Supramolecular Recognition and Selective Protein Uptake by Penetrating Peptide Hybrids

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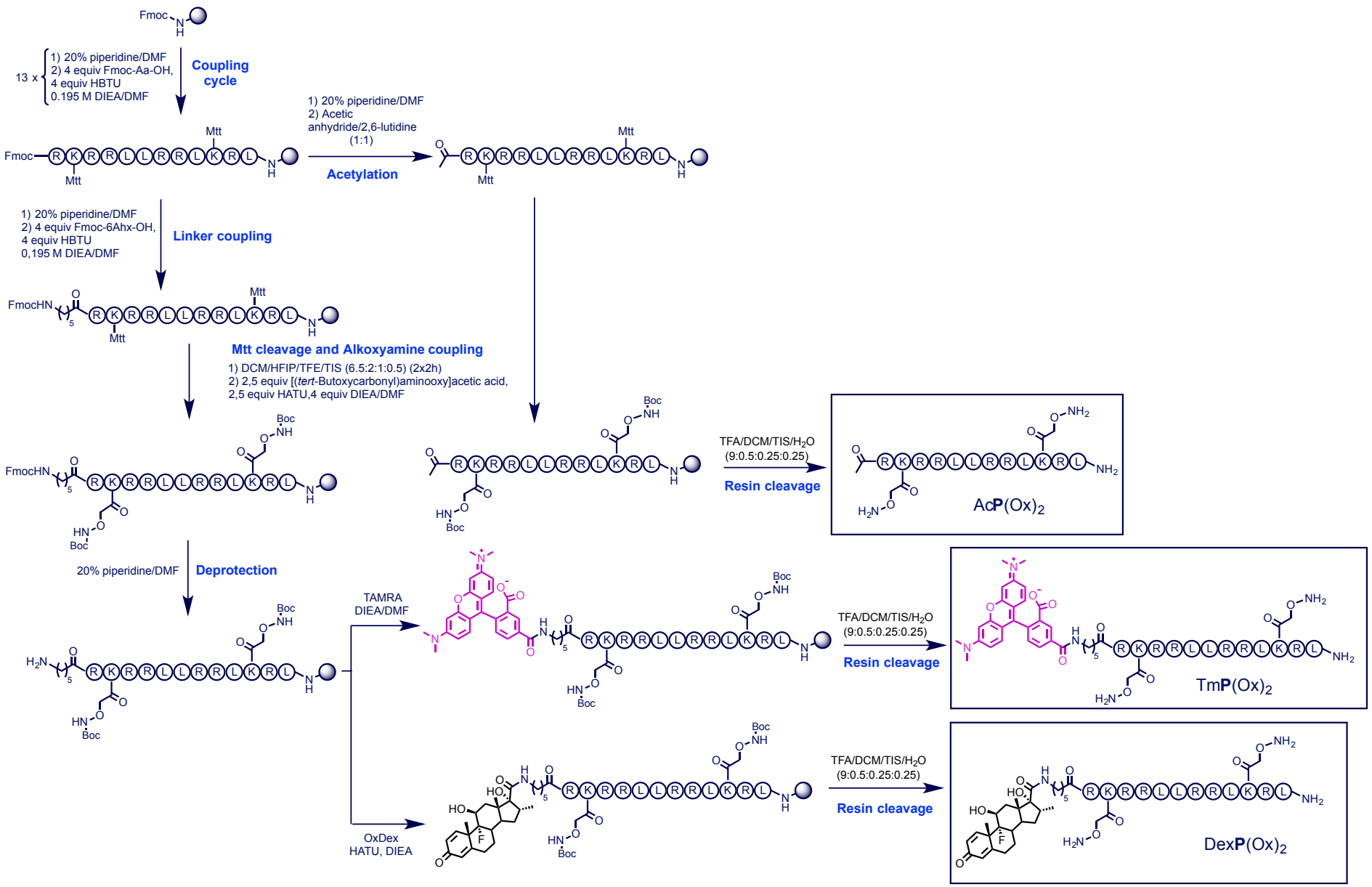


Figure S1. General synthetic scheme for the Solid Phase Peptide Synthesis (SPPS).

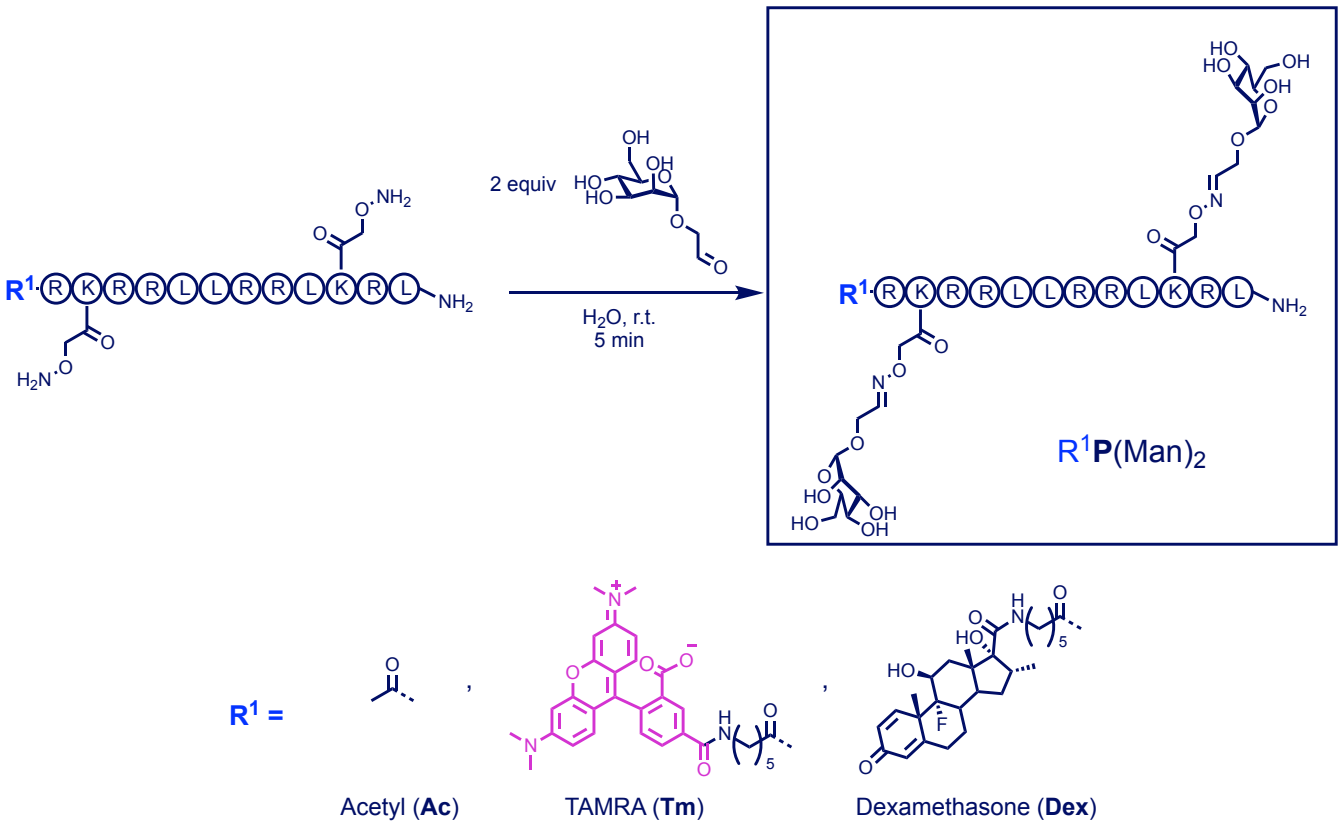


Figure S2. General synthetic scheme for the mannose aldehyde coupling. Peptides were obtained with an overall yield of: 28 % for Ac**P**$(Man)_2$, 5.6 % for Tm**P**$(Man)_2$ and 5 % for Dex**P**$(Man)_2$.

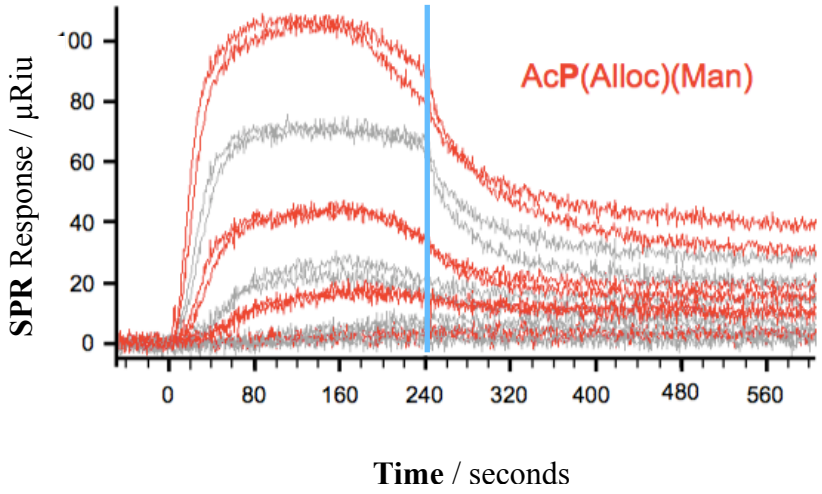


Figure S3. SPR. Sensorgrams of the interaction of ConA (9668 µRiU) in the concentration range [Ac**P**(Alloc)(Man)] = 6-2000 nM.

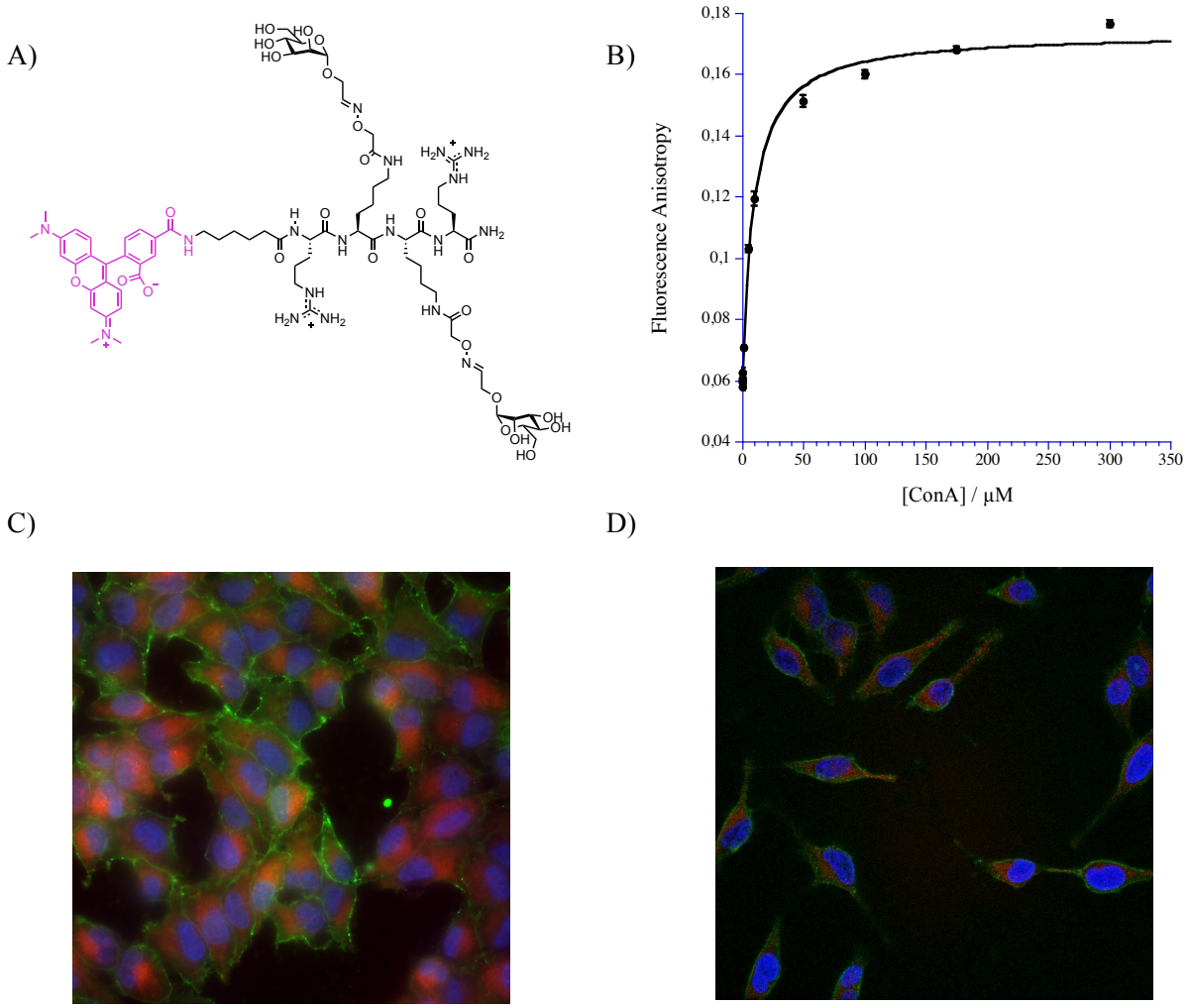
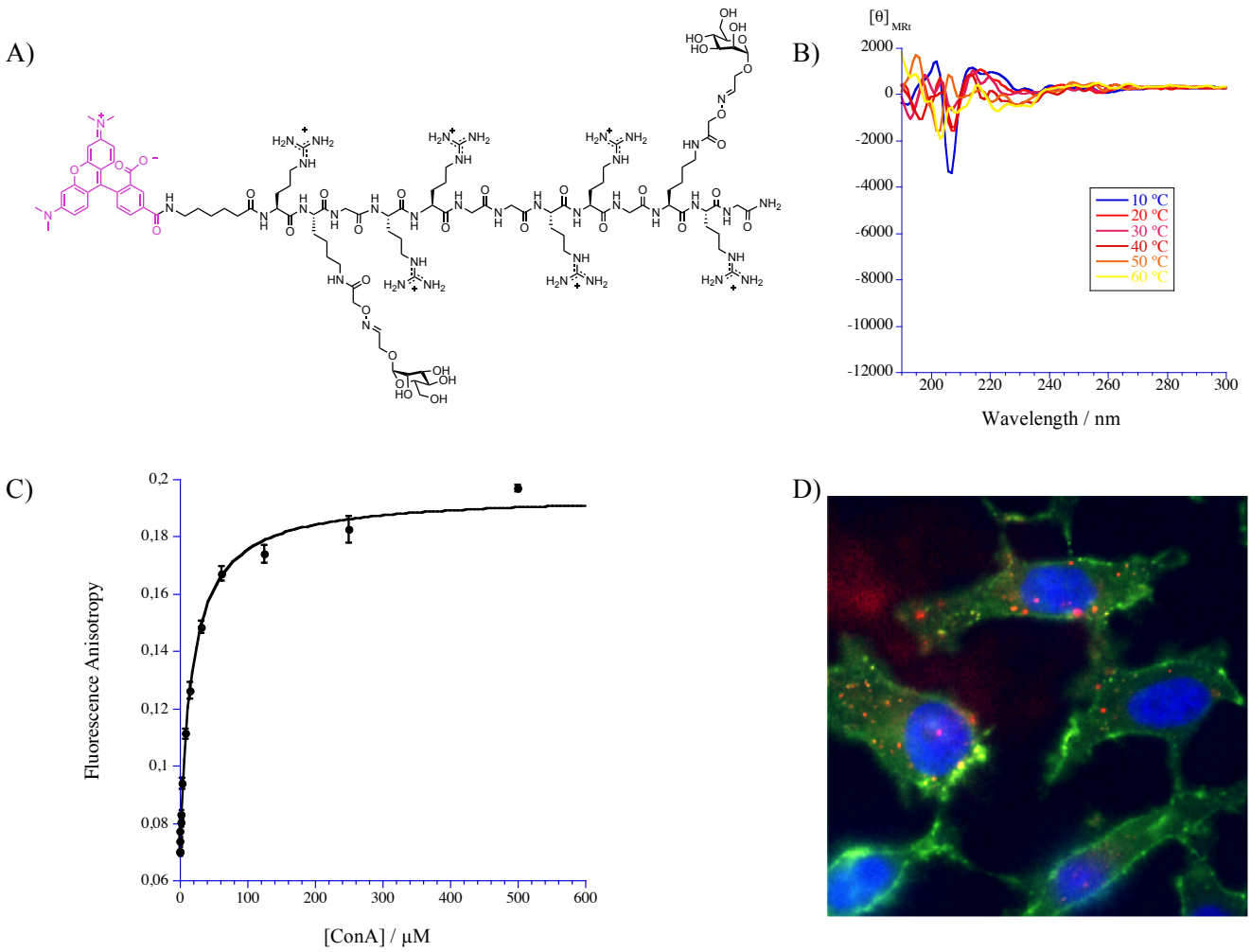


