Glycosylated Cell Penetrating Peptides, GCCPs

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Supporting figures

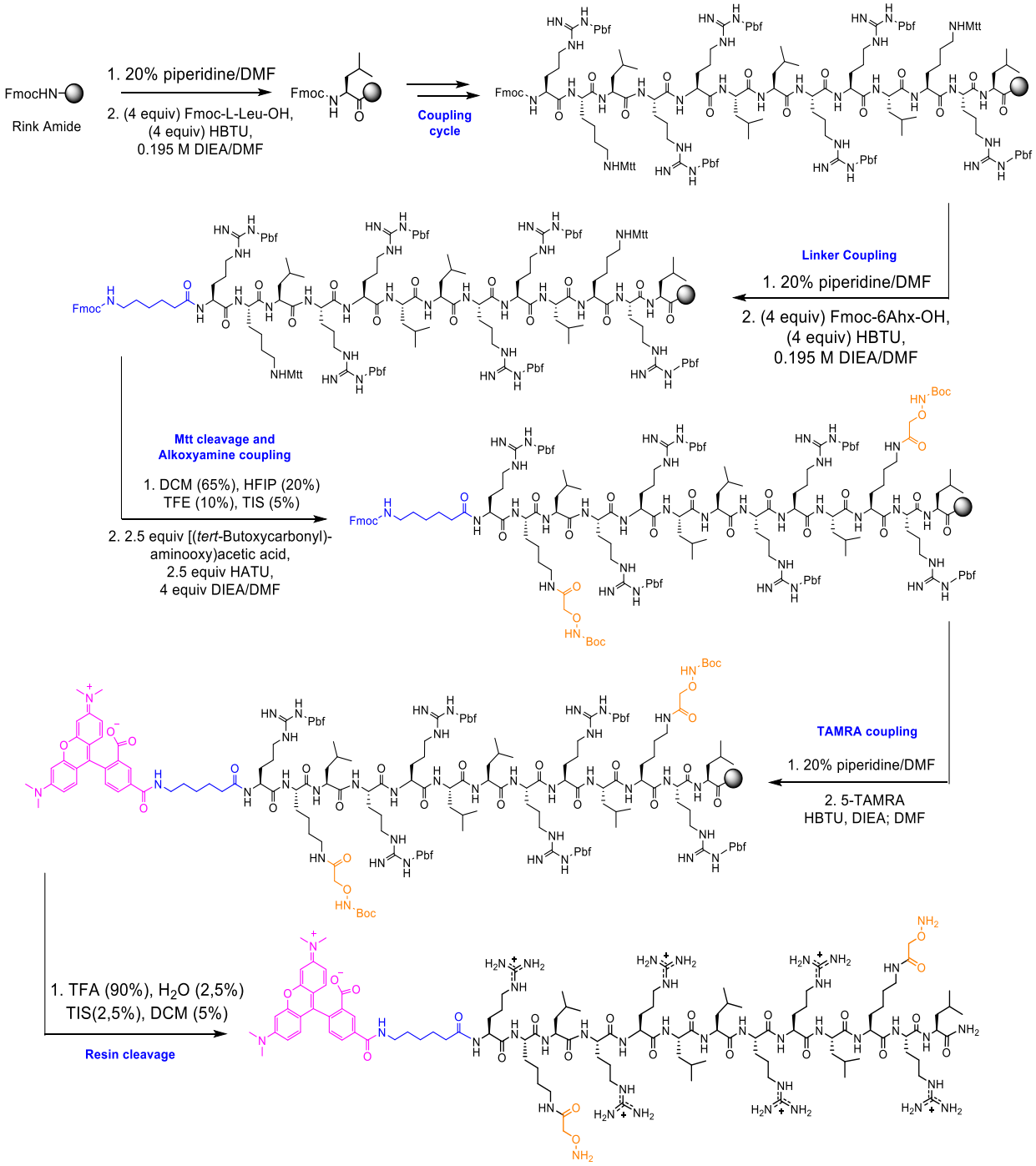

Figure S1. General synthetic scheme for the Solid Phase Peptide Synthesis (SPPS).

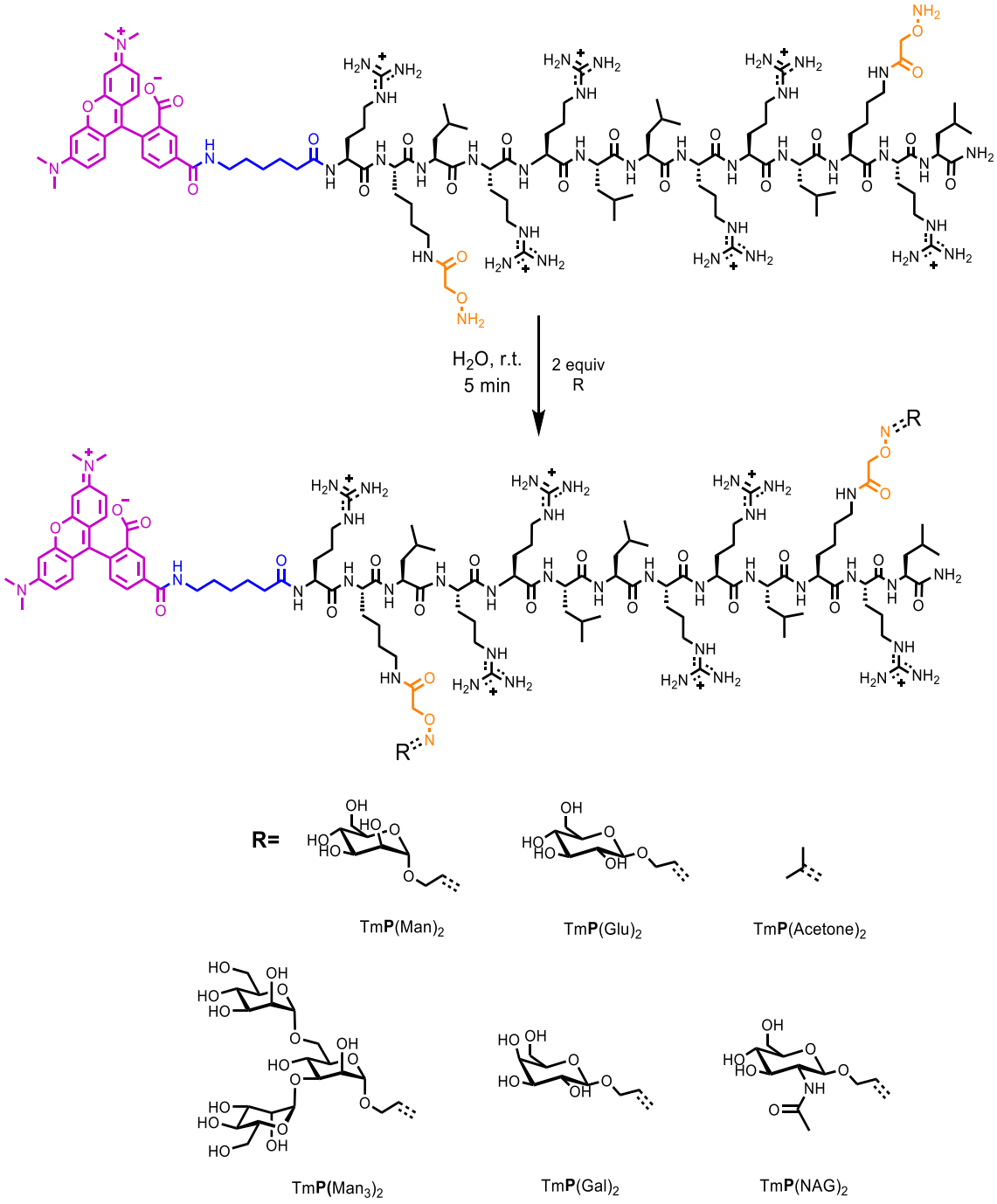

Figure S2. General synthetic scheme for ligands coupling.

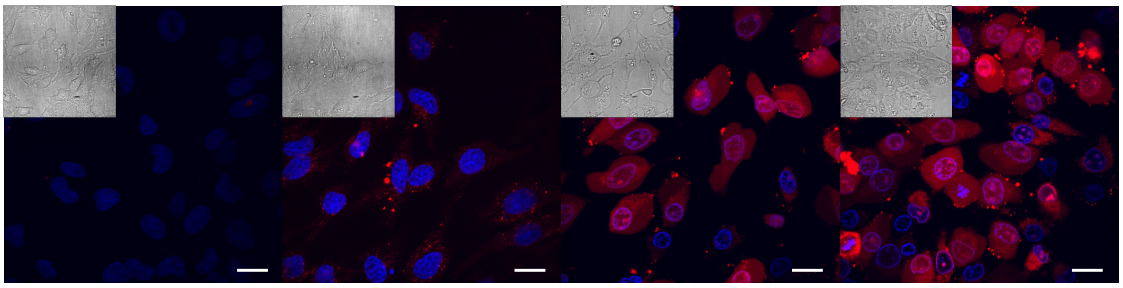

Figure S3. These pictures show a dose-response microscopy images of Tm**P**(Acetone)$_2$ internalization capacity in HeLa cells. (0,5, 1, 3, 5) μM of this control peptide in HKR buffer was incubated for 30 min and 37ºC. Scale bar: 25 μm.

