduction of diyne **5** with deuterium gas and palladium on carbon, the benzyl ester remained intact, yielding deuterated **6**. Contrary to the results of reduction of several analogs of **5**,[17] no isotopic scrambling had taken place as judged from the isotope distribution in the products. Finally, the benzyl ester was hydrolyzed[18] using DCl in deuterium oxide and dioxane to give isotopically labeled Fmoc-Ahx-OH-D8 (**7**).



Scheme 2. Synthesis of azidooxaheptanioc acids **14a** and **14b**. Reagents and conditions: a) TrCl, Et_3N, DMAP, 23% yield, 68% recovered **8**; b) TsCl, Et_3N, 95% yield; c) NaN_3, DMF, 50°C, quantitative; d) TFA, TES, CH_2Cl_2, 83% yield; e) tert-butyl bromoacetate, Bu_4NBr, NaOH, H_2O, toluene, 61% (**13a** and **13b**) yield; f) TFA/CH_2Cl_2 (1/1 v/v), 81% (**14a**) and 87% (**14b**) yield.

The syntheses of N_3-Aoh-OH-D0 (**14a**) and N_3-Aoh-OH-D8 (**14b**) were accomplished as follows (Scheme 2). Upon tritylation of deuterated **8**, monotrityl ether **9** was obtained in 23% yield, while the unreacted deuterated butanediol was recovered easily (68%). Tosylation of the primary alcohol followed by the replacement of the tosylate by azide gave protected azidoalcohol **11**. Detritylation (TFA/TES in CH_2Cl_2) afforded azidoalcohol **12b**. Both known 4-azido-1-butanol[19] **12a** and deuterated **12b** were alkylated under phase transfer conditions with *tert*-butyl bromoacetate to furnish the corresponding *tert*-butyl ester **13a** and **13b**. Subsequent acidolysis of the *tert*-butyl esters employing TFA/CH_2Cl_2 (1/1 v/v) yielded azido acid **14a** in 49% over two steps and **14b** in an overall yield of 42% over the last five steps.



Scheme 3. Synthesis of activity based probes **1ab** and **2ab**. Reagents and conditions: a) 20% piperidine in NMP; b) Fmoc-Lys(Mtt)-OH, HCTU, DiPEA, NMP; c) 1% TFA in CH_2Cl_2, then biotin, HCTU, DiPEA, NMP; d) Fmoc-Ahx-OH or **7**, HCTU, DiPEA, NMP; e) **14a** or **14b**, HCTU, DiPEA, NMP; f) Me_3P, 20% H_2O in dioxane; g) 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-Tyr(tBu)-OH, Fmoc-Leu-OH, **21**; h) 5% H_2O in TFA.

The incorporation of spacers **7** and **14ab** into the respective cysteine protease inhibitors **1b** and **2ab** is shown in Scheme 3. Immobilization of biocytin on a Rink linker was accomplished as described in chapter 3. Standard solid phase peptide synthesis (SPPS) with the sequential addition of a spacer (**7**, **14a** and **14b,** respectively), Fmoc-Tyr(tBu)-OH, Fmoc-Leu-OH and ethyl (2S,3S)oxirane-2,3-dicarboxylate **21**,[20] acidic cleavage from the resin and purification by repeated precipitation afforded target compounds **1b**, and **2a** and **2b** in 34%, 29% and 40%, respectively in 80-90% purity as judged by LC/MS. A small portion of each product was purified to homogeneity by RP-HPLC.

Inhibitory results

To establish the inhibition profile of the newly synthesized probes, a set of labeling experiments with cell lysates of the mouse macrophage cell line J774 was performed. Cell lysates were incubated with DCG-04 (**1a**) as a control and with the new probes **1b**, **2a** and **2b** for 60 minutes at 37°C. The resulting mixtures were separated by SDS-PAGE. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane,

followed by chemiluminescence induced by horseradish peroxidase–streptavidin conjugate (Figure 2). Probes **2ab** label the cysteine proteases Cat B, L, S, and Z in a cell lysate with the same efficiency as DCG-04, which has been shown previously to effectively target these proteolytic enzymes.[12] This suggests that both sets of isotopic coded activity based probes **1ab** and **2ab** are viable quantitative functional proteomics tools for the cathepsin family of cysteine proteases.