Figure S4. A) $TmArg_2(Man)_2$ peptide structure. B) Fluorescence anisotropy titration and best fitting to a simple 1:1 binding model of $TmArg_2(Man)_2$ with increasing amounts of ConA in HKR buffer (pH 7.4) at 22 ºC. Calculated K_d = 9 ± 1 µM. C) Epifluorescence and D) CLSM images of $TmArg_2(Man)_2$ (3 µM, red) treated with $ConA_{FITC}$ (30 nM, green) and incubated with HeLa cells. Nuclei were counterstained with Hoechst (blue).



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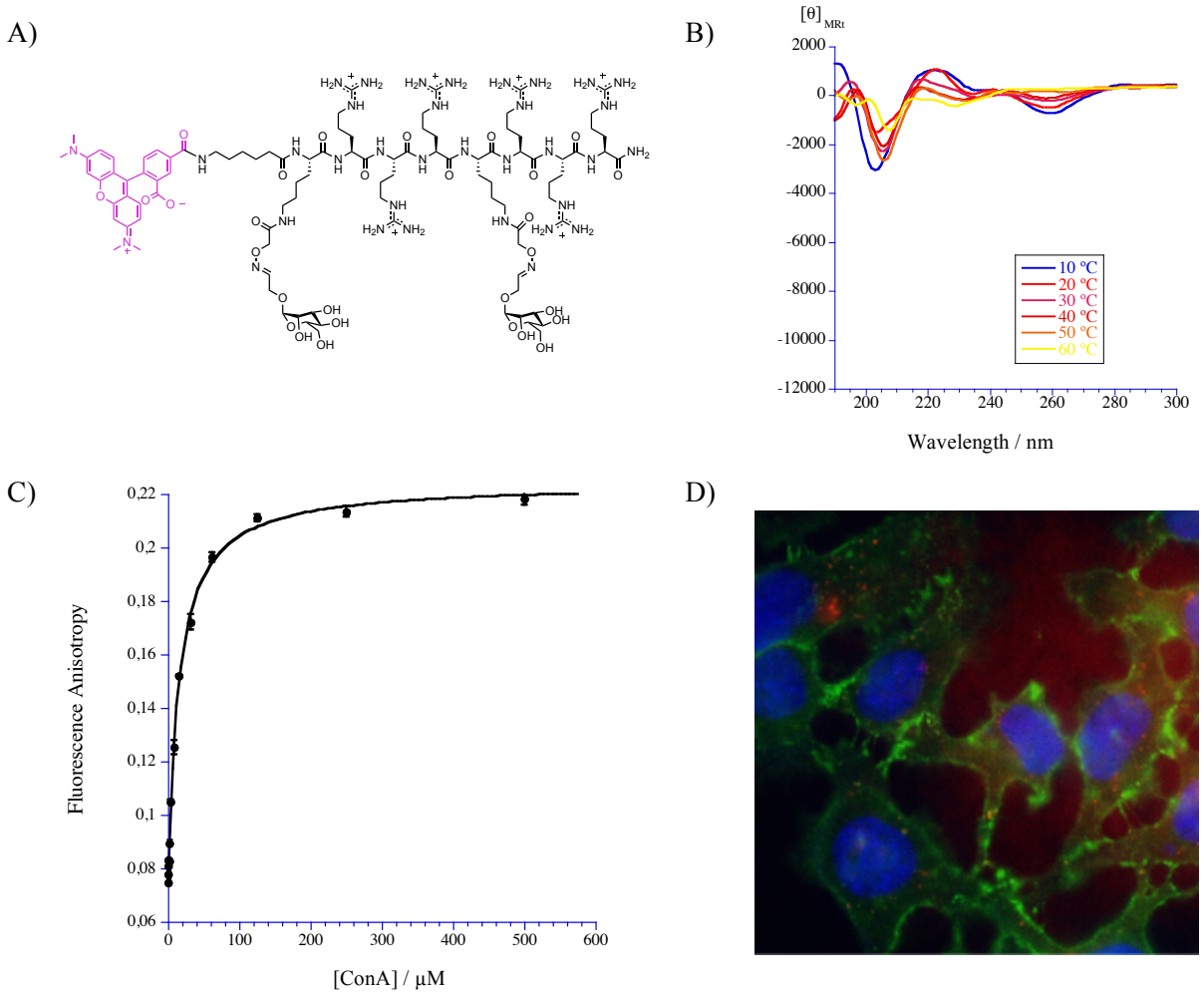


Figure S6. A) $TmArg_6(Man)_2$ peptide structure. B) Circular dichroism of $TmArg_6(Man)_2$ measured in HKR buffer at different temperatures. C) Fluorescence anisotropy titration and best fitting to a simple 1:1 binding model of $TmArg_6(Man)_2$ with increasing amounts of ConA in HKR buffer (pH 7.4) at 22 ºC. Calculated K_d = 16 ± 1 µM. D) Epifluorescence image of $TmArg_6(Man)_2$ (3 µM, red) treated with $ConA_{FITC}$ (30 nM, green) and incubated with HeLa cells. Nuclei were counterstained with Hoechst (blue).

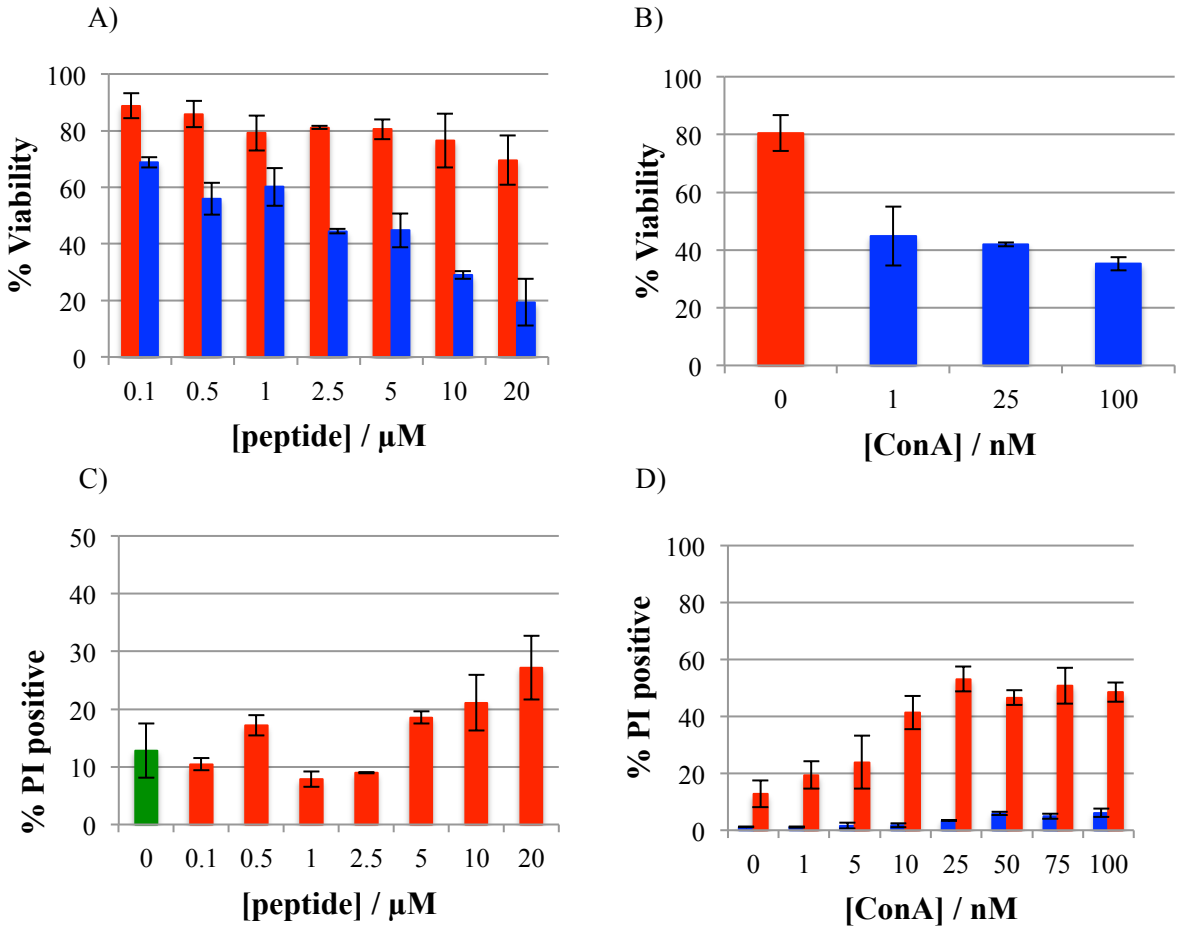


Figure S7. Cell viability in HeLa Cells. A) MTT assay at different concentrations of Ac**P**$(Man)_2$ (red) and Ac**P**$(Acetone)_2$ (blue); B) MTT assay at different concentrations of ConA using 5 µM of Ac**P**$(Man)_2$, the viability for Ac**P**$(Man)_2$ alone is shown in red at 0 nM of ConA; C) Propidium iodide staining of dead cells for flow cytometry at different concentrations of Ac**P**$(Man)_2$. The staining of untreated cells (control) is represented in green; D) Propidium iodide staining of dead cells for flow cytometry at different concentrations of ConA using 5 µM of Ac**P**$(Man)_2$ (red). Blue bars represent the cytotoxicity of the ConA alone.

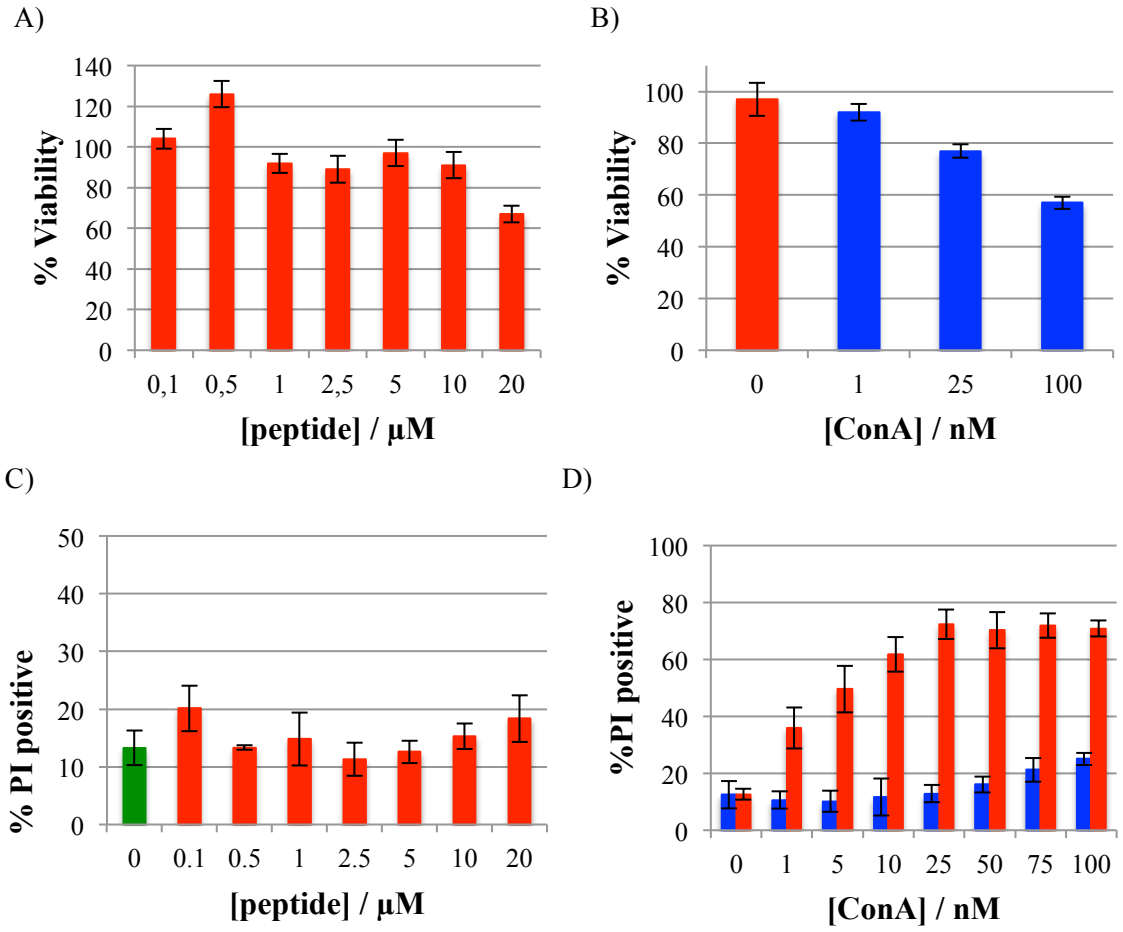


Figure S8. Cell viability in HepG2 Cells. A) MTT assay at different concentrations of Ac**P**$(Man)_2$ (red); B) MTT assay at different concentrations of ConA using 5 µM of Ac**P**$(Man)_2$, the viability for Ac**P**$(Man)_2$ alone is shown in red at 0 nM of ConA; C) Propidium iodide staining of dead cells for flow cytometry at different concentrations of Ac**P**$(Man)_2$. The staining of untreated cells (control) is represented in green; D) Propidium iodide staining of dead cells for flow cytometry at different concentrations of ConA using 5 µM of Ac**P**$(Man)_2$ (red). Blue bars represent the cytotoxicity of the ConA alone.

