Electronic Supplementary Material (ESI) for Chemical Communications

Cleavable trifunctional biotin reagents for protein labeling, capture and release

Yinliang Yang, Steven H. L. Verhelst

Supporting Information

1 General methods

Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification. Reactions were analyzed by thin-layer chromatography on 0.20 mm silica plates with fluorescent indicator. High resolution mass spectrometry analysis was performed on an Agilent 6210 LC–MS equipped with an electrospray TOF. Preparative HPLC purification was performed on a Waters 515 HPLC system using an X-bridge C_{18} column. Purifications were performed at room temperature and compounds were eluted with increasing concentration of acetonitrile (solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in acetonitrile).

2 Synthesis biotin-diol-tamra-azide (1)

See also Scheme S1.

The cleavable diol building block (methyl 2,3-O-isopropylidene-L-tartrate) was synthesized as described before.[1] The synthesis of building block **7** was performed on Rink resin, using Fmoc-amino acid/DIC/HOBt (3eq/3eq/3eq with respect to the loading reported by the supplier; final concentration ~ 0.3 M in DMF) and reacted with the resin for 3-5h at room temperature. After the coupling of methyl 2,3-O-isopropylidene-L-tartrate under the same conditions as for the amino acids, 1,8-diamino-3,6-dioxaoctane (1 ml) was added and the resin was shaken overnight. Biotin (1.5 eq) was coupled with DIC/HOBt overnight. The resin-bound intermediate was cleaved by incubation with a solution of TFA: TIS: H_2O (90%: 2.5%: 7.5%) for 3 hours. The peptide was precipitated in cold diethyl ether and purified by HPLC yielding a white solid (18 mg, 23%). ESI-MS: $[M+H]^+$ calculated for $C_{32}H_{57}N_{11}O_{10}S$ 788.4011, found 788.4051. The building block 2 (3 mg; 2.8 µmol) was reacted overnight with 5-(and6-) carboxytetramethylrhodamine, succinimidyl ester (2.5 mg; 1.2 eq) and DIEA 1.72 µl (10 µmol) in DMSO (100 µL) . The solution was purified by HPLC to give a target compound (2 mg; yield: 60%). ESI-MS: $[M+H]^+$ calculated for $C_{57}H_{77}N_{13}O_{14}S$ 1200.5434, found 1200.5389.

3 Synthesis of biotin-diazobenzene-tamra-azide (2)

See also Scheme S2.

The diazobenzene cleavable building block **8** was synthesized following literature procedures.[2] The synthesis compound **9** was performed on Rink resin. First, Fmoc-Lys(azide)-OH, Fmoc-

PEG-OH and Fmoc-Lys(Boc)-OH were coupled as described above. The Fmoc group of Fmoc-lys(boc) was removed on the resin by treatment with 20 % piperidine in DMF (20 min). The diazobenzene cleavable building block (1.5 eq), HBTU, DIEA was reacted with the resin overnight. After deprotection of the Fmoc, biotin (1.5 eq) was coupled with DIC/HOBt overnight. The resin-bound building block was cleaved by incubation with a solution of TFA: TIS: H_2O (95%: 2.5%: 2.5%) for 2 hours. The peptide was precipitated in cold diethyl ether and purified by HPLC yielding orange solid (10.5 mg, yield 5.6%). ESI-MS: $[M+H]^+$ calculated for $C_{43}H_{63}N_{13}O_9S$ 938.4592, found 938.4686. The building block 5 (10 mg, 0.011 mmol) was reacted overnight in DMSO (200 µl) with 5-(and-6)carboxytetramethylrhodamine, succinimidyl ester (4.68 mg, 0.009 mmol) and DIEA (7.6 µL, 0.044 mmol). The solution was purified by HPLC to give a red compound (10 mg; yield 83%). ESI-MS: $[M+H]^+$ calculated for $C_{68}H_{83}N_{15}O_{13}S$ 1350.6015, found 1350.6037.

4 Synthesis of biotin-bisaryl-hydrazone-tamra-azide (3)

See also Scheme S3.

The synthesis of 6-hydrazinonicotinic acid (**10)** was performed using a reported procedure.[3] 6-chloronicotinic acid (1.5 g) was added 10 ml 80% hydrazine in water. The reaction was heated at 100 °C for 4h. The homogeneous reaction mixture was cooled to room temperature and concentrated to give a white solid. The solid was dissolved in water and acidified to pH 5.5 with HCl. The formed precipitate was isolated by filtration. The solid was washed with 95% ethanol and ether, and dried under vacuum resulting in 1.24 g of a light yellow compound.

4-Formyl-benzoic acid NHS ester (**11**): carboxylbenzaldehyde 450 mg (3 mmol) was dissolved in a mixture of THF (15 ml) and DCM (20 ml). *N*-hydroxysuccinimide 345 mg (3 mmol) and EDCI 1.14g (6 mmol) were added. The reaction was maintained at room temperature for 6 h. The solvent was evaporated and DCM 20 ml was added. The excess EDCI was removed by washing with 1M $KHSO_4$ and the organic layer was washed with brine, dried with $MgSO_4$ and concentrated *in vacuo.* The residue was directly used in next step without purification.

Synthesis of **12**: Boc-lys-OH (246 mg, 1 mmol) was dissolved in 10 ml DMF and 5 ml water. 4-Formyl-benzoic acid NHS ester (271 mg, 1.1 mmol) and DIEA 345 µL (2 mmol) were added. The reaction was maintained at room temperature for 5h. The solvent was evaporated and EtOAc (20 ml) was added. The organic layer was washed with a pH 2.5 citrate solution. The organic

layer was dried by $MgSO_4$ and the solvent was evaporated, resulting in 380 mg of crude material. This compound was dissolved in THF (10 ml). *N*-hydroxysuccinimide (127 mg, 1 mmol) and EDCI (380 mg, 2 mmol) were added. The reaction stirred for 6 h at room temperature. The solvent was evaporated and DCM was added. The organic layer was washed with 1M $KHSO_4$ and dried by $MgSO_4$. Evaporation of solvent resulted in 500 mg of crude material, which was used in the next step without further purification. It was reacted for 3 h with DIEA (369 µL, 0.5 mmol) and 3-azido-propylamine (120 mg, 1.5 mmol) in DCM (20 mL) . The reaction mixture was washed with 1 M $KHSO_4$, saturated $NaHCO_3$ and brine. The organic layer was dried on $MgSO_4$ and concentrated under reduced pressure. Silica column chromatograph (66–100% EtOAc in PE) afforded the title compound (301 mg, 65%) overall 4 steps. ESI-MS: $[M+H]^+$ calculated for $C_{22}H_{32}N6O_5$ 461.2434, found 461.2497.

Synthesis of **13**: compound **12** (138 mg, 300 µmol) was dissolved in DMF (5 ml) and 6-hydrazinonicotinic acid (46 mg, 300 µmol) was added. The reaction was stirred overnight and the solvent was evaporated. The residue was used in solid phase synthesis without purification. ESI-MS: $[M+H]^+$ calculated for $C_{28}H_{37}N_9O_6$ 596.2867, found 596.2884.

The synthesis of building block **14** was performed on Rink resin by first coupling of Fmoc-Lys(biotin)-OH and Fmoc-PEG-OH. The Fmoc-group was removed by treatment with 20% piperidine in DMF (20 min). The hydrazone cleavable building block **13** (44.6 mg, 0.075 mmol), HBTU (28.4 mg 0.075 mmol), DIEA (26 µl, 0.15 mmol) were added and reacted overnight. The compound was cleaved from the resin by incubation with a solution of TFA: TIS: H_2O (95%: 2.5%: 2.5%) for 2 hours, precipitated in cold diethyl ether and purified by HPLC yielding a yellow solid (10 mg, yield 20%). ESI-MS: $[M+H]^+$ calculated for $C45H67N_{15}O_9S$ 994.4967, found 997.4740. The building block 10 (5 mg, 0.005 mmol) was dissolved in DMSO (200 µl). 5-(and 6-) carboxytetramethylrhodamine, succinimidyl ester (2.65 mg, 0.005 mmol) and DIEA (1.74 µL, 0.01 mmol) were added and the reaction mixture was shaken overnight. The solution was purified by HPLC to give a red compound (6 mg; yield 85%). ESI-MS: $[M+3H]^+/3$ calculated for $C_{70}H_{87}N_{17}O_{13}S$ 469.5463, found 469.5505.

