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Supporting Information

# Generation of the UFM1 Toolkit for Profiling UFM1-Specific Proteases and Ligases

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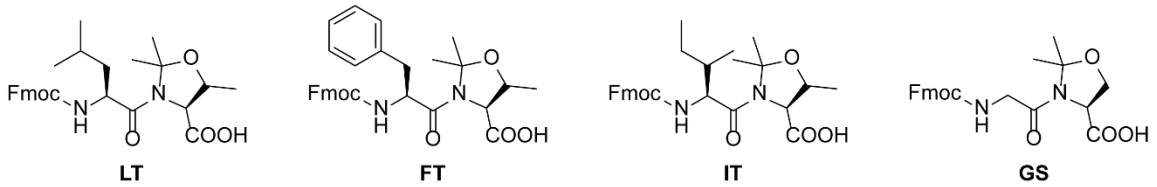
# Table of Contents

A.




B.

| | |
|---|---|
| UFM1 | MSKVSFKIT**L_T**SDPRLPYKV LSVPESTP**FT** AVLKFAAEEF |
| (83AA) | KVPAATSAI**I_T**NDGIGINPA QTAGNVFLKH **GS**ELRIIPRD RVG |

**Figure S1: A)** *Building blocks (dipeptides) used in synthesis of UFM1.* ***B)*** *Amino acid sequence of UFM1. The position of the incorporated dipeptides in the sequence are indicated bold in the sequence.*

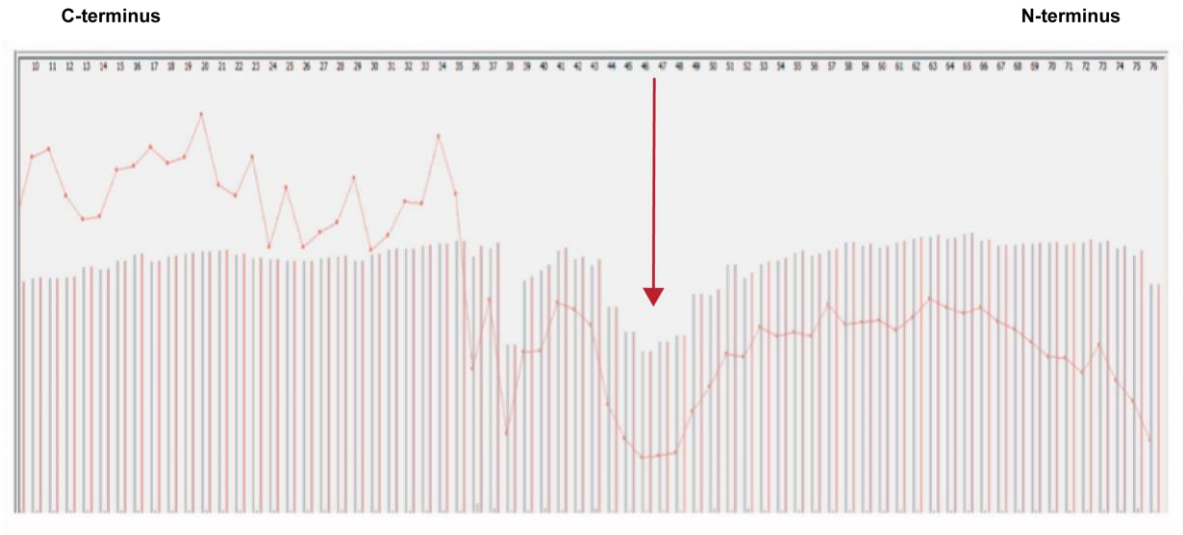



***Figure S2:*** **A)** *UV- traces of the FMOC deprotection during linear SPPS synthesis of UFM1. Red arrow indicates major inefficient coupling-position and start of synthesis is indicated by a black arrow. To circumvent this issue, we synthesized two fragments: a N-terminal fragment (AA 1-44) and a C-terminal fragment (AA 45-83) and ligated them using native chemical ligation (NCL). (LC-MS analysis of UFM1-PA generated by linear synthesis is shown in Figure S10).*

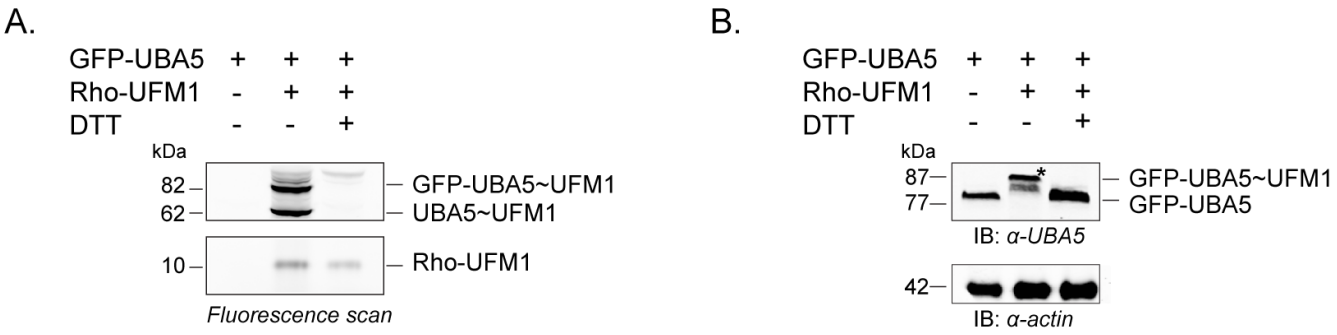



***Figure S3:*** *UBA5~Rho-UFM1 thioester formation* visualized *by* ***A)*** *in-gel fluorescence scanning and* ***B)*** *corresponding immunoblots against Uba5.*

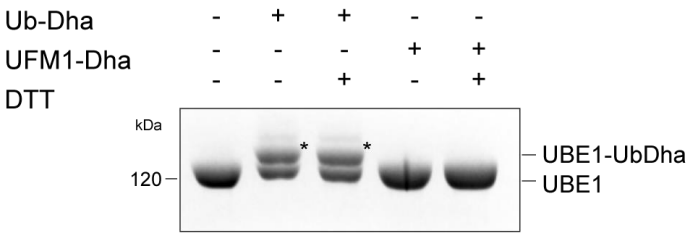



**Figure S4:** *UBE1 does not recognize UFM1-Dha. Coomassie-stained gel showing that UBE1 reacts with Ub-Dha but not with UFM1-Dha.*

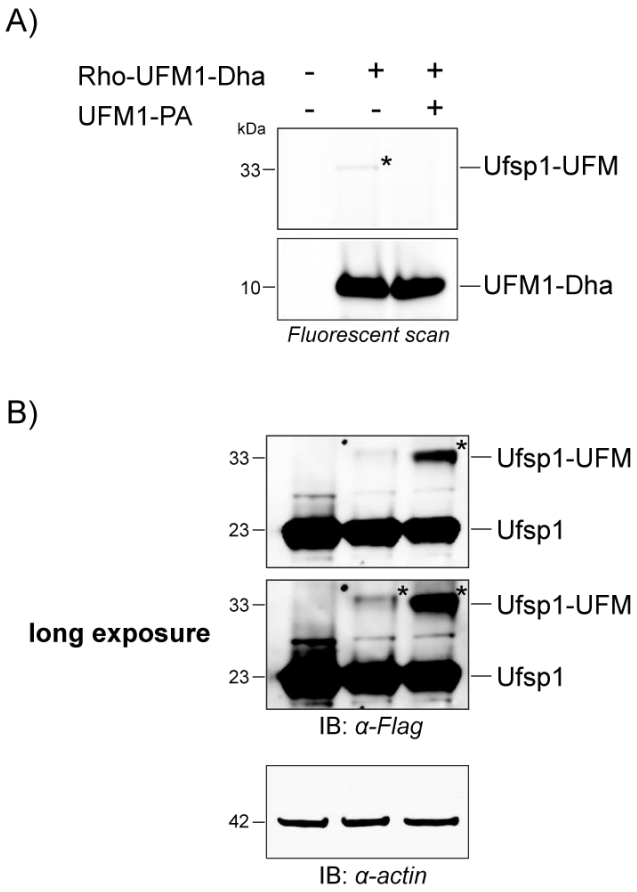



***Figure S5:*** *Competition experiments reveal preference for UFM1-PA over UFM1-Dha. A) Fluorescence scan showing that only a minor portion of ectopically overexpressed Flag-Ufsp1 reacts with Rho-UFM1-Dha (8). B) Corresponding immunoblot against the Flag-tagged protease reveals that the enzyme prefers UFM1-PA (9) over Rho-UFM-Dha (8), as becomes clear after prolonged exposure of the immunoblot. Differences in reactivity of Ufsp1 towards UFM1-PA and UFM1-Dha may be due to the unique active-site configuration of this protease[1]. Asterisks (*) denote the labeled enzymes.*