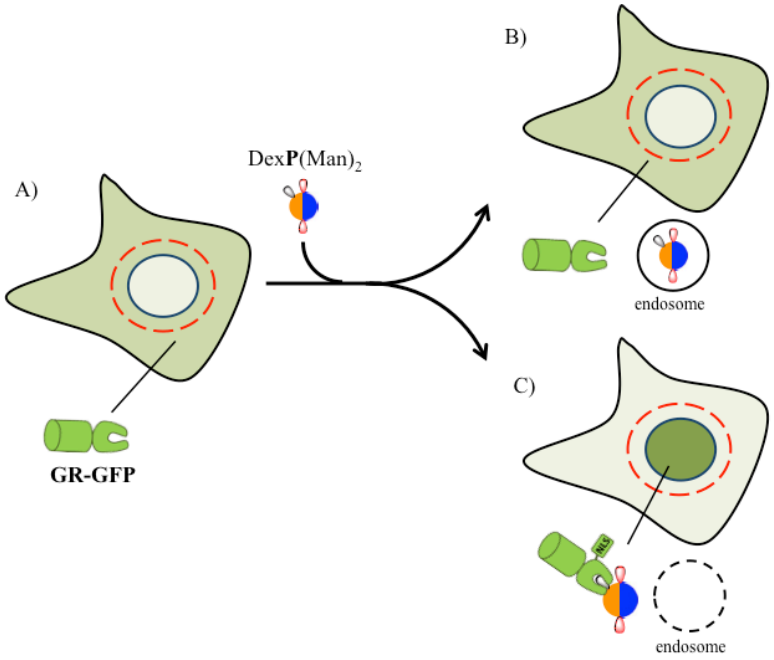


Figure S9. Glucocorticoid induced GFP translocation assay (GIGT). A) Cells transfected with the plasmid pK7-GR-GFP show green fluorescence mostly on the cytoplasm, and so, the ratio between the median fluorescence in the nucleus and in the surrounding cytosolic region (red circle) will be low. Upon incubation with dexamethasone-labelled peptides, if the peptide remains trapped in the endosome (B) or it does not enter the cell, the glucocorticoid receptor will not be able to bind the dexamethasone and the translocation ratio will remain low. However, if the peptide is able to reach the cytosol, by direct translocation or endosomal escape (C), the binding to the glucocorticoid receptor triggers a conformational change that exposes NLSs. This causes the accumulation of GR-GFP in the nucleus and the increase in the translocation ratio.

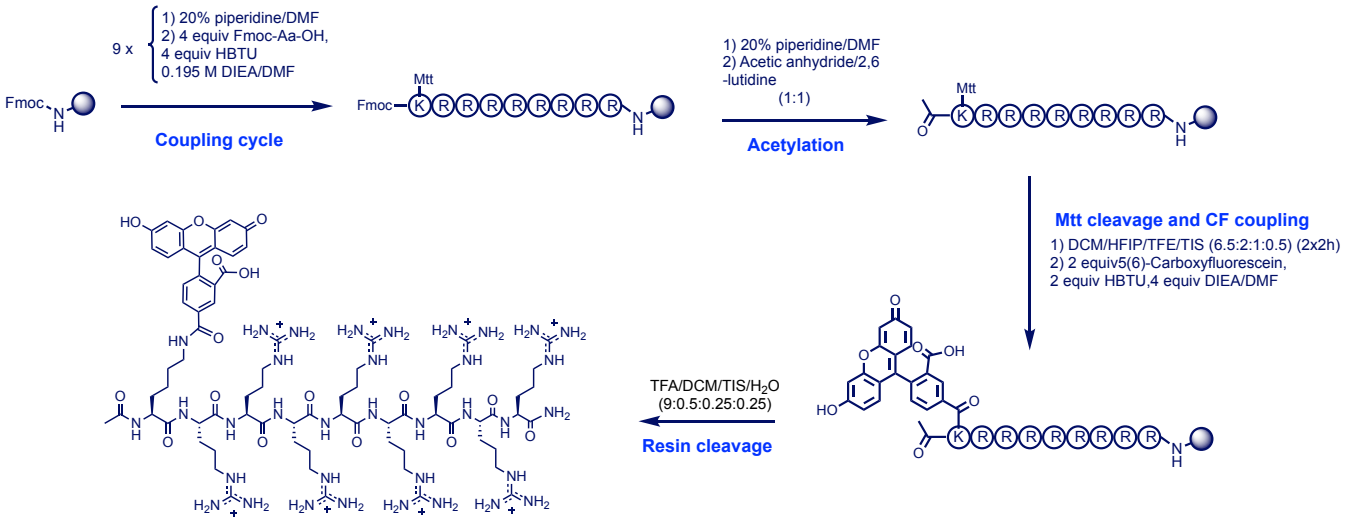


Figure S10. General synthetic scheme for the Solid Phase Peptide Synthesis (SPPS) for synthesizing the CFArg$_8$ peptide.

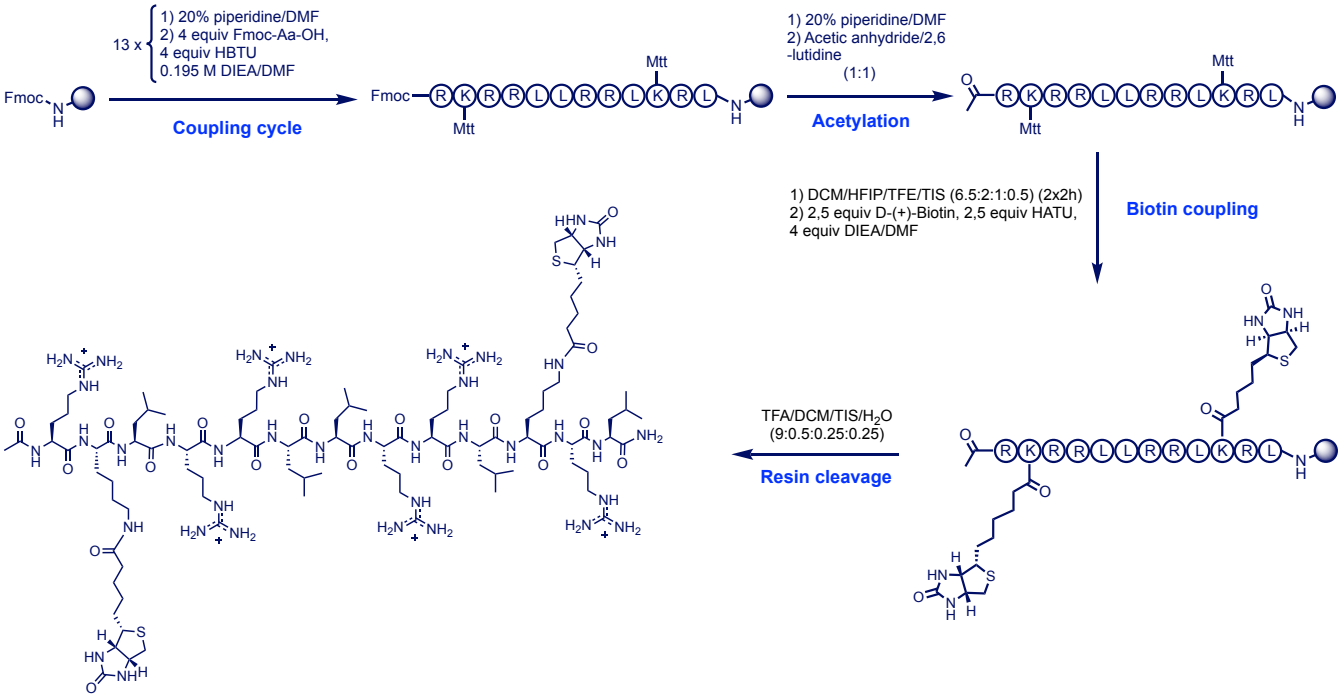


Figure S11. General synthetic scheme for the Solid Phase Peptide Synthesis (SPPS) for synthesizing the Ac**P**$(Biot)_2$ peptide.

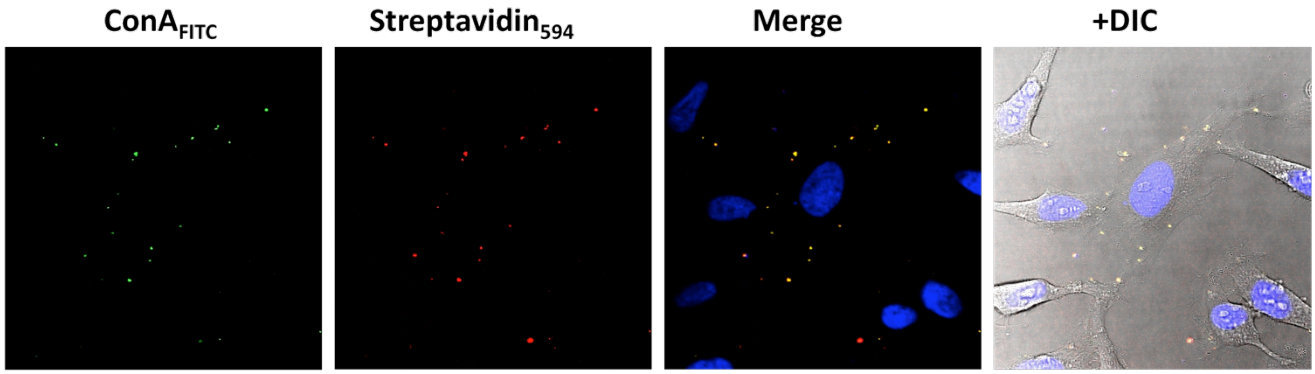


Figure S12. Lipofectamine is not a selective carrier. CLSM images of HeLa cells incubated with a mixture of 30 nM $ConA_{FITC}$, 30 nM $Streptavidin_{594}$ and 2 ng/μL of Lipofectamine 2000 (protein/Lipofectamine complex formation was done by incubation in a tenth of the final volume for 20 min) and incubated 30 min at 37 ºC. Both proteins co-localize in punctate structures, located at the perifery of the cell. Nuclei were counterstained with Hoechst (blue).

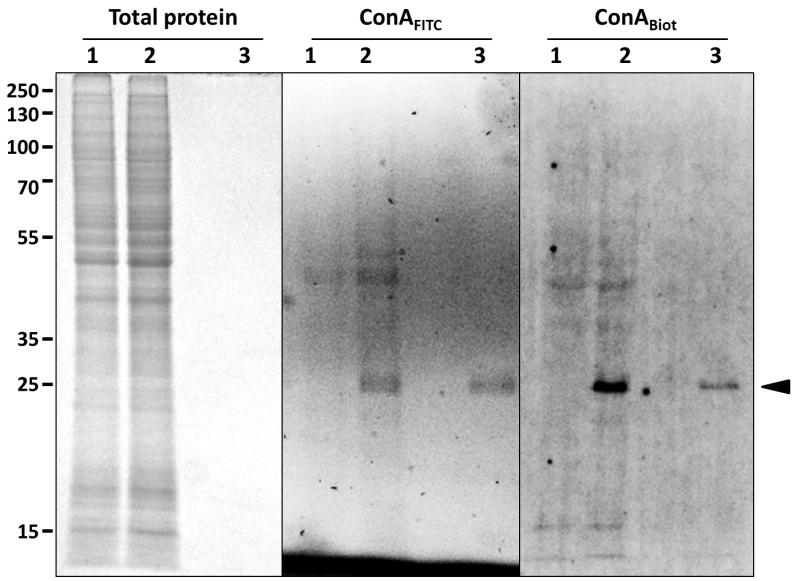


Figure S13. Gel electrophoresis to confirm integrity of the cargo after delivery. HeLa cells untreated (lane 1) or incubated with 3 μM of AcP(Man)$_2$ and 30 nM of FITC- or biotin-labeled ConA (as indicated over the panels) (lane 2) for 30 min at 37 ºC were washed, trypsinized and lysed, and proteins in the lysates resolved by SDS-PAGE. A small amount of the original protein was loaded as a control (lane 3). ConA$_{FITC}$ was imaged in the unfixed gel under UV light (ConA$_{FITC}$ panel) and afterwards the gel was fixed and stained with Coomassie Brilliant Blue to confirm that similar amounts of protein were loaded in lanes 1 and 2 (Total protein panel). ConA$_{Biot}$ was detected after protein transfer to PVDF membrane and incubation with Streptavidin$_{488}$ (Alexa Fluor-488). Arrowhead indicates the expected position of the ConA monomer (~25 kDa).

