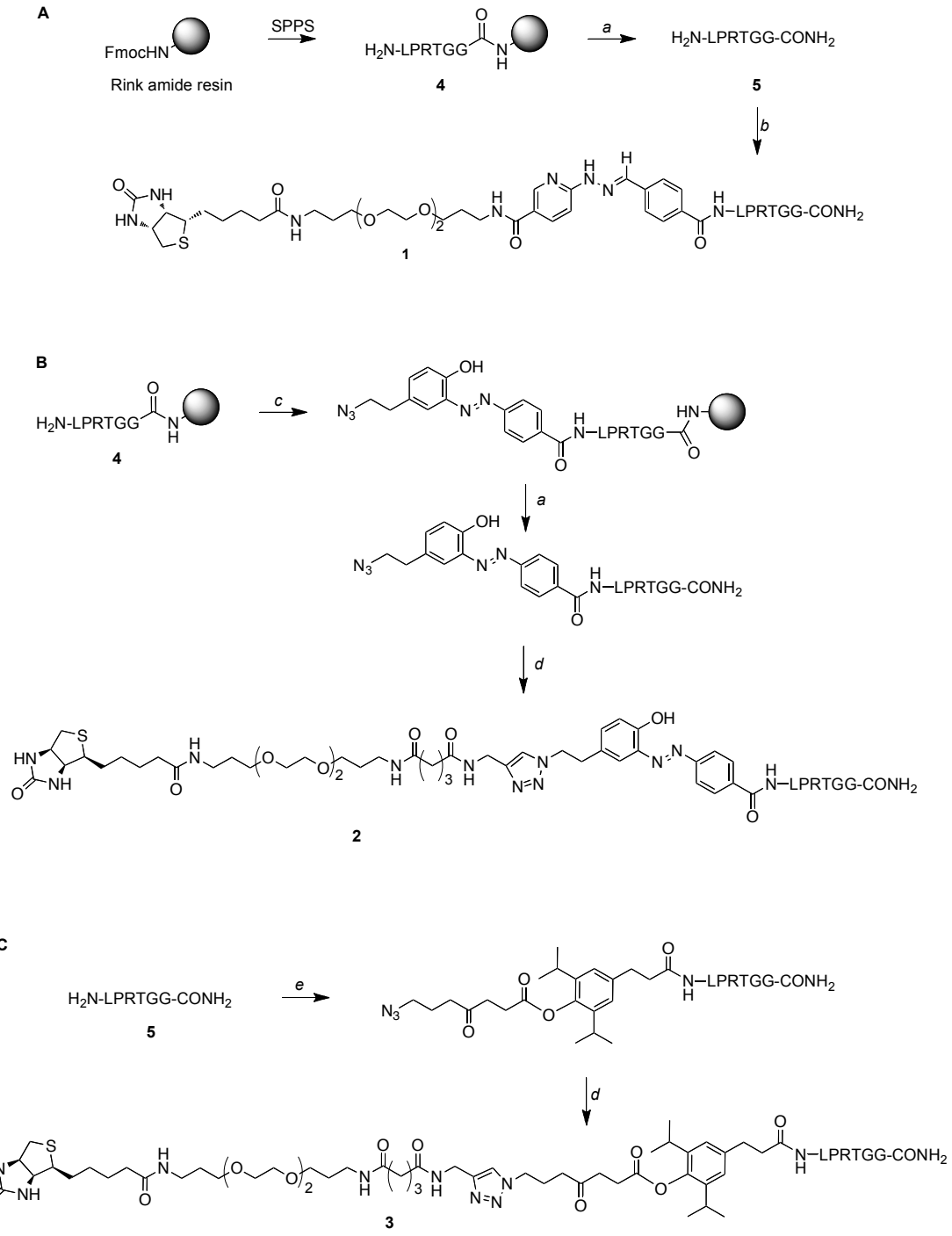
SUPPORTING INFORMATION

Synthesis of the peptides

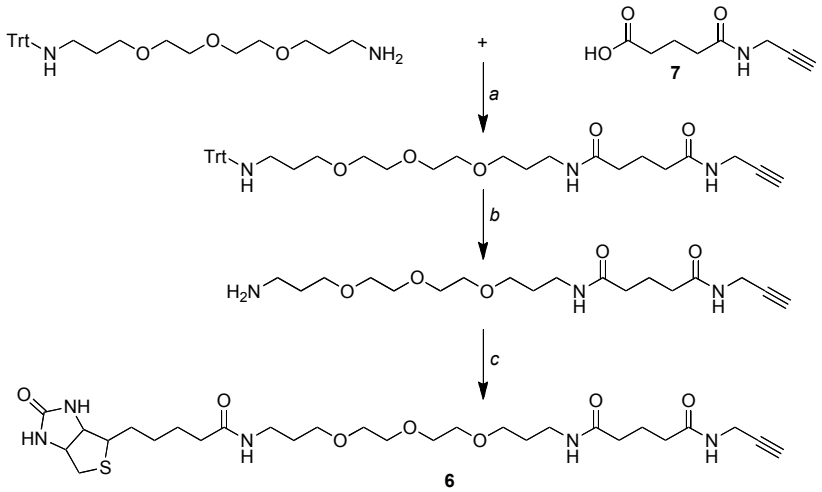
Peptides **1-3** were synthesized by acylating the N-terminal amine of peptides **4** and **5**, which were prepared by standard solid phase peptide synthesis, with the appropriate cleavable linkers and subsequent introduction of the biotin group. Acylation of LPRTGG peptide **5** with the commercially available *N*-hydroxysuccinimide ester of the bisarylhydrazone cleavable linker afforded peptide **1** (Scheme 1A). Peptides **2** (Scheme 1B) and **3** (Scheme 1C) were synthesized by coupling azide-bearing versions of previously reported azobenzene and levulinoyl ester linker to resin-bound peptide **4** or in solution to peptide **5**, respectively. Conjugation of biotin-PEG-alkyne **6** (synthesis see scheme 2) to the resulting azido precursors gave peptides **2** and **3**.

Scheme 1. Synthesis of cleavable linker containing peptides.



Reagents and conditions: (a) 95% TFA, 2.5% TIS, 2.5% H_2O, 2h, rt; (b) CBL-354, DiPEA, DMF, 16h, rt; (c) azobenzene, HBTU, HOBt, DiPEA, DMF, 16h, rt; (d) alkyne **6**, tBuOH, H_2O, $CuSO_4$, sodium ascorbate, 16h, 37°C; (e) Lev-OSu, DiPEA, DMF, 16h, rt.