5 Synthesis of biotin-disulfide-tamra-azide (4)

See also Scheme S4.

The synthesis of building block **15** was performed on Rink resin by coupling Fmoc-Lys(azide)-OH, Fmoc-Lys(Boc)-OH and Fmoc-PEG-OH. The N-terminal Fmoc group was removed by treatment with 20% piperidine in DMF (20 min). Then, sulfo-NHS-SS-Biotin (1.5 eq) and DIEA (2 eq) were added and the mixture was shaken overnight. The compound was cleaved by incubation with a solution of TFA: TIS: H_2O (95%: 2.5%: 2.5%) for 2 hours and precipitated in cold diethyl ether yielding a white solid (21 mg). The crude building block **15** (3.4 mg) was dissolved in DMSO (200 µl). 5-and 6-carboxytetramethylrhodamine, succinimidyl ester (1.41 mg) and DIEA (5 µL, 0.029 mmol) were added and the reaction was shaken overnight. The final product was purified by HPLC to give a red compound (0.12 mg; yield 3.7 %). ESI-MS: $[M+2H/]^+/2$ calculated for $C_{58}H_{80}N_{13}O_{12}S$ 623.7567, found 623.7559.

6 Synthesis of biotin-dde-tamra-azide (5)

See also Scheme S5.

Synthesis of biotin(dde)

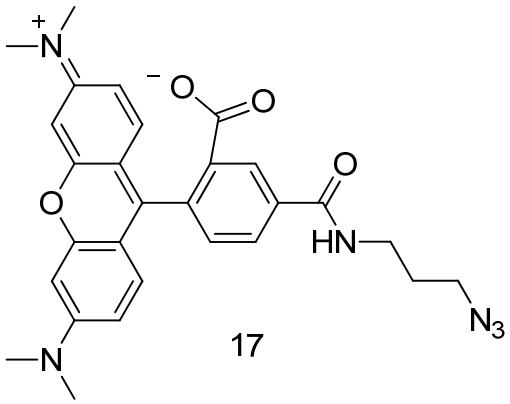
Biotin(dde) was made according to a literature procedure.[4] Dimedone (154 mg, 1.1 mmol) was dissolved in DMF (5 ml). Biotin (244 mg, 1.0 mmol), DIC (156 µl; 1.0 mmol) and DMAP (122 mg; 1.0 mmol) were added. The solution was stirred for 48h. DMF was removed under reduced pressure and EtOAc was added. The organic layer was washed with 1M $KHSO_4$ and extracted with saturated $NaHCO_3$. The solution was acidified with HCl and extracted with DCM. The organic layer was dried by $MgSO_4$ and concentrated under reduced pressure. The residue was recrystallized in MeOH/H2O (1:1) yielding 200 mg of the target compound (55%). ESI-MS: $[M+H]^+$ calculated for $C_{18}H_{26}N_2O_4S$ 367.1613, found 367.1746.

Biotin-dde-tamra-azide

The title compound was synthesized on Rink resin by coupling Fmoc-Lys(azide)-OH, Fmoc-Lys(Mtt)-OH, and Fmoc-PEG-OH**.** The Fmoc group was removed on the resin by treatment with 20 % piperidine in DMF (20 min). 5-(and 6-)carboxytetramethylrhodamine (1.5 eq) and DIEA (1.5 eq) were added and the mixture was shaken overnight. After loading the 5-(and 6-)carboxytetramethylrhodamine, Mtt was deprotected with 1% TFA in DCM (8x 10 min). Then the resin was washed with DMF 3 times and biotin(dde) (0.09 mmol, 32.94 mg) and DIEA (15.6 µl, 0.09 mmol) were reacted with the resin overnight. The compound was cleaved from the resin by incubation with a solution of TFA: TIS: H_2O (95%: 2.5%: 2.5%) for 2 hours. The solvent was

evaporated under reduced pressure. The crude peptide was purified by HPLC to give a red compound (15.28 mg; yield 51 %). ESI-MS: $[M+2H]^{2+}$ calculated for $C_{61}H_{80}N_{12}O_{12}S$ 603.2870, found 603.3004.

7 Structures of Rh-N_3 and alkyne-E64



17

8 Cell culture and lysate preparation

RAW 264.7 cells were cultured in DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin and maintained in a humidified 37 °C incubator with 5% CO_2. To generate lysates, cells were washed twice with phosphate-buffered saline (PBS), harvested by use of a cell scraper, and collected by centrifugation. Cell pellets were then washed with PBS and lysed with 0.1% Triton-X 100 in sodium acetate buffer (2 mM DTT, 50 mM NaOAc pH 5.5 and 5 mM $MgCl_2$) at 4^{o}C for 30min. The mixture was then centrifuged at high speed (eppendorf centrifuge, 15000 rpm, 4 ^{o}C, for 20 minutes) to remove cell debris. The supernatant was snap-frozen in liquid nitrogen and stored at -80 ^{o}C. Protein concentration was determined by the DC protein assay.

9 ABP labeling in cell lysates

The cell lysate (2.5 ml; 2 mg/ml) in 50 mM sodium acetate buffer (pH 5.5) was incubated with alkyne-E64 (10 μM final concentration) for 2 h. The reaction was stopped by running a PD-10 gel filtration column. The elution (in 100 mM Na_2HPO_4, pH 7.4) was adjusted to 5 ml with Na_2HPO_4 buffer. Alkyne-E64 labeled lysate was stored at -20 ^{o}C until further usage in click chemistry experiments.

10 Click chemistry-mediated labeling

To alkyne-E64 labeled lysate in 100 mM sodium phosphate buffer, pH 7.4 were added the azide tags (25-50 μM), TBTA (50 μM), TCEP (1 mM) and $CuSO_4$ (1 mM) and the mixture was incubated at room temperature for 1h. Four volumes of cold acetone were added in order to precipitate the proteins and remove excess reagents. This process was repeated one time. The protein pellet was dissolved in 1% SDS in PBS and diluted with PBS to make the final concentration of SDS 0.25% and the protein concentration 1 mg/ml. For the Dde trifunctional biotin tag and TMR-azide, a gel filtration (Zeba spin column; Pierce) was used instead of acetone precipitation to get rid of the excess reagents.

11 Fluorescent gel scanning and biotin-streptavidin blotting

Samples were resolved on a 15% SDS polyacrylamide gel. The labeled protein bands were visualized with fluorescent scanning on a Typhoon Trio+. For detection by streptavidin, gels were transferred to a nitrocellulose membrane. The membrane was blocked with 3% milk in PBST (0.1% Tween in PBS) for 1h at room temperature. Then the membrane was washed with PBST (3x 5 min) and incubated with streptividin-peroxidase (diluted 3500x from a 1 mg/ml stock solution) for 1h. The membrane was washed with PBST (3x 15min), treated with 1 mL ECL plus solution (GE Healthcare) and exposed to Kodak X-omat LS film.

12 Capture and release of proteins

General methods: Trifunctional tag labeled proteome was incubated with streptavidin beads (Ultralink streptavidin, Pierce) for 1.5h. Immobilized beads were separated from the unbound fraction by centrifugation. The supernatant was collected. The beads were washed with 1% SDS (3x) and PBS (3x), except for the biotin-dde-tmr-N_3 sample, which was only washed with PBS. Next, the beads were divided into equal parts for chemical release or SDS boiling. It was made sure that the same amount of sample was loaded in each gel lane.

For the samples containing the diol trifunctional tag, the chemical release was performed with $NaIO_4$ (25 μL of 1 or 10 mM in 100 mM Na_2HPO_4 pH 7.4) while shaken for 20 min. The cleavage solution was collected and added to 4xSB buffer. This process was repeated 2 more times. At last the beads were washed with 1% SDS in PBS (2x), and the cleavage solution and wash solution were combined. The beads were subsequently washed with PBS (3X). 1xSB

(equal to the total amount of the combined cleavage solutions) was added to the beads and boiled for 5 min to release any uncleaved protein targets. 30µl of each sample was loaded onto a 15% SDS gel and visualized by fluorescent scanning and biotin-streptavidin blot.