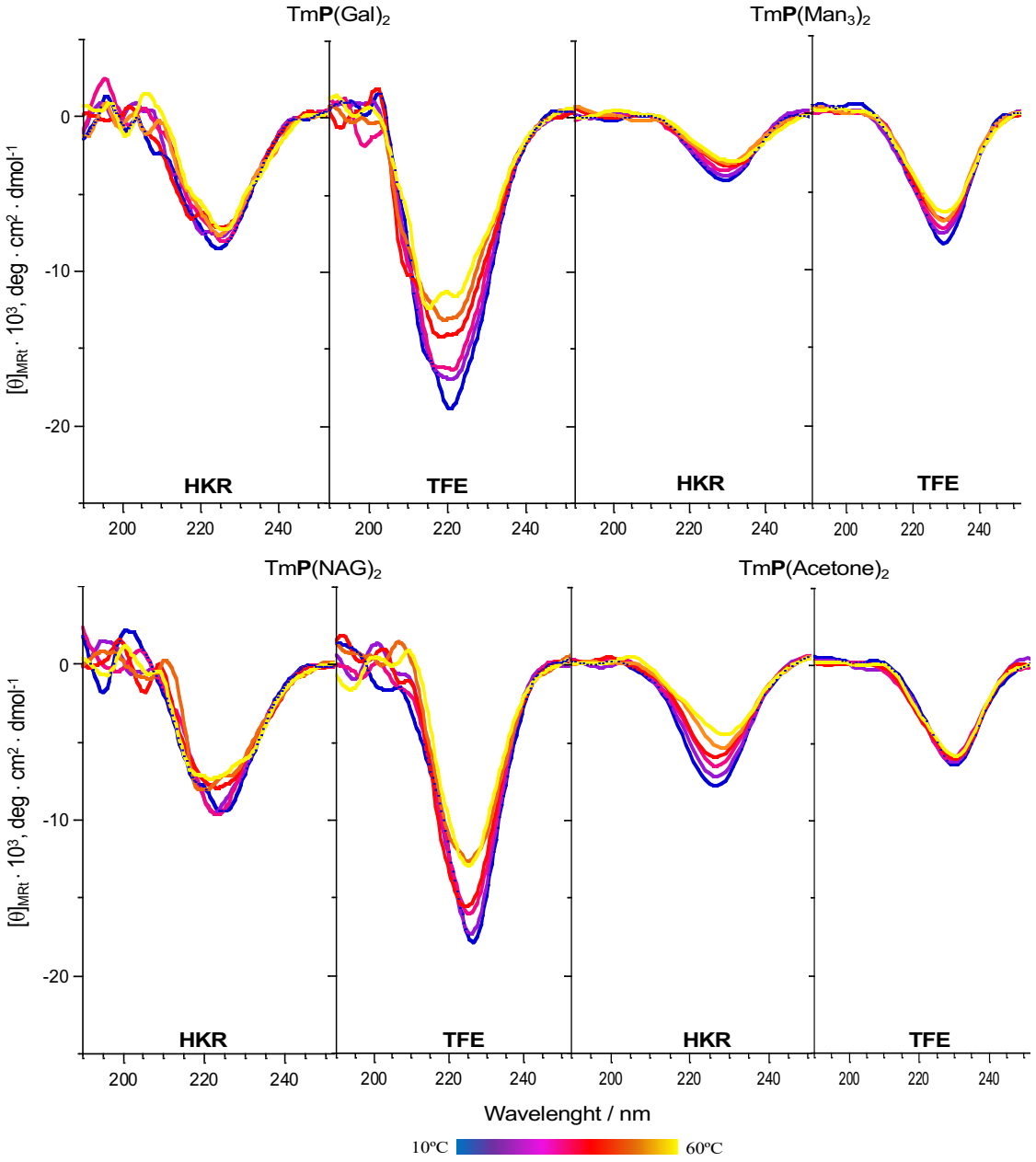

Figure S4. CD spectra of Tm**P**(Gal)$_2$, Tm**P**(NAG)$_2$, Tm**P**(Man$_3$)$_2$ and Tm**P**(Acetone)$_2$ in different conditions, TFE (trifluoroethanol) and HKR buffer (pH 7.4).

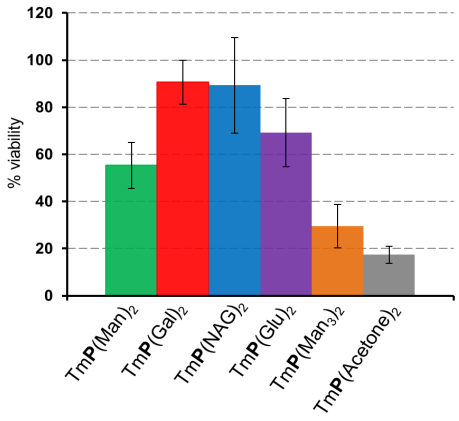

Figure S5. MTT assay. Cytotoxicity of each GCPP and control peptide Tm**P**(Acetone)$_2$ were checked using MTT assay. HeLa cells were incubated at 5 µM during 30 min and 37ºC.

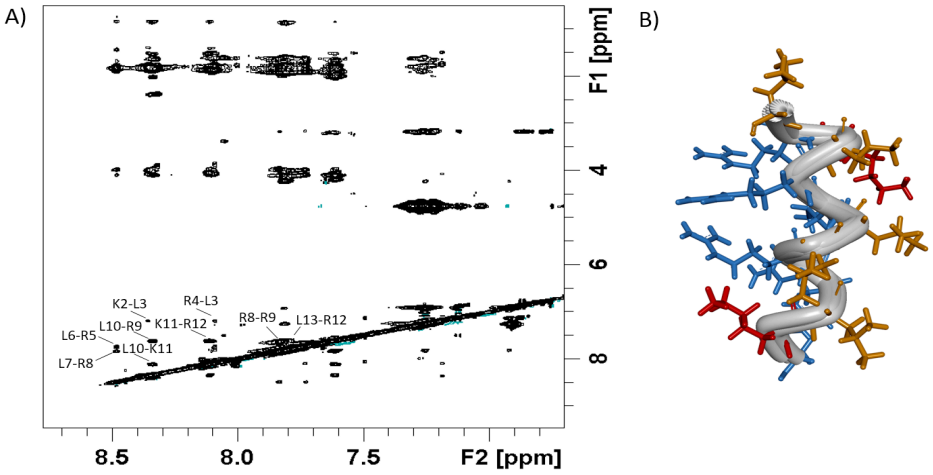

Figure S6. A) 2D NOESY spectrum of Tm**P**(Man)$_2$, acquired in phosphate buffer 20 mM containing 10% D2O and 30% TFE, at 600 MHz. B) Peptide structure by NMR applying the CYANA software.

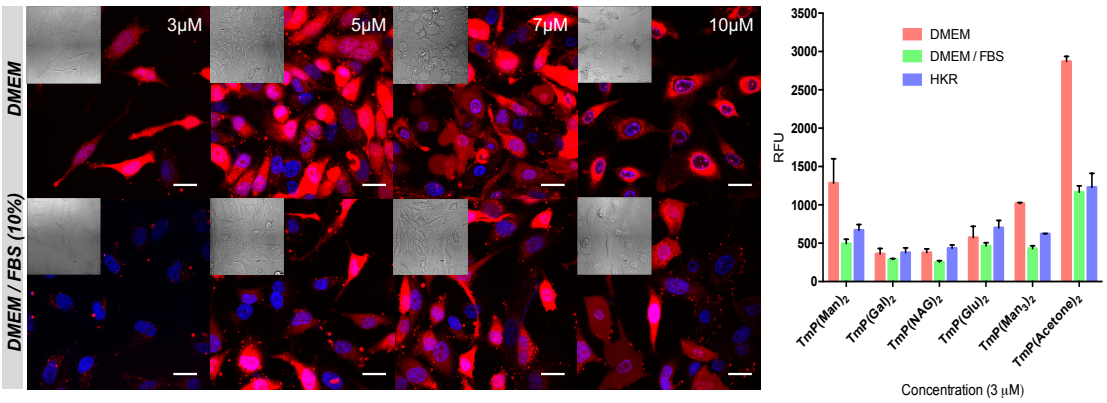

Figure S7. Dose-response microscopy images of Tm**P**$(Glu)_2$ internalization capacity in HeLa cells. (3, 5, 7, 10) μM of this peptide in DMEM and DMEM/FBS (10%) was incubated for 30 min and 37ºC. Scale bar: 25 μm. A) Dose-response confocal experiments. B) Flow cytometry.