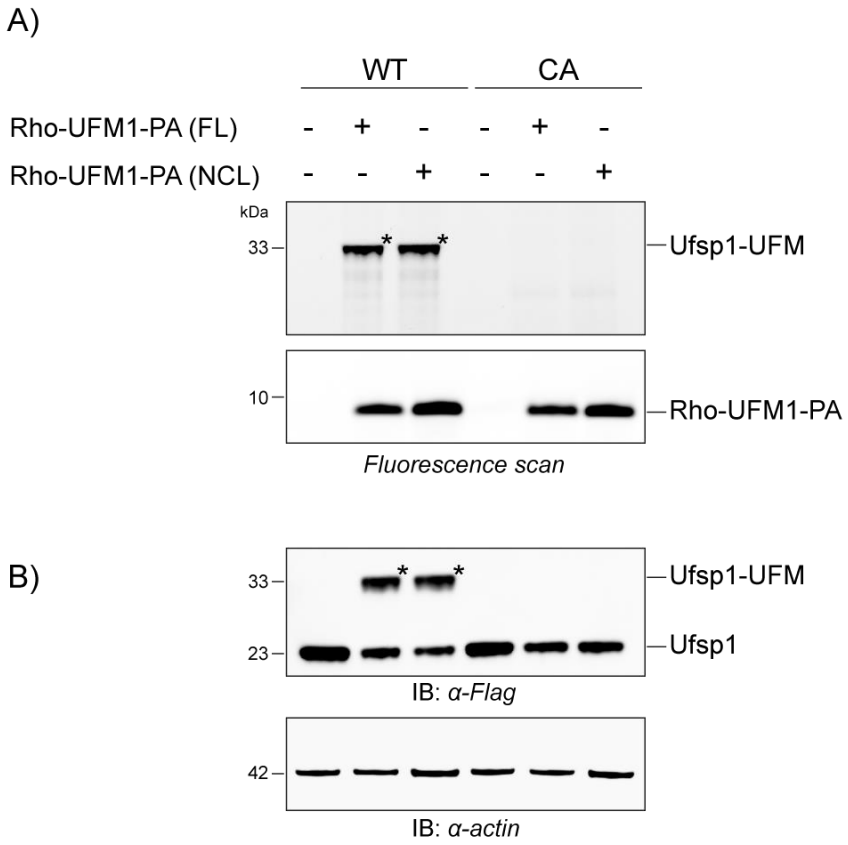



***Figure S6:*** *Both UFM1 PA generated by native chemical ligation (NCL) and by linear synthesis (FL) react with catalytically active murine Flag-Ufsp1. FL= UFM1 generated by linear synthesis; NCL = UFM1 generated by NCL* ***(6)****.*

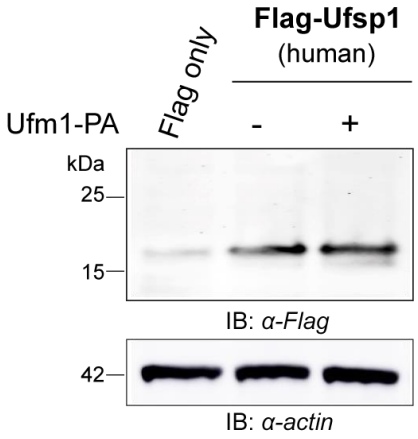



***Figure S7:*** *Human Ufsp1 is unreactive towards UFM1-PA as confirmed by immunoblot.*

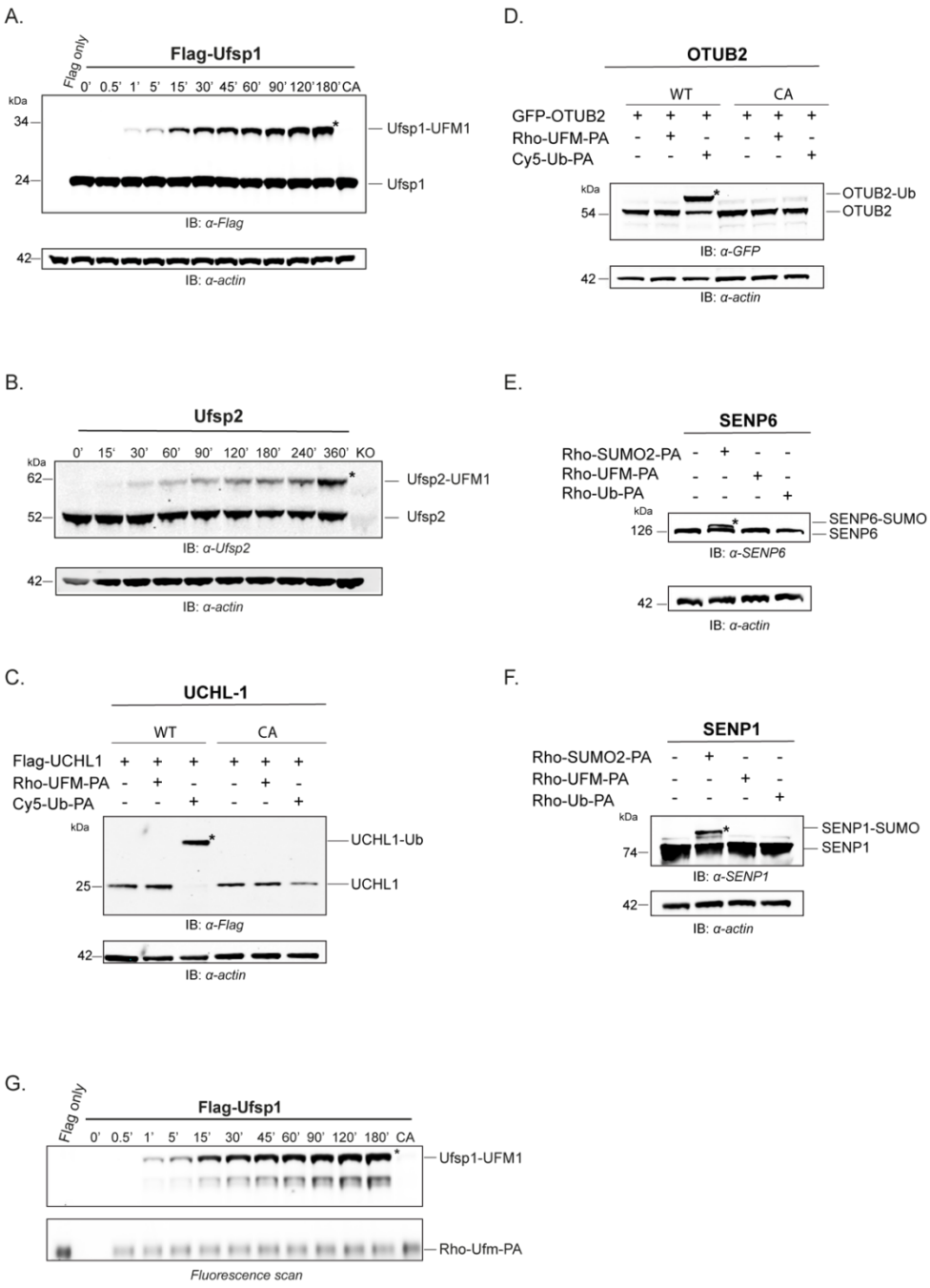



***Figure S8:*** *Assessment of UFM1-PA reactivity and specificity against a panel of cysteine protease subfamilies.* ***A)*** *Immunoblot showing time-dependent reactivity of Flag-Ufsp1 (murine) towards Rho-UFM1-PA (10) and time-dependent labeling of endogenous Ufsp2 (human).* ***C-D)*** *Flag-UCHL1 and GFP-OTUB2 don't react with UFM1-PA.* ***E-F)*** *Flag-SENP6 and -SENP1 are unreactive towards UFM1-PA.* ***G)*** *Corresponding fluorescent scans for time-dependent Flag-Ufsp1 labeling with Rho-UFM1-PA.*

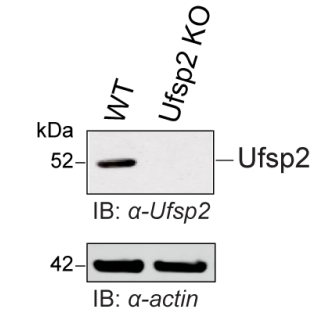



***Figure S9:*** *Validation of the Ufsp2 knockout cell line (Ufsp2 KO) assessed by immunoblotting against Ufsp2 confirming the CRISPR-CAS mediated knockout.*