For the samples containing the diazobenzene trifunctional tag, the chemical release was done by treatment of 25 µL of $Na_2S_2O_4$ (200 mM in 100 mM Na_2HPO_4 pH 7.4) while shaking for 20 min. The process was repeated 2 more times. The beads were washed with 1% SDS in PBS (2x) and the wash and cleavage solutions were combined and added to 4xSB. The beads were washed with PBS (3x) and boiled with 1xSB to release any uncleaved protein targets. 30µl of each sample was loaded onto a 15% SDS gel and visualized by fluorescent scanning and biotin-streptavidin blot.

For the samples containing the bisaryl hydrazone trifunctional tag, the chemical release was done with either 100 mM NH_2OH (in 100 mM Na_2HPO_4, pH 4.6), 100 mM NH_2OH and 100 mM aniline (in 100 mM Na_2HPO_4, pH 4.6) or 1 M NH_2OH and 100 mM aniline (in 100 mM Na_2HPO_4, pH 4.6). The solution was shaken for 4h. The cleavage solution was collected and added to 4xSB buffer. The beads were washed with 1% SDS in PBS and the cleavage solution and wash solution were combined. The beads washed with PBS (3X) and boiled with 1xSB to release any uncleaved protein targets. 30 µl of each sample was loaded onto a 15% SDS gel and visualized by fluorescent scanning and biotin-streptavidin blot.

For samples containing the disulfide trifunctional tag, the chemical release was performed with 0.5 M DTT in water. Then, the beads were washed with 1% SDS in PBS (2x) and the solutions were combined with 4xSB. The beads washed with PBS (3X) and boiled with 1xSB to release any uncleaved protein targets. 30µl of each sample was loaded onto a 15% SDS gel and visualized by fluorescent scanning and biotin-streptavidin blot.

The chemical release of the samples containing the Dde trifunctional tag was performed under 3 different conditions: (1) 200 mM Tris (pH 8.5), (2) 200 mM Tris (pH 8.5) with 0.05% SDS or (3) 0.05% SDS in water. The mixtures were shaken for 2h and the cleavage solutions were added to 4xSB. The beads were washed with PBS (2x), which solutions were combined with the cleavage solution. The beads were then washed with PBS (3x) and boiled with 1xSB to release any uncleaved protein targets. 30 µl of each sample was loaded onto a 15% SDS gel and visualized by fluorescent scanning and biotin-streptavidin blot.

13 Tryptic digestion of cleavable probe

Biotin-dde-tmr-azide (1 µl of a 5 mM solution in DMSO) was added to 100 mM hydrazine in H_2O (1 ml). The solution was stirred 14h at room temperature. The solution was concentrated with a C18 stage tip and eluted with 100 µl of 50% MeCN/H_2O, 0.1% FA. 1 µl of solution was analyzed by LC-MS to check for full cleavage of the cleavable bond. After analysis, the solution was evaporated in a speed vac. Acetonitrile (10 µl) was added to dissolve the residue. 100 mM NH_4HCO_3 (1 ml) and trypsin (1µg) were added. The solution was incubated overnight at 37 ^oC. The solution was concentrated with a C18 stage tip and eluted with 100 µl of 50% MeCN/H_2O, 0.1% FA. 1 µl of solution was analyzed by LC-MS. Al LC-MS runs were performed on an Agilent 6210 LC–MS equipped with an electrospray TOF, using an Agilent Zorbax-SB C18 column (150 x 0.5 mm) and a gradient of 0-100% MeCN in water + 0.1% formic acid.

References

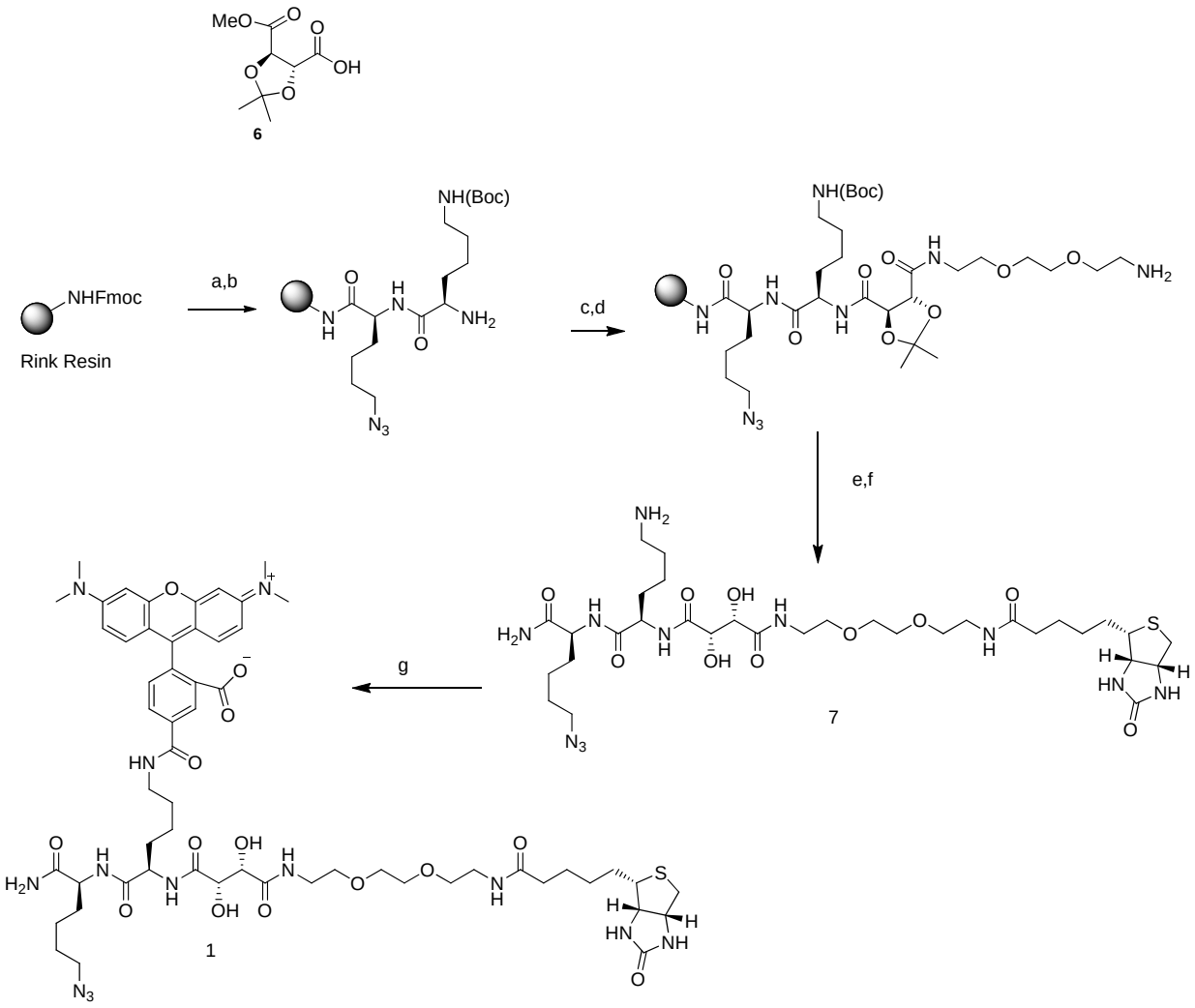
1. Y. Yang, H. Hahne, B. Kuster and S. H. L. Verhelst, *Mol. Cell. Proteomics*, 2013,12, 237-244.