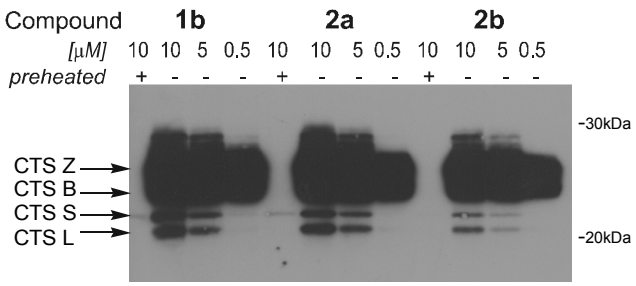


Figure 2. Derivatization of the DCG-04 molecule does not alter targeting of active protease species in J774 cells. Crude extracts prepared from J774 cells were labeled for one hour at 37°C with different concentrations of compounds **1b**, **2a** and **2b**. As a control, extracts were pre-heated for 5 minutes at 100°C prior to labeling (lanes 1, 5 and 9). Proteins were then separated by SDS-PAGE on 12.5% gels and labeled polypeptides visualized by streptavidin blotting. Polypeptide species corresponding to Cat Z, B, S and L are indicated based on previous studies.[10]

Attempts to synthesize labeled Cat B active-site fragment

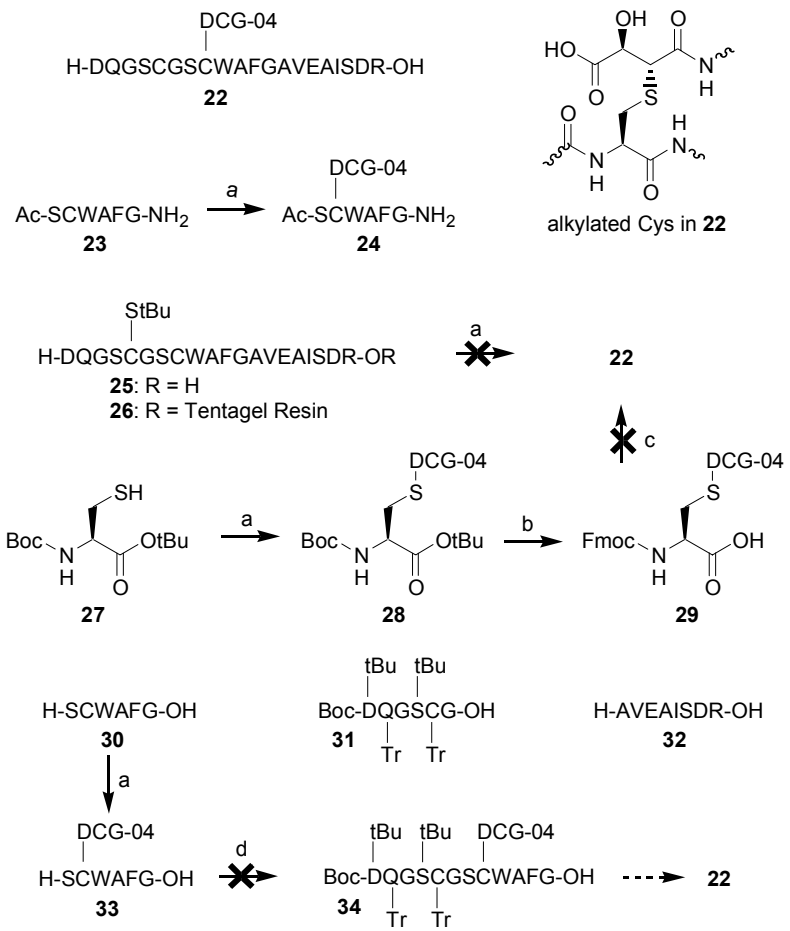
Next an experiment was set up to identify the labeled active site of a cysteine protease. Recombinant Cat B was labeled with epoxide **1a** and treated with trypsin. The labeled catalytic cysteine residue, Cys^{105}, is expected to be in the DQGSCGSC^{105}WAGGAVEAISDR sequence, originating from proteolytic cleavage after Arg^{100} and Arg^{120}. The tryptic digest was analyzed by mass spectrometry, but the labeled active-site fragment could not be found. In order to gain as much information on the expected labeled tryptic fragment, it was decided to embark on the synthesis of this fragment **22** (Scheme 4).

The key step in the synthesis of **22** would be the alkylation of Cys^{105} with epoxide **1a**. To investigate the feasibility of such an alkylation, a series of base catalyzed alkylations was performed on pentapeptide **23**. The use of solvents such as ethanol, DMF, THF and mixtures thereof with DMSO, as well as the use of inorganic bases such as KOH or Na_2CO_3 effected hydrolysis of the ethyl ester present in **1a**. The reaction proved to proceed with the best result using DMSO as solvent and DiPEA as base to afford **24** in moderate efficiency as judged by LC/MS analysis. As only a small amount of **22** was needed for mass spectrometry experiments, it was decided to use these conditions to react peptide **25**, in

which Cys[102] was protected as an tert butyl disulfide, with probe **1a**. However, no product formation could be detected by LC/MS analysis. Subjecting the resin-bound and protected peptide **26**, with only Cys[105] liberated, to the same conditions did not afford target compound **22** either.