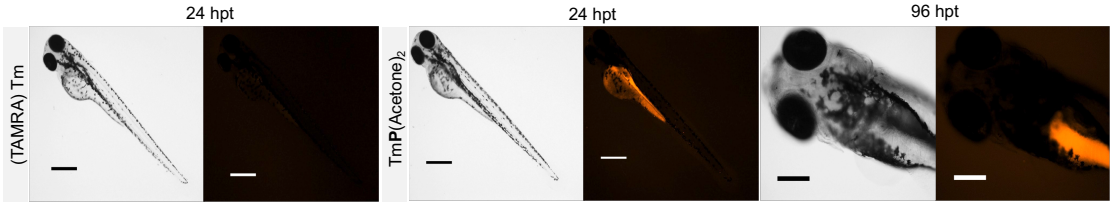

Figure S8. Images of zebra fish showing no fluorescence of the TAMRA control and strong yolk internalization of Tm**P**$(Acetone)_2$ 24 hours post treatment. 96 hours post treatment the Tm**P**$(Acetone)_2$ did not cross the yolk and higher concentrations of this peptide (> 3 μM) showed strong toxicity and complete morbidity to the zebrafish. *In vivo* experiments were performed at 1 μM with Tm (TAMRA) and control peptide Tm**P**$(Acetone)_2$. Scale bar 500 μm (24 hpt) and 250 μm (96 hpt).

Materials and methods

Commercially available Fmoc-Rink Amide AM resin, N-HBTU and Fmoc-L-Lys(Mtt)-OH were used as obtained from Iris. [Tert-butoxycarbonyl)aminooxy]acetic acid were purchased from TCI Chemicals. Fmoc-L-Leu-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-6Ahx-OH, Heparin sodium salt were purchased from Sigma-Aldrich®. 5(6)-Carboxytetramethlyrhodamine succinimidyl ester were purchased from Carbosynth. Hoechst 33342 Trihydrochloride Trihydrate were purchased in ThermoFisher. Deuterated solvents (D_2O) were from EMD Millipore Corporation. N,N-Dimethylformamide, for peptide synthesis, was purchased from Scharlau. All the other solvents were HPLC grade, purchased from Sigma-Aldrich® or Fisher Scientific® and used without further purification.

High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) analyses were carried out on Agilent Technologies 1260 Infinity II associated with a 6120 Quadrupole LC-MS using an Agilent SB-C18 column or an DIONEX Ultimate 3000 U-HPLC$^+$ (Thermo Scientific) with an Acclaim RSLC 120-C18 column with Solvent A: Solvent B gradients between 5:95 (Solvent A: H_2O with 0.1% TFA; Solvent B: CH_3CN with 0.1% TFA). High-performance liquid chromatography (HPLC) preparative purification was carried out on Waters 1525 composed by a binary pump with a dual Waters 2489 detector with a Phenomenex Luna C18(2) 100Å column. A JASCO with an Agilent Eclipse XDB-C18 column was used for semi-preparative purification using gradient 5:95 (Solvent A: H_2O with 0.1% TFA; Solvent B: CH_3CN with 0.1% TFA). Nuclear Magnetic Resonance (NMR) spectra were recorded on either a Varian Mercury 300 MHz or a Varian Inova 500 MHz spectrometer. Chemical shifts are reported in ppm (δ

units) referenced to the following solvent signals: D_2O δH 4.79. Spin multiplicities are reported as a singlet (s), doublet (d), triplet (t) with coupling constants (J) given in Hz, or multiplet (m). Accurate mass determinations (HR-MS) using ESI-MS were performed on a Sciex QSTAR Pulsar mass spectrometer and are reported as mass-per-charge ratio (m/z). Recalculation of the labelled peptides concentrations was performed by measuring the absorbance on a Biochrom Libra S60 UV-VIS Spectrophotometer. Circular Dichroism (CD) measurements were performed with a Jasco J-1100 CD Spectrometer equipped with a Jasco MCB-100 Mini Circulation Bath. Cell microscopy images were acquired with an Andor Zyla 4.2 digital camera mounted on a Nikon Eclipse Ti-E epifluorescence microscope and with a Leica SP5 confocal microscope. A Guava EasyCyteTM cytometer (EMD Millipore) was used for all flow cytometry experiments.

Abbreviations

Peptide Abbreviations: Tm**P**$(X)_2$ (Tm = TAMRA and X = Man, Glu, Acetone, Man_3, Gal and NAG); Aa: Amino acid; Arg: Arginine; Boc: tert-Butoxycarbonyl; CPP: Cell-Penetrating Peptide; DCM: Dichloromethane; DIEA: N,N-Diisopropylethylamine; DMF: N,N-Dimethylformamide; FBS: Fetal Bovine Serum; Fmoc: 9-fluorenylmethoxycarbonyl; HFIP: 1,1,1,3,3,3-Hexafluoro-2-propanol; HKR: HEPES-Krebs-Ringer buffer; HRMS (ESI): High resolution mass spectrometry (electrospray ionization); Lys: Lysine; Mtt: 4-Methyltrityl; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N-HATU: N-[(Dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methyl methanaminiun hexafluorophosphate N-oxide; N-HBTU: N-[(1HBenzotriazol-1-yl)4-(dimethylamino)methylene]-N-methyl methanaminiumhexafluoro phosphate N-oxide; Pbf: 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl; RP: Reverse Phase; SPPS: Solid Phase Peptide Synthesis; TAMRA: 5(6)-Carboxytetramethlyrhodamine succinimidyl ester; TFE: Trifluoroethanol; TIS: Triisopropylsilane; TNBS: 2,4,6-Trinitrobenzenesulfonic acid; 6Ahx: 6-aminohexanoic acid. DMEM: Dulbecco's Modified Eagle Medium. FBS: Fetal Bovine Serum.

General protocols

General protocols for the SPPS

All peptides were synthesized by manual Fmoc solid-phase peptide synthesis[1,2] using Rink Amide resin (loading 0.71 mmol/g). The resin (0.1 mmol) was swelled in DMF (peptide synthesis grade, 2 mL) for 20 min in a peptide synthesis vessel prior synthesis. Coupling cycle consisted of the removal of Fmoc protecting group with a solution of piperidine in DMF (20%, 2mL) for 10 min and then the mixture was filtered and the resin was washed with DMF (3x2 mL, 1 min). The amino acid coupling was carried out by treatment with a solution of α-amino acids (4 equiv), N-HBTU (3.95 equiv) in DMF (2 mL), which was mixed with DIEA (0.195 M solution in DMF, 1.2 equiv) 1 min before the addition and the resulting mixture was shaken by bubbling Ar for 15 min. Finally, the resin was washed with DMF (3x2 mL, 1 min). The efficiency of each amino acid coupling and deprotection was monitored employing the TNBS test[3]. Once the linear peptide was finished, the ending protocol used was “linker coupling”, after Fmoc cleavage with piperidine/DMF (20%, 2mL), the linear peptide was treated with a solution of N-Fmoc-6-aminohexanoic acid (4 equiv), N-HBTU (3.95 equiv) and DIEA (0.195 M solution in DMF, 1.2 equiv) in DMF.

The resin was washed with DCM (2x2 mL, 5 min), and the Mtt protecting group was selectively removed by mechanical shaking of the resin with a mixture of DCM/HFIP/TFE/TIS (6.5:2:1:0.5, 2x2 mL, 2 h). Finally, the mixture was filtered and the resin was washed with DCM (2x2 mL, 2

min) and DMF (2 mL, 20 min). A solution of [(tert-butoxycarbonyl) aminooxy] acetic acid (2.5 equiv per free amine) and N-HATU (2.5 equiv) in DMF (1 mL) was added to the resin followed by the dropwise addition of a solution of DIEA (4 equiv) in DMF (0.5 mL). The resin was shaken by bubbling Ar for 30 min and finally washed with DMF (3x2 mL, 2 min) and DCM (3x2 mL, 2 min).