2. S. H. L. Verhelst, M. Fonovic and M. Bogyo, *Angew. Chem. Int. Ed. Engl.*, 2007, **46**, 1284-1286.

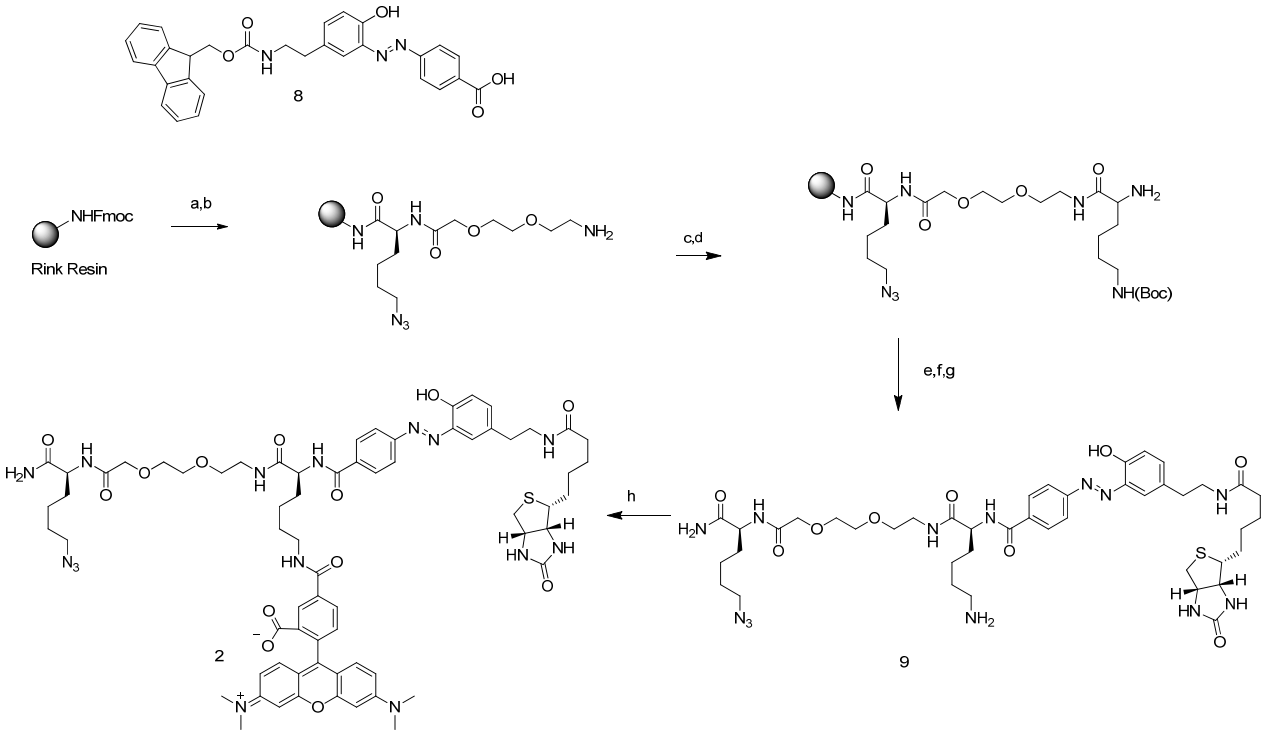
3. M. J. Abrams, M. Juweid, C. L. tenKate, D. A. Schwartz, M. M. Hauser, F. E. Gaul, A. J. Fuccello, R.H. Rubin, H.W. Strauss and A. J. Fischman, *J. Nucl. Med*., 1990, **31**, 2022-2028.

4. B. Kellam, W. C. Chan, S. R. Chhabra and B. W. Bycroft, *Tetrahedron Lett.*, 1997, **38**, 5391-5394.

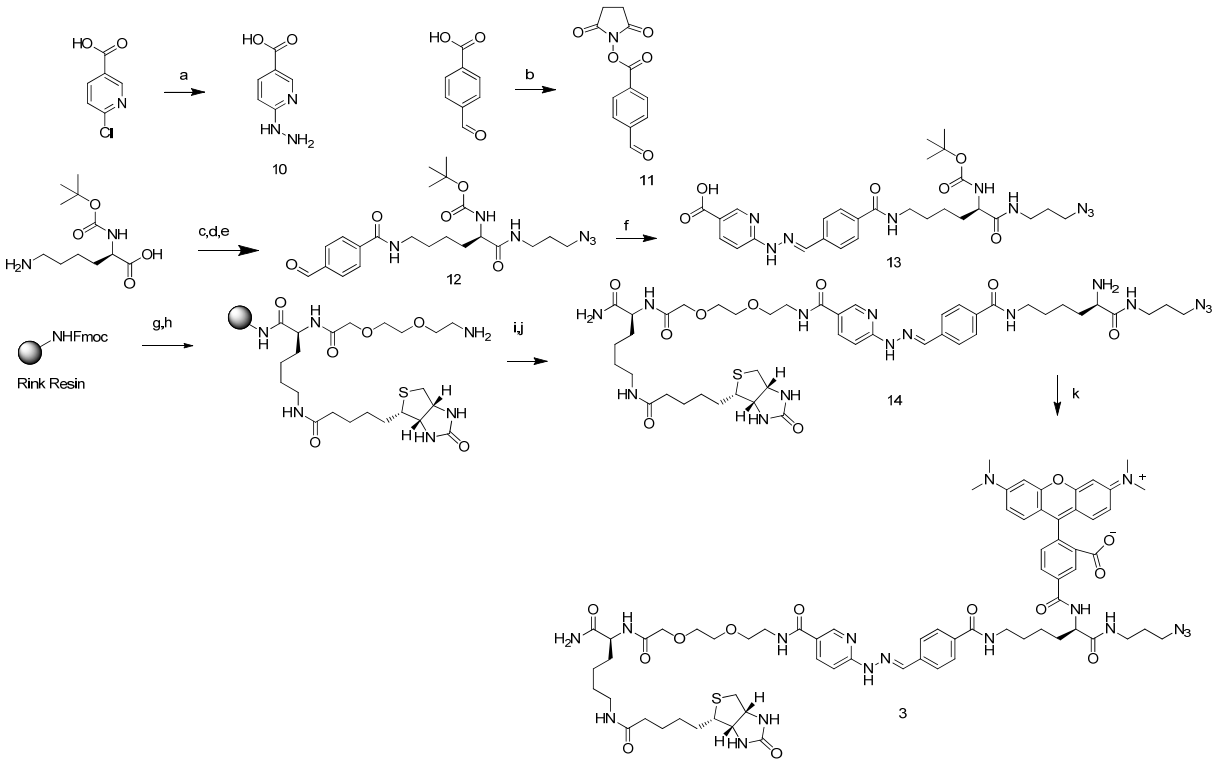
Schemes



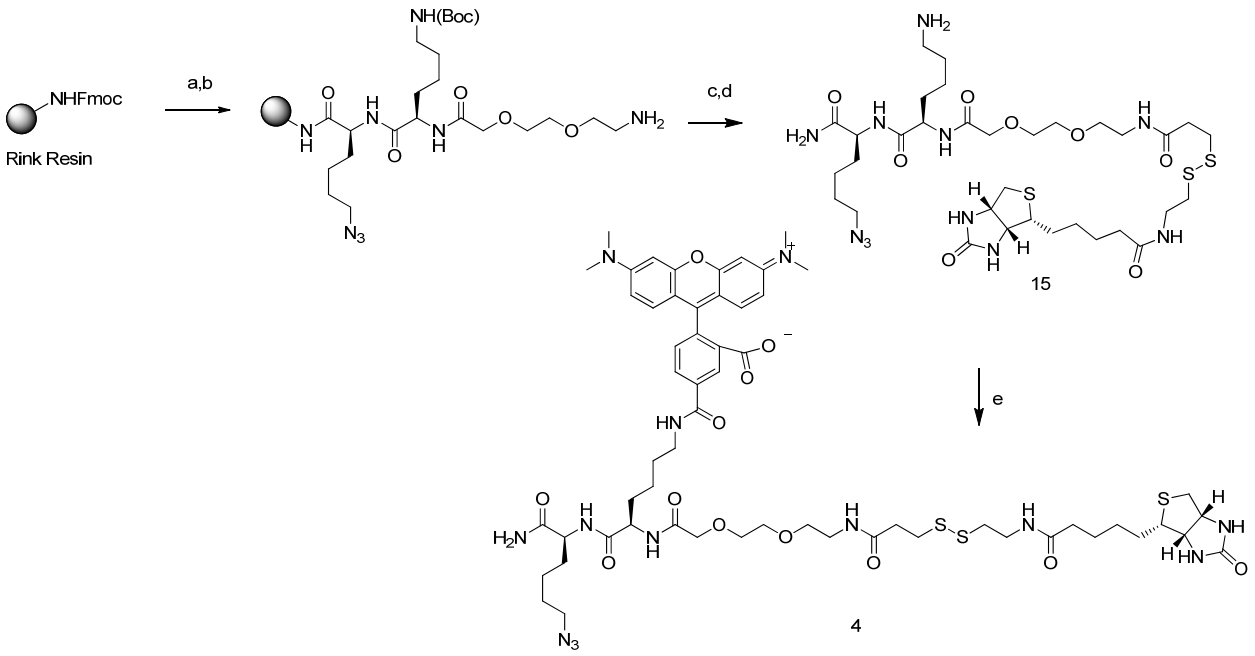
Scheme S1. Synthesis of biotin-diol-tmr-azide (**1**). a) 20% piperdine in DMF, then Fmoc-lys(azide)-OH,DIC, HOBt; b) 20% piperdine in DMF, then Fmoc-Lys(Boc)-OH, DIC, HOBt; c) 20% piperdine in DMF; then building block **6**, DIC, HOBt; d) 1,8-diamino-3,6-dioxaoctane, overnight; e) biotin, DIC, HOBt; f) TFA : TIS: H_2O (90% : 2.5% : 7.5%) 2h, then precipitation with cold diether and purification by HPLC; g) tamra-succinimidyl ester, DIEA, then HPLC purification.