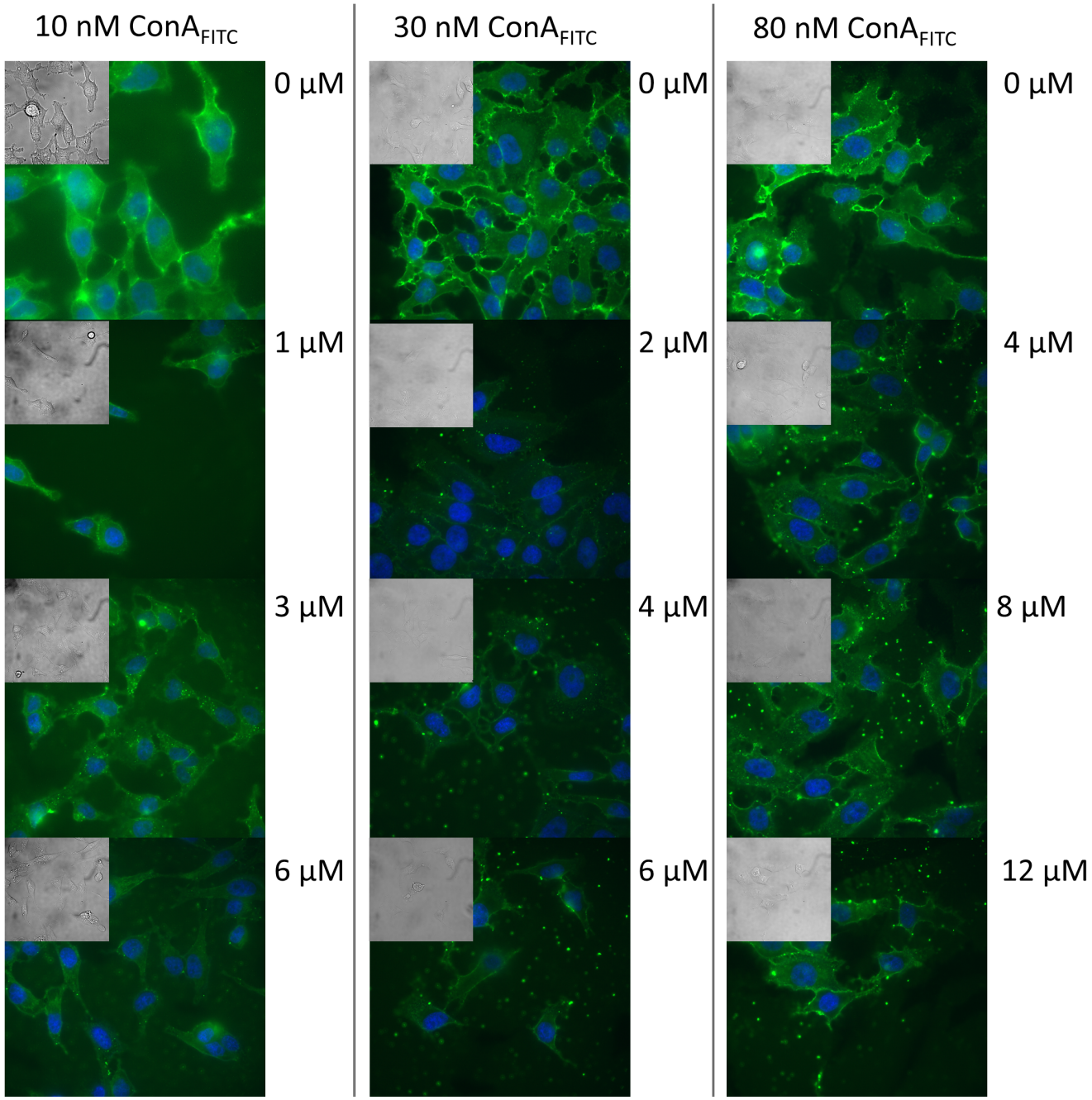


Figure S14. Importance of peptide/lectin ratio. Different amounts of ConA$_{FITC}$, as indicated above the panels, were incubated for 30 min with HeLa cells in the presence of the concentrations of Ac**P**(Man)$_2$ indicated at the right of the panels. Nuclei were counterstained with Hoechst (blue).

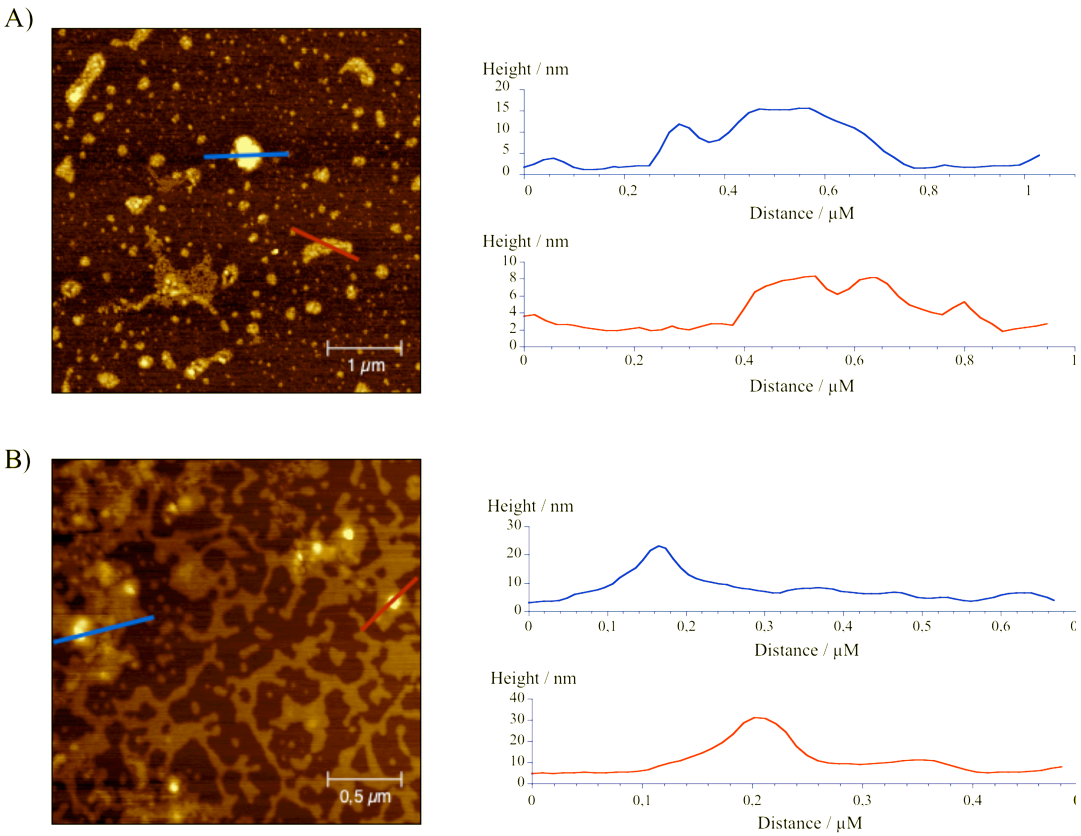


Figure S15. AFM topography images of A) Ac**P**$(Man)_2$ (2 µM) and B) Ac**P**$(Man)_2$ (4 µM) with ConA (30 nM) aqueous dispersions deposited on mica surface; images showed highly disperse aggregates. Dispersions prepared and deposited analogously as described in methods.

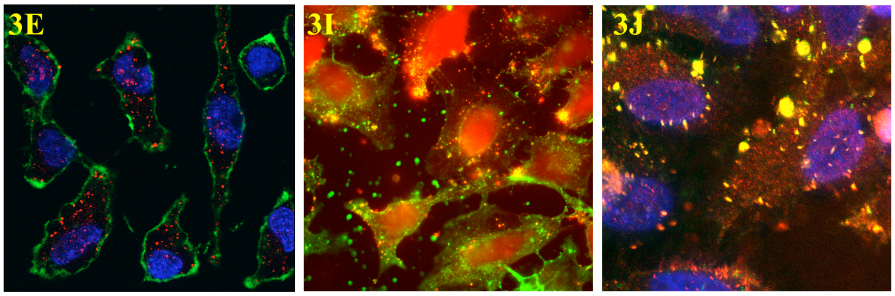


Figure	Manders' tM_1 (Peptide)	Manders' tM_2 (Protein)
3E	0.03	0.04
3I	0.52	0.64
3J	0.66	0.63

Table S1. Co-localization parameters calculated for figures 3E, 3I, and 3J. 3E) CLSM image of $CFArg_8$ (red) and $ConA_{647}$ (green); 3I) epifluorescence image of Tm**P**$(Man)_2$ (red) and $ConA_{647}$ (green); 3J) CLSM image of Tm**P**$(Man)_2$ (red) and $ConA_{FITC}$ (green). Co-localization parameters were calculated with ImageJ using the plugin Coloc2 and the Costes method for the estimation of the threshold.

Peptide	K_d
Tm**P**$(Man)_2$	14 ± 1
Tm**P**(Alloc)(Man)	27 ± 4
Tm**P**$(Acetone)_2$	151 ± 7
$TmArg_2(Man)_2$	9 ± 1
$TmArg_6Gly_5(Man)_2$	19 ± 2
$TmArg_6(Man)_2$	16 ± 1
Methyl α-D-mannopyranoside	137 ± 29

Table S2. Equilibrium dissociation constants obtained by fluorescence anisotropy after fitting to a simple 1:1 binding model.

Video S1. Internalization kinetics. To study the internalization kinetics, Tm**P**$(Man)_2$ (3 μM) and $ConA_{FITC}$ (30 nM) were incubated for 7 min in HKR to generate peptide/protein complexes. This solution was added to HeLa cells, previously washed with HKR, and the plate was immediately imaged on an epifluorescence microscope. Images for both peptide (red) and protein (green) channels were taken at intervals of 3 min for 20 min.

Materials and Methods

Commercially available Rink Amide-resin, *N*-HBTU, EDC and Fmoc-*L*-Lys(Mtt)-OH were used as obtained from Iris. [(*tert*-butoxycarbonyl)aminooxy]acetic acid, Chlorpromazine and Dexamethasone were purchased from TCI Chemicals. D-(+)-Biotin was used as obtained from Alfa Aesar. Dimethylaminopyridine, N-Hydroxysuccinimide, Fmoc-*L*-Gly-OH, Fmoc-*L*-Leu-OH, Fmoc-*L*-Arg(Pbf)-OH, Fmoc-6Ahx-OH, 5(6)-Carboxyfluorescein, Concanavalin A from *Canavalia ensiformis*, Concanavalin A from *Canavalia ensiformis* (Jack bean) FITC conjugate, propidium iodide, Ammonium chloride, Chloroquine, Heparin sodium salt and 5-(N-Ethyl-N-isopropyl)amiloride were purchased from Sigma-Aldrich®. 5(6)-Carboxytetramethlyrhodamine succinimidyl ester, 4-methylumbelliferyl-α-*D*-mannopyranoside (4-MU-α-D-Man) and Methyl-β-cyclodextrin were purchased from Carbosynth. Hoechst 33342 Trihydrochloride Trihydrate, Concanavalin A Alexa Fluor 647 conjugate, Lysotracker green DND-26, Streptavidin Alexa Fluor 594, Streptavidin Alexa Fluor 488, Lipofectamine 2000 and Coomassie (Bradford) protein assay kit were purchased in ThermoFisher. Wortmannin was obtained from Fluorochem. Egg yolk *L*-α-phosphatidylcholine (EYPC) was purchased from Avanti Polar Lipids, Inc. 1-α-formylmethyl-mannopyranoside[1] , Ox-Dexamethasone (Ox-Dex)[2] were synthesized according to protocols described in the literature[1,2]. Deuterated solvents (D_2O and $CDCl_3$) 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 on DIONEX Ultimate 3000 U-HPLC$^+$ (Thermo Scientific) with an Acclaim RSLC 120-C18 column with *Solvent A:Solvent B* gradients between 5:95 and 5:75 (*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) 100A column. An Agilent 1200 with an Agilent Eclipse XDB-C18 column was used for semi-preparative purification using gradients between 5:95 and 5:75 (*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 δ H 4.79 and $CDCl_3$ δ H 7.26. 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 Dynamica HALO XB-10 UV-VIS Single Beam Spectrophotometer. Fluorescence measurements were performed with a FluoroMax-2 spectrofluorometer (Jobin-Yvon Spex) equipped with a stirrer and a temperature controller. 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.

Absorbance and fluorescence of cellular extracts were measured using a microplate reader Tecan Infinite F200Pro. Absorbance of labelled Concanavalin A was measured using a NanoDrop 1000 spectrophotometer. AFM images were acquired in a Park NX10 microscope in non-contact mode using ACTA silicon cantilevers with 37 N/m nominal spring constant (k) and 300 kHz nominal resonance frequency.

Abbreviations

Peptide Abbreviations: Tm**P**$(Man)_2$ (Tm = TAMRA and Man = Mannose aldehyde); Aa: Amino acid; AFM: Atomic Force Microscope; Arg: Arginine; BSA: Bovine Serum Albumin; Boc: *tert*-Butoxycarbonyl; Calcd: Calculated; CF: 5(6)-Carboxyfluorescein; CLSM: Confocal laser scanning microscopy; ConA: Concanavalin A; CPP: Cell-Penetrating Peptide; DCM: Dichloromethane; DIEA: N,N-Diisopropylethylamine; DMAP: 4-Dimethylaminopyridine; DMEM: Dulbecco's Modified Eagle Medium; DMF: N,N-Dimethylformamide; DMSO: Dimethylsulfoxide; EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; EIPA: 5-(N-Ethyl-N-isopropyl)amiloride; EYPC: Egg yolk phosphatidylcholine; FBS: Fetal Bovine Serum; Fmoc: 9-fluorenylmethoxycarbonyl; GFP: Green Fluorescent Protein; GR-GFP: Glucocorticoid receptor-Green Fluorescent Protein; 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-methylmethanaminiun hexafluorophosphate N-oxide; *N*-HBTU: N-[(1H-Benzotriazol-1-yl)4-(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate N-oxide; NHS: N-Hydroxysuccinimide; NLS: nuclear localization signal; Ox: Oxime; Ox-Dex: Dexamethasone Acid; Pbf: 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl; PI: propidium iodide; 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.

General protocols

General protocol for the SPPS

All peptides were synthesized by manual Fmoc solid-phase peptide synthesis[3] 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%, 2 mL) for 10 min and then the mixture was filtered and the resin was washed with DMF (3 x 2 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 (3 x 2 mL, 1 min). The efficiency of each amino acid coupling and deprotection was monitored employing the TNBS test[4].

Once the linear peptide was finished, two different ending protocols were used:

A) Acetylation: the acetylation capping of N-terminal group was performed by standard Fmoc removal conditions (20% piperidine in DMF) followed by treatment with a solution of acetic anhydride and 2,6-lutidine (1:1, 1 mL) for 30 min.

B) Linker coupling: after Fmoc cleavage with piperidine/DMF (20%, 2 mL), 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 (2 x 2 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, 2 x 2 mL, 2 h). Finally, the mixture was filtered and the resin was washed with DCM (2 x 2 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 (3 x 2 mL, 2 min) and DCM (3 x 2 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 (3 x 3 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 (3 x 3 mL) and DCM (3 x 3 mL).

Ox-Dex terminating peptide, the Fmoc-protecting group of the linker was removed by using a solution of piperidine in DMF (20%, 4 mL) during 15 min and the resin was washed with DMF (3 x 3 mL). A solution of Ox-Dex (3 equiv), *N*-HATU (2.9 equiv) and DIEA (0.195 M, 3 equiv) in DMF (2 mL) was added and the mixture was shaken by bubbling Ar for 30 min. Finally, the resin was washed with DMF (3 x 3 mL) and DCM (3 x 3 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 (R^1**P**$(Ox)_2$) in H_2O (5 mM) was reacted with a solution of corresponding ligands (2 equiv per alkoxyamine) [1-α-formylmethyl-mannopyranoside or acetone] 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 a C18 RP-HPLC [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 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 analytical HPLC, ^{1}H-NMR and low and high resolution mass spectrometry.