General protocol for N-terminal functionalization

Fluorescently labelled peptides: the Fmoc-protecting group of the previously attached linker was removed by using a solution of piperidine in DMF (20%, 4 mL) for 15 min and the resin was washed with DMF (3x3 mL). The coupling was carried out by the addition of a solution of 5(6)-Carboxytetramethylrhodamine succinimidyl ester (1 equiv) and DIEA (0.195 M, 1 equiv) in DMF (2 mL) and the mixture was stirred by bubbling Ar for 30 min. Finally, the resin was washed with DMF (3x3 mL) and DCM (3x3 mL).

General protocol for peptide cleavage

Finally, peptides were deprotected and cleaved from the resin by standard TFA cleavage procedure at rt by using TFA/DCM/H_2O/TIS (90:5:2.5:2.5, 1 mL per 70 mg of resin) for 2 h. Then, the mixture was filtered, washed with TFA (1 mL) and the peptide was precipitated with ice-cold Et_2O (25 mL). The precipitate was centrifuged and dissolved in H_2O (5 mL). Peptides were obtained following the previously described procedure, and were treated with the different ligands without purification.

General protocol for ligand coupling

A solution of peptide in H_2O (5 mM) was reacted with a solution of corresponding aldehyde ligands[4] (2 equiv. per alkoxyamine) in H_2O (120 mM) for 5 min. Then, peptides were purified by RP-HPLC for removing the ligand excess. The purification was carried by Agilent Eclipse XDB-C18 column H2O (0.1% TFA)/CH_3CN (0.1% TFA), 95:5→5:95 (5→35 min)] with a binary gradient of Solvent A and Solvent B, the collected fractions were lyophilized and stored at -20 °C. Purity and identity were confirmed by HPLC, ^{1}H-NMR and low and high resolution mass spectrometry.

Synthesis of peptides

*Synthesis of Tm**P**$(Man)_2$*

Following the general protocol of the SPPS for synthesizing a TAMRA labelled peptide with two α-D-mannoses, Tm**P**$(Man)_2$ was obtained after RP-HPLC purification [Agilent Eclipse XDB-C18, 9.4x250 mm, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→35 min)] with an overall yield of 6% (17 mgs) and 99% purity. R_t 7.62 min (Fig. S6) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→12 min)]. The spectroscopic data matched those previously reported.[2] **^{1}H-NMR** (500 MHz, D_2O, δ): 8.21 (s, 1H), 7.92 (d, *J* = 8.1 Hz, 1H), 7.58 (m, 1H), 7.36 (d, *J* = 8.1 Hz, 1H), 7.11 (t, *J* = 9.9 Hz, 2H), 6.97 (m, 1H), 6.83 (d, *J* = 9.6 Hz, 2H), 6.75 (s, 2H), 4.80-4.73 (m, 2H), 4.68 (s, 4H), 4.40 (d, *J* = 24.1 Hz, 4H), 4.28-3.96 (m, 13H), 3.87-3.24 (m, 12H), 3.13 (s, 12H), 3.10-2.95 (m, 16H), 2.91-2.80 (m, 2H), 2.25 (t, *J* = 7.1 Hz, 2H), 1.79-1.11 (m, 57H), 0.87-0.67 (m, 30H). **MS** (ESI, H_2O): 1657 (7, $[M+2H+4TFA]^{+2}$), 1599 (17, $[M+2H+3TFA]^{+2}$), 1542 (15, $[M+2H+2TFA]^{+2}$), 1027 (100, $[M+3H+2TFA]^{+3}$), 989 (99, $[M+3H+TFA]^{+3}$), 953 (28, $[M+3H+]^{+3}$), 743 (62, $[M+4H+TFA]^{+4}$), 716 (53, $[M+4H]^{+4}$). **HRMS** (ESI): Calculated for $C_{129}H_{217}N_{39}O_{34}$: 1428.3220; found: 1428.3209 ($[M+2H]^{+2}$).

*Synthesis of Tm**P**$(Gal)_2$*

Following the general protocol of the SPPS for synthesizing a TAMRA labelled peptide with two β-D-galactoses, Tm**P**$(Gal)_2$ was obtained after RP-HPLC purification [Agilent Eclipse XDB-C18, 9.4x250 mm, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→35 min)] with an overall yield of 9% (26 mgs) and 97% purity. R_t 7.81 min (Fig. S7) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→12 min)]. **^{1}H-NMR** (500 MHz, D_2O, δ): δ 8.47 (s, 1H), 8.08-7.96 (m, 1H), 7.63-7.53 (m, 1H), 7.25 (s, 1H), 7.06-6.91 (m, 2H), 6.89-6.83 (m, 1H), 6.79-6.62 (m, 2H), 6.57 (s, 2H), 4.81-4.68 (m, 4H), 4.66-4.58 (m, 4H), 4.38 (s, 2H), 4.28-3.90 (m, 13H), 3.89-3.31 (m, 12H), 3.19-3.05 (m,16H), 3.04 (s, 12H), 2.91-2.71 (m, 2H), 2.37-2.11 (m, 2H), 1.98-1.04 (m, 57H), 0.90-0.53 (m, 30H). **MS** (ESI, H_2O): 1599 (7, $[M+2H+3TFA]^{+2}$), 1543 (8, $[M+2H+2TFA]^{+2}$), 1029 (75, $[M+3H+2TFA]^{+3}$), 991 (100, $[M+3H+TFA]^{+3}$), 953 (20, $[M+3H]^{+3}$), 744 (35, $[M+4H+TFA]^{+4}$), **HRMS** (ESI): Calculated for $C_{129}H_{218}N_{39}O_{34}$: 952.5504; found: 952.5499 ($[M+3H]^{+3}$).

*Synthesis of Tm**P**$(NAG)_2$*

Following the general protocol of the SPPS for synthesizing a TAMRA labelled peptide with two N-acetyl-β-D-glucosamine, Tm**P**$(NAG)_2$ was obtained after RP-HPLC purification [Agilent Eclipse XDB-C18, 9.4x250 mm, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→35 min)] with an overall yield of 9% (26 mgs) and 98% purity. R_t 7.63 min (Fig. S8) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→12 min)]. **^{1}H-NMR** (500 MHz, D_2O, δ): 8.37 (s, 1H), 8.08 (s, 1H), 7.97-7.86 (m, 1H), 7.77 (s, 1H), 7.49 (t, J = 5.4 Hz, 2H), 6.97-6.91 (m, 1H), 6.91-6.81 (m, 2H), 6.67-6.52 (m, 2H), 4.54-4.46 (m, 2H), 4.47-4.32 (m, 19.7 Hz, 4H), 4.29-3.89 (m, 13H), 3.77-3.29 (m, 12H), 3.09 (s, 12H), 3.08-2.95 (m, 16H), 2.85-2.71 (m, 2H), 2.34-2.22 (m, 2H), 1.94-1.85 (m, 6H), 1.81-1.29 (m, 57H), 0.86-0.57 (m, 30H). **MS** (ESI, H_2O): 1638 (10, $[M+2H+3TFA]^{+2}$), 1581 (5, $[M+2H+2TFA]^{+2}$), 1055 (100, $[M+3H+2TFA]^{+3}$), 1016 (80, $[M+3H+TFA]^{+3}$), 979 (15, $[M+3H]^{+3}$),762 (30, $[M+4H+TFA]^{+4}$), 734 (25, $[M+4H]^{+4}$), **HRMS** (ESI): Calculated for $C_{133}H_{224}N_{41}O_{34}$: 979.9014; found: 979.9011 ($[M+3H]^{+3}$).

*Synthesis of Tm**P**$(Glu)_2$*

Following the general protocol of the SPPS for synthesizing a TAMRA labelled peptide with two β-D-glucose, Tm**P**$(Glu)_2$ was obtained after RP-HPLC purification [Agilent Eclipse XDB-C18, 9.4x250 mm, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→35 min)] with an overall yield of 15% (42 mgs) and 99% purity. R_t 7.59 min (Fig. S9) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→12 min)]. **^{1}H-NMR** (300 MHz, D_2O, δ): 8.21 (s, 1H), 7.96-7.91 (m, 1H), 7.83 (s, 1H), 7.69-7.57 (m, 1H), 7.19-7.12 (m, 2H), 7.09-7.00 (m, 1H), 6.75 (s, 2H), 6.65-6.57 (m, 2H), 4.46 (s, 2H), 4.41-4.33 (m, 4H), 4.27-4.00 (m, 13H), 3.86-3.27 (m, 12H), 3.13 (s, 12H), 3.10-3.02 (m, 16H), 2.76 (d, J = 7.4 Hz, 2H), 2.31-2.18 (m, 2H), 1.96-1.09 (m, 57H), 0.95-0.60 (m, 30H). **MS** (ESI, H_2O): 1598 (10, $[M+2H+3TFA]^{+2}$), 1544 (8, $[M+2H+2TFA]^{+2}$), 1029 (50, $[M+3H+2TFA]^{+3}$), 991 (100, $[M+3H+TFA]^{+3}$), 953 (23, $[M+3H]^{+3}$), 744 (47, $[M+4H+TFA]^{+4}$), 715 (43, $[M+4H]^{+4}$), **HRMS** (ESI): Calculated for $C_{129}H_{218}N_{39}O_{34}$: 952.5504; found: 952.5505 ($[M+3H]^{+3}$).