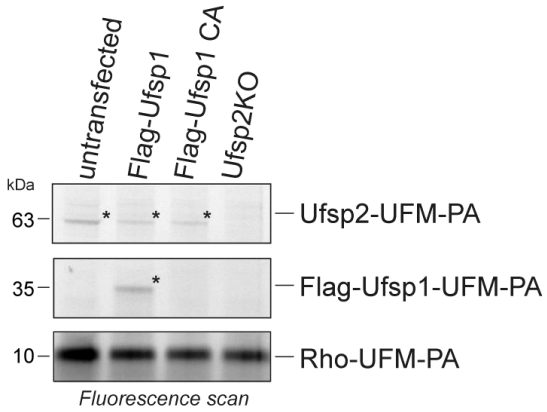



***Figure S10:*** *In-gel Fluorescence scans visualizing labeling of murine Flag-Ufsp1 or inactive Flag-Ufsp1 (C53A) following electroporation with Rho-UFM1-PA into HeLa cells ectopically expressing murine Flag-Ufsp1 or Flag-Ufsp1(C53A). Additionally, labeling of endogenous Ufsp2 can be detected, while no reactivity is observed in the Ufsp2 KO cells. Probe-labeled enzymes are indicated with an asterisk (*).*

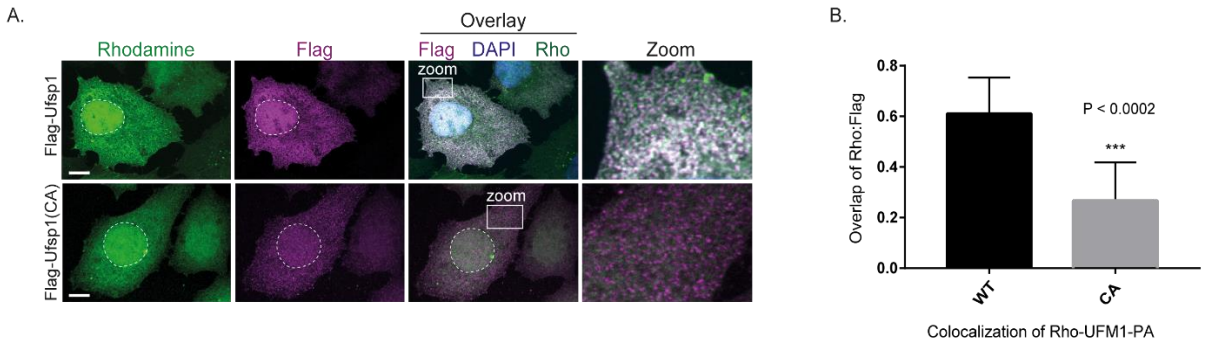



***Figure S11:*** *Reactivity of HeLa cells electroporated with Rho-UFM1-PA in the absence or presence of ectopically expressed murine Flag-Ufsp1.* ***A)*** *Confocal images of HeLa cells in the presence of Flag-Ufsp1 or Flag-Ufsp1 (C53A) after probe electroporation.* ***B)*** *Quantification of colocalization (Mander's overlap coefficient) of Rho-UFM1-PA with Flag-Ufsp1 (WT) or the mutant Flag-Ufsp1 (CA)(n= 2, error bars correspond to SD, with significance (p) calculated using a two-sided t-test).*

# Chemical synthesis

## Solid Phase Peptide Synthesis (SPPS)

### Linear Synthesis Strategy

Solid Phase Peptide Synthesis (SPPS) was performed using standard 9-fluorenylmethoxycarbonyl (Fmoc)-based solid phase chemistry on a 25 µmol scale on an Syro II (Multisyntech) peptide synthesizer. Synthesis was initiated on an Fmoc-Val-TentagGel R TRT resin (Rapp Polymere, Cat# RA1201). Fmoc-protected amino acids were coupled using four-fold excess relative to the pre-loaded Fmoc amino acid trityl resin (0.2 mmol/g) using double couplings in N-Methyl-2-pyrrolidone (NMP) for 40 min followed by a second coupling of 60 min using PyBOP (4 eq) and DiPEA (8 eq) at room temperature. After coupling each amino acid, Fmoc deprotection was performed in 20% piperidine in NMP for 3x4/15 min in cycle 1 and 2x10/10 min for the rest of the cycles at RT. To facilitate synthesis, dipeptides were incorporated wherever possible: Fmoc-Leu-Thr(Novabiochem/Merck Millipore; Fmoc-Ile-Thr(psiMe,Mepro)-OH; Cat#8521930025), Fmoc-Gly-Ser (Novabiochem / Merck Millipore; Fmoc-Gly-Ser-(psiMe, Mepro)-OH; Cat#: 8.52200.0005), Fmoc-Gln-Thr (Novabiochem /Merck Millipore; Fmoc-Leu-Ser(psiMe,Mepro)-OH; Cat#8.52179.0005 CAS 339531-50-9), and Fmoc-Glu-Gly Novabiochem / Merck Millipore; Fmoc-Asp(OtBu)-(Dmb)Gly-OH; Cat#8521150005 CAS 900152-72-9). In the case of the UFM1-PA probe, Gly83 was omitted to allow coupling of propargylamine, which was performed as described previously[2].

### Native Chemical Ligation Strategy

Solid Phase Peptide Synthesis (SPPS) was performed using standard 9-fluorenylmethoxycarbonyl (Fmoc)-based solid phase chemistry on a 25µmol scale on an Intavis MultipPep CF peptide synthesizer. For the synthesis of the N-terminal UFM1 fragment ($UFM1_{1-44}$), synthesis was initiated on an Fmoc-Ala-Tentagel R TRT resin (Rapp Polymere, Cat# RA1201). Fmoc-protected amino acids were coupled using four-fold excess relative to the pre-loaded Fmoc amino acid trityl resin (0.2 mmol/g) using couplings in N-Methyl-2-pyrrolidone (NMP) for 40 min followed by a second coupling of 60 min using four-fold excess of Fmoc protected amino acid, PyBOP (4 eq) and DiPEA (8 eq) at room temperature. After coupling each amino acid, Fmoc

deprotection was performed in 20% piperidine in NMP for 3x4/15 min in cycle 1 and 2x10/10 min for the rest of the cycles at RT. To facilitate synthesis, Leu 10 and Thr 11 were coupled as a dipeptide LT (Novabiochem/Merck Millipore; Fmoc-Ile-Thr(psiMe,Mepro)-OH; Cat#8521930025). Fmoc-L-norleucine (CHEM IMPEX INT'L, Cat#02440; CAS 77284-32-3) was incorporated at the N-terminus to circumvent methionine oxidation during downstream chemistry. For the C-terminal fragment of UFM1 ($UFM1_{45-82}$), synthesis was initiated on an Fmoc-Val-Tentagel TRT resin (Rapp Polymere, Cat#RA1227) followed by double couplings and Fmoc-deprotection as described above. Dipeptides were incorporated wherever possible: Fmoc-Gly-Ser (Novabiochem/Merck Millipore; Fmoc-Gly-Ser-(psiMe,Mepro)-OH; Cat#: 8.52200.0005), Gln-Thr (Novabiochem/Merck Millipore; Fmoc-Leu-Ser(psiMe,Mepro)-OH; Cat#8.52179.0005 CAS 339531-50-9), and Fmoc-Glu-Gly Novabiochem /Merck Millipore; Fmoc-Asp(OtBu)-(Dmb)Gly-OH; Cat#8521150005 CAS 900152-72-9). To permit native chemical ligation, Ala45 in the wild type sequence was replaced by cysteine. For the UFM1-PA and UFM1-Dha activity-based probes, Gly83 was omitted to allow coupling of propargylamine, or a S-benzyl-L-cysteine methyl ester (Cys(Bn)-OMe) in the case of UFM1-Dha.

**Native Chemical Ligation reactions:**

UFM1-thioester peptide **(1)** (1.3 mg) and cysteinyl-UFM1 peptide **(3)** (1.5 mg) were dissolved in 100 µL Gdn•HCl buffer (8M) and 10 µL MPAA (1M) and 10 µL TCEP (1M) were added after which the pH was adjusted to 7.6. The reaction was agitated at $37^0$C for 30 min after which LC-MS analysis showed complete consumption of the N-terminal thioester and formation of the NCL-product.

**Desulphurisation reactions:**

The reaction was diluted to 10 mL in water and spun down using an Amicon spinfilter (MWCO 10 kD) to 1 mL. This procedure was repeated two times after which the remaining suspension was taken up in 4 mL (Gdn•HCl (8M)/ TCEP (1M), 4:1 v/v). Glutathione (32 mg/mL) and VA044 (31 mg/mL) were added, the pH adjusted to 6.5 and desulphurization was accomplished by agitation overnight at 37 $^0$C. HPLC purification followed by lyophilisation of the appropriate fractions, followed by SEC purification using 20 mM TRIS, 150 mM NaCl buffer at pH 7.6 yielded the final compounds.