As alkylation of Cys[105] within the context of the tryptic fragment proved to be unsuccessful, an attempt was made to incorporate S-alkylated cysteine into the growing peptide chain on the solid support. Reaction of protected cysteine **27** with epoxide **1a** afforded **28**. Removal of the acid labile protecting groups and installation of an Fmoc protecting group yielded **29**, which was purified by recrystallization. This building block proved unsuitable for coupling to the growing tryptic digest peptide chain. Where coupling of a trityl protected cysteine building block to the N-terminal Trp on the resin was difficult and needed double couplings, **29** could not be coupled to the Trp residue at all.

A third route that might lead to **22** was found in a double block coupling strategy. As alkylation of **22** went in moderate yield, it was expected that reaction of **30** with **1a** would afford some **33**, which, after HPLC purification, could be reacted with the succinimidyl ester of protected N-terminal fragment **31**. Activation of **31** *via* the same method and coupling to unprotected C-terminal



Scheme 4. Synthetic routes toward alkylated tryptic digest fragment of Cat B. Reagents and conditions: a) **1a**, DMSO, DiPEA; b) TFA/H_2O (95/5 v/v), then Fmoc-OSu, DiPEA, DMSO; c) Fmoc based SPPS; d) **31**, HOSu, DIC, DMF, then **33**.

fragment **32** would eventually lead to target **22**. However, alkylation of **30** gave product **33** in a marginal 2% yield after HPLC purification. Transformation of **31** to the corresponding succinimidyl ester went uneventful, but subsequent reaction with **33** did not yield any product.

Conclusions

In summary, the efficient synthesis of two pairs of isotopic coded spacers is presented and it is shown that their incorporation into a known cysteine protease inhibitor does not alter the inhibitory profile of the label. This opens the way to quantitative functional proteomics studies on a functional subset of the proteome, namely the cathepsin family of cysteine proteases. One hurdle that remains to be taken is the identification of the labeled tryptic digest fragment of the Cat B active site in a mass spectrometric experiment. Synthesis of this fragment has not been succesfull, but the shorter peptide fragment that was S-alkylated with the cysteine protease probe could be of use as well. Importantly, this concept may be extended towards other isotopic coded spacers (^{13}C, ^{15}N) and activity based probes targeting other proteins.[7]

Experimental section

General methods and materials: All solvents used in synthesis were of p.a. grade and stored over molecular sieves. Pyridine, CH_2Cl_2, toluene, triethylamine, DiPEA, DMF, NMP, DMSO and TFA were bought at Biosolve. Tritylchloride, tosylchloride, 4-(dimethylamino)pyridine, deuterium chloride (20 wt% solution in D_2O) and triethylsilane were purchased from Acros. 1,1,2,2,3,3,4,4-Octadeutero-1,4-butanediol, deuterium (99.8% D) and *tert*-butyl bromoacetate were purchased from Aldrich. Sodium azide was bought at Merck. Fmoc-Leu-OH, Fmoc-Tyr(tBu)-OH and Fmoc-Lys(Mtt)-OH were bought at Senn Chemicals.

TLC analysis was performed on Merck 25DC Plastikfolien Kieselgel 60 F_{254}, with detection by UV absorption (254 nm) and spraying with one of the following solutions: *a* 20% H_2SO_4 in EtOH followed by charring, *b* ammonium molybdate (25 g/l) in 10% H_2SO_4 followed by charring, *c* $KMnO_4$ (10 g/l in 2% aq. Na_2CO_3). Fluka silica gel (230-400 mesh) was used for column chromatography. ^1H-NMR and ^{13}C-NMR spectra were recorded with a Bruker AC200 instrument at 200 and 50.1 MHz, respectively. Chemical shifts (δ) are given in ppm, relative to tetramethylsilane as an internal standard. Mass spectra were recorded with a Perkin Elmer Sciex API 165 mass instrument equipped with a custom-made Electrospray Interface (ESI). 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, 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. High resolution mass spectra were recorded on a Finnigan LTQ-FT (Thermo electron).

(N-Fluorenylmethyloxycarbonyl)-propargylamine (3): The title compound was prepared according to the literature procedure.[15] ^1H and ^{13}C NMR analyses were in agreement with the reported values.

Benzyl propiolate (4): The title compound was prepared according to the literature procedure.[16] ^1H and ^{13}C NMR analyses were in agreement with the reported values.