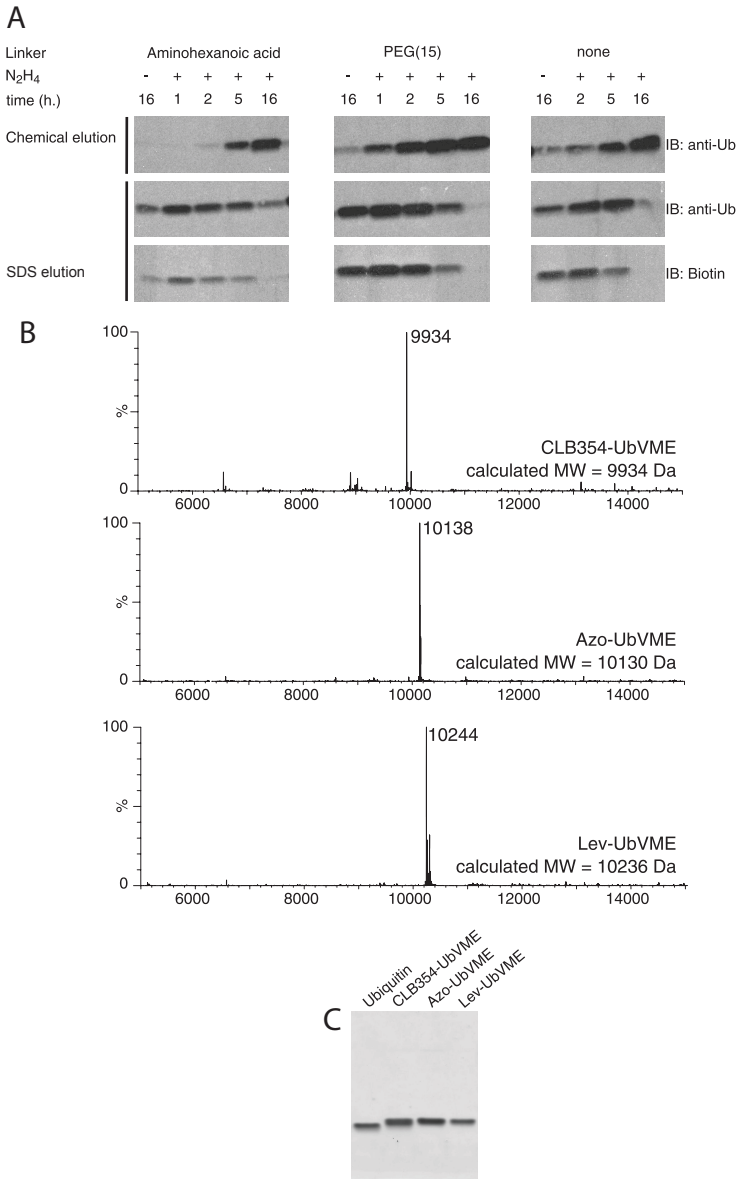
Scheme 2. Synthesis of alkyne **6**.



Reagents and conditions: (a) EDC(I), HOBt, DiPEA, DMF, 16h, rt; (b) TFA/CH_2Cl_2, TIS; (c) EDC(I), HOBt, DiPEA, DMF, 16h, rt.

Similar probes were synthesized without spacer, with an aminohexanoic acid spacer or with the described PEG spacer (synthesis not shown). These probes were sortagged onto ubiquitin and the resulting cleavable linker containing ubiquitin derivatives were used in a pulldown experiment (SI Fig 1A). The ubiquitin was incubated with streptavidin-agarose and subsequently washed to remove any unbound ubiquitin. Next, the resin was treated with hydrazine to initiate cleavage. The eluted product was loaded on gel and blotted using an anti-ubiquitin antibody (top panel SI Fig 1A). Boiling in sample buffer eluted the material remaining of the streptavidin-sepharose beads and the resulting material was also run on SDS-PAGE (lower panels SI Fig 1A). These experiments revealed that 80% of Biotin-PEG-ubiquitin eluted after 5h, whereas the Biotin-ubiquitin construct and the Biotin-Ahx-ubiquitin construct only showed ~50% cleavage. We therefore decided to add an PEG spacer to all the different biotin-[cleavable-linker]-UbVME probes. We transacylated peptides **1-3** onto G_3UbVME as described in the main manuscript. Purification of the resulting product by reverse phase HPLC was followed by LC/MS analysis of the products (SI Fig 1B) and we subsequently analyzed the purity of products by SDS-PAGE (SI Fig 1C).

SI Fig 1 (A) Influence of the spacer length on the cleavage efficiency. Analysis of Cleavable linker containing UbVME by (B) mass-spectrometry and (C) gel electrophoresis.



General experimental.

All chemicals were of commercial sources and were used as received. Fmoc-Gly-OH, Fmoc-Thr-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, *O*-benzotriazole-*N*,*N*,*N*',*N*'-tetramethyl-uronium hexafluorophosphate (HBTU), benzotriazol-1-yl oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from

EMD Biosciences/Novabiochem. Rink amide resin was purchased from Advanced Chemtech. (E)-4-((5-(2-azidoethyl)-2-hydroxyphenyl)diazenyl)benzoic acid was synthesized as described by Hang and co-workers.[32] Bisaryl hydrazone linker (Chromalink™ biotin labeling reagent) was purchased from solulink. Levulinoyl cleavable linker was a generous gift from the Overkleeft lab.