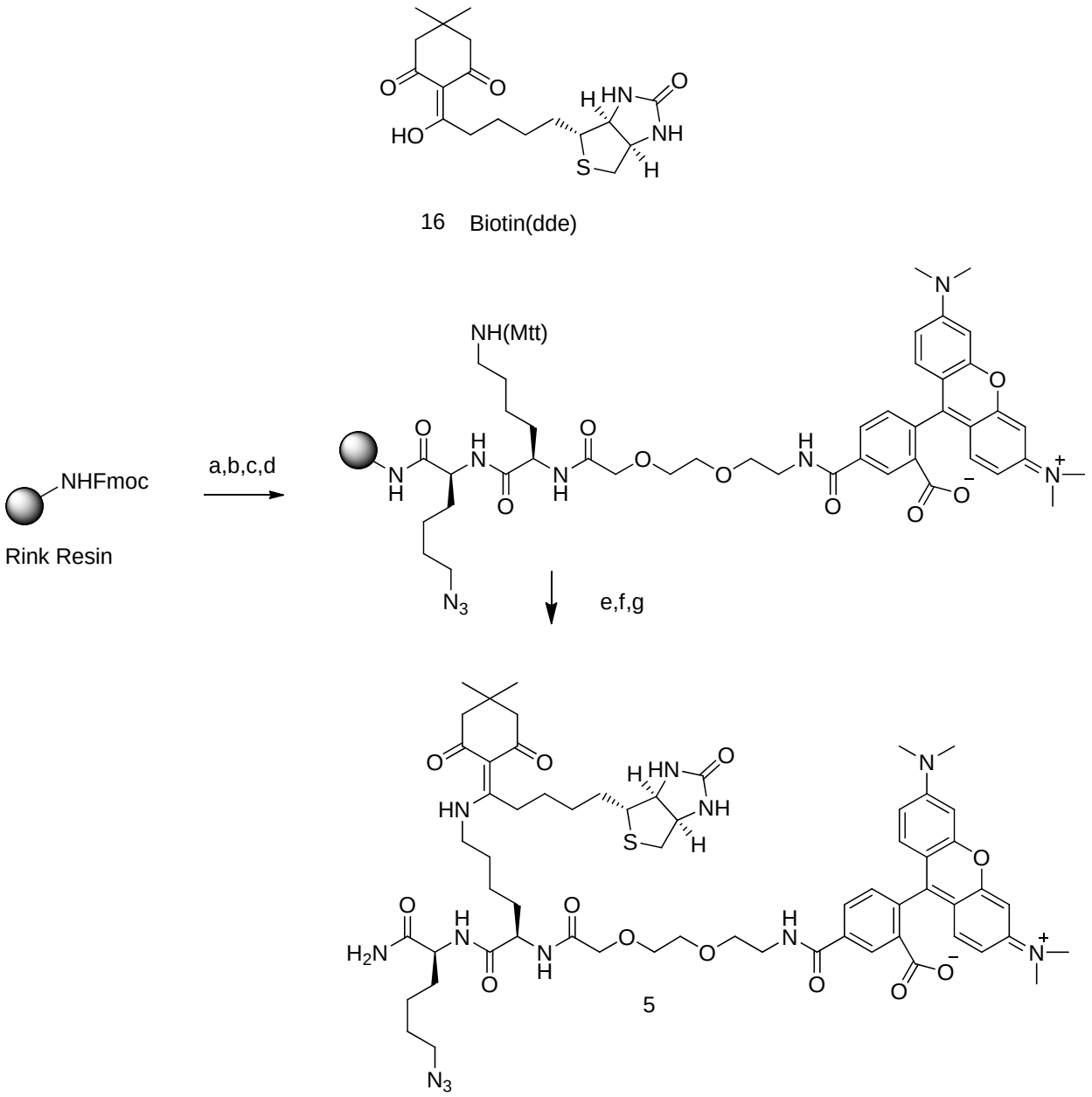
Scheme S2. Synthesis of biotin-diazobenzene-tmr-azide (**2**). a) 20% piperdine in DMF, then Fmoc-lys(azide)-OH,DIC, HOBt; b) 20% piperdine in DMF, Fmoc-8-amino-3,6-dioxaoctanoic acid, DIC, HOBt; c) 20% piperdine in DMF; then Fmoc-Lys(Boc)-OH, DIC, HOBt; d) 20% piperdine in DMF; e) building block 8, DIEA, HBTU; f) 20% piperdine in DMF, then biotin, DIC, HOBt; g) TFA : TIS: H_2O (95% : 2.5% : 2.5%) 2 h, then precipitation with cold diether and purification by HPLC; h) tamra-succinimidyl ester, DIEA, then HPLC purification.



Scheme S3. Synthesis of biotin-bisaryl hydrozone –tmr-azide (**3**). a) 80% hydrazine in H_2O, 100 ^oC, 4 h; b) EDCI, *N*-hydroxysuccinimid; c) building block 11, DIEA; d) EDCI, *N*-hydroxysuccinimid; e) DIEA, 3-azido-propylamine; f) building block 10, overnight in the dark; g) 20% piperdine in DMF, then Fmoc-lys(biotin)-OH,DIC, HOBt; h) 20% piperdine in DMF, Fmoc-8-amino-3,6-dioxaoctanoic acid, DIC, HOBt, then 20% piperdine in DMF; i) building block 13,DIEA, HBTU; j) TFA : TIS: H_2O (95% : 2.5% : 2.5%) 2 h, then precipitation with cold diether and purification by HPLC; k) tamra-succinimidyl ester, DIEA, then HPLC purification.



Scheme S4. Synthesis of biotin-disulfide-tmr-azide (**4**). a) 20% piperdine in DMF, then Fmoc-lys(azide)-OH,DIC, HOBt; b) 20% piperdine in DMF, Fmoc-8-amino-3,6-dioxaoctanoic acid, DIC, HOBt; c) 20% piperdine in DMF; then biotin-succinimidyl ester, DIEA; d) TFA : TIS: H_2O (95% : 2.5% : 2.5%) 2 h, then precipitation with cold diether and lyophilization; e) tamra-succinimidyl ester, DIEA, then HPLC purification.



Scheme S5. Synthesis of biotin-dde-tmr-azide **5**. a) 20% piperdine in DMF, then Fmoc-lys(azide)-OH, DIC, HOBt; b) 20% piperdine in DMF, Fmoc-Lys(Mtt)-OH, DIC, HOBt; c) 20% piperdine in DMF, Fmoc-8-amino-3,6-dioxaoctanoic acid, DIC, HOBt; d) 20% piperdine in DMF, tamra-succinimidyl ester, DIEA e) 1% TFA in DCM (10 min x 8), then biotin(dde), DIEA; f) TFA : TIS: H_2O (95% : 2.5% : 2.5%) 2 h, g) Precipitation with cold diether and purification by HPLC.