6-(Fluorenylmethyloxycarbonylamino)-hexa-2,4-diynoic acid benzyl ester (5): In a 100 ml three necked reaction flask were placed, Cu(I)Cl (180 mg, 1.79 mmol), TMEDA (0.260 ml, 1.79 mmol), THF (20 ml) and *N*-Fmoc propargyl amine (1.03 g, 3.72 mmol). Oxygen was bubbled through the suspension and at 30°C a solution of benzyl propiolate (2.58 g, 16.1 mmol) in THF (20 ml) was added dropwise over a 1.5 h period. The reaction was stirred for another 2 h at 40°C while bubbling oxygen. The resulting black mixture was diluted with EtOAc (100 ml) and washed subsequently with 0.1 M HCl (60 ml) and brine (60 ml). After drying ($MgSO_4$), evaporation of the solvent and column chromatography of the black residue (hexanes:EtOAc 95:5 → 9:1 → 3:1), the title compound was isolated as an orange/red solid (590 mg, 36 %). 0.60 g *N*-Fmoc-propargyl amine was recovered. ^1H-NMR ($CDCl_3$); δ 4.12 (d, 2H, *J*=5.1 Hz, CH_2N); 4.21 (t, 1H, *J*=6.6 Hz, CH); 4.45 (d, 2H, *J*=6.6 Hz, CH_2O); 4.98 (broad s, 1H, NH); 5.22 (s, 2H, CH_2O); 7.25-7.44 (m, 9H, arom); 7.56 (d, 2H, *J*=7.3 Hz, arom); 7.76 (d, 2H, *J*=7.3 Hz, arom). ^{13}C-NMR ($CDCl_3$); δ 31.2, 46.8, 65.8, 66.9, 67.3, 67.9, 68.1, 70.7, 119.8, 124.8, 126.9, 127.6, 128.3, 128.5, 134.2, 141.0, 143.4, 152.2, 155.7.

6-(Fluorenylmethyloxycarbonylamino)–2,2,3,3,4,4,5,5-octadeuterohexanoic acid benzyl ester (6): Benzyl ester **5** (307 mg, 0.705 mmol) was dissolved in a mixture of dry 1,4-dioxane (50 ml) and D_2O (2 ml). The solvents were evaporated *in vacuo* and the procedure was repeated once. Then the residue was dissolved in EtOAc (12 ml) and Pd/C-10% (14 mg) was added. The mixture was put in a Parr apparatus and shaken under D_2 (2.5 bar) for 4 h. The solution was filtered, concentrated and purified by column chromatography (hexanes:EtOAc 9:1 → 3:1), to afford the title compound as a pale yellow solid (267 mg, 84 %). ^1H-NMR ($CDCl_3$); δ 3.15 (d, 2H, *J*=5.8 Hz, CH_2N); 4.21 (t, 1H, *J*=6.6 Hz, CH); 4.40 (d, 2H, *J*=6.6 Hz, CH_2O); 4.74 (m, 1H, NH); 5.11 (s, 2H, CH_2O); 7.25-7.39 (m, 9H, arom); 7.58 (d, 2H, *J*=7.3 Hz, arom); 7.76 (d, 2H, *J*=7.3 Hz, arom). ^{13}C-NMR ($CDCl_3$); δ 20.1-21.7 (m), 22.0-23.1 (m), 25.3-26.4 (m), 30.0-31.4 (m), 38.1, 44.8, 63.6, 63.9,

117.4, 122.5, 124.5, 125.1, 125.7, 126.0, 133.5, 138.8, 141.5, 154.0, 170.9. ESI-MS *m/z* 452.1 $[M+H]^+$, 474.2 $[M+Na]^+$, 903.5 $[2M+H]^+$, 925.6 $[2M+Na]^+$.

6-(Fluorenylmethyloxycarbonylamino)–2,2,3,3,4,4,5,5-octadeuterohexanoic acid (7): Under argon benzyl ester **6** (205 mg, 0.455 mmol) was dissolved in dry 1,4-dioxane (8 ml) and DCl/D_2O (0.30 ml, 1/4 w/w)) was added. The reaction mixture was stirred at 90°C and after 4 hours TLC showed complete conversion of the benzyl ester. The mixture was cooled and the solvents were evaporated *in vacuo*. Purification by column chromatography (hexanes:EtOAc 9:1 → 3:1 → 1:1 +0.2% HOAc), afforded the target compound as a white solid (140 mg, 85 %). ^{1}H-NMR (CD_3OD); δ 2.99 (s, 2H, CH_2N); 4.10 (t, 1H, *J*=6.7 Hz, CH); 4.26 (d, 2H, *J*=6.7 Hz, CH_2O); 7.17-7.33 (m, 4H, arom); 7.55 (d, 2H, *J*=7.3 Hz, arom); 7.70 (d, 2H, *J*=7.3 Hz, arom). ^{13}C-NMR (CD_3OD); δ 23.7-24.9 (m), 25.3-26.7 (m), 28.7-30.1 (m), 33.2-34.7 (m), 41.3, 48.3, 67.4, 120.8, 126.0, 128.0, 128.6, 142.4, 145.2, 158.7, 177.6. ESI-MS *m/z* 362.2 $[M+H]^+$, 723.6 $[2M+H]^+$. HRMS $[M+H]^+$ calculated: 362.220, found: 362.220.