Water used in biological procedures or as a reaction solvent was purified using a MilliQ purification system (Millipore). DriSolv® anhydrous CH_2Cl_2, DriSolv® anhydrous MeOH, DriSolv® anhydrous DMF were purchased from EMD Chemicals. Redistilled, anhydrous *N,N'*- diisopropylethylamine (DiPEA), trifluoroacetic acid (TFA), triisopropylsilane (TIS) *N*-methylpyrrolidone (NMP) was obtained from Sigma-Aldrich.

Mass Spectrometry. LC-ESI-MS analysis was performed using a Micromass LCT mass spectrometer (Micromass® MS Technologies, USA) and a Paradigm MG4 HPLC system equipped with a HTC PAL autosampler (Michrom BioResources, USA) and a Waters Symmetry 5 μm C8 column (2.1 x 50 mm, MeCN:H_2O (0.1% formic acid) gradient mobile phase, 150 μL/min).

HPLC/FPLC. HPLC purifications were achieved using an Agilent 1100 Series HPLC system equipped with a Waters Delta Pak 15 μm, 100 Å C18 column (7.8 x 300 mm, MeCN:H_2O gradient mobile phase, 3 mL/min) using the gradient indicated below. Cation exchange chromatography was performed on a Pharmacia AKTA Purifier system equipped with a Mono S 5/50 GL column (Amersham).

UV-vis Spectrocopy. UV-vis spectroscopy was performed on a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA).

General procedure for the solid phase peptide synthesis of the probes.

Rink-amide resin was solvated in NMP and after removal of the Fmoc-group by treating the resin with 20% piperidine in NMP, the resin was loaded and elongated using the consecutive steps. (I) The resin was washed with NMP (3x), CH_2Cl_2 (3x) and NMP. (II) Fmoc-protected amino acids were condensed under the agency of HOBt (3 equiv.), PyBOP (3 equiv.) and DiPEA (6 equiv.). (III) The resin was washed

again using the same conditions as in step (I). (IV) The coupling was monitored using Kaiser test and if complete, (V) the Fmoc-protective group was removed using 20% piperidine in NMP.

In the final step, the peptides were cleaved off resin by agitating the resin in the presence 95%TFA, 2.5% TIS, 2.5% H_2O for 3h. Ice-cold Et_2O was added to the cleavage solution and the formed precipitate was collected by centrifugation of the solution for 30 min at 4°C. The crude pellet was purified by reverse phase HPLC purification (buffers used: A: H_2O, B: ACN, C: 10% TFA in H_2O).

Biotin-PEG(15)-alkyne

Glutaric acid propargylamide **7**(89 mg, 0.525 mmol) was dissolved in DMF (2.5 mL). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC(I)) (101 mg, 0.525 mmol) and hydroxybenzotriazole (HOBt) (71 mg, 0.525 mmol) were added. The reaction was stirred for 10 min and subsequently *O*-(*N*-Trt-3-aminopropyl)-*O*'-(3-aminpropyl)-diethyleneglycol (231 mg, 0.5 mmol) was added. TLC analysis revealed complete consumption of the starting material after stirring overnight. The solution was concentrated *in vacuo*, redissolved with EtOAc and washed with 1M aqueous HCl, saturated aqueous $NaHCO_3$. The organic layer was dried, concentrated in vacuo, redissolved in TFA/CH_2Cl_2 containing 1% triisopropylsilane. The reaction was stirred until TLC analysis revealed complete conversion of the starting material. The solution was concentrated in vacuo, coevaporated with toluene to remove traces of TFA. The crude product was added to a premixed solution of biotin (134 mg, 0.55 mmol), EDC (101 mg, 0.525 mmol) and HOBt (71 mg, 0.525 mmol) in DMF. The reaction was stirred overnight, diluted with EtOAc, and washed with 1M aqueous HCl, saturated aqueous $NaHCO_3$. The organic layer was dried, concentrated in vacuo and the product was precipitated form Et_2O and used as crude in the following reactions.