**RP-HPLC purifications**

Shimadzu semi-preparative RP-HPLC system, equipped with a Waters C18-Xbridge 5 µm OBD (10 x 150 mm) column at a flowrate of 6.5 mL/min. using 2 mobile phases: A: MQ + 0.05% TFA, B: $CH_3CN$ + 0.05 % TFA. Gradient: 10 -> 70% B.

**Gel filtration**

Size Exclusion Chromatography was performed on a Sephadex S75 10/300 column (GE Healthcare), using a 20 mM TRIS, 150 mM NaCl buffer at pH 7.6. Appropriate fractions were pooled and concentrated using an Amicon spinfilter (MWCO 10 kD) to a final concentration of ca. 1 mg/mL

**LC-MS measurements:**

Waters 2795 Separation Module (Alliance HT) using a Phenomenex Kinetex C18-column (2.1x50, 2.6 µm), Waters 2996 Photodiode Array Detector (190-750 nm) and LCT$^{TM}$ ESI-Mass Spectrometer. Samples were run using 2 mobile phases: A = 1% $CH_3CN$, 0.1% formic acid in water and B = 1% water and 0.1% formic acid in $CH_3CN$. Flow rate= 0.8 mL/min, runtime= 6 min, column T= 40°C. Gradient: 0 - 95% B. Data processing was performed using Waters MassLynx Mass Spectrometry Software 4.1 (deconvolution with MaxEnt1 function).

**HRMS-measurements:**

High resolution mass spectra were recorded on a Waters Acquity H-class UPLC with XEVO-G2 XS Q-TOF mass spectrometer equipped with an electrospray ion source in positive mode (source voltage 3.0 kV, desolvation gas flow 900 L/hr, temperature 250°C) with resolution R = 22000 (mass range m/z = 50-2000) and 200 pg/uL Leu-Enk (m/z = 556.2771) as a lock mass.

**Cysteinyl peptides:**

**Cys-UFM$_{46\text{-}83}$ (3)**

ESI MS$^+$ (amu) calcd: 4105.70, found 4105.83, rt: 1.29 min. **HRMS**: $[C_{177}H_{295}N_{55}O_{55}S + 3H]^{3+}$: found 1369.4028, calc. 1369.3995, $[C_{177}H_{295}N_{55}O_{55}S + 4H]^{4+}$: found

1027.3020, calc. 1027.3016, $[C_{177}H_{295}N_{55}O_{55}S + 5H]^{5+}$: found 822.0464, calc. 822.0428, $[C_{177}H_{295}N_{55}O_{55}S + 6H]^{6+}$: found 685.2041, calc. 685.2037.

**Cys-UFM$_{46-82}$PA (5)**
ESI MS$^+$ (amu) calcd: 4085.71, found 4085.92, rt: 1.30 min. **HRMS**: $[C_{177}H_{295}N_{55}O_{55}S + 3H]^{3+}$: found 1362.7411, calc. 1362.7363, $[C_{177}H_{295}N_{55}O_{55}S + 4H]^{4+}$: found 1022.3049, calc. 1022.3042, $[C_{177}H_{295}N_{55}O_{55}S + 5H]^{5+}$: found 818.0503, calc. 818.0449, $[C_{177}H_{295}N_{55}O_{55}S + 6H]^{6+}$: found 681.8771, calc. 681.8721.

**Thioester peptides:**

**UFM$_{1-45}$S-methylpropionate (1)**
ESI MS$^+$ (amu) calcd: 4955.88, found 4956.18, rt: 1.47 min. **HRMS**: $[C_{233}H_{370}N_{52}O_{64}S + 3H]^{3+}$: found 1652.9109, calc. 1652.9113, $[C_{233}H_{370}N_{52}O_{64}S + 4H]^{4+}$: found 1239.6851, calc. 1239.6847, $[C_{233}H_{370}N_{52}O_{64}S + 5H]^{5+}$: found 991.9526, calc. 991.9493, $[C_{233}H_{370}N_{52}O_{64}S + 6H]^{6+}$: found 826.9634, calc. 826.9595, $[C_{233}H_{370}N_{52}O_{64}S + 7H]^{7+}$: found 708.9703, calc. 708.9664.

**RHO-UFM$_{1-45}$S-methylpropionate (2)**
ESI MS$^+$ (amu) calcd: 5312.22, found 5312.68, rt: 1.49 min. **HRMS**: $[C_{254}H_{382}N_{54}O_{68}S + 3H]^{3+}$: found 1771.6080, calc. 1771.6045, $[C_{254}H_{382}N_{54}O_{68}S + 4H]^{4+}$: found 1328.9521, calc. 1328.9553, $[C_{254}H_{382}N_{54}O_{68}S + 5H]^{5+}$: found 1063.3638, calc. 1063.3658, $[C_{254}H_{382}N_{54}O_{68}S + 6H]^{6+}$: found 886.3071, calc. 886.3062, $C_{254}H_{382}N_{54}O_{68}S + 7H]^{7+}$: found 759.8373, calc. 759.8350.

**UFM1 derivatives:**

**UFM1 (6)**
Isolated yield: 2.05 mg, 0.22 µmol (85.7%). ESI MS$^+$ (amu) calcd: 8909.37, found 8911.00, rt: 1.43 min. **HRMS**: $[C_{406}H_{657}N_{107}O_{117} + 5H]^{5+}$: found 1782.7858, calc. 1782.7856, $[C_{406}H_{657}N_{107}O_{117} + 6H]^{6+}$: found 1485.8250, calc. 1485.8226, $[C_{406}H_{657}N_{107}O_{117} + 7H]^{7+}$: found 1273.7136, calc. 1273.7063, $[C_{406}H_{657}N_{107}O_{117} + 8H]^{8+}$: found 1114.6235, calc. 1114.6190, $[C_{406}H_{657}N_{107}O_{117} + 9H]^{9+}$: found 990.8897, calc. 990.8844, $[C_{406}H_{657}N_{107}O_{117} + 10H]^{10+}$: found 891.9011, calc. 891.8967, $[C_{406}H_{657}N_{107}O_{117} + 11H]^{11+}$: found 810.9100, calc. 910.9069, $[C_{406}H_{657}N_{107}O_{117} +$

12H]$^{12+}$: found 743.4201, calc. 743.4153, [$C_{406}H_{657}N_{107}O_{117}$ + 13H]$^{13+}$: found 686.3077, calc. 686.3070.

**RHO[110]-UFM1 (7)**

Isolated yield: 2.06 mg, 0.22 µmol (78.6%). ESI MS$^+$ (amu) calcd: 9265.02, found 9266.00, rt: 1.43 min. **HRMS**: [$C_{427}H_{669}N_{109}O_{121}$ + 6H]$^{6+}$: found 1545.1616, calc. 1545.1693, [$C_{427}H_{669}N_{109}O_{121}$ + 7H]$^{7+}$: found 1324.5773, calc. 1324.5748, [$C_{427}H_{669}N_{109}O_{121}$ + 8H]$^{8+}$: found 1159.1305, calc. 1159.1289, [$C_{427}H_{669}N_{109}O_{121}$ + 9H]$^{9+}$: found 1030.4490, calc. 1030.4489, [$C_{427}H_{669}N_{109}O_{121}$ + 10H]$^{10+}$: found 927.5087, calc. 927.5047, [$C_{427}H_{669}N_{109}O_{121}$ + 11H]$^{11+}$: found 843.2809, calc. 943.2777, [$C_{427}H_{669}N_{109}O_{121}$ + 12H]$^{12+}$: found 773.0917, calc. 773.0886, [$C_{427}H_{669}N_{109}O_{121}$ + 13H]$^{13+}$: found 713.6972, calc. 713.6978, [$C_{427}H_{669}N_{109}O_{121}$ + 14H]$^{14+}$: found 662.7930, calc. 662.7913.

**UFM1-PA (9)**

Isolated yield: 1.28 mg, 0.14 µmol (32.5%). ESI MS$^+$ (amu) calcd: 8921.44, found 8921.00, rt: 1.44 min. **HRMS**: [$C_{407}H_{657}N_{107}O_{115}S$ + 6H]$^{6+}$: found 1487.8208, calc. 1487.8197, [$C_{407}H_{657}N_{107}O_{115}S$ + 7H]$^{7+}$: found 1275.4229, calc. 1275.4180, [$C_{407}H_{657}N_{107}O_{115}S$ + 8H]$^{8+}$: found 1116.1199, calc. 1116.1167, [$C_{407}H_{657}N_{107}O_{115}S$ + 9H]$^{9+}$: found 992.2168, calc. 992.2157, [$C_{407}H_{657}N_{107}O_{115}S$ + 10H]$^{10+}$: found 893.0991, calc. 893.0949, [$C_{407}H_{657}N_{107}O_{115}S$ + 11H]$^{11+}$: found 811.9999, calc. 811.9961, [$C_{407}H_{657}N_{107}O_{115}S$ + 12H]$^{12+}$: found 744.4134, calc. 744.4138, [$C_{407}H_{657}N_{107}O_{115}S$ + 13H]$^{13+}$: found 687.2245, calc. 687.2287.