1,1,2,2,3,3,4,4-Octadeutero-4-trityloxybutan-1-ol (9): A mixture of 1,1,2,2,3,3,4,4-octadeutero-1,4-butanediol (2.0 g, 20.4 mmol), trityl chloride (1.9 g, 6.8 mmol), triethylamine (0.95 ml, 6.8 mmol) and about a spatula tip of 4-(dimethylamino)pyridine was stirred until TLC-analysis indicated consumption of tritylchloride. EtOAc (10 ml) was added and the mixture was filtered and concentrated in vacuo. Purification over silica gel (hexanes:EtOAc 6:1→1:1, then EtOAc:MeOH 9:1) yielded 1.57 g (4.6 mmol, 68% yield to TrCl, 23% conversion of diol) of the title compound. ^{1}H NMR ($CDCl_3$): δ, 7.35-7.15 (m, 15H, H_{arom}), 1.8 (br. s. 1H OH); ^{13}C NMR ($CDCl_3$): δ, 143.9 ($C_{q,arom}$) 128.1, 127.2, 126.3 (C_{arom}), 86.0 (C_q Tr), 63-59 (m CD_2O 2x) 29-27 (m, CD_2), 26-24 (m, CD_2). HRMS $[M+Na]^+$ calculated: 363.217, found: 363.218. 1.36 g (13.8 mmol, 68%) of the deuterated diol was recovered. Overall, 91% of the deuterated starting material was converted or recovered.

1,1,2,2,3,3,4,4-Octadeutero-4-trityloxybutanol tosylate (10): To a solution of **9** (1.57 g, 4.6 mmol) in CH_2Cl_2 were added triethylamine (1.28 ml, 9.2 mmol, 2 eq.), a catalytic amount of 4-(dimethylamino)pyridine and tosyl chloride (1.31 g, 6.9 mmol, 1.5 eq.) and the mixture was stirred for 3 h, after which TLC-analysis indicated a completed reaction. Excess tosyl chloride was quenched by addition of MeOH (5 ml) and the mixture was concentrated *in vacuo*. The residue was taken up in EtOAc, filtered and the filtrate was concentrated *in vacuo*. The crude product was purified over silica gel (hexanes:EtOAc 9:1) to yield 2.16 g (4.4 mmol, 95%) of a white solid. ^{1}H NMR ($CDCl_3$): δ, 7.74 (d, 2H, *J*=9.7 Hz, H_{arom}), 7.4-7.1 (m, 17H, H_{arom}), 2.42 (s, 3H, Me); ^{13}C NMR ($CDCl_3$): δ, 144.4, 143.9, 132.9 ($C_{q,arom}$), 129.6, 128.3, 127.8, 127.5, 126.7 (C_{arom}), 86.1 (C_q Tr), 21.3 (Me). HRMS $[M+Na]^+$ calculated: 517.229, found: 517.227.

1,1,2,2,3,3,4,4-Octadeutero-4-trityloxybutane azide (11): To a solution of **10** (2.2 g, 4.4 mmol) in DMF was added sodium azide (426 mg, 6.55 mmol, 1.5 eq.) and the mixture was stirred at 50°C until TLC analysis indicated consumption of tosylate **2**. The reaction mixture was concentrated *in vacuo*, taken up in Et_2O and washed (H_2O, 3×). The combined water layers were extracted with Et_2O. The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo* to yield 1.68 g (quant.) of a colourless oil. ^{1}H NMR ($CDCl_3$): δ, 7.5-7.1 (m, 15H, H_{arom}); ^{13}C NMR ($CDCl_3$): δ, 143.8 ($C_{q,arom}$) 128.0, 127.2, 126.4 (C_{arom}), 85.9 (C_q Tr), 62-60 (m, CD_2O), 51-49 (m, CD_2N_3), 26-24 (m, CD_2 2×).

1,1,2,2,3,3,4,4-Octadeutero-4-azidobutan-1-ol (12b): To a stirred solution of **11** (1.6 g, 4.37 mmol) and triethylsilane (1 ml, 6.5 mmol, 1.5 eq.) in CH_2Cl_2 (2.5 ml) was added TFA dropwise, until no more yellow colour of trityl cations was observed, and the mixture was stirred for 15 min. The mixture was concentrated *in vacuo*, and purified over silica gel (hexanes:EtOAc 9:1, 4cv, then hexanes:EtOAc 1:1, 4cv) to yield 445 mg (3.6 mmol, 83%) of a yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ, 5.11 (br s, 1H, OH); ^{13}C NMR ($CDCl_3$): δ, 61-59 (m, CD_2OH), 51-49 (m, CD_2N_3), 29-27 (m, CD_2), 25-23 (m, CD_2).