Biotin-CLB-LPRTGG-CONH$_2$ (1)

H_2N-Leu-Pro-Arg-Thr-Gly-Gly-$CONH_2$ (16.3 mg, 22.9 μmol), synthesized according the general procedure, was dissolved in DMF (250 μL). CLB (10 mg, 12.3 μmol) and N,N-diisopropylethylamine (20 μL, 115 μmol) were added. The reaction was stirred overnight, concentrated *in vacuo.* RP-HPLC purification (19-34% B in 20 min (5 CV)) gave the title compound (7.9 mg, 6.1 μmol, 50%) as a white solid.
LC/MS: R_t 8.04 min; linear gradient 5→45% B in 10 min; ESI/MS: *m/z* = 1294.69 $(M+H)^+$

Biotin-Azo-LPRTGG-$CONH_2$ (2)

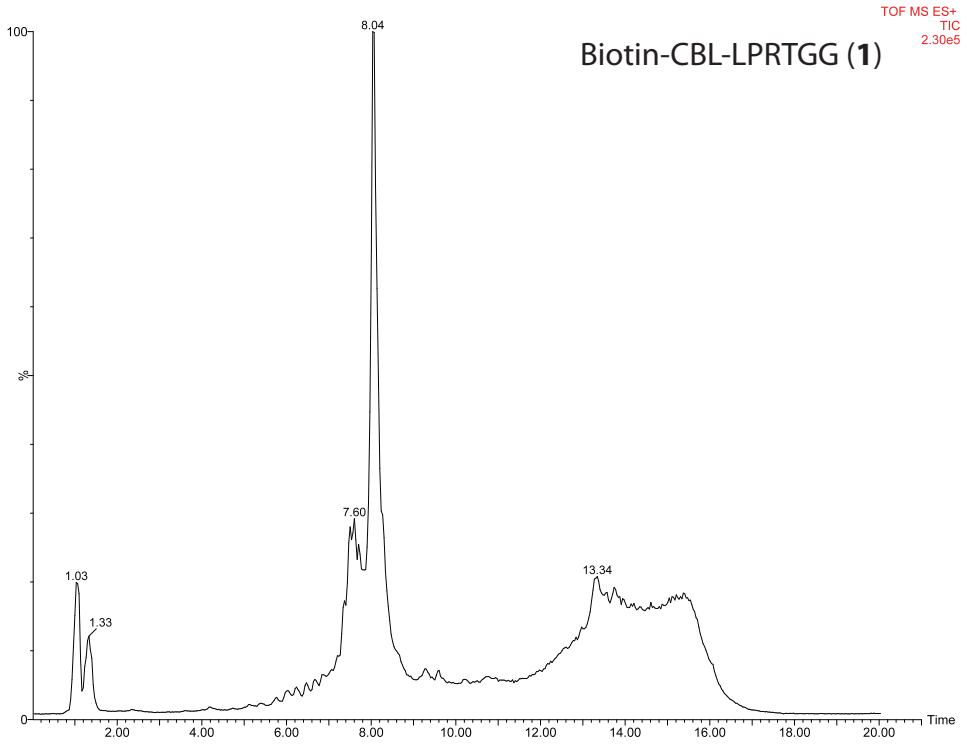
Rink amide resin (50 μmol) was loaded with Fmoc-Glyc-OH, elongated with the appropriately protected amino acids and cleaved off the resin as described in the general method. For the final coupling (E)-4-((5-(2-azidoethyl)-2-hydroxyphenyl)diazenyl)benzoic acid was used. After coupling overnight, the resin was washed with NMP, 20% piperidine in NMP, NMP and CH_2Cl_2. The peptide was cleaved from the resin using the conditions described in the general method affording the crude peptide (63%, 35.5 mg, 31.7 μmol). The crude peptide (16.6 mg, 20 μmol) was dissolved in *tert*-butanol (0.25 mL) and H_2O (0.25 mL). Biotin-PEG-alkyne **4** (15 mg, 25 μmol), $CuSO_4$ (8 μL, 1M in H_2O) and sodium ascorbate (12 μL, 1M in H_2O) were added and the reaction was incubated overnight at 37°C. The peptide was purified by RP-HPLC (22-34% B in 16 min (4 CV)) gave the title compound **2** (9.7 mg, 6.5 μmol, 33%) as an orange solid.
LC/MS: R_t 9.00 min; linear gradient 5→45% B in 10 min; ESI/MS: *m/z* = 1489.72 $(M+H)^+$.