*Synthesis of Tm**P**$(Man_3)_2$*

Following the general protocol of the SPPS for synthesizing a TAMRA labelled peptide with two α-D-mannose trisaccharide, Tm**P**$(Man_3)_2$ was obtained after RP-HPLC purification [Agilent Eclipse XDB-C18, 9.4x250 mm, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→35 min)] with an overall yield of 11% (38 mgs) and 99% purity. R_t 7.73 min (Fig. S10) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→12 min)]. **^{1}H-NMR** (500 MHz, D_2O, δ): 8.21 (s, 1H), 8.03-7.87 (m, 1H), 7.72-7.58 (m, 1H), 7.44-7.35 (m, 1H), 7.23-7.12 (m, 2H), 7.04-6.95 (m, 2H), 6.95-6.86 (m, 2H), 6.83 (s, 2H), 5.07-4.93 (m, 2H), 4.86-4.70 (m,

4H), 4.51-4.40 (m, 4H), 4.33-4.09 (m, 13H), 4.07-3.34 (m, 62.5 Hz, 36H), 3.18 (s, 12H), 3.14-3.03 (m, 16H), 2.94-2.78 (m, 2H), 2.36-2.11 (m, 2H), 1.95-1.03 (m, 57H), 0.95-0.58 (m, 30H). **MS** (ESI, H_2O): 1244 (100, $[M+3H+2TFA]^{+3}$), 1207 (78, $[M+3H+TFA]^{+3}$), 1169 (10, $[M+3H]^{+3}$), 905 (55, $[M+4H+TFA]^{+4}$), 604 (47, $[M+6H+TFA]^{+6}$), **HRMS** (ESI): Calculated for $C_{153}H_{258}N_{39}O_{54}$: 1168.6208; found: 1168.6204 ($[M+3H]^{+3}$).

*Synthesis of Tm**P**(Acetone)$_2$*

Following the general protocol of the SPPS for synthesizing a TAMRA labelled peptide capped with acetone, Tm**P**(Acetone)$_2$ was obtained after RP-HPLC purification [Agilent Eclipse XDB-C18, 9.4x250 mm, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→35 min)] with an overall yield of 12% (30 mgs) and 99.9% purity. R_t 8.21 min (Fig. S11) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→12 min)]. **^{1}H-NMR** (500 MHz, D_2O, δ): 8.07-7.89 (m, 2H), 7.55 (s, 1H), 7.03 (d, *J* = 9.2 Hz, 2H), 6.89-6.69 (m, 4H), 4.34 (d, *J* = 7.9 Hz, 4H), 4.27-3.93 (m, 13H), 3.40-3.22 (m, 2H), 3.10 (s, 12H), 3.07-2.98 (m, 16H), 2.31-2.11 (m, 2H), 1.89-1.63 (m, 12H), 1.60-1.10 (m, 57H), 0.88-0.64 (m, 30H). **MS** (ESI, H_2O): 1492 (8, $[M+2H+4TFA]^{2+}$), 1434 (10, $[M+2H+3TFA]^{2+}$), 1376 (8, $[M+2H+2TFA]^{2+}$), 918 (100, $[M+3H+2TFA]^{3+}$), 881 (100, $[M+3H+TFA]^{3+}$), 690 (18, $[M+4H+2TFA]^{4+}$), 662 (40, $[M+4H+TFA]^{4+}$), 632 (18, $[M+4H]^{4+}$). **HRMS** (ESI): Calculated for $C_{119}H_{201}N_{39}O_{22}$: 1264.2899; found: 1264.2902 ($[M+2H]^{2+}$).

General procedure for circular dichroism

Circular dichroism measurements were carried out using the following settings: acquisition range: 300-190 nm; band width: 1.0 nm; accumulation: 3 scans; data pitch: 1 nm; CD scale: 200 mdeg/1.0 dOD; D.I.T. (Data Integration Time): 1s; scanning mode: continuous; scanning speed: 200 nm/min. Measurements were done from 10°C to 60°C (data interval: 10°C; temp. gradient: 5°C /min) in a quartz cell of 0.2 cm path length at a final volume of 0.5 mL (HKR buffer or TFE) with a final peptide concentration of 200 μM.

The results are expressed as the mean residue molar ellipticity $[\theta]_{MRt}$ with units of degrees·cm^2·dmol^{-1} and calculated using the equation S1:

$$[\theta]_{MRt} = \frac{100 \cdot \theta}{C \cdot l \cdot N^o of\ residues} \qquad (S1)$$

where θ is the ellipticity (deg), C is the peptide concentration (M) and l is the cell path length (cm).[5,6]

NMR measurements

NMR experiments were acquired with 1.5 mM peptide samples prepared in phosphate buffer 20 mM containing 30% of deuterated trifluoroethanol (TFE-d_2) and 10 % of D_2O. 2D-TOCSY and 2D-NOESY spectra were acquired on a Bruker 500 MHz spectrometer at 298 K, with mixing times of 75 ms and 500 ms, respectively. Both experiments were recorded with 4096 data points in F2 and 256 data points in F1.

Peptide assignment were carried out by using CARA software[7] and structure calculations were performed by using CYANA 3.0 with distance restraints derived from the experimental NOEs.[8]

Cells lines and culture

Cell lines were incubated at 37°C, 5% CO_2, 95% humidity in an INCO 108 incubator (Memmert) with Dulbecco's Modified Eagle's Medium (4500 mg/L glucose, L-glutamine, sodium pyruvate

and sodium bicarbonate; Sigma-Aldrich), supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% of Penicillin-Streptomycin-Glutamine Mix (Fisher). Each cell line was grown on four chamber glass bottom dishes and washed with HEPES–Krebs–Ringer (HKR) buffer (5mM HEPES, 137 mM NaCl, 2.68 mM KCl, 2.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4) and nuclei were stained by incubation with 1µM Hoechst 33342 in HKR for 30 min. The peptide was diluted in HKR, DMEM or DMEM/FBS (10%) to obtain the different concentrations that were recalibrated by UV. Cells were incubated with the peptide for 30 min and then washed to remove excess peptide using an heparin solution (2x500 µL, 0.1 mg/ml) and HKR buffer (2x500 µL) before performing epifluorescence microscopy using a Nikon Eclipse Ti-E inverted microscope or confocal microscopy with a Leica SP5 microscope. Images were analyzed with ImageJ.

Cell viability: MTT assay

Cell viability was established by a standard MTT assay (Fig. S5). One day before the assay, a suspension of HeLa cells was plated in 96-well tissue culture plates (Costar 96 Flat Bottom Transparent Polystyrol) by adding 100 µL (150.000 cells/mL) per well. The next day, the medium was aspirated and cells were incubated with different concentrations of peptide diluted in HKR (50 µL/well). After 1 hour of incubation at 37ºC, the medium was aspirated and cells were washed with HKR buffer (2x100 µL/well). Then fresh medium (DMEM) containing 10% FBS (100 µL) was added to the cells during 3h. Control cells 100% were permorfed with only cell culture medium (100 µL final medium) and control cell 0% with TRITON solution. The viability was measured by quantifying the cellular ability to reduce the water-soluble tetrazolium dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) to its insoluble formazan salt as follows. MTT (5 mg/mL in PBS, 10 µL/well) was added to the wells and the cells were further incubated for 4 h. The supernatant was carefully removed and the water-insoluble formazan salt was dissolved in DMSO (100 µL/well). The absorbance was measured at 570 nm using a microplate reader (Infinite F200pro, Tecan). Data points were collected in triplicate and expressed as normalized values for untreated control cells (100% and 0%).

General protocol for flow cytometry

One day before the assay, different cell lines were plated in 96-well tissue culture plates (Costar 96 Flat Bottom Transparent Polystyrol) by adding 100 µL (150.000 cells/mL) per well. The next day, the medium was aspirated and cells were incubated with different concentrations of peptide (50 µL/well) in HKR buffer. After 30 min of incubation at 37ºC, the buffer was aspirated; cells were washed with heparin solution (1x100 µL, 0.1 mg/mL) and HKR buffer (2x100 µL). Finally trypsin 50 µL was added in each well and cells were incubated for 15 min at 37ºC. After this, 150 µL of a solution of 2% FBS and 5 mM EDTA in PBS were added to the cells. Cells were analyzed in a Guava EasyCyte (Millipore) cytometer and the results were processed in an InCyte software (GuavaSoft 3.2 Millipore).