7-Azido-3-oxoheptanoic acid tert-butyl ester (13a): To a solution of known[21] 4-azido-butane-1-ol (1.7 g, 15 mmol), *tert*-butyl bromoacetate (4.2 ml, 30 mmol, 2 eq.), tetrabutylammonium bromide (490 mg, 3 mmol, 10 mol%) and a catalytic amount of tetrabutylammonium iodide in toluene (60 ml) was added aq. NaOH (60 ml, 50% by weight) and the mixture was stirred vigorously at ambient temperature until TLC-analysis indicated consumption of **12a**. Water (600 ml) was added to the mixture, and stirring was continued for 1 h. The organic layer was separated and the aq. layer extracted with toluene (3×). The combined organic layers were washed (sat. aq. NH_4Cl, sat. aq. $NaHCO_3$, brine), dried over $MgSO_4$ and concentrated *in vacuo*. Purification of the crude product over silica gel (hexanes:EtOAc 9:1) yielded 2.1 g (9.1 mmol, 61%) of a yellow liquid. ^{1}H NMR ($CDCl_3$): δ, 3.95 (s 2H, CH_2C=O), 3.6-3.5 (m, 2H, CH_2O), 3.25-3.15 (m, 2H, CH_2N_3), 1.8-1.65 (m, 4H, CH_2 2x), 1.48 (s, 9H, tBu); ^{13}C NMR ($CDCl_3$): δ, 168.7 (C=O), 80.3 (C_q tBu), 69.8 (CH_2O), 67.8 (CH_2C=O), 50.3 (CH_2N_3), 26.0, 24.9 (m, CH_2). HRMS $[M+H]^+$ calculated: 230.150, found: 230.150.

4,4,5,5,6,6,7,7-octadeutero-7-azido-3-oxaheptanoic acid tert-butyl ester (13b): To a solution of **12b** (445 mg, 3.6 mmol), *tert*-butyl bromoacetate (1.06 ml, 7.2 mmol, 2 eq.), tetrabutylammonium bromide (119 mg, 0.36 mmol, 10 mol%) and a catalytic amount of tetrabutylammonium iodide in toluene (15 ml) was added aq. NaOH (15 ml, 50% by weight) and the mixture was stirred vigorously at ambient temperature until TLC-analysis indicated consumption of **12b**. Water (150 ml) was added to the mixture, and stirring was continued for 1 h. The organic layer was separated

and the aq. layer extracted with toluene (3×). The combined organic layers were washed (sat. aq. NH_4Cl, sat. aq. $NaHCO_3$, brine), dried over $MgSO_4$ and concentrated *in vacuo*. Purification of the crude product over silica gel (hexanes:EtOAc 9:1) yielded 530 mg (2.2 mmol, 61%) as a colourless oil. ^1H NMR ($CDCl_3$): δ, 3.94 (s 2H, CH_2), 1.48 (s, 9H, tBu); ^{13}C NMR ($CDCl_3$): δ, 169.1 (C=O), 80.7 (C_q tBu), 70-68 (m CD_2O), 68.0 (CH_2), 50-48 (m CD_2N_3), 26-23 (m, CD_2 2×).

7-azido-3-oxoheptanoic acid (14a): *tert*-Butyl ester **13a** (1 g, 4.3 mmol) was treated with TFA/CH_2Cl_2 (20 ml, 1/1 v/v) for 2 h at ambient temperature. Toluene (20 ml) was added, and the mixture was concentrated to half its volume. Toluene (20 ml) was added again and the mixture was coevaporated with toluene 3× to remove residual TFA. The resulting yellow oil was dissolved in EtOAc and extracted with sat. aq. $NaHCO_3$ (3×). The organic layer was discarded, the aqueous layer acidified with conc. HCl to pH 1 and extracted with Et_2O (6×). The combined Et_2O layers were dried ($MgSO_4$), filtered and concentrated *in vacuo* to yield 610 mg (3.5 mmol, 81%) of a yellowish oil. ^1H NMR ($CDCl_3$): δ, 10.68 (br s, 1H, COOH), 4.15 (s 2H, CH_2C=O), 3.65-3.55 (m, 2H, CH_2O), 3.35-3.25 (m, 2H, CH_2N_3), 1.8-1.6 (m, 4H, CH_2 2x); ^{13}C NMR ($CDCl_3$): δ, 175.1 (C=O), 70.8 (CH_2O), 67.1 (CH_2C=O), 50.6 (CH_2N_3), 26.1, 25.0 (m, CH_2). HRMS $[M+H]^+$ calculated: 174.087 found: 174.088.

4,4,5,5,6,6,7,7-octadeutero-7-azido-3-oxaheptanoic acid (14b): *tert*-Butyl ester **13b** (530 mg, 2.2 mmol) was treated with TFA/CH_2Cl_2 (10 ml, 1/1 v/v) for 2 h at ambient temperature. Toluene (10 ml) was added, and the mixture was concentrated to half its volume. Toluene (10 ml) was added again and the mixture was coevaporated with toluene 3× to remove residual TFA. The resulting yellow oil was dissolved in EtOAc and extracted with sat. aq. $NaHCO_3$ (3×). The organic layer was discarded, the aqueous layer acidified with conc. HCl to pH 1 and extracted with Et_2O (6×). The combined Et_2O layers were dried ($MgSO_4$), filtered and concentrated *in vacuo* to yield 346 mg (1.9 mmol, 87%) of a yellowish oil. ^1H NMR ($CDCl_3$): δ, 9.86 (br s, 1H, COOH), 4.14 (s 2H, CH_2); ^{13}C NMR ($CDCl_3$): δ, 175.0 (C=O), 71-69 (m CD_2O), 66.9 (CH_2), 51-49 (m CD_2N_3), 26-23 (m, CD_2 2×). ESI-MS:[M+Na]=204.0.