Biotin-Lev-LPETGG-$CONH_2$ (3)

H_2N-Leu-Pro-Arg-Thr-Gly-Gly-$CONH_2$ (10 mg, 16.8 μmol), synthesized according the general procedure, was dissolved in CH_2Cl_2 (250 μL). 4-(3-((2,5-dioxopyrrolidin-1-yl)oxy)-3-oxopropyl)-2,6-diisopropylphenyl 7-azido-4-oxoheptanoate (6.5 mg, 12.6 μmol) and N,N-diisopropylethylamine (13 μL, 74.6 μmol) were added. When LC/MS showed complete conversion, the reaction was concentrated *in vacuo*, redissolved in

DMF and Biotin-PEG-alkyne **4** (18 mg, 30 μmol), $CuSO_4$ (20 μL, 1M in H_2O) and sodium ascorbate (30 μL, 1M in H_2O) were added. After incubating overnight at 37°C, the product was purified by RP-HPLC (25-40% B in 20 min (5 CV)) gave the title compound **3** (8.0 mg, 5.7 μmol, 34%) as a white solid.

LC/MS: R_t 9.84 min; linear gradient 5→45% B in 10 min; ESI/MS: *m/z* = 1595.87 $(M+H)^+$.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14.00
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18.00
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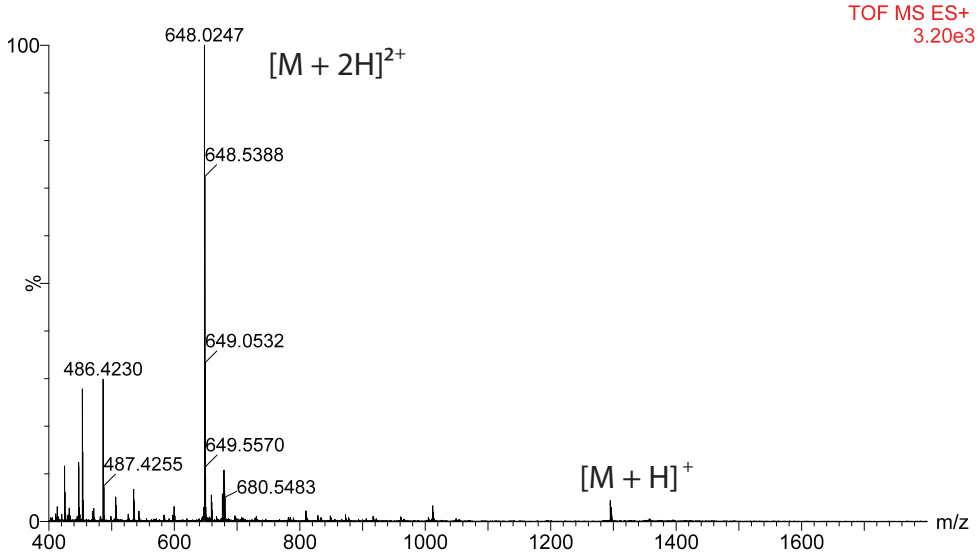
Time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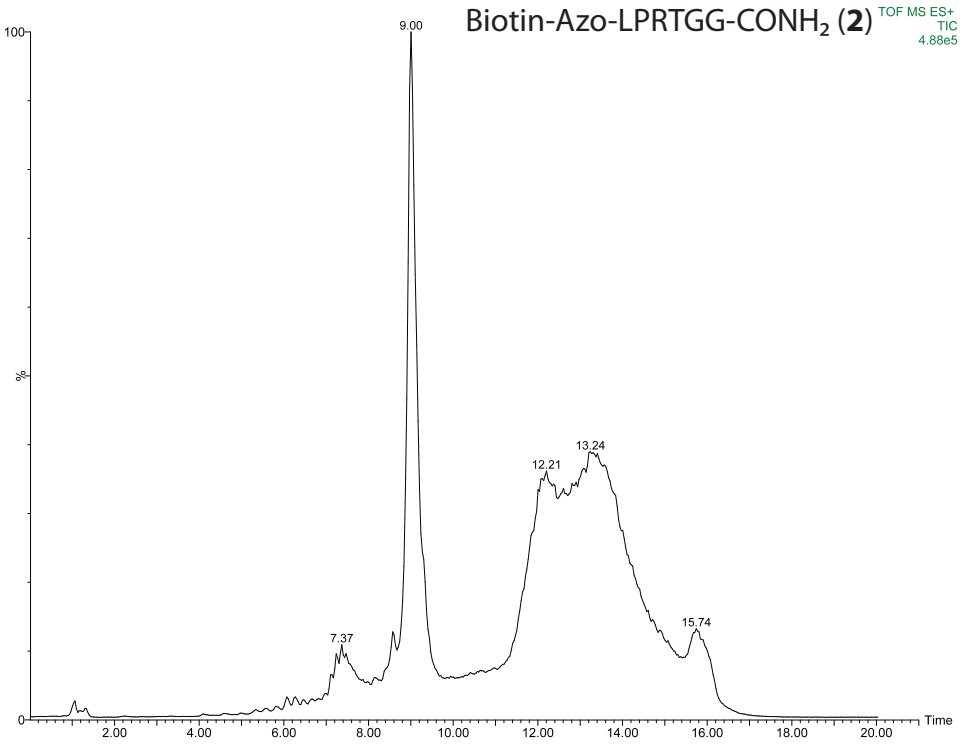
m/z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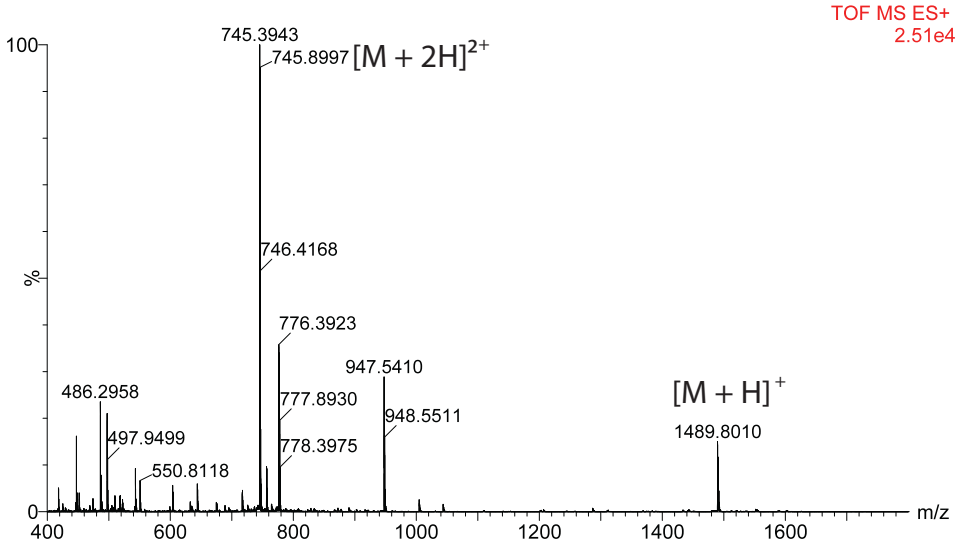
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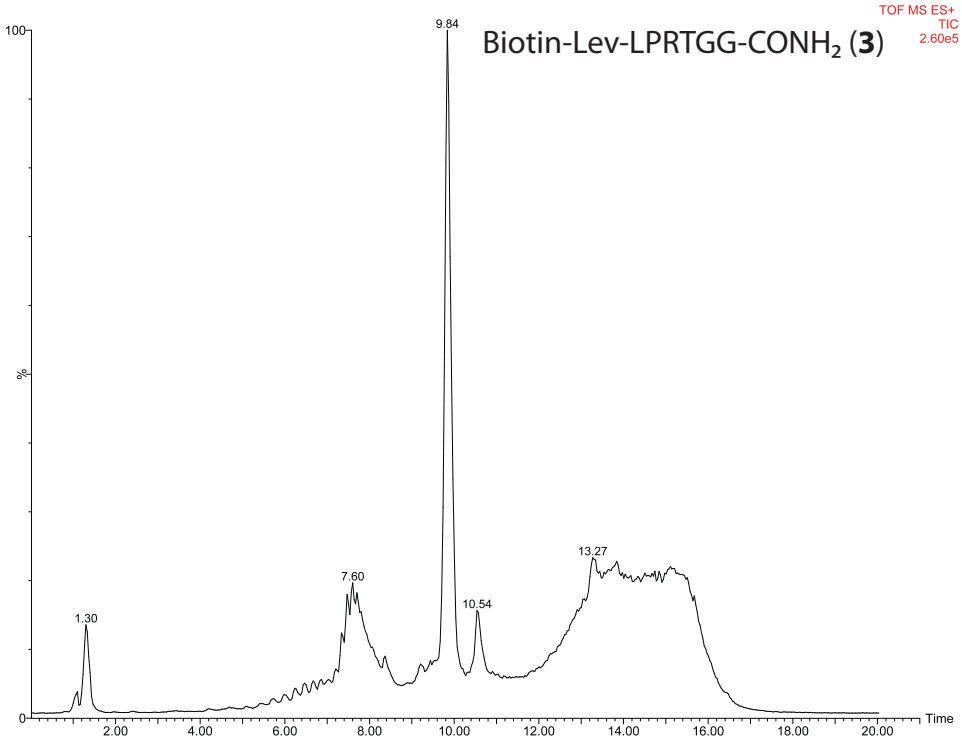
m/z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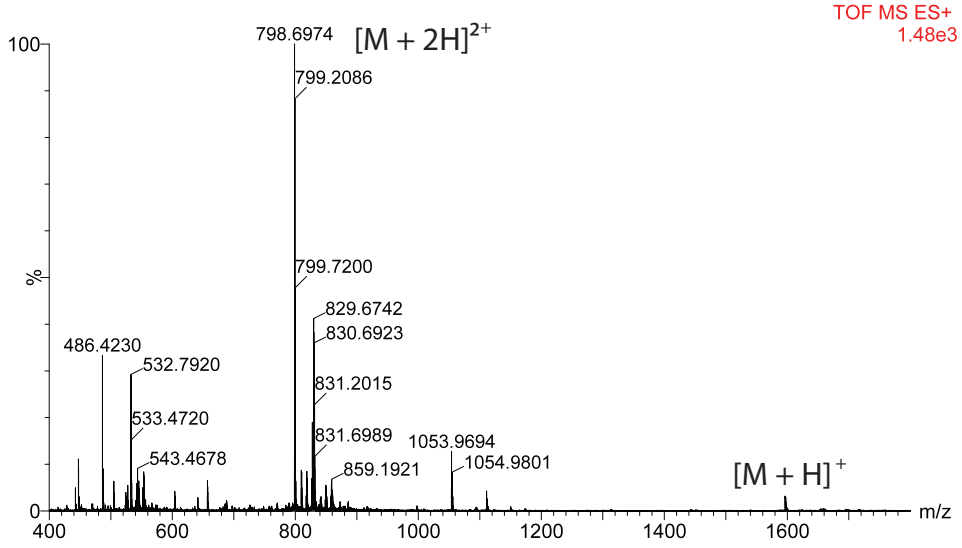
Time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m/z