Zebrafish experiments

One-year-old adult zebrafish (Danio rerio) were maintained in 30 L aquaria at a rate of 1 fish per liter of water on a recirculating system, in a controlled environment of 14-h light/10-h dark cycle at 28 ºC. Zebrafish embryos were obtained by massive spawning from breeding stocks of adult zebrafish.

48 hours post-fertilization (hpf) zebrafish embryos were exposed to 1 µM concentration of glycopeptides (namely Tm**P**$(Gal)_2$ and Tm**P**$(Man_3)_2$) in order to evaluate their uptake capacity into the embryo and, if possible, their biodistribution. This was possible because of fluorescence characteristics of glycopeptides analysed and transparency of fish embryos. Exposition took

place for 96 h, being fishes checked every 24 h. Pictures were taken with a Nikon AZ100 Zoom fluorescence microscope and analyzed with NIS Elements software (Nikon).

The protocols used in this study were performed in compliance with the EU animal experimentation regulation (EU, 2010) and were approved by the Bioethics Committee for Animal Experimentation CEEA-LU (Universidade de Santiago de Compostela, Spain). When needed, embryos were euthanized by tricaine (MS-222) overdose

Supporting figures for characterization

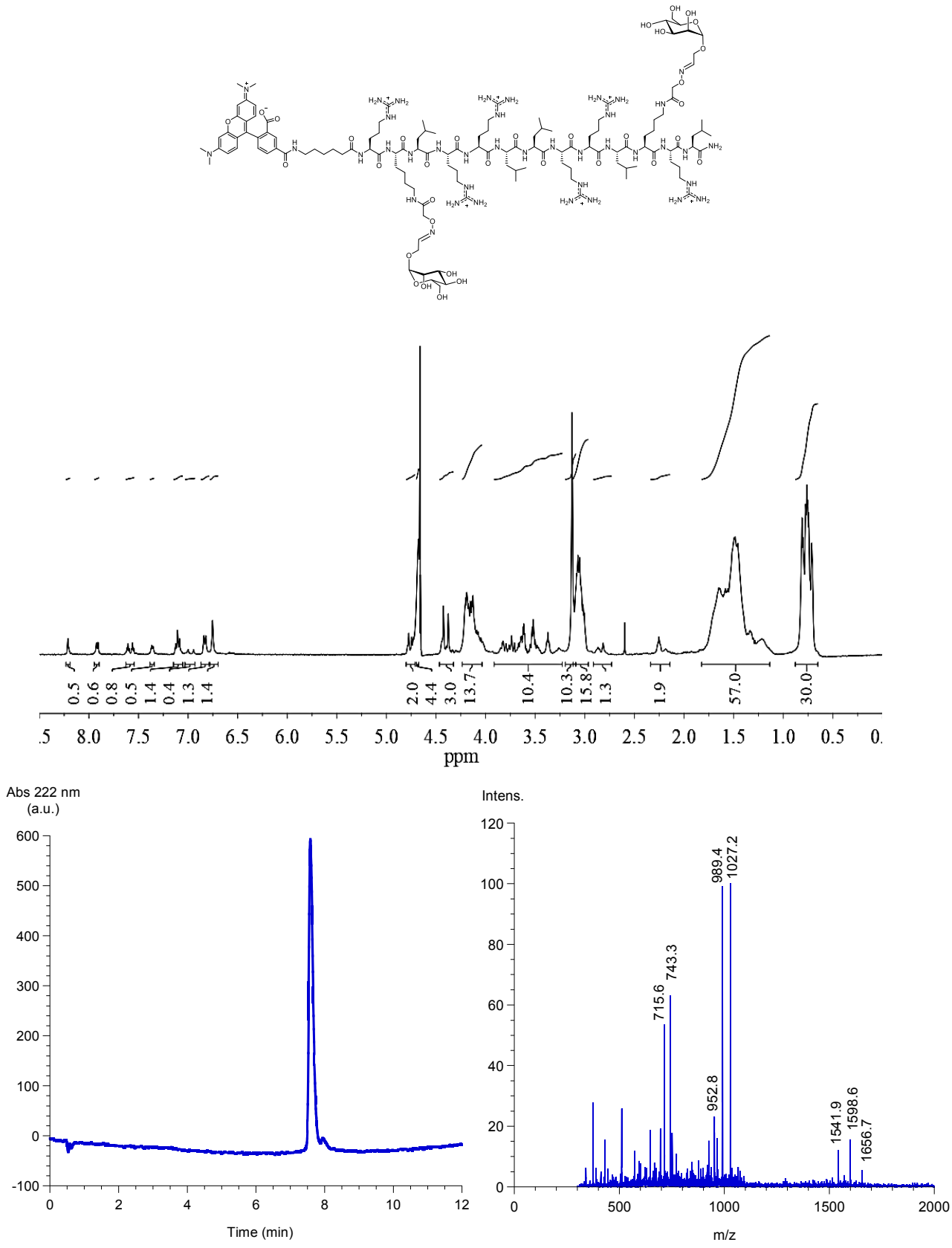

Figure S9. A) ^{1}H-NMR spectra in D_2O of Tm**P**$(Man)_2$. B) RP-HPLC-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→12 min)]. (R_t 7.62 min) and ESI-MS of Tm**P**$(Man)_2$.

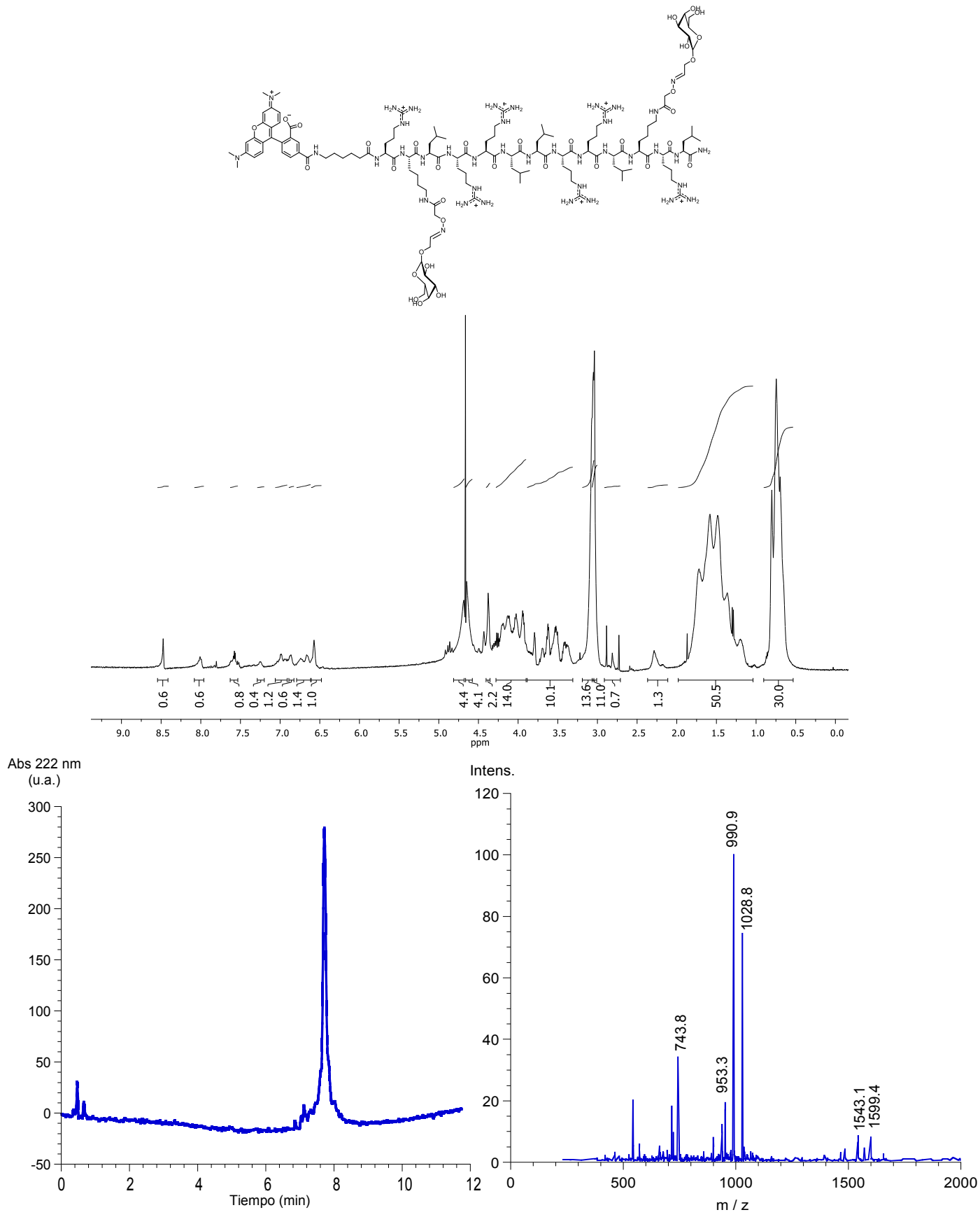

Figure S10. A) ^{1}H-NMR spectra in D_2O of Tm**P**$(Gal)_2$. B) RP-HPLC-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→12 min)]. (R_t 7.81 min) and ESI-MS of Tm**P**$(Gal)_2$.