Ethyl (2S,3S)oxirane-2,3-dicarboxylate (21): The title compound was prepared according to the literature procedure.[20] ^1H and ^{13}C analysis were in agreement with the reported value. α_D^{22} +105 (*c* 1.2, Et_2O). As a control, Ethyl (2R, 3R)oxirane-2,3-dicarboxylate was prepared following the same procedure. α_D^{22} -114 (*c* 1.2, Et_2O).

Synthesis of the novel DCG-04 analogues: Fmoc Rink amide resin (78 mg, 50 μmol) was elongated by standard Fmoc-based SPPS. 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 minutes. 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 h 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 DMF (5×). Lysine side chain Mtt protecting groups were removed by treatment of the resin 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. Azides were reduced to amines 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×). Deuterated building blocks **7** and **14b** were coupled using 2 eq. of the appropriate acid. After coupling of known **21** to the immobilised peptide, the resin was washed extensively (alternating CH_2Cl_2-MeOH 3×, alternating CH_2Cl_2-Et_2O 3×. The resin was transferred into a clean vial, washed with CH_2Cl_2 and treated with TFA/H_2O (1 ml, 95/5 v/v) for 2 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*. To the residue, toluene was added and the concentration was repeated. The residue was taken up in MeOH pored into Et_2O. The precipitate was filtered off and washed with Et_2O. This precipitation was repeated to give the DCG-04 analogues in 29-40% yield. A portion of every analogue was purified to homogeneity by RP-HPLC, applying a linear gradient (20-40% B in 3 column volumes). **1b** was isolated in 34% yield. HRMS $[M+H]^+$ calculated: 933.497, found: 933.505. **2a** was isolated in 29% yield. HRMS $[M+H]^+$ calculated: 941.441, found: 941.449. **2b** was isolated in 40% yield. HRMS $[M+H]^+$ calculated: 949.492, found: 949.499.

Peptide 24: Fmoc Rink amide resin (78 mg, 50 µmol) was elongated by manual solid phase peptide synthesis to give Ac-SCWAFG-NH_2 after cleavage from the resin. **23** was obtained as indicated above. 9 mg (2.5 µmol) of the crude product and **1a** (6 mg, 6 µmol) were dissolved in DMSO saturated with argon and DiPEA (5 µl) was added. The reaction mixture was stirred under argon atmosphere, and after 3 h, LC/MS analysis indicated formation of target **24**, as well as saponified **1a**, saponified **24** and the disulfide of **24**. Longer reaction times resulted in more saponifiaction, whereas shorter reaction times resulted in less product formation as well. As judged by LC/MS, the yield of the reaction was estimated around 30%.

Peptide 25: Tentagel HMB resin preloaded with Fmoc-Arg (50 µmol) was elongated using automated standard Fmoc-based SPPS, except for the two difficult couplings after the Trp residue, where double couplings of 2 h were applied. The peptide was cleaved from the resin with TFA/H_2O/TIS (95/2.5/2.5 v/v/v), purified by RP-HPLC and reacted with **1a** as described for compound **24**. LC/MS analysis gave no indication for formation of **22**. Also, removal of the Mmt protecting group of the target cysteine residue on the solid support followed by on-resin alkylation with **1a** did not result in productive alkylation.

Boc-Cys(DCG-04)-OtBu (28): To a solution of Boc-Cys-OtBu (**26**) (28 mg, 0.1 mmol), **1a** (90 mg, 0.1 mmol) in DMSO (2 ml) saturated with argon, DiPEA (68 µl, 0.4 µmol) was added and the mixture was stirred for 3 h. The reaction mixture was poured into Et_2O/EtOAc (30 ml, 1/1 v/v), cooled (0°C) and agitated to aid precipitation. The white precipitate was collected by centrifugation and washed (Et_2O) to yield 111 mg (94 µmol, 94%) of a white powder, with a purity of 90% as judged from LC/MS. $[M+H]^+$ calculated for $C_{55}H_{89}N_9O_{15}S_2$: 1180.5, found: 1180.9.