Figures

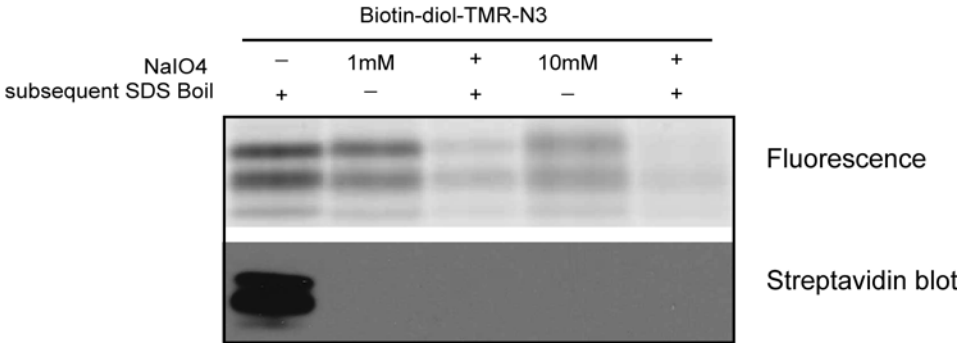


Fig. S1. Elution of enriched cathepsins by the diol linker. Both 1 mM and 10 mM $NaIO_4$ can release the protein targets. Fluorescent scanning showed a lower intensity for the release by 10 mM periodate, probably due to oxidative damage to the fluorophore.

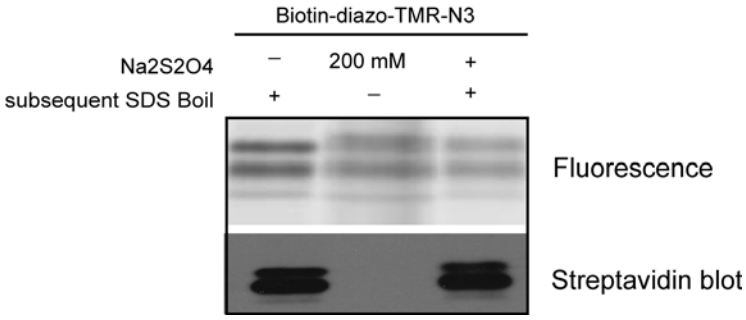


Fig. S2. Elution of enriched cathepsins by the diazobenzene linker. The streptavidin blot shows that the targets that are eluted by 200 mM $Na_2S_2O_4$ have lost their biotin moiety.

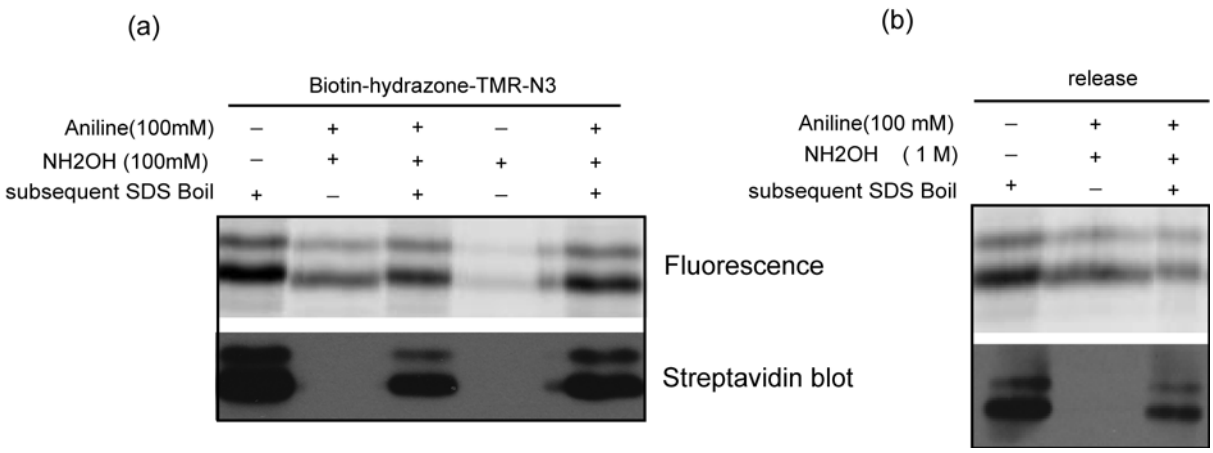


Fig. S3. Elution of enriched cathepsins by the bisaryl hydrazone linker. (a) NH_2OH with or without aniline cannot efficiently elute the protein targets. However, the streptavidin blot shows that the elution is specific, as the chemically eluted targets have lost their biotin moiety. (b) An increase of the NH_2OH concentration to 1 M led to a slightly higher cleavage efficiency.

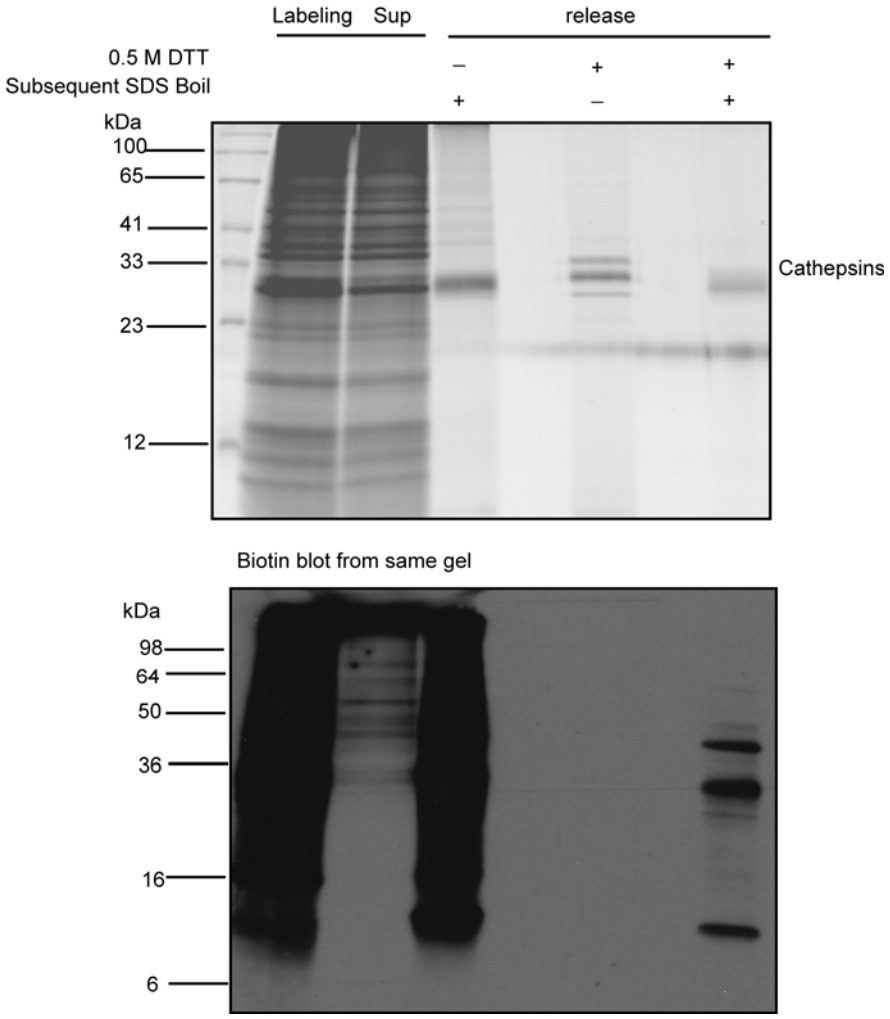


Fig. S4. Labeling and capture of cathepsins from RAW 264.7 cell lysate with the trifunctional tag biotin-disulfide-tmr-N_3. Proteins were separated by non-reducing 15% SDS-PAGE and visualized with fluorescent scanning (upper panel) and streptavidin-biotin blot (lower panel). First lane: labeling after click chemistry with biotin-disulfide-tmr-N_3 tag. Second lane: supernatant after capture with streptavidin beads. Third lane: release of immobilized proteins by boiling with SDS sample buffer. Fourth lane: release of immobilized proteins by 0.5 M DTT in H_2O for 1 h. Last lane: boiling the beads after chemical release to see the chemical release efficiency. Clearly, the multiple bands in the input and supernatant of the fluorescent gel (langes 1 and 2) and in the input and release of the biotin blot (lanes 1, 3 and 6; the latter due to incomplete S-S bond reduction by the DTT) indicate the extensive disulfide exchange that has taken place.