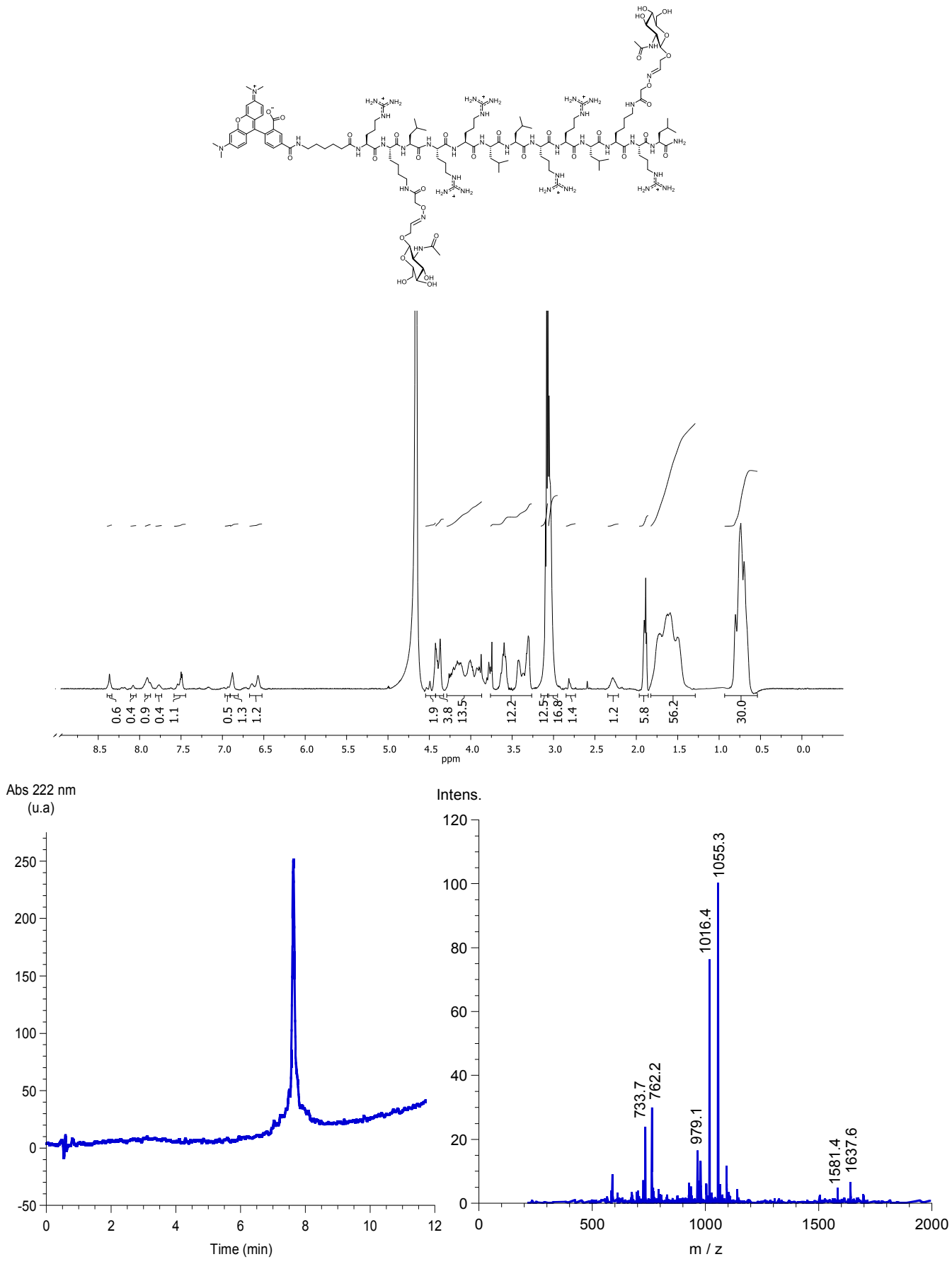

Figure S11. A) ^{1}H-NMR spectra in D_2O of Tm**P**$(NAG)_2$. B) RP-HPLC-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→12 min)]. (R_t 9.90 min) and ESI-MS of Tm**P**$(NAG)_2$.

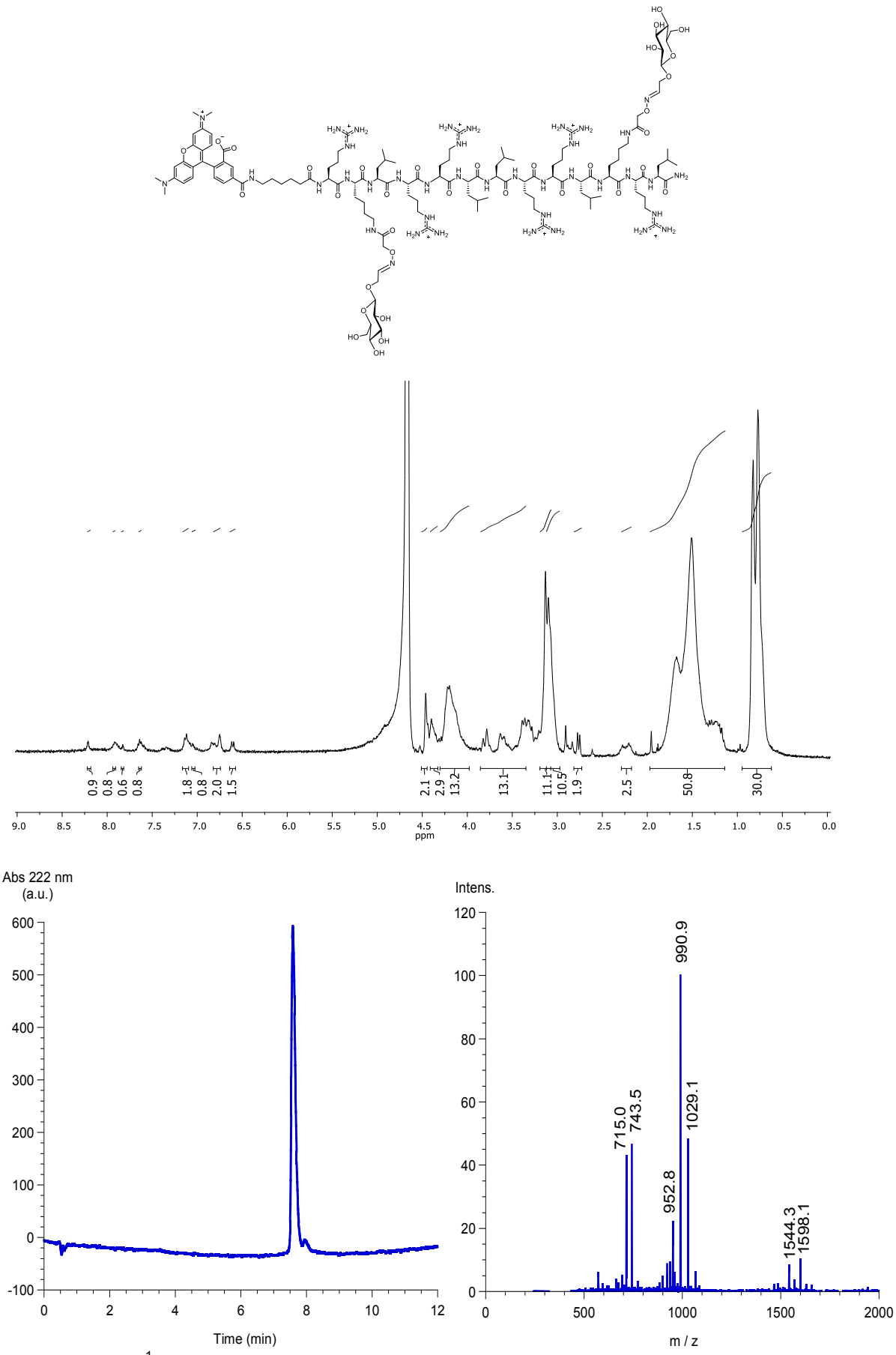

Figure S12. A) ^{1}H-NMR spectra in D_2O of Tm**P**$(Glu)_2$. B) RP-HPLC-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→12 min)]. (R_t 7.59 min) and ESI-MS of Tm**P**$(Glu)_2$.