Fmoc-Cys(DCG-04)-OH (29): Crude **28** (94 µmol) was treated with TFA/H_2O (1 ml, 95/5 v/v) for 3 h. The product was precipitated with cold Et_2O (35), aided by vortexing. The white precipitate was collected by centrifugation and washed (Et_2O), and redissolved in DMSO (1 ml) with Fmoc-OSu (95 mg) and DiPEA (124 µl). After 3 h of stirring, the mixture was poured into Et_2O (30 ml) and left for precipitation for 16 h at 4°C. The precipitate was collected by centrifugation and washed (Et_2O), to yield 78 mg (47 µmol, 50%) of the target compound. LC/MS $[M+H]^+$ calculated for $C_{61}H_{83}N_9O_{15}S_2$: 1245.6, found: 1245.5. Attempts to couple this alkylated cysteine to the Trp residue of growing peptide chain WAFGAVEAISDR remained fruitless.

Boc-D(tBu)Q(Tr)GS(tBu)C(Tr)G-OH (31): Peptide **31** was synthesized on hyper acid labile Fmoc-Gly-HMPB tentagel resin. The protected peptide was cleaved from the resin by batchwise treatment with 1% TFA in CH_2Cl_2 (20 × 1 ml) for 0.5 min and immediate neutralization of the acidic batches in MeOH/pyridine (10 ml, 9/1 v/v). The solution was concentrated *in vacuo* and **31** was purified to homogeneity by RP-HPLC on a semi-preperative Alltima CN column (Alltech, 10 × 250 mm, 5 µm particle size) and the A-buffer replaced by H_2O/MeOH (1/1 v/v) to yield 5 mg (4 µmol, 8%) of the target compound. LC/MS $[M+H]^+$ calculated for $C_{70}H_{83}N_7O_{13}S$: 1262.6, found: 1262.7.

H-SC(DCG-04)WAFG-OH (33): Peptide **30** was synthesized on 50 µmol resin using automated standard Fmoc-based SPPS and cleavage of the product from resin by treatment with TFA/H_2O/TIS (95/2.5/2.5 v/v/v). Peptide **30** was precipitated (Et_2O) and used without any further purification. LC/MS $[M+H]^+$ calculated for $C_{31}H_{39}N_7O_8S$: 670.2, found: 670.4. **30** (50 µmol) was dissolved in DMSO (1.5 ml) and **1a** (54 mg, 60 µmol) was added. The mixture was saturated with argon before DiPEA (250 µmol, 40 µl) was added, and the reaction mixture was stirred under an argon atmosphere for 16 h. LC/MS analysis of the reaction mixture indicated formation of **33**, as well as peptide disulfide formation. **33** was purified to homogeneity by RP-HPLC to yield 1.5 mg (1 µmol, 2%) of the target compound. LC/MS $[M+H]^+$ calculated for $C_{74}H_{105}N_{15}O_{19}S_2$: 1572.7, found: 1572.9.

34: Activation of **31** (4 µmol) with DIC (1 µl, 4 µmol) and *N*-hydroxysuccinimide (1.2 mg, 10 µmol) in DMF (0.5 ml) was confirmed by LC/MS analysis after 2 h. LC/MS $[M+H]^+$ calculated for

$C_{74}H_{86}N_8O_{15}S$: 1359.6, found: 1359.9. The activated ester solution and DiPEA (4 μmol) were added to **33** (1 μmol) and the mixture was stirred for 16 h. LC/MS analysis did not reveal the presence for target **34**.

Active site labeling of cysteine proteases in J774 cell lysates and detection by streptavidin-blotting: The mouse macrophage cell line J774 was cultured in Dulbecco's Modified Eagle's medium containing 10% fetal calf serum (FCS), 1% penicillin/streptomycine and 1% glutamine. Cells were harvested by centrifugation at 4°C, washed in phosphate buffered saline (PBS) pH 6.8 and cell pellets corresponding to 5×10^7 cells were frozen at –80°C. Cell pellets were thawed on ice and lysed in 100 μl lysis buffer pH 5 (50 mM NaOAc pH 5, 5 mM $MgCl_2$, 0.5% Nonidet-P40), incubated for 30 minutes and centrifuged for 15 min at 13.000 × g to remove nuclei. Protein concentration of the cell extract was measured using the BioRad Protein Assay with bovine serum albumin as standard. Cell lysate (25 μg protein) was incubated with DCG-04, **1b**, **2a** and **2b** respectively for 60 min at 37°C. The reaction was stopped by addition of double concentrated, reducing Laemmli sample buffer and boiling for 10 min. Samples were analyzed by SDS-PAGE and transferred to a polyvinylidene membrane (Immobilon P; Millipore). After blocking over-night in phosphate-buffered saline (PBS) pH 7.2 containing 10% non-fat dry milk, the membrane was incubated with strept-HRP (1:2500; Amersham) in PBS containing 0.2% Tween 20 for 60 min at room temperature followed by extensive washing in PBS-Tween. Polypeptide species reactive to the compounds were detected by Enhanced Chemiluminescence (ECL NEN; Perkin Elmer).

References and notes

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