Synthesis of peptides

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

Following the general protocol of the SPPS for synthesizing an acetylated peptide with two mannoses, Ac**P**$(Man)_2$ was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 28% and 99.3% purity. R_t 3.8 min (Fig. S16) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)]. **^{1}H NMR** (300 MHz, D_2O, δ): 7.77 (t, J = 5.3 Hz, 1H), 7.17 (t, J = 3.9 Hz, 1H), 4.93 (s, 2H), 4.59 (s, 4H), 4.45-4.22 (m, 17H), 4.07-3.64 (m, 12H), 3.33-3.16 (m, 16H), 2.07 (s, 3H), 1.98-1.32 (m, 51H), 1.03-0.82 (m, 30H). **MS** (ESI, H_2O): 1300 (19, $[M+2H+2TFA]^{2+}$), 1243 (21, $[M+2H+TFA]^{2+}$), 1187 (23, $[M+2H]^{2+}$), 867 (42, $[M+3H+2TFA]^{3+}$), 827 (67, $[M+3H+TFA]^{3+}$), 791 (100, $[M+3H]^{3+}$), 593 (38, $[M+4H]^{4+}$). **HRMS** (ESI): Calcd for $C_{100}H_{188}N_{36}O_{30}$: 1186.7139; found: 1186.7141 ($[M+2H]^{2+}$).

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

Following the general protocol of the SPPS for synthesizing a TAMRA labelled peptide with two mannoses, Tm**P**$(Man)_2$ was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 6% and 100% purity. R_t 4.1 min (Fig. S17) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)]. **^{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, $[M+2H+TFA]^{2+}$), 1027 (100, $[M+3H+2TFA]^{3+}$), 989 (99, $[M+3H+TFA]^{3+}$), 743 (62, $[M+4H+TFA]^{4+}$), 716 (53, $[M+4H]^{4+}$). **HRMS** (ESI): Calcd for $C_{129}H_{217}N_{39}O_{34}$: 1428.3209; found: 1428.3220 ($[M+2H]^{2+}$).

Synthesis of Ac**P**(Alloc)(Man)

Following the general protocol of the SPPS for synthesizing an acetylated peptide with one mannose, and using Alloc protected Lysine, Ac**P**(Alloc)(Man) was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 15% and 100% purity. R_t 4.1 min (Fig. S18) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)]. **^{1}H NMR** (300 MHz, D_2O, δ): 7.62 (t, J = 5.3 Hz, 1H), 7.02 (t, J = 3.7 Hz, 1H), 5.90-5.73 (m, 1H), 5.23-5.06 (m, 2H), 4.78 (s, 1H), 4.48-4.39 (m, 5H), 4.31-4.04 (m, 15H), 3.89-3.46 (m, 6H), 3.15-2.95 (m, 16H), 1.92 (s, 3H), 1.78-1.12 (m, 51H), 0.87-0.70 (m, 30H). **MS** (ESI, H_2O): 1260 (18, $[M+2H+3TFA]^{2+}$), 1203 (27, $[M+2H+2TFA]^{2+}$), 1147 (20, $[M+2H+TFA]^{2+}$), 803 (38, $[M+3H+2TFA]^{3+}$), 763 (100, $[M+3H+TFA]^{3+}$), 727 (42, $[M+3H]^{3+}$), 574 (10, $[M+4H+TFA]^{4+}$), 547 (27, $[M+4H]^{4+}$). **HRMS** (ESI): Calcd for $C_{94}H_{175}N_{35}O_{24}$: 1090.6868; found: 1090.6871 ($[M+2H]^{2+}$).

Synthesis of Tm**P**(Alloc)(Man)

Following the general protocol of the SPPS for synthesizing a TAMRA labelled peptide with one mannose, and using Alloc protected Lysine, Tm**P**(Alloc)(Man) was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 10% and 98.4% purity. R_t 4.3 min (Fig. S19) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)]. **^{1}H NMR** (300 MHz, D_2O, δ): 8.24 (s, 1H), 8.08-7.87 (m, 2H), 7.55 (m, 1H), 7.03 (s, 2H), 6.88-6.61 (m, 3H), 5.71 (m, 2H), 5.07 (m, 3H), 4.52-4.30 (m, 4H), 4.29 (s, 2H), 4.26-3.92 (m, 13H), 3.86-3.44 (m, 6H), 3.30 (m, 2H), 3.09 (s, 12H), 3.08 (s, 16H), 2.22 (m, 2H), 1.96-1.12 (m, 57H), 0.95-0.59 (m, 30H). **MS** (ESI, H_2O): 965 (43, $[M+3H+2TFA]^{3+}$), 927 (100, $[M+3H+TFA]^{3+}$), 668 (40, $[M+4H]^{4+}$), 533 (10, $[M+5H]^{5+}$). **HRMS** (ESI): Calcd for $C_{123}H_{204}N_{38}O_{28}$: 888.5326; found: 888.5330 ($[M+3H]^{3+}$).

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

Following the general protocol of the SPPS for synthesizing an acetylated peptide capped with acetone, Ac**P**$(Acetone)_2$ was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 4% and 100% purity. R_t 4.3 min (Fig. S20) **[**RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)]. **^{1}H NMR** (300 MHz, D_2O, δ): 4.36 (s, 4H), 4.27-4.05 (m, 13H), 3.20-3.00 (m, 16H), 1.92 (s, 3H), 1.83 (s, 6H), 1.76 (s, 6H), 1.71-1.17 (m, 51H), 0.85-0.69 (m, 30H); **MS** (ESI, H_2O): 1193 (30, $[M+2H+3TFA]^{2+}$), 1133 (39, $[M+2H+2TFA]^{2+}$), 1077 (23, $[M+2H+TFA]^{2+}$), 757 (30, $[M+3H+3TFA]^{3+}$), 720 (100, $[M+3H+TFA]^{3+}$), 680 (61, $[M+3H]^{3+}$), 513 (30, $[M+4H]^{4+}$). **HRMS** (ESI): Calcd for $C_{90}H_{172}N_{36}O_{18}$: 1022.6820; found: 1022.6820 ($[M+2H]^{2+}$).

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 [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 12% and 99.9% purity. R_t 4.4 min (Fig. S21) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)]. **^{1}H NMR** (300 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): Calcd for $C_{119}H_{201}N_{39}O_{22}$: 1264.2902; found: 1264.2899 ($[M+2H]^{2+}$).

Synthesis of Ac$Arg_2(Man)_2$

Following the general protocol of the SPPS for synthesizing an acetylated tetrapeptide with two mannoses, Ac$Arg_2(Man)_2$ was obtained after RP- HPLC purification [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 15% and 100% purity. R_t 3.1 min (Fig. S22) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)]. **^{1}H NMR** (300 MHz, D_2O, δ): 7.62 (t, J = 5.3 Hz, 1H), 7.02 (t, J = 3.9 Hz, 1H), 4.78 (s, 2H), 4.44 (s, 4H), 4.25-4.07 (m, 8H), 3.90-3.47 (m, 12H), 3.20-2.98 (m, 8H), 1.91 (s, 3H), 1.83-1.13 (m, 20H). **MS** (ESI, H_2O): 1183 (17, $[M+H]^+$), 590 (100, $[M+2H]^{2+}$). **HRMS** (ESI): Calcd for $C_{46}H_{84}N_{15}O_{21}$: 1182.5963; found: 1182.5961 ($[M+H]^+$).

Synthesis of Tm$Arg_2(Man)_2$

Following the general protocol of the SPPS for synthesizing a TAMRA labelled tetrapeptide with two mannoses, Tm$Arg_2(Man)_2$ was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 26% and 100% purity. R_t 3.8 min (Fig. S23) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)]. **^{1}H NMR** (300 MHz, D_2O, δ): 8.35 (d, J = 14.1 Hz, 1H), 7.99 (t, J = 5.5 Hz, 1H), 7.56 (t, J = 10.4 Hz, 1H), 7.43 (d, J = 7.8 Hz, 1H), 7.11-6.98 (m, 2H), 6.95 (s, 1H), 6.80 (d, J = 9.5 Hz, 2H), 6.64 (d, J = 7.0 Hz, 2H), 4.73 (s, 2H), 4.38 (s, 4H), 4.22-3.99 (m, 4H), 3.85-3.33 (m, 12H), 3.10 (s, 12H), 3.08-2.98 (m, 12H), 2.86 (t, J = 7.5 Hz, 2H), 2.24 (t, J = 7.0 Hz, 2H), 1.81-1.12 (m, 26H). **MS** (ESI, H_2O): 834 (100, $[M+2H]^{2+}$), 556 (95, $[M+3H]^{3+}$). **HRMS** (ESI): Calcd for $C_{75}H_{114}N_{18}O_{25}$: 833.4088; found: 833.4096 ($[M+2H]^{2+}$).

Synthesis of Tm$Arg_6Gly_5(Man)_2$

Following the general protocol of the SPPS for synthesizing a TAMRA labelled peptide with two mannoses, Tm$Arg_6Gly_5(Man)_2$ was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H_2O

(0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 9% and 100% purity. R_t 3.4 min (Fig. S24) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)]. **^{1}H NMR** (500 MHz, D_2O, δ): 8.42 (s, 1H), 8.02 (s, 1H), 7.66-7.49 (m, 1H), 7.41 (s, 1H), 7.09-6.88 (m, 3H), 6.71 (s, 2H), 6.29 (s, 2H), 4.77 (s, 2H), 4.74 (s, 4H), 4.46-4.30 (m, 4H), 4.27-4.04 (m, 10H), 3.98-3.73 (m, 16H), 3.75-3.45 (m, 12H), 3.37 (d, J = 5.3 Hz, 2H), 3.17-3.01 (m, 12H), 2.98 (s, 8H), 2.24 (s, 2H), 1.85-1.10 (m, 42H). **MS** (ESI, H_2O): 1402 (7, $[M+2H+2TFA]^{2+}$), 973 (42, $[M+3H+3TFA]^{3+}$), 936 (100, $[M+3H+2TFA]^{3+}$), 898 (65, $[M+3H+TFA]^{3+}$), 702 (60, $[M+4H+2TFA]^{4+}$), 674 (95, $[M+4H+TFA]^{4+}$), 645 (35, $[M+4H]^{4+}$), 516 (45, $[M+5H]^{5+}$). **HRMS** (ESI): Calcd for $C_{109}H_{177}N_{39}O_{34}$: 1288.1651; found: 1288.1655 ($[M+2H]^{2+}$).

Synthesis of $TmArg_6(Man)_2$

Following the general protocol of the SPPS for synthesizing a TAMRA labelled peptide with two mannoses, $TmArg_6(Man)_2$ was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 9% and 100% purity. R_t 3.4 min (Fig. S25) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)]. **^{1}H NMR** (300 MHz, D_2O, δ): 8.41 (s, 1H), 7.99 (d, J = 8.5 Hz, 1H), 7.57-7.29 (m, 2H), 7.00-6.85 (m, 3H), 6.68 (d, J = 9.3 Hz, 2H), 6.33 (s, 2H), 4.84-4.66 (m, 2H), 4.70-4.65 (m, 4H), 4.32 (d, J = 15.0 Hz, 4H), 4.17-3.91 (m, 8H), 3.88-3.37 (m, 12H), 3.34-3.24 (m, 2H), 2.98 (s, 12H), 2.96 (s, 16H), 2.23-2.08 (m, 2H), 1.77-1.03 (m, 42H). **MS** (ESI, H_2O): 840 (90, $[M+3H+2TFA]^{3+}$), 803 (100, $[M+3H+TFA]^{3+}$), 765 (30, $[M+3H]^{3+}$), 629 (30, $[M+4H+2TFA]^{4+}$), 602 (25, $[M+4H+TFA]^{4+}$). **HRMS** (ESI): Calcd for $C_{99}H_{162}N_{34}O_{29}$: 1145.6119; found: 1145.6118 ($[M+2H]^{2+}$).

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

Following the general protocol of the SPPS for synthesizing an Dex labelled peptide with two mannoses, Dex**P**$(Man)_2$ was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 5% and 99.6% purity. R_t 4.2 min (Fig. S26) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)]. **^{1}H NMR** (300 MHz, D_2O, δ): 7.62 (s, 1H), 7.38 (d, J = 10.4 Hz, 1H), 7.00 (s, 1H), 6.27 (d, J = 9.9 Hz, 1H), 6.07 (s, 1H), 4.77 (s, 2H), 4.43 (s, 8H), 4.13 (m, 13H), 3.87-3.44 (m, 12H), 3.23 (s, 2H), 3.15-2.90 (m, 16H), 2.84 (m, 2H), 2.26 (m, 2H), 2.03-1.11 (m, 70H), 0.93-0.62 (m, 36H). **MS** (ESI, H_2O): 1573 (18, $[M+2H+3TFA]^{2+}$), 1517 (20, $[M+2H+2TFA]^{2+}$), 1049 (22, $[M+3H+3TFA]^{3+}$), 1011 (90 $[M+3H+2TFA]^{3+}$), 974 (100, $[M+3H+TFA]^{3+}$), 938 (50, $[M+3H]^{3+}$). **HRMS** (ESI): Calcd for $C_{125}H_{222}FN_{37}O_{34}$: 1402.3371; found: 1402.3376 ($[M+2H]^{2+}$).

Synthesis of $CFArg_8$

For the preparation of the control peptide $CFArg_8$, the carboxyfluorescein was coupled in the amino group of a lysine Mtt localized at the end of the peptide sequence. Therefore, following the general protocol of the SPPS for

growing the peptide, the Mtt protecting group was selectively removed by mechanically stirring the resin with a mixture of DCM/HFIP/TFE/TIS (6.5:2:1:0.5, 2 x 1 mL per 70 mg of resin) for 2 h. Finally, the mixture was filtered and the resin was washed with DCM (2 x 2 mL, 2 min) and DMF (2 mL, 20 min). Then, a solution of 5(6)-carboxyfluorescein (2 equiv) and *N*-HBTU (2 equiv) in DMF (1 mL) was added to the vessel followed by the drop wise addition of DIEA (4 equiv). The resulting mixture was shaken by bubbling Ar for 30 min and finally the filtered resin was washed with DMF (3 x 2 ml, 2 min) and DCM (3 x 2 ml, 2 min) (Fig. S10).