**RHO[110]-UFM1-PA (10)**

Isolated yield: 3.04 mg, 0.33 µmol (76.7%). ESI MS$^+$ (amu) calcd: 9277.78, found 9278.00, rt: 1.45 min. **HRMS**: [$C_{428}H_{669}N_{109}O_{119}S$ + 5H]$^{5+}$: found 1856.3859, calc. 1856.3981, [$C_{428}H_{669}N_{109}O_{119}S$ + 6H]$^{6+}$: found 1547.1647, calc. 1547.1663, [$C_{428}H_{669}N_{109}O_{119}S$ + 7H]$^{7+}$: found 1326.2830, calc. 1326.2865, [$C_{428}H_{669}N_{109}O_{119}S$ + 8H]$^{8+}$: found 1160.6285, calc. 1160.6267, [$C_{428}H_{669}N_{109}O_{119}S$ + 9H]$^{9+}$: found 1031.7826, calc. 1031.7802, [$C_{428}H_{669}N_{109}O_{119}S$ + 10H]$^{10+}$: found 928.7054, calc. 928.7029, [$C_{428}H_{669}N_{109}O_{119}S$ + 11H]$^{11+}$: found 844.3685, calc. 844.3670, [$C_{428}H_{669}N_{109}O_{119}S$ + 12H]$^{12+}$: found 774.0875, calc. 774.0871, [$C_{428}H_{669}N_{109}O_{119}S$ + 13H]$^{13+}$: found 714.6212, calc. 714.6194. **CD-measurements**

Validation of the structural integrity of our synthetic UFM1 was done using circular dichroism (CD). After dissolving synthetic UFM1 (40 mg/mL) in DMSO, it was diluted into dd$H_2O$, vortexed briefly and added to 20 mM sodium phosphate buffer (pH 7.0) and buffer exchanged to 20 mM sodium phosphate buffer (pH 7.0) using a 3 kDa cut-off Amicon Ultra-15 Centrifugal Filter Unit (Millipore). In case of the expressed UFM1 and the UFM1 generated by native chemical ligation, proteins were directly buffer exchanged into 20mM sodium phosphate buffer (pH 7.0) using a 3 kDa cut-off Amicon Ultra-15 Centrifugal Filter Unit (Millipore). Final concentrations of synthetic and expressed UFM1 were determined by gel-quantification. Circular dichroism was measured using a JASCO J-815 CD Spectrometer at 25°C using samples diluted to approximately 10 µM final concentration. CD spectra were recorded ranging from 250 to 190 nm at a scan rate of 20 nm per minute and a scan width of 1 nm using a quartz cuvette with a 1 mm path length. Three cumulative measurements were averaged and plotted using Graphpad PRISM.

**Protein expression and purification**

UFM1 and UBA5 were expressed as N-terminal His-SUMO fusions in *E. coli* BL21(DE3) using autoinduction[3]. After reaching $OD_{600}$ 0.6, the temperature was lowered to 18°C and the bacteria were grown an additional 18h. Enzymes were purified using TALON beads (Clontech) equilibrated in Buffer A (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT), washed twice with Buffer A and eluted with 100mM imidazole (pH 7.5) For further purification, the enzymes were subjected to anion exchange (Resource Q, GE Healthcare) with a gradient of 1 M NaCL in Buffer A, followed by size exclusion chromatography (S75, 16/600, GE Healthcare). In the case of UFM1, the N-terminal His-SUMO tag was cleaved by incubation with 5 µM SENP2 for 1h at 4°C, followed by further purification using TALON beads (Clontech) and size exclusion chromatography using a Superdex S75 16/600 column (GE Healthcare).
Pure proteins were concentrated, aliquoted, flash frozen in liquid nitrogen, and stored at -80°C.

**Constructs**

Murine Ufsp1 was subcloned into a 2xFLAG-C1 vector (Clontech) at BglII/PstI restriction sites. Similarly, human Ufsp2 amplified from cDNA was cloned into a GFP-C1 vector (Clontech) at EcoRI/XhoI sites. UFM1 and UBA5 were amplified from cDNA, and cloned into an N-terminal $His_6$-SUMO LIC vector according to the standard protocol. The active-site cysteine (C53) in murine Ufsp1 was mutated to alanine according to the protocol of the Quik Change Site-directed Mutagenesis Kit (Invitrogen). All constructs were verified by sequencing. Human Ufsp1 was amplified from cDNA and cloned into a 2x-Flag-C1 vector (Clontech) using BglII/ PstI restriction sites.

**Mammalian cell lines**

HEK293T and HeLa cell lines used in this study originated from ATCC and were cultured under standard conditions in DMEM (Gibco) supplemented with 10% FCS (Sigma-Aldrich) at 37°C with 5% $CO_2$. CRISPR-mediated Ufsp2 depletion in Hela cells was performed by co-transfecting confluent HeLa with a vector harbouring the gRNA and the Cas9 and a construct conferring blasticidin resistance[4]. After blasticidin selection and clonal expansion, Ufsp2 depletion was verified immunoblotting against using anti-Ufsp2 antibody (1:1000 dilution, Abcam ab185965). Ufsp2 guide RNA (gRNA) was designed using the CRSIPR Design tool (http://crispr.mit.edu/), subcloned into a pX330-U6-Chimeric-BB-CBh-hSpCas9 vector (Addgene, plasmid # 42230)[5].
All cell lines were routinely tested for mycoplasma contamination with consistently negative outcome.

**Labeling of purified enzymes**

Thioester-formation of His-SUMO-UBA5 and UFM1, Rho-UFM1 or UFM1-Dha were assessed by incubating 1µM of His-SUMO-UBA5 with 5µM UFM1 in labeling buffer (50 mM Bis-Tris, pH 6.5, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM DTT and 0.4 mM ATP) at 30°C for 30 min. Reactions were quenched by addition of 3x SDS-PAGE loading Dye without addition of reducing agents and subsequently resolved by standard SDS-PAGE gel electrophoresis. Enzymes were visualized by in-gel fluorescence scanning ($\lambda_{em}$ / $\lambda_{ex}$ = 480/530 nm) followed by Coomassie staining. Labeling of recombinant $His_6$-UBE1 with Ub-Dha or UFM1-Dha was performed as described previously[6].

**Labeling of overexpressed enzymes in cell lysates**

For overexpression of UFM1 specific proteases and DUBs, Flag-Ufsp1 and the GFP-tagged DUBs[2] were transfected into HEK293T cells using polyethylenimine (PEI, Polysciences, Inc.) according to the manufacturer's recommendations. In case of the SENPs and the human Flag-Ufsp1, transfection of the DNA was performed using confluent HeLa cells using Effectene (Qiagen) according to the manufacturer's instructions. After 24h, cells were scraped into ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5% NP-40, 2 mM DTT, and a Protease inhibitor tablet (Roche)), briefly sonicated on ice and clarified by centrifugation. For all labeling reactions, 25 µL of lysate was incubated with 0.5 µg of Rho-UFM-PA at 37°C for the indicated time. Reactions were terminated by the addition of 3x SDS-PAGE loading buffer supplemented with 2-β-mercaptoethanol and boiled for 10 min at 95°C. Samples were resolved using standard SDS-PAGE gel and visualized by both fluorescence scanning ($\lambda_{em}$ / $\lambda_{ex}$ = 480/530 nm) and by immunoblotting. Following gel transfer onto nitrocellulose membranes, immunoblotting was performed using mouse anti-Flag (1:1000 dilution, Sigma) and mouse anti-β-actin (1:10000 dilution, Sigma) and probed using fluorescent secondary antibodies anti-mouse 800 (1:10000 dilution, LiCOR, 926-3210) and anti-rabbit-800 (1:10000, LiCOR, 926-3211) and visualized on the LICOR Odyssey system v3.0.