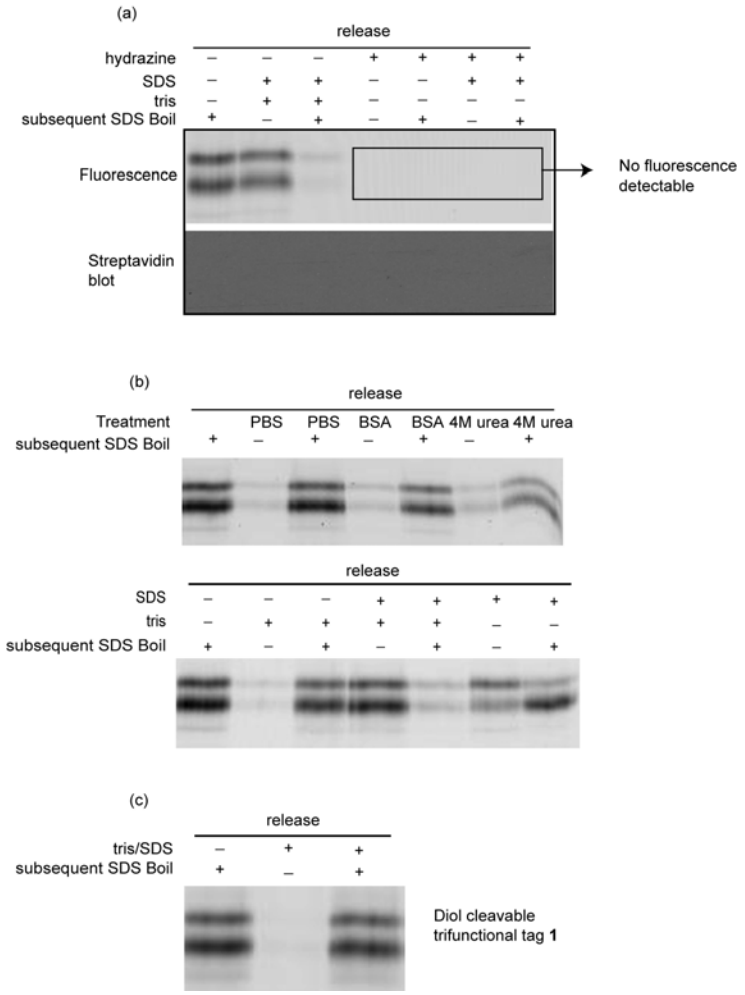


Fig. S5. Elution of enriched cathepsins by the Dde-based linker. (a) Top panel: treatment with 0.05% SDS and 200 mM Tris pH 8.5 (lane 2) elute the target proteins. Subsequent boiling with SDS sample buffer shows that almost all target proteins are released (third lane). Hydrazine treatment (with or without 0.05% SDS) leads to the loss of fluorescence. Lower panel: a streptavidin blot shows that the biotin is cleaved either by the hydrazine treatment or the Tris/SDS (treatment prior to gel analysis or as part of the Laemmli running buffer). (b) Different mild conditions (PBS treatment, BSA (1 mg/mL in PBS) treatment, 4 M urea treatment, 2 h) did not lead to an effective elution (top panel). Tris treatment alone or SDS treatment alone did not lead to effective elution (bottom panel). (c) Treatment of the diol cleavable linker under the same elution conditions (0.05% SDS and 200 mM Tris pH 8.5) did not lead to release of the protein targets, illustrating that the cleavage is dependent on the Dde linker.