$CFArg_8$ was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 11% and 100% purity. R_t 3.2 min (Fig. S27) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)]. **^{1}H NMR** (500 MHz, D_2O, δ): 8.16 (d, *J* = 1.8 Hz, 1H), 7.93 (t, *J* = 7.9 Hz, 1H), 7.58 (s, 1H), 7.36 (d, *J* = 7.9 Hz, 1H), 7.17-7.06 (m, 2H), 6.86-6.77 (m, 1H), 6.72 (td, *J* = 9.3 and 1.8 Hz, 1H), 6.60 (dd, *J* = 9.2 and 2.4 Hz, 1H), 4.24-3.86 (m, 9H), 3.32 (m, 2H), 3.12-2.87 (m, 16H), 1.88 (d, *J* = 10.2 Hz, 3H), 1.76-1.16 (m, 38H). **MS** (ESI, H_2O): 675 (50, $[M+3H+2TFA]^{3+}$), 638 (100, $[M+3H+TFA]^{3+}$), 599 (45, $[M+3H]^{3+}$), 477 (50, $[M+4H+TFA]^{4+}$), 450 (58, $[M+4H]^{4+}$), 360 (17, $[M+5H]^{5+}$). **HRMS** (ESI): Calcd for $C_{77}H_{125}N_{35}O_{16}$: 898.0008; found: 898.0016 ($[M+2H]^{2+}$).

Synthesis of Ac**P**$(Biot)_2$

For the preparation of the biotinylated peptide, D-(+)-Biotin was coupled in the amino group of the lysines Mtt localized at the peptidic sequence. Therefore, following the general protocol of the SPPS for growing the peptide, the Mtt protecting group was selectively removed by mechanically stirring the resin with a mixture of DCM/HFIP/TFE/TIS (6.5:2:1:0.5, 2 x 1 mL per 70 mg of resin) for 2 h. Finally, the mixture was filtered and the resin was washed with DCM (2 x 2 mL, 2 min) and DMF (2 mL, 20 min). Then, a solution of D-(+)-Biotin (2 .5 equiv) and N-HATU (2.5 equiv) in DMF (1 mL) was added to the vessel followed by the drop wise addition of DIEA (4 equiv). The resulting mixture was shaken by bubbling Ar for 30 min and finally the filtered resin was washed with DMF (3 x 2 ml, 2 min) and DCM (3 x 2 ml, 2 min) (Fig. S11).

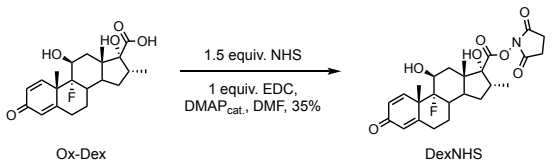
Ac**P**$(Biot)_2$ was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 11% and 100% purity. R_t 4.1 min (Fig. S28) [RP-HPLC Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)]. **^{1}H NMR** (500 MHz, D_2O, δ): 4.52-4.39 (m, 2H), 4.34-4.22 (m, 2H), 4.22-3.83 (m, 13H), 3.22-2.95 (m, 20H), 2.90-2.74 (m, 2H), 2.69-2.53 (m, 2H), 2.10 (s, 3H), 1.97-1.88 (m, 2H), 1.88-1.14 (m, 63H), 0.84-0.65 (m, 30H). **MS** (ESI, H_2O): 1362 (10, $[M+2H+4TFA]^{2+}$), 1306 (32, $[M+2H+3TFA]^{2+}$), 1249 (30, $[M+2H+2TFA]^{2+}$), 1191 (7, $[M+2H+TFA]^{2+}$), 870 (18, $[M+3H+3TFA]^{3+}$), 832 (80, $[M+3H+2TFA]^{3+}$), 795 (100, $[M+3H+TFA]^{3+}$), 756 (35, $[M+3H]^{3+}$), 624 (20, $[M+4H+2TFA]^{4+}$), 596 (25, $[M+4H+TFA]^{4+}$), 567 (40, $[M+4H]^{4+}$). **HRMS** (ESI): Calcd for $C_{100}H_{186}N_{38}O_{18}S_2$: 1135.7120; found: 1135.7119 ($[M+2H]^{2+}$).

Preparation of Dexamethasone labelled Concanavalin A

Synthesis of Dexamethasone-NHS

Dexamethasone-NHS was prepared by the reaction of Ox-Dex[S2] (0.106 mmol) with N-Hydroxysuccinimide (1.5 equiv) in DMF, using EDC (1 equiv) and DMAP (cat.) for 4 h. The DMF was removed by rotary evaporation and the oil obtained was dried. The compound was washed with 1 M solution of HCl and after that with a saturated solution of $NaHCO_3$. Purification by silica gel column chromatography (DCM/MeOH, 99:1) provided final product with an overall yield of 35%.

^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.22 (d, *J* = 10.1 Hz, 1H), 6.32 (dd, *J* = 10.1, 1.9 Hz, 1H), 6.12 (s, 1H), 4.37 (d, *J* = 8.1 Hz, 1H), 3.22 (s, 1H), 3.12-2.97 (m, 1H), 2.84 (s, 4H), 2.63 (td, *J* = 13.8, 13.0, 6.1 Hz, 1H), 2.49-2.17 (m, 4H), 1.87-1.72 (m, 3H), 1.63-1.56 (m, 1H), 1.55 (s, 3H), 1.35-1.28 (m, 2H), 1.22 (s, 3H), 1.07 (d, *J* = 7.1 Hz, 3H). **^{13}C NMR (**500 MHz, $CDCl_3$, δ): 186.56 (q), 168.40 (q), 166.04 (q), 152.12 (CH), 129.81 (CH), 125.13 (CH), 101.02 (q), 99.62 (q), 86.46 (q), 71.99 (CH), 48.62 (q), 48.17 (q), 42.96 (CH), 37.01 (CH), 35.88 (CH_2), 34.32 (CH), 32.35 (CH_2), 30.99 (CH_2), 29.70 (CH_2), 25.67 (CH_2), 22.90 (CH_3), 16.70 (CH_3), 14.57 (CH_3). (Fig. S29). **HRMS** (ESI): Calcd for $C_{25}H_{31}FNO_7$: 476.2082; found: 476.2079.



Synthesis of ConA-Dex

ConA-Dex was prepared by the reaction of Concanavalin A (3 mg/mL) with Dexamethasone-NHS (50 equiv) in $NaHCO_3$ buffer (pH 9) at room temperature for 2 h. The resulting solution was dialysed overnight in HKR buffer. To quantify the number of dexamethasones per Concanavalin A the absorbance of the mixture was measured using a NanoDrop UV-Vis Spectrophotometer. Absorbance values at 242 nm (maximum absorbance for dexamethasone) and 280 nm (maximum absorbance for the protein) were measured for various dilutions of the dialysed ConA-Dex, and several known concentrations of ConA or dexamethasone. Data in the linear range were analysed using the following system of equations:

$$A_{242}^{\mathrm{ConA-Dex}} = x \cdot A_{242}^{\mathrm{ConA}} + y \cdot A_{242}^{\mathrm{Dex}}$$

$$A_{280}^{\mathrm{ConA-Dex}} = x \cdot A_{280}^{\mathrm{ConA}} + y \cdot A_{280}^{\mathrm{Dex}}$$

Where the values *x* and *y* were used to estimate the concentration of each component in ConA-Dex by multiplying the concentration of pure ConA or dexamethasone by *x* or *y* respectively, and the extent of labelling was calculated as the ratio between dexamethasone and ConA concentrations. A value of around 16 Dex per ConA tetramer was obtained.

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): 1 s; scanning mode: continuous; scanning speed: 200 nm/min. Measurements were done from 10 ºC to 60 ºC (data interval: 10 ºC; temp. gradient 5 ºC/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.

For the measurements in liposomes, samples were prepared by drying under reduced pressure L-α-phosphatidylcholine (8.5 μL, 100 mg/mL solution in $CHCl_3$) and peptide (325 μL, 200 μM) in TFE to obtain a ratio lipid/peptide of 18:1. Lipids were suspended in HKR buffer (650 μL) and sonicated for 45 min until a clear solution was obtained. Spectra were recorded in a 0.2 cm path length quartz cell.

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,

$$[\theta]_{MRt} = \frac{100 \cdot \theta}{C \cdot l \cdot No.of\ residues} \qquad \text{(S1a)}$$

where θ is the ellipticity (mdeg), C is the peptide concentration (M) and l is the cell path length (cm).

$$[\theta]\% = \frac{\theta MRt_{222} - 2340}{30300} \times 10 \qquad \text{(S1b)}$$

Formula to calculate the percent of helicity in where the molar ellipticity at 222 nm is an absolute value.

Cells Lines and Culture

HeLa cells were incubated at 37 ºC/ 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).

Cell transport experiments in HepG2 cells

Internalization experiments in HepG2 cells were done as described for HeLa cells (see Methods in the manuscript). In the case of streptavidin, incubation time was increased to 5 h.

Cell viability: MTT Assay

Cell viability was established by a standard MTT assay (Fig. S7 and S8). One day before the assay, a suspension of HeLa or HepG2 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 and peptide/protein complexes diluted in HKR (50 µL/well). After 30 min of incubation at 37 ºC, the medium was aspirated and replaced by fresh medium (DMEM) containing 10% FBS (100 µL). Control cells were given only cell culture medium (100 µL final medium). 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 6 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%).

Propidium Iodide Assay

Cell viability was established by a standard PI assay (Fig. S7 and S8). One day before the assay, a suspension of HeLa or HepG2 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 and peptide/protein complexes diluted in DMEM (50 µL/well). After 30 min of incubation at 37 ºC, the medium was aspirated; cells were washed with fresh medium and then 100 µL of trypsin were added in each well and cells were incubated for 15 min at 37 ºC. After this, 100 µL of a solution of 2% FBS and 5 mM EDTA in PBS containing PI (0.25 µg/mL) were added and the plate was incubated for 5 min in the dark. Samples were analysed on a Guava EasyCyte^{TM} cytometer. Propidium iodide was measured by excitation at 532 nm and collecting emission at 695/50 nm. Data points were collected in triplicate and analysis was performed with InCyte software included in GuavaSoft 3.2 (Millipore).

Quantification of the uptake by fluorometry

For internalization assays, a modification of the quantification of CPP uptake by fluorometry protocol described in Holm *et al.*[5] was used. Briefly, semiconfluent monolayers of HeLa cells seeded the day before were washed twice with HKR before incubation with 4 µM of TAMRA labelled peptides, with Tm**P**$(Man)_2$/ConA or with Ac**P**$(Man)_2$/$ConA_{FITC}$ complexes (previously prepared by co-incubation for 7 min rt) diluted in the same buffer for 30 min at 37 ºC (unless otherwise stated). Then, cells were washed twice with HKR and trypsinized, and after the addition of 1 mL of HKR, centrifuged for 5 min at 1000 g. The resultant pellets were lysed by incubation in aqueous solution of NaOH (0.1 M) at 4 ºC for 60 min and frozen until analysis.

Where indicated, cells were pretreated for 30 min before the incubation of the peptide and during the incubation with the following inhibitors: Wortmannin (200 nM), chlorpromazine (30 µM), methyl-ß-cyclodextrin (5 mM), EIPA (50 µM), ammonium chloride (50 mM), chloroquine (100 µM) or heparin (5 µg/mL). For the incubation at low temperature, cells were placed on ice 15 min before the incubation with the peptides, and ice-cold solutions were used for the washes and incubation.

Fluorescence levels of the lysates were measured using a microplate reader (Infinite F200Pro, Tecan), both for TAMRA (λ_{ex} 560 nm, λ_{em} 610 nm) and fluorescein (λ_{ex} 485 nm, λ_{em} 535 nm) labelled compounds, and the concentration of the peptides or ConA was calculated by comparison to a standard curve. After neutralization with a volume of an aqueous solution of HCl (0.1 M), protein concentration in the lysates was determined using a Coomassie (Bradford) protein assay kit following the manufacturer instructions, and measuring the absorbance at 570 nm (Infinite F200Pro, Tecan). The uptake was calculated as pmol of peptide/mg of total protein, and normalized to the uptake in untreated cells in the case of the inhibition studies.

Glucocorticoid assay

The ability of the peptide to reach the cytosol was determined with a glucocorticoid induced GFP translocation assay (GIGT)[6]. In this assay, a GFP protein fused to the steroid binding domain of the glucocorticoid receptor (GR) accumulates in the nucleus of the cell in response to dexamethasone binding, so the translocation ratio (the ratio between the nuclear and cytoplasmic fluorescence) can be used as an indicator of the presence of dexamethasone-labelled peptides in the cytosol (Fig. S9).

HeLa cells grown in four chamber glass bottom dishes were transfected with the plasmid pK7-GR-GFP (a gift from Ian Macara[7], Addgene plasmid #15534) using Lipofectamine 2000 and, 24 h post-transfection, cells were washed with HKR and incubated for 30 min with Hoechst 33342 (1 μM), and endocytosis inhibitors where indicated, at the following concentrations: chlorpromazine (50 μM), methyl-ß-cyclodextrin (5 mM), Wortmannin (200 nM), chloroquine (100 μM), NH_4Cl (50 mM), heparin (5 μg/mL) or EIPA (50 μM). Cells were then incubated for 1 h with Dex**P**$(Man)_2$ (4 μM), in the presence or absence of unlabelled ConA (30 nM), with dexamethasone (1 μM) (as positive control) or just with HKR (as negative control) and immediately imaged. To study the cytosolic release of ConA-Dex, cells were incubated with 7 nM of Dex-labelled ConA (equivalent to around 100 nM Dexamethasone) previously incubated with 4 μM of Ac**P**$(Man)_2$. As controls, cells were incubated with the same amount of ConA-Dex in the absence of peptide or after incubation for 20 min with 1 μg of Lipofectamine 2000. Twenty to thirty images of each sample were acquired with an Andor Zyla 4.2 digital camera mounted on a Nikon Eclipse Ti-E microscope at 60x magnification and the translocation ratio (the ratio of the median intensities of GFP in the nucleus and in the 2 μm wide surrounding region) was calculated with CellProfiler[8] as follows. Nuclei were identified as Hoechst stained objects using the three-class thresholding Otsu method and the cytoplasmic region was defined as the 2 μm surrounding area. To ensure a better separation of the cytoplasmic and nuclear region, nuclei were shrunk 0.5 μm before measuring object intensity. Cells falling below the 20% of the maximum intensity of the image were considered untransfected and discarded for the analysis. A total of 40 to 80 cells were analyzed for each sample.

Statistical analysis of the data was performed with R software[S9]. Results were subjected to pairwise two-tailed Student's t-test and p-values were adjusted using Bonferroni's correction.

Time-lapse videos

For time-lapse experiments, HeLa cells incubated with the peptide/protein complexes were placed in an incubator chamber at 37 ºC on a Nikon Eclipse Ti-E inverted microscope. The acquisition of the images was controlled with NIS-Elements software (Nikon), using the indicated time intervals. Videos were assembled using ImageJ.