Transfection of the UFM1 conjugating enzyme GFP-UBA5 was performed in confluent HeLa cells using Effectene (Qiagen) according to the manufacturer's instructions. After 24h, cells were scraped into Co-IP buffer (50 mM Tris-HCl, pH 7.5, 5% glycerol, 10 mM $MgCl_2$, 0.5% NP-40, 1 mM DTT and Complete protease inhibitors (Roche)), resuspended and lysed by sonication on ice. Following clarification by centrifugation at maximum speed (4°C, 20 min, 20.000g), 30 µL cell lysate was incubated with 1µg Rho-UFM1, 0.4 mM ATP for 30 min at 37°C. Reactions were quenched by addition of either 3x SDS-PAGE loading buffer with or without the addition of 100mM 2-β-mercaptoethanol. Enzyme reactivity was visualized by resolving the samples using a standard SDS-PAGE gel and subsequent fluorescence scanning ($\lambda_{em}$ / $\lambda_{ex}$ = 480/530 nm). To visualize the proportion of labelled enzyme, proteins were transferred onto nitrocellulose and immunoblotted using rabbit anti-UBA5 (1:1.000 dilution, Abcam ab177478) or HRP-Flag (1:10.000 dilution, Sigma) and probe using either fluorescent secondary antibody anti-rabbit 800 (1:10.000 dilution, LiCOR, 926-3211) and visualized on the LiCOR Odyssey system or with Super Signal West Dura Extended

Duration Signal Substrate (ECL, Thermo Fisher) with subsequent visualization using the Amersham Imager AI600.

**Labeling of endogenous enzymes**

Cell lysates were prepared by resuspending cell pellets in three pellet volumes of HR Buffer (50 mM TRIS-HCl, pH 7.4, 5 mM $MgCl_2$, 250 mM sucrose, 1 mM DTT) and lysed by sonication on ice. After clarification by centrifugation (20,000 rpm, 4°C, 20 min), total protein concentration was determined using Nanodrop. For cell lysate experiments, 100 µg of lysate was incubated with 0.5 µg of Rho-UFM1-PA for the indicated time at 37°C. Reactions were quenched by the addition of 3x SDS-PAGE loading buffer supplemented with 2-β-mercaptoethanol and boiled for 10 min at 95°C. Samples were resolved by SDS-PAGE gel and probe reactivity assessed by fluorescence scanning ($\lambda_{em}$ / $\lambda_{ex}$ = 480/530 nm). Visualization of endogenous Ufsp2 was performed by immunoblotting flowing gel transfer to nitrocellulose membrane and probing with rabbit anti-Ufsp2 antibody (1:1000 dilution, Abcam ab185965).

Labeling with Rho-UFM1 or UFM1-Dha was performed using 30 µl cell lysates prepared as previously described and incubated with 1 ug Rho-UFM1 or Rho-UFM1-Dha in the presence or absence of 0.4 mM ATP for 60 minutes at 37°C. In the case of the Rho-UFM1-Dha labeling, 1mM ATP and 1mM $MgCl_2$ were added every 20 minutes to replenish consumed ATP. As negative controls, cell lysates were depleted of ATP by pre-incubation with Apyrase (Sigma Aldrich) at 37°C for 20 minutes prior to Rho-UFM1-Dha addition (ATP and $MgCl_2$ were omitted). After quenching the reactions using 3x SDS-PAGE sample buffer, the samples were resolved using a standard SDS-PAGE gel and subsequently imaged using fluorescence scanning ($\lambda_{em}$ / $\lambda_{ex}$ = 480/530 nm). To determine the proportion of active enzyme, the gels were transferred to nitrocellulose membranes and probed with rabbit anti-UBA5 antibody (1:1000 dilution, Abcam ab177478) and fluorescent secondary antibodies anti-rabbit-800 (1:10000, LiCOR, 926-3211).

**Electroporation experiments**

For electroporation experiments, 80.000 HeLa cells were seeded into a 6-well plate and transfected with murine Flag-Ufsp1 and the corresponding catalytically inactive mutant using Effectene (Qiagen), according to the manufacturer's instructions. To facilitate the incorporation of the probe, the growth medium was replaced 4-6 h after

transfection and refreshed again 1-2 h prior to electroporation. Following removal of the growth medium, cells were kept on ice for the duration of the protocol. Cells were washed twice with cold electroporation buffer (2 mM HEPES, pH 7.4, 15 mM $K_2HPO_4/KHPO_4$, 250 mM mannitol, 1 mM $MgCl_2$). 1.5 mL of a solution of Rho-UFM1 (or Rho-UFM1-PA, Rho-UFM1-Dha) in electroporation buffer (0.4 mg/mL) was added to each of the wells and electroporation was performed on ice using a Biorad GenePulser Xcell with CE and PE module Pulse Generator equipped with a Petri Pulser electroporation applicator (BTX) using the following settings: square wave, voltage = 75V, pulse length = 3ms, pulse interval = 1.5s, number of pulses = 5, cuvette width = 2 mm. The electroporation applicator was turned 90 degrees, and electroporation was repeated once. The probe solution was replaced by cold electroporation buffer, and cells were allowed to recover on ice for 2 min. After electroporation, cells were washed twice with ice-cold PBS and allowed to recover for 120 min under standard growth conditions. For gel-based analysis, samples were lysed using reducing SDS-PAGE loading buffer followed by brief sonication and heating at 98°C for 10 min before being separated on SDS-PAGE gel followed by visualization by fluorescence scanning ($\lambda_{ex}$ / $\lambda_{em}$ = 480/530 nm). Subsequently, western-blotting was performed as previously described, and membranes were probed with mouse anti-Flag (1:1000 dilution, Sigma) and mouse anti-β-actin (1:10000 dilution, Sigma).

**Confocal microscopy.** For microscopy experiments, the samples were fixed in 4% formaldehyde (Merck) in PBS and mounted onto glass slides (Thermo Scientific) using Prolong Gold mounting medium with DAPI (Invitrogen). Images were collected on a Leica SP8 confocal microscope equipped with HyD detectors, using a 63x oil-immersion magnification lens in combination with 2-4x digital zoom. Image processing and fluorescence intensity analysis were performed using ImageJ software and expressed in the form of Mander's overlap coefficients calculated using JaCoP.

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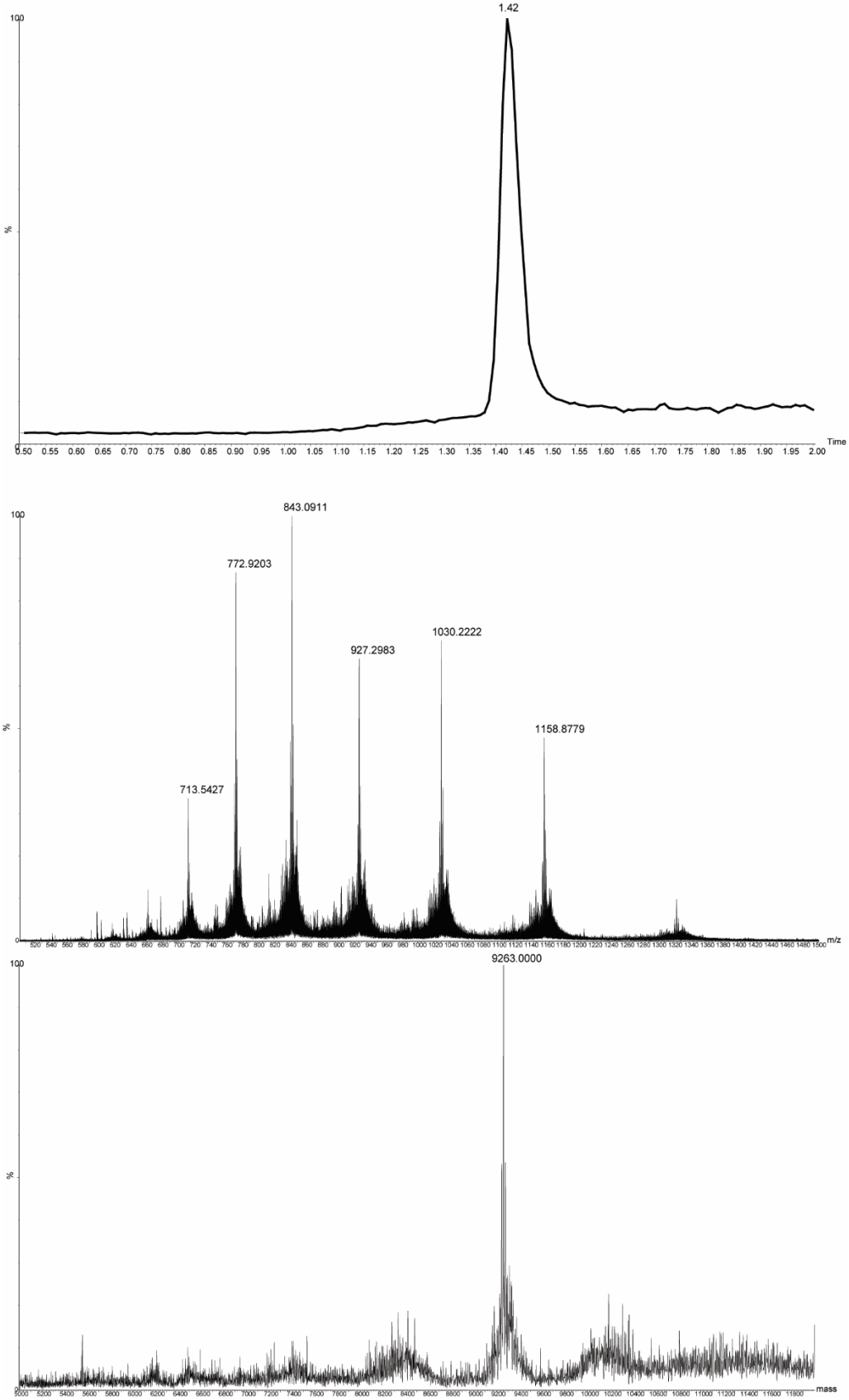