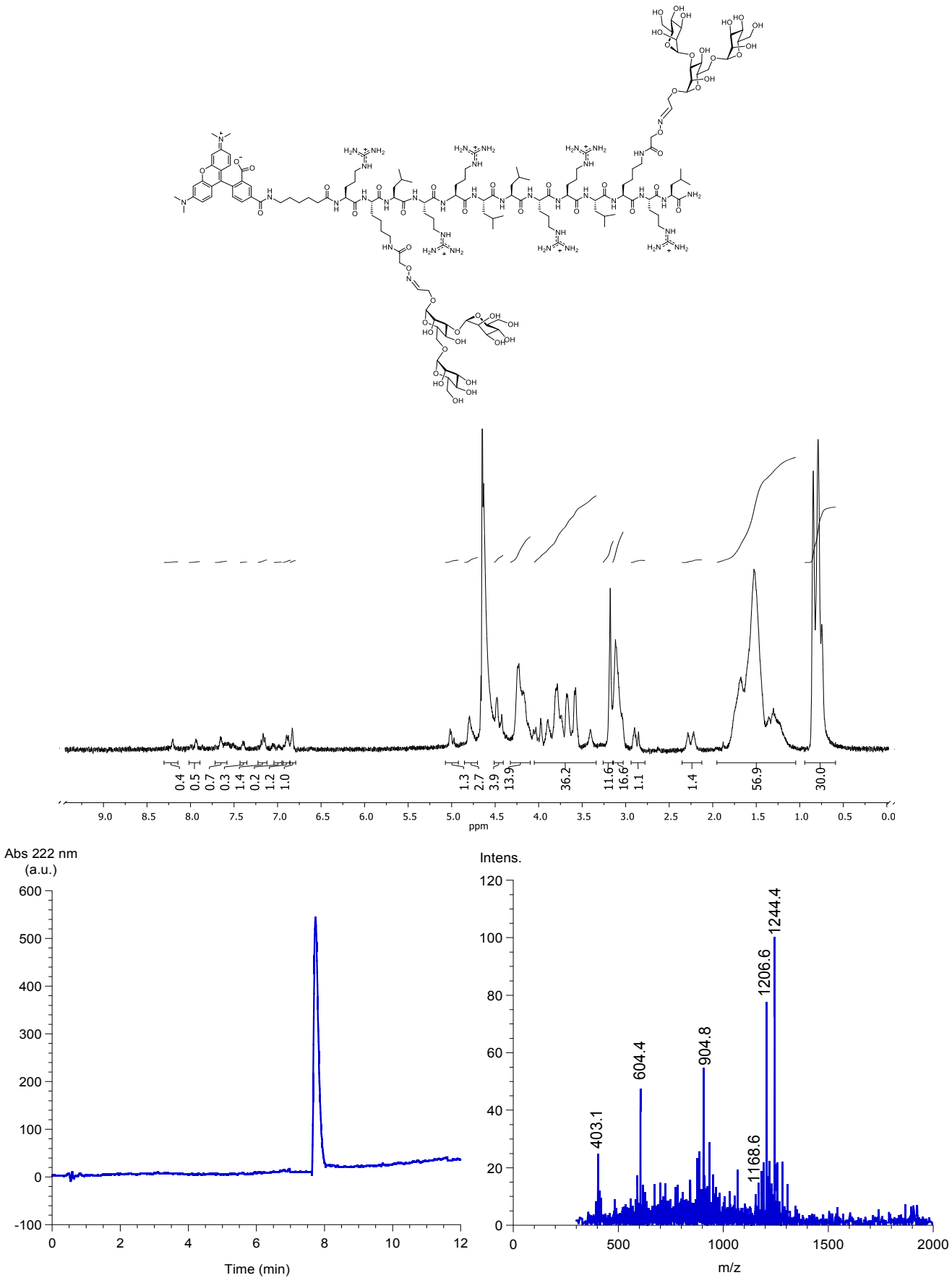

Figure S13. A) ^{1}H-NMR spectra in D_2O of Tm**P**$(Man_3)_2$. B) RP-HPLC-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→12 min)]. (R_t 7.73 min) and ESI-MS of Tm**P**$(Man_3)_2$.

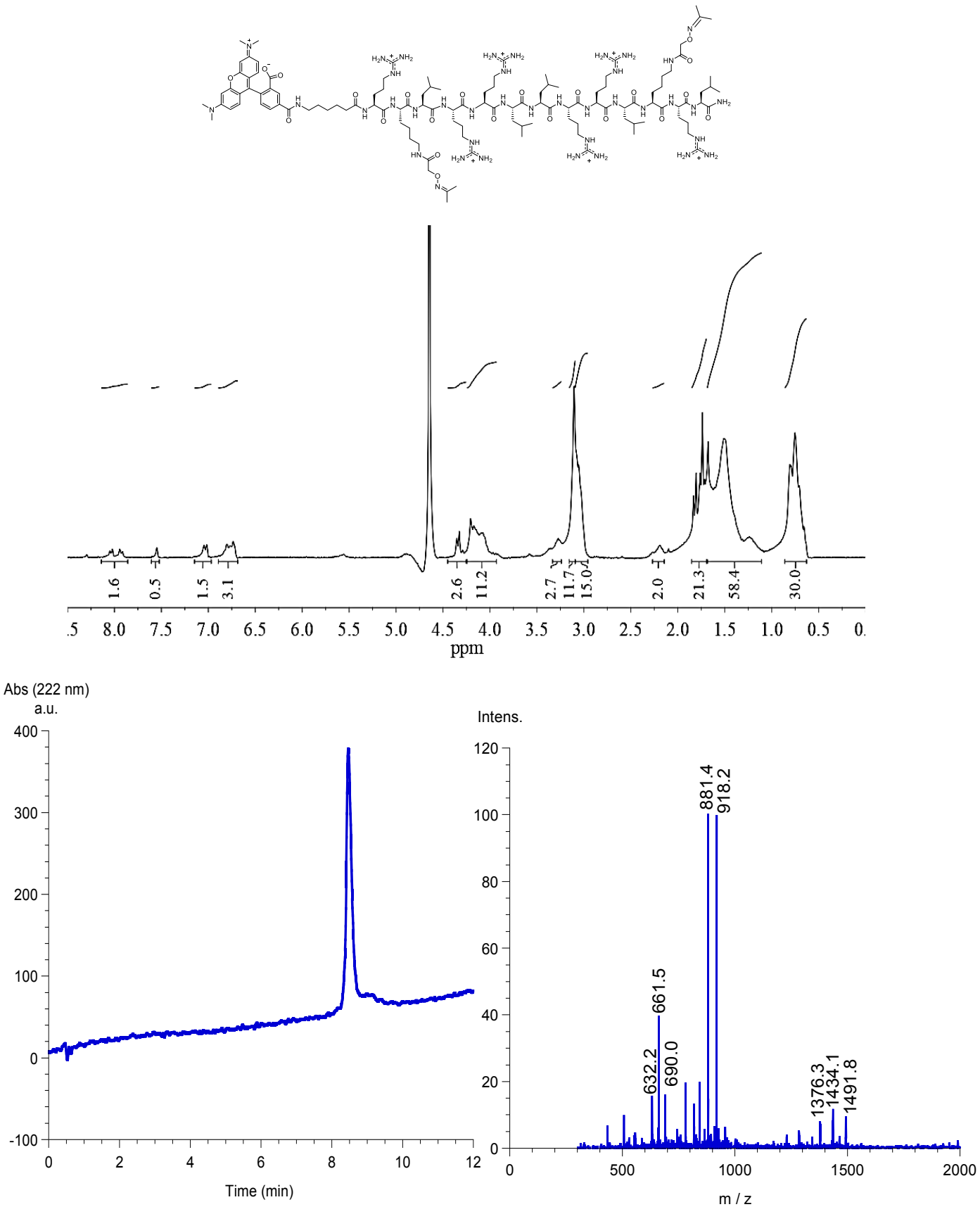

Figure S14. A) ^{1}H-NMR spectra in D_2O of Tm**P**(Acetone)$_2$. B) RP-HPLC-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→12 min)]. (R_t 8.21 min) and ESI-MS of Tm**P**(Acetone)$_2$.

Supporting references

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