Gel electrophoresis

To study protein degradation, HeLa cells were incubated with the mixtures of ConA (FITC- or biotin-labelled) and Ac**P**$(Man)_2$ for 30 min in HKR at 37 ºC. Cells were washed with HKR, trypsinized and centrifuged for 5 min at 1000 g, 4 ºC. Pellets were re-suspended in Laemmli buffer 1x (60 mM Tris-HCl pH 6.8, 2 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol, 0.0010 % bromophenol blue) and boiled for 5 min. Proteins were separated on 12.5 % SDS-PAGE gels.

Gels containing FITC-labelled protein were directly imaged under UV light in a Gel Doc system (Bio-Rad), and then stained with Coomassie blue to ensure similar amounts of protein were loaded in each lane. For the detection of the biotinylated protein, proteins were transferred to a PVDF membrane (Immobilon-P, Millipore), blocked with 5 % BSA in PBS with 0.05 % Tween-20 for 1 h and incubated with 4 μg/mL of $Streptavidin_{488}$ diluted in blocking solution. After several washes, bands were detected under UV light using a Gel Doc system.

Atomic Force Microscopy (AFM).

Standard AFM measurements (Fig. S15) were conducted in ambient atmosphere at room temperature. Shortly before mica deposition, samples were prepared by incubating a 2 μM and 4 μM solution of Ac**P**$(Man)_2$ with a 30 nM solution of ConA in HKR buffer, for 10 min. For AFM imaging, 10 μL of the sample were dropped onto freshly exfoliated mica and after 10 min the mica was thoroughly washed with Milli-Q water, and dried under nitrogen flow.

Supporting Figures for Characterization

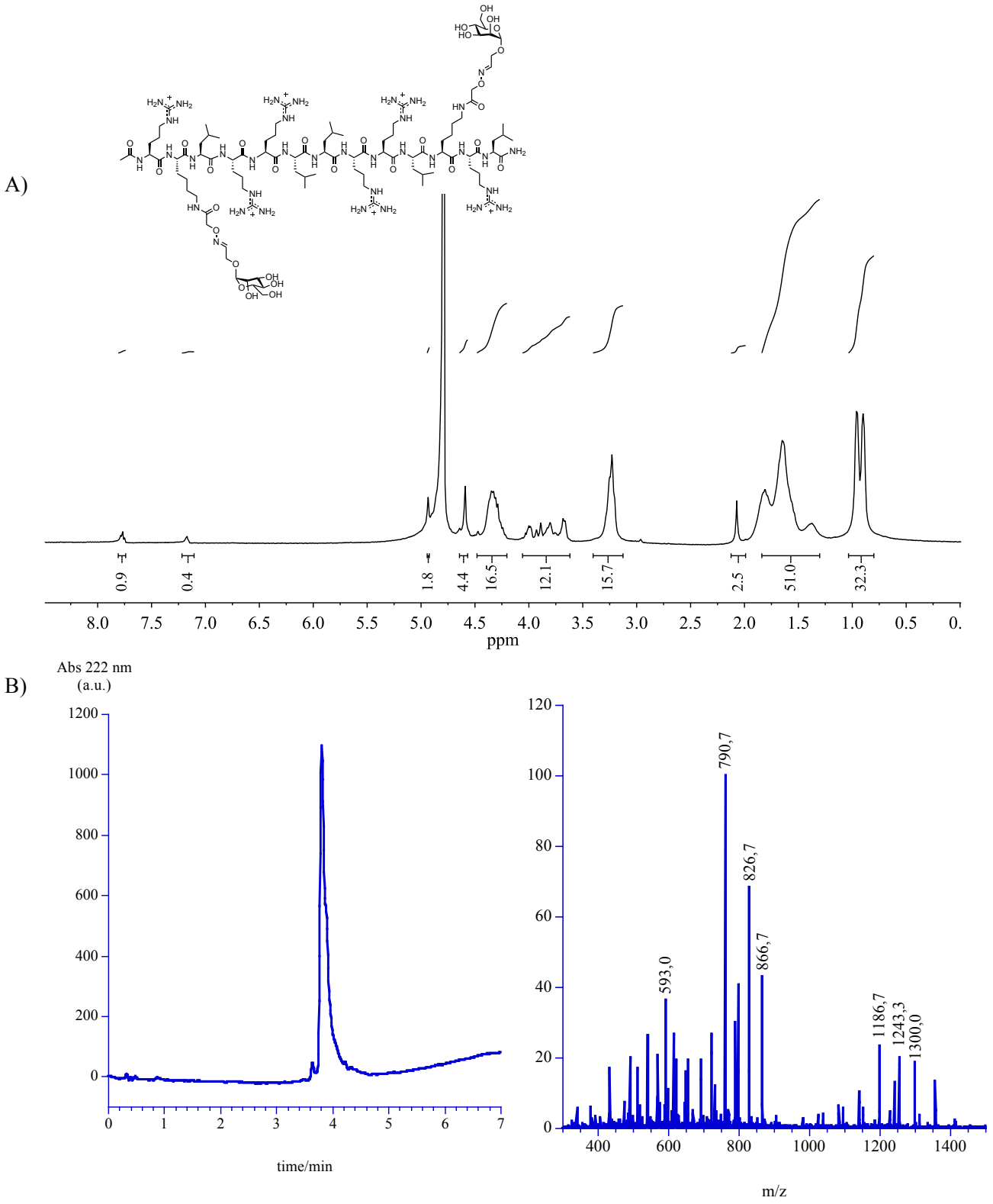


Figure S16. A) ^{1}H-NMR spectra in D_2O of Ac**P**$(Man)_2$. B) RP-HPLC [Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)] (R_t 3.8 min) and ESI-MS for Ac**P**$(Man)_2$.

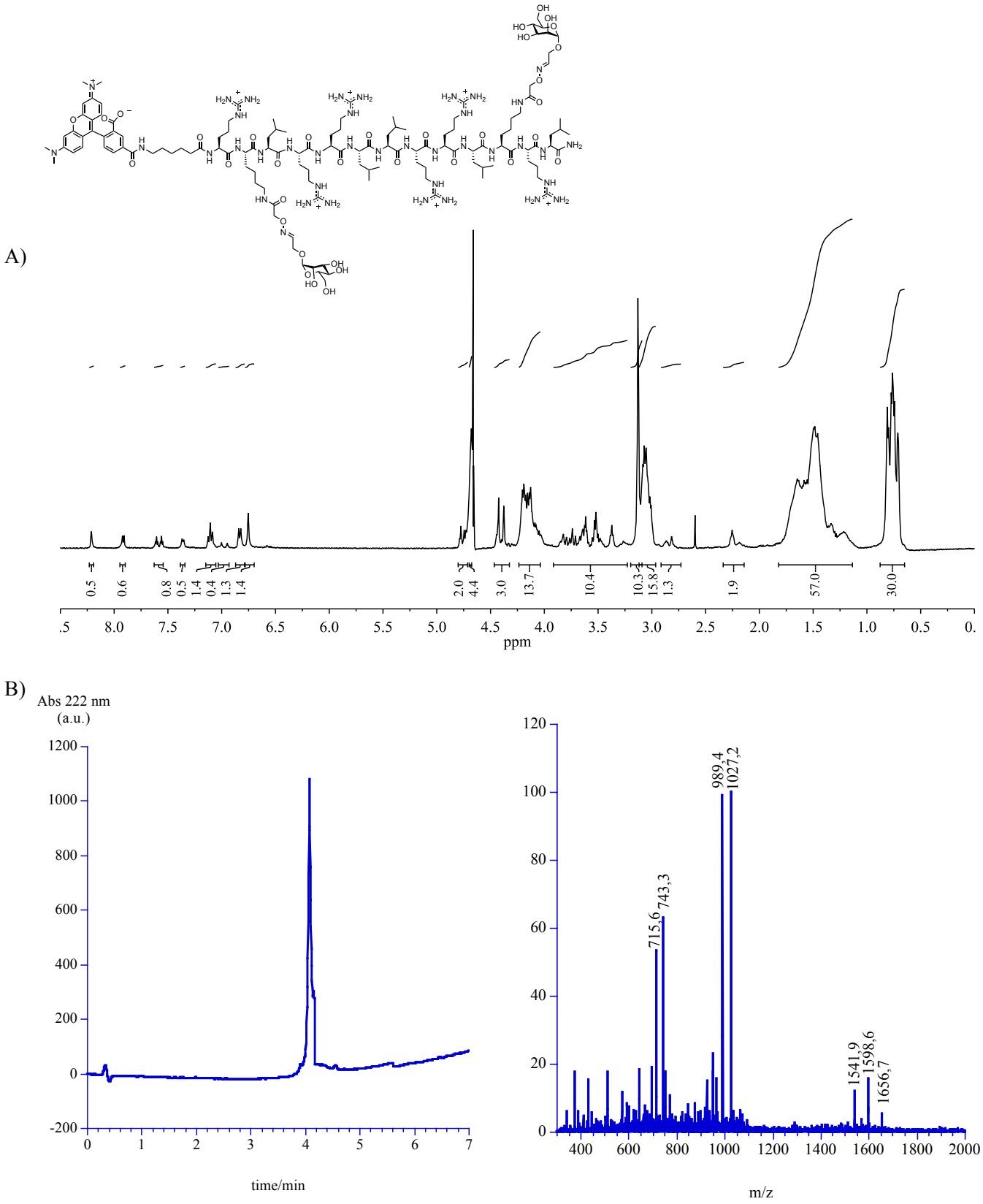


Figure S17. A) ^{1}H-NMR spectra in D_2O of Tm**P**$(Man)_2$. B) RP-HPLC [Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)] (R_t 4.1 min) and ESI-MS for Tm**P**$(Man)_2$.

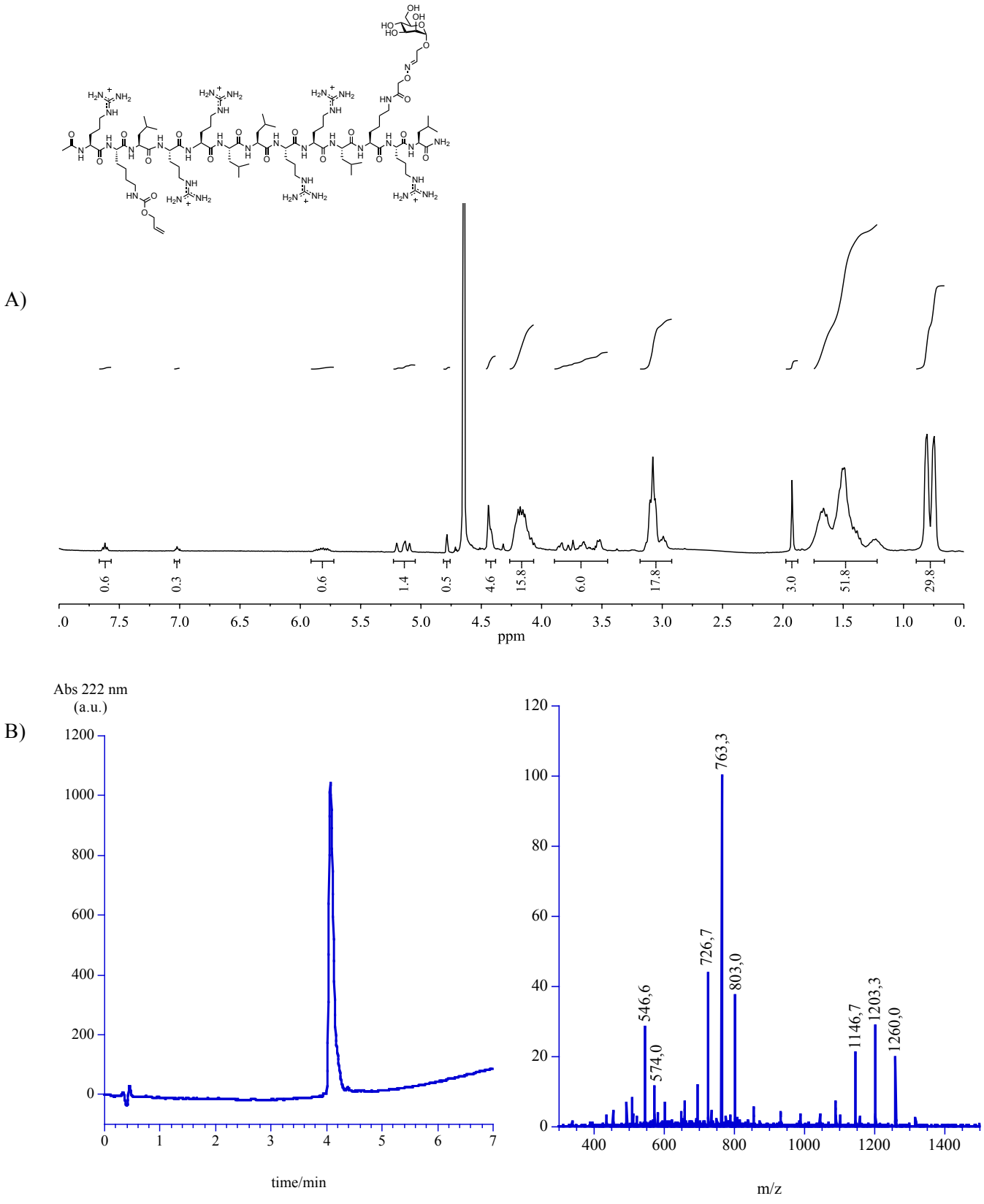


Figure S18. A) ^{1}H-NMR spectra in D_2O of Ac**P**(Alloc)(Man). B) RP-HPLC [Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)] (R_t 4.1 min) and ESI-MS for Ac**P**(Alloc)(Man)**.**