***Figure S12:*** *(HR)LC-MS analysis of Rho-UFM1-PA prepared by linear SPPS. Total Ion Count spectrum (top panel), ESI-MS spectrum (middle) and the deconvoluted mass (bottom panel). Calculated mass: 9263.94; Mass found: 9263.00.*

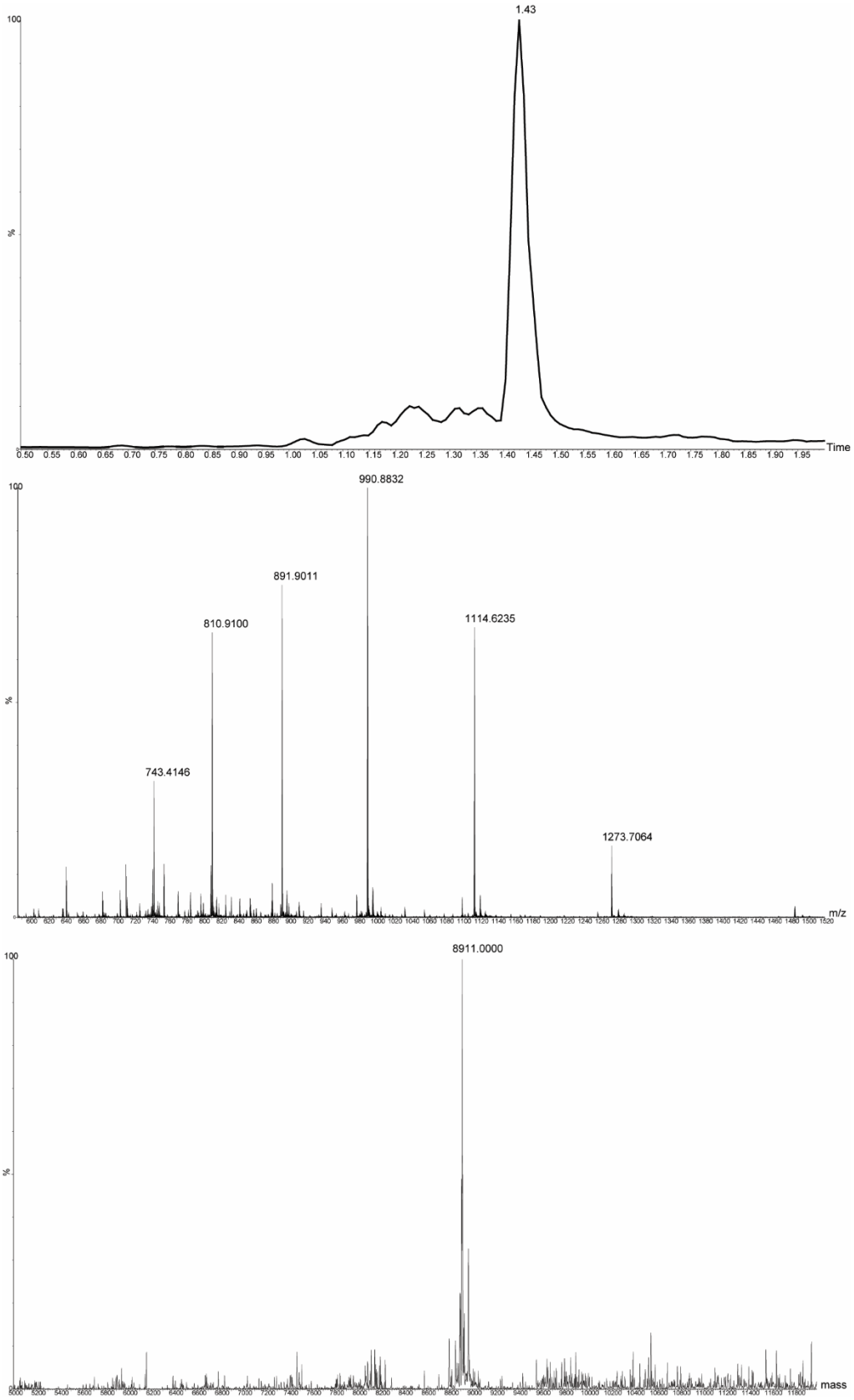


***Figure S13:*** *(HR)LC-MS analysis of UFM1 (6) prepared by NCL. Total Ion Count spectrum (top panel), ESI-MS spectrum (middle) and the deconvoluted mass (bottom panel). Calculated mass: 8909.37; Mass found: 8911.00*

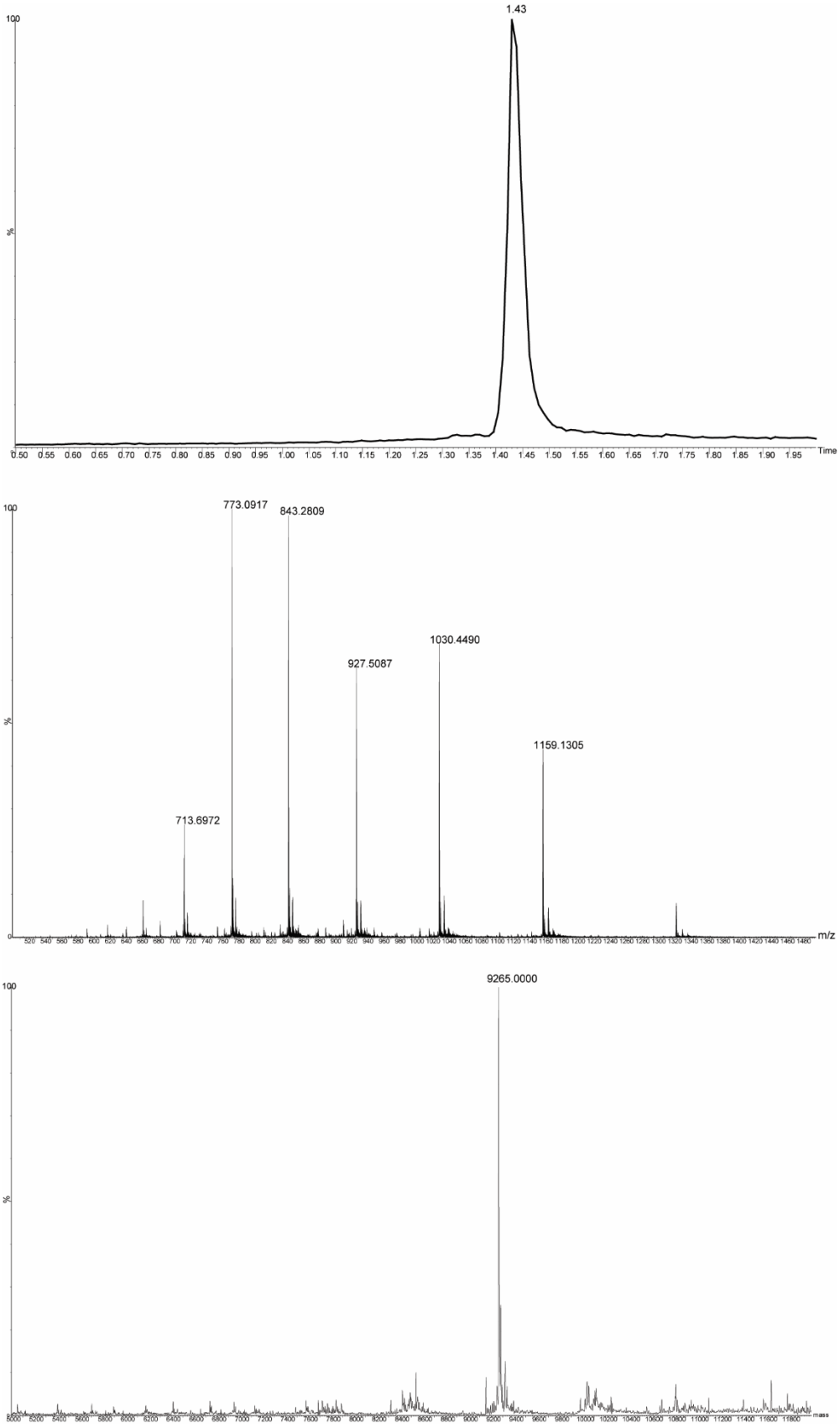


***Figure S14:*** *(HR)LC-MS analysis of Rho-UFM1 (7) prepared by NCL. Total Ion Count spectrum (top panel), ESI-MS spectrum (middle) and the deconvoluted mass (bottom panel). Calculated mass: 9265.02; Mass found: 9265.00*