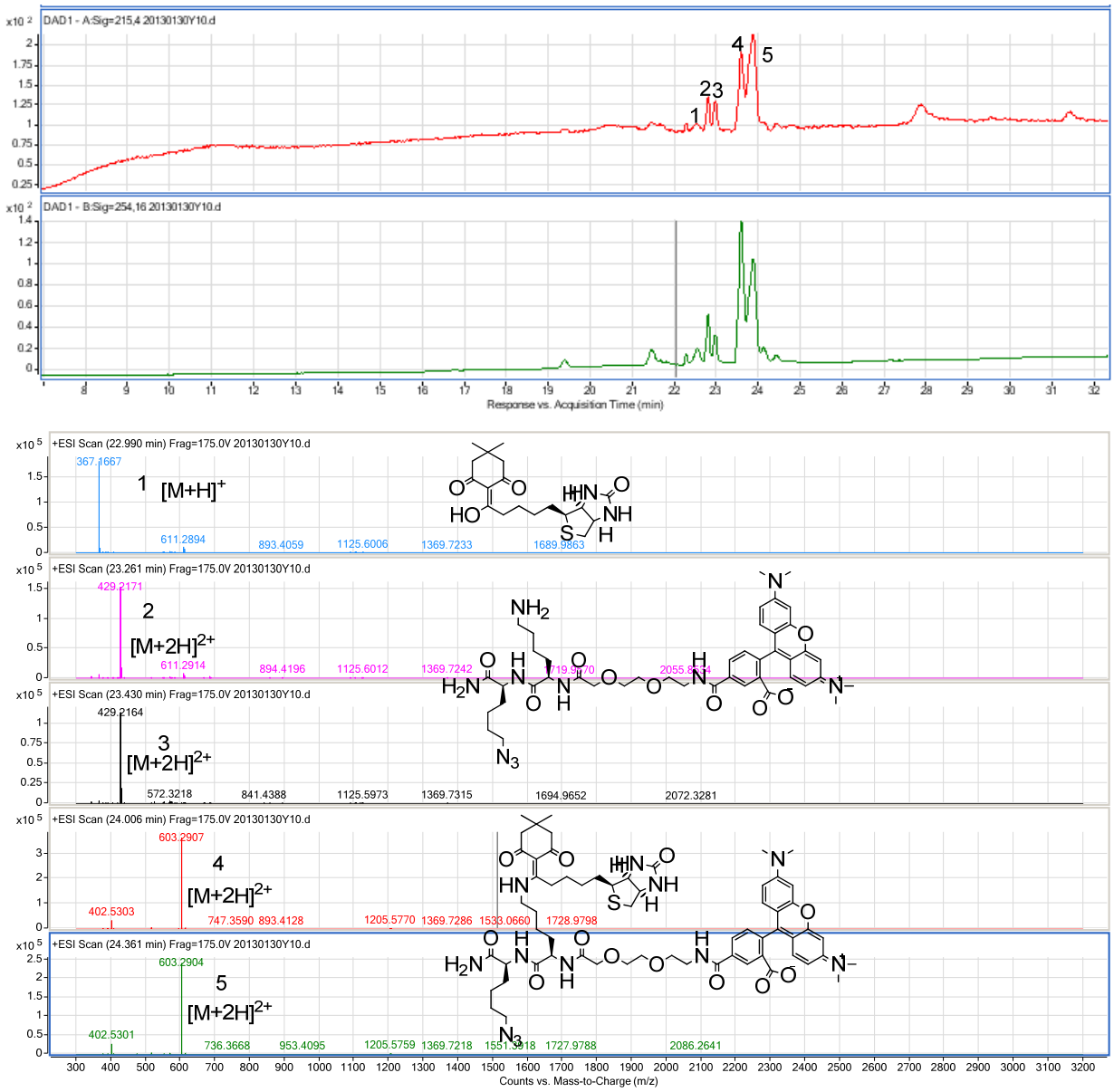
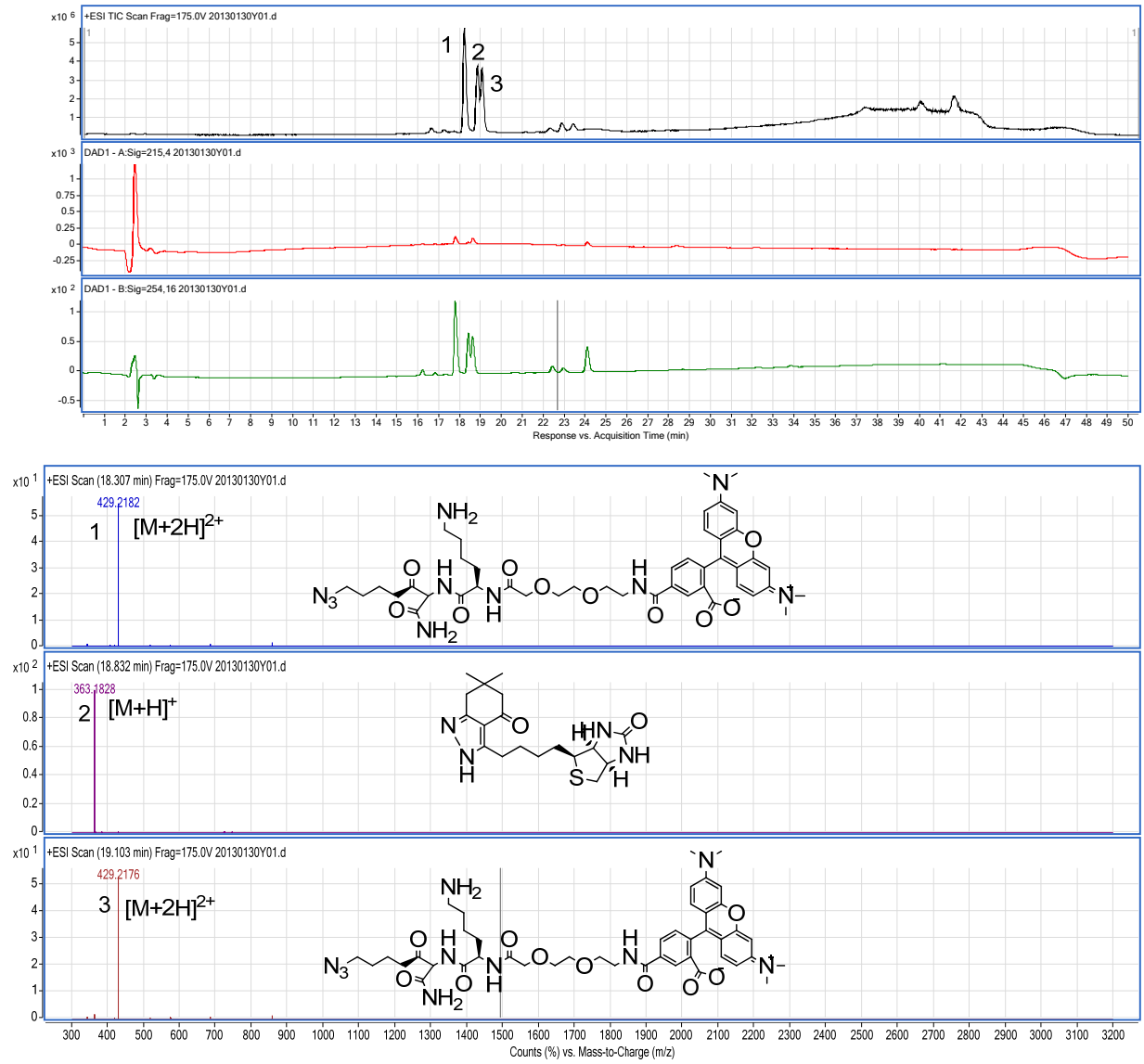
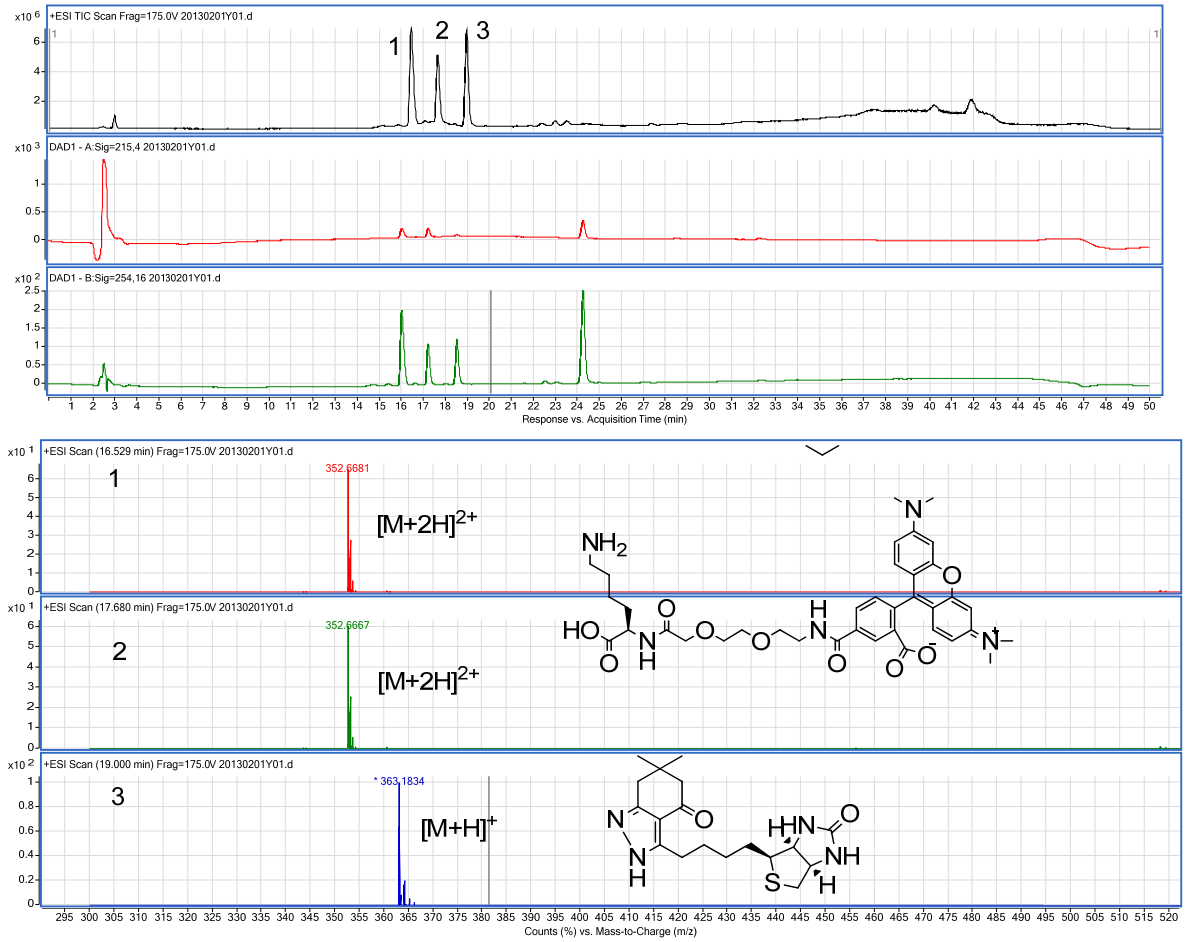


Fig. S6. Treatment of the Dde-trifunctional tag **5** with Tris/SDS and detection of the products by LC-MS. Peak 1 shows a mass of $[M+H]^+$ 367.1667. Calculated for $C_{18}H_{27}N_2O_4S^+$: 367.1686.

Figure S7. Digestion of the masked trypsin-cleavable site in the Dde-trifunctional tag and detection by LC-MS.



A. The Dde-trifunctional tag was cleaved with hydrazine. Peak 1 and 3 contain the two different regioisomers of the TAMRA fluorophore. Peak 2 contains the cleaved-off biotin moiety.



B The Dde-trifunctional tag was digested with trypsin after cleavage with hydrazine. Peak 1 and 2 contain the two regioisomers of the trypsin digested TAMRA tag. Peak 3 contains the cleaved-off biotin.

Table S1

Immobilization and release efficiency of different trifunctional tags. MW= molecular weight

	Diol (**1**)	Diazo (**2**)	Hydrazone (**3**)	SS (**4**)	Dde (**5**)
Pull down efficiency[a]	82%	78%	84%	73%	72%
Optimized cleavage conditions	1mM $NaIO_4$ pH7.4 3x20 min	200 mM $Na_2S_2O_4$ pH7.4, 3x20 min	100 mM NH_2OH 100 mM aniline pH 4.6, 4 h	0.5 M DTT 1 h	200 mM Tris 0.05% SDS pH 8.5, 2 h
Cleavage efficiency[b]	74%	58%	47%	60%	92%
MW of residual tag left after cleavage and trypsin digestion	767.83	976.09	771.86	945.09	171.20

[a] the pull down efficiencies were determined by gel band densitometry as determined with ImageJ (version 1.46) and calculated by the ratio of the intensity of the bands in the supernatant (middle lanes in Figure 3b) and the intensity of the bands of the labeling (input; left lanes in Figure 3b)

[b] the cleavage efficiencies were determined by gel band densitometry, and calculated by the ratio of the intensity of the bands by chemical cleavage (middle lanes, Figure 4) and the total amount released (sum of intensities of the middle and right lanes, Figure 4).