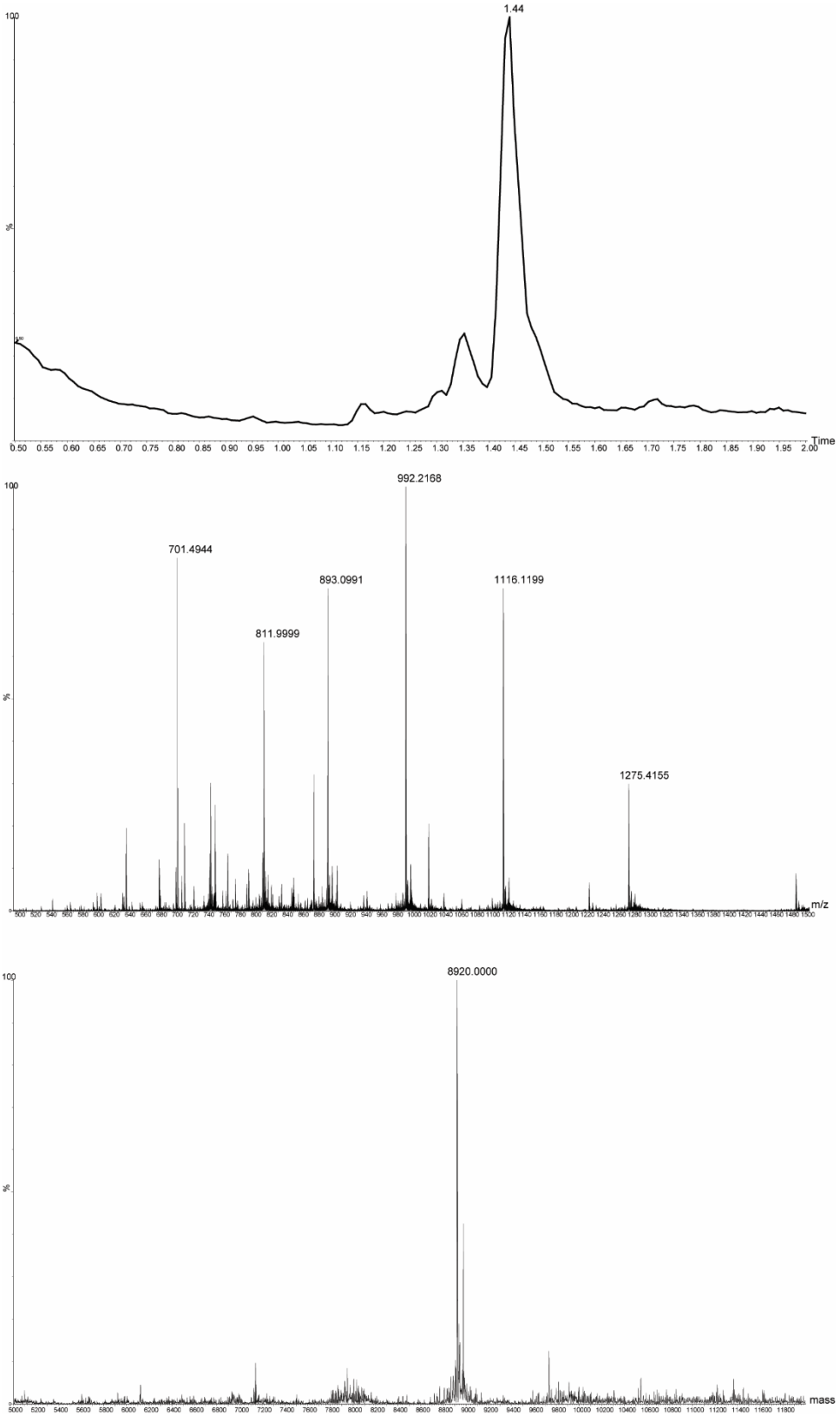


***Figure S15:*** *(HR)LC-MS analysis of UFM1-PA (9) prepared by NCL. Total Ion Count spectrum (top panel), ESI-MS spectrum (middle) and the deconvoluted mass (bottom panel). Calculated mass: 8921.44; Mass found: 8920.00*

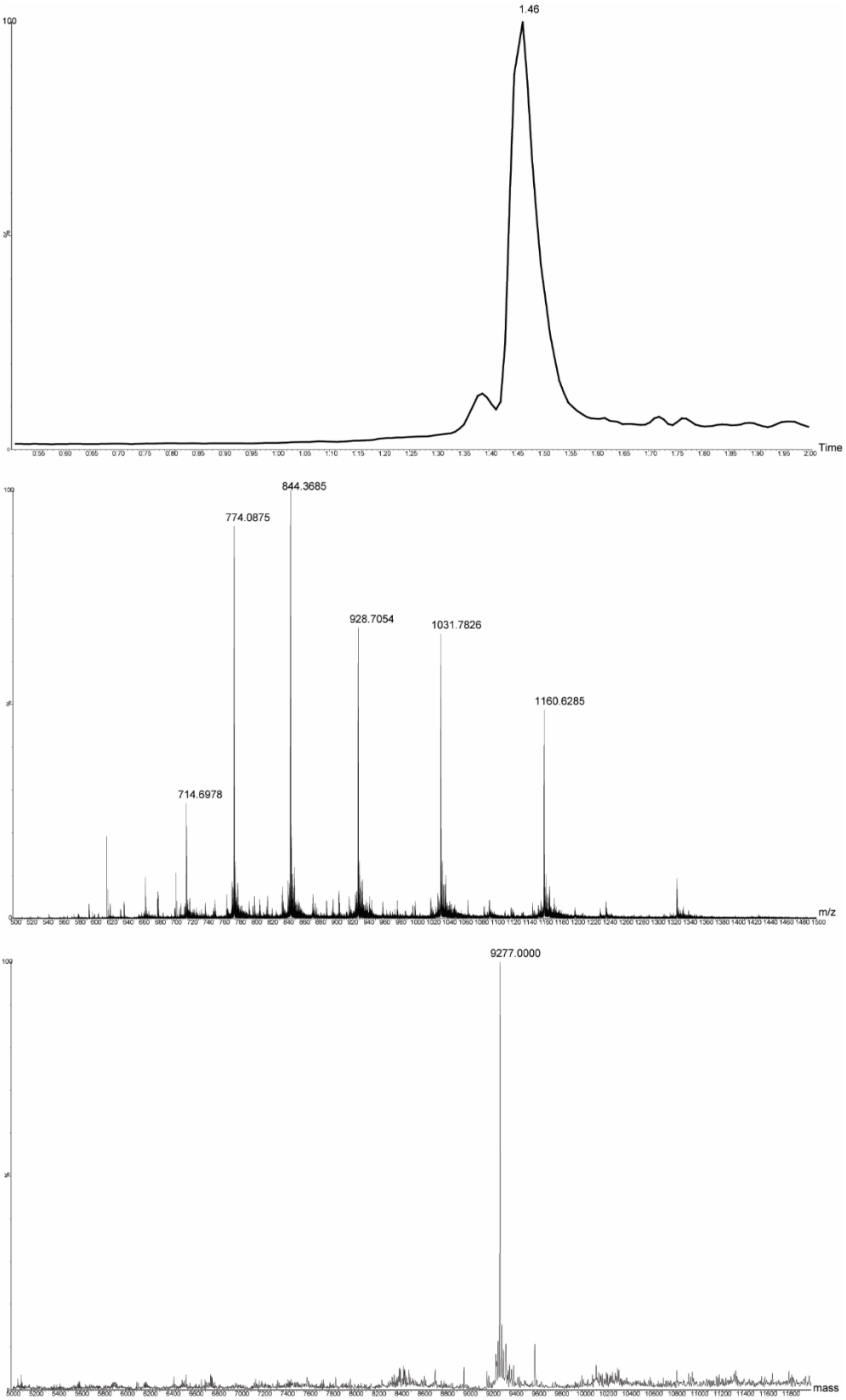


***Figure S16:*** *(HR)LC-MS analysis of Rho-UFM1-PA (10) prepared by NCL. Total Ion Count spectrum (top panel), ESI-MS spectrum (middle) and the deconvoluted mass (bottom panel). Calculated mass: 9277.78; Mass found: 9277.00*