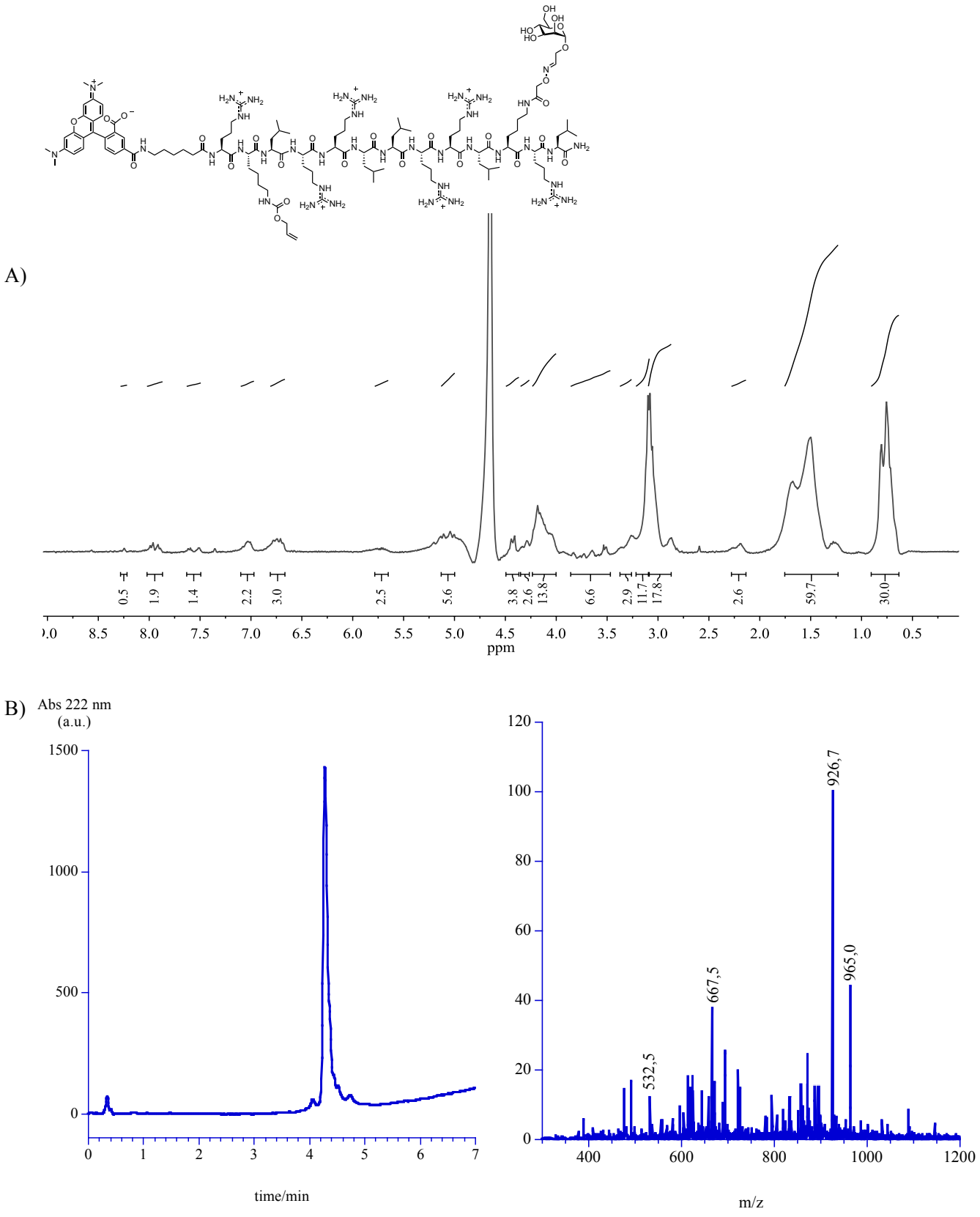


Figure S19. A) ^{1}H-NMR spectra in D_2O of Tm**P**(Alloc)(Man). B) RP-HPLC [Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)] (R_t 4.3 min) and ESI-MS for Tm**P**(Alloc)(Man).

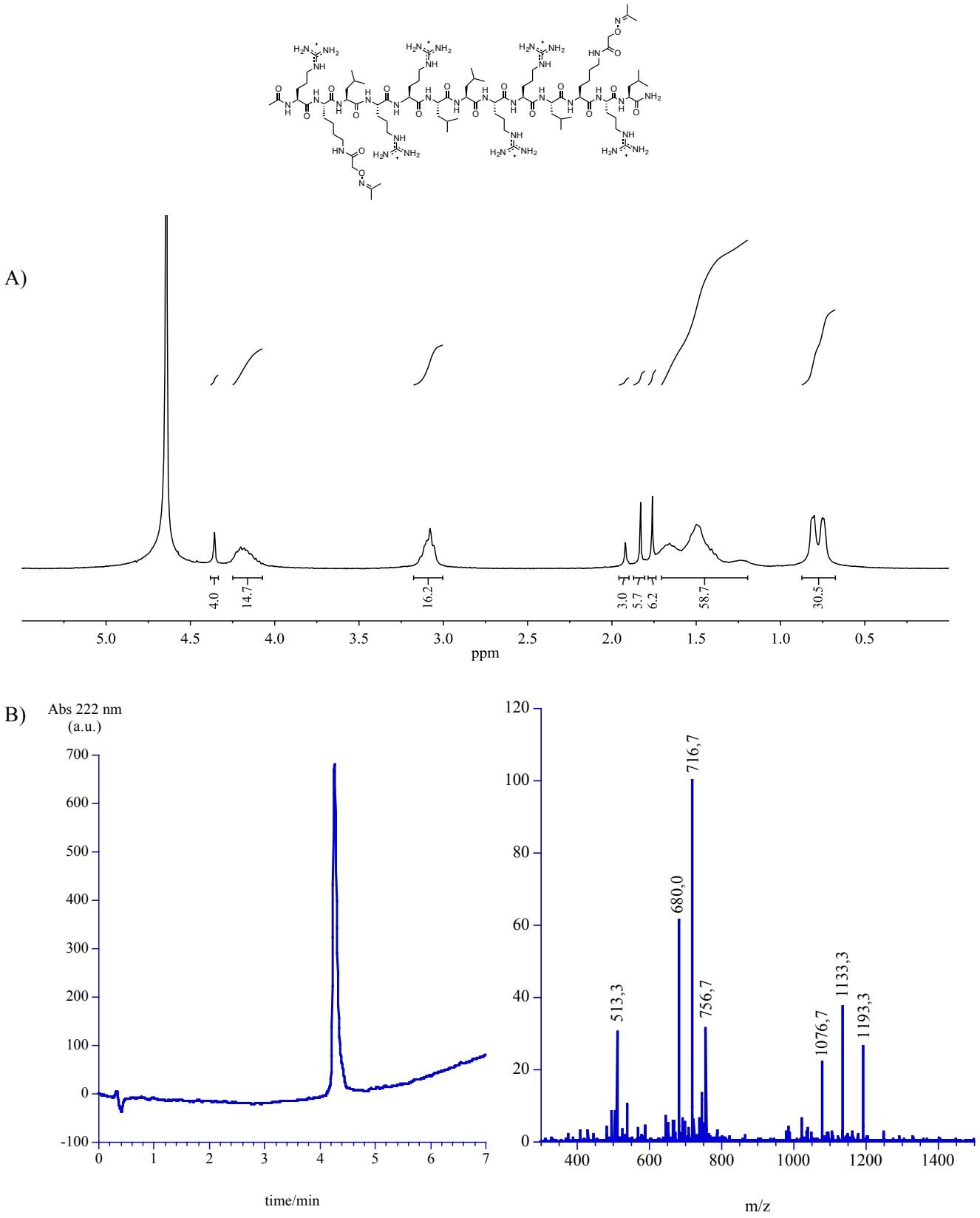


Figure S20. A) ^{1}H-NMR spectra in D_2O of Ac**P**(Acetone)$_2$. B) RP-HPLC [Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)] (R_t 4.3 min) and ESI-MS for Ac**P**(Acetone)$_2$.

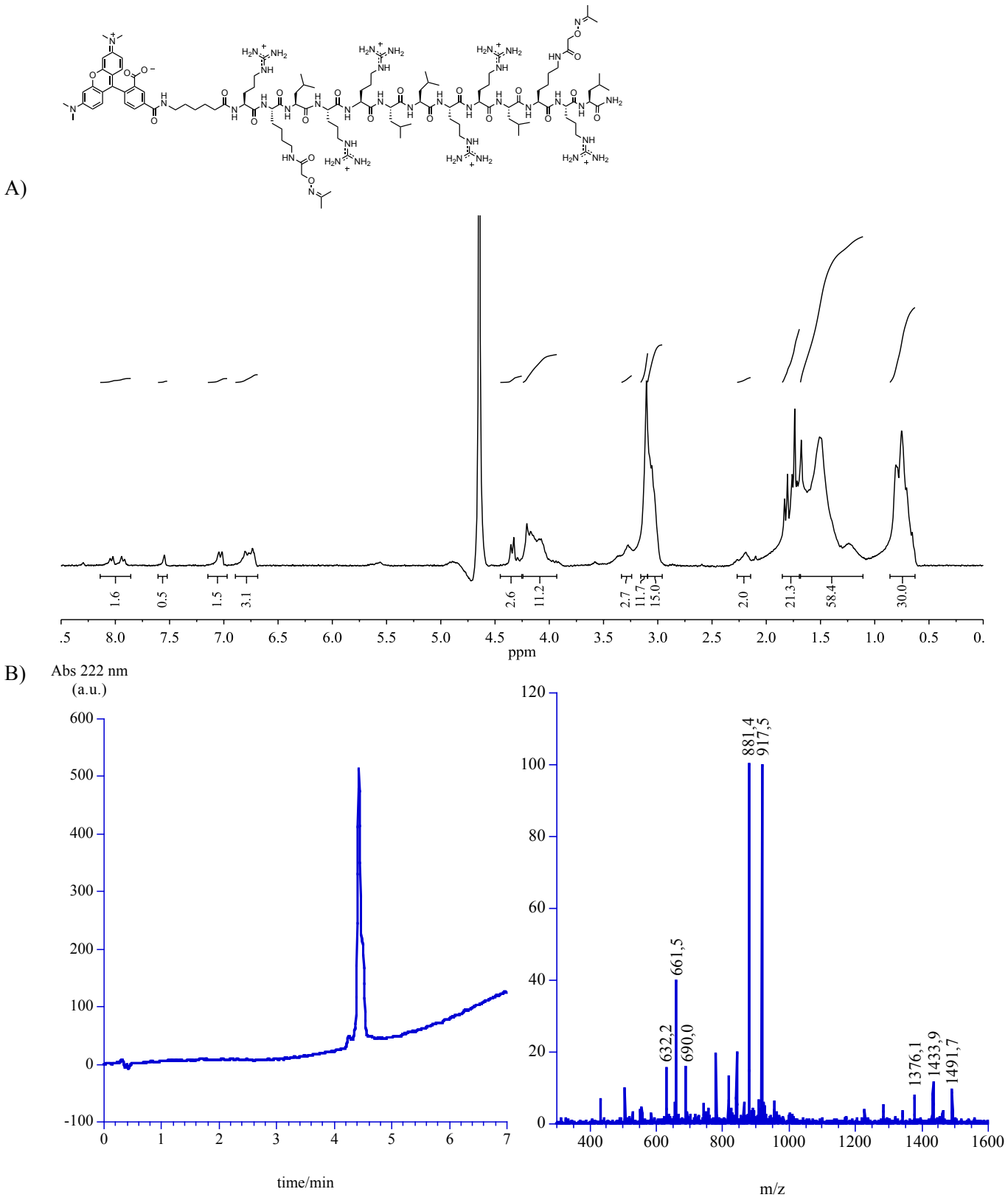


Figure S21. A) ^{1}H-NMR spectra in D_2O of Tm**P**(Acetone)$_2$. B) RP-HPLC [Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)] (R_t 4.4 min) and ESI-MS for Tm**P**(Acetone)$_2$.

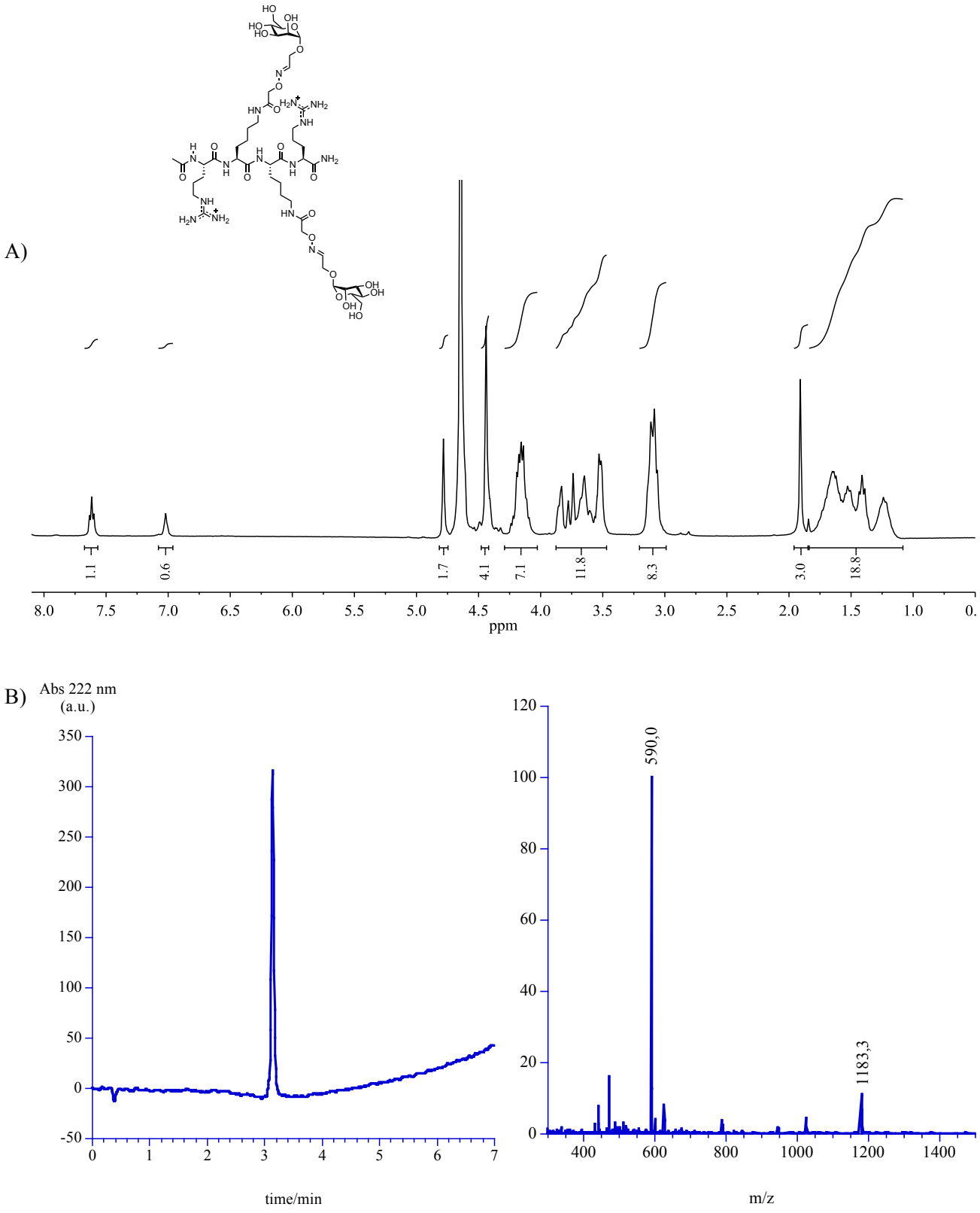


Figure S22. A) ^{1}H-NMR spectra in D_2O of $AcArg_2(Man)_2$. B) RP-HPLC [Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)] (R_t 3.1 min) and ESI-MS for $AcArg_2(Man)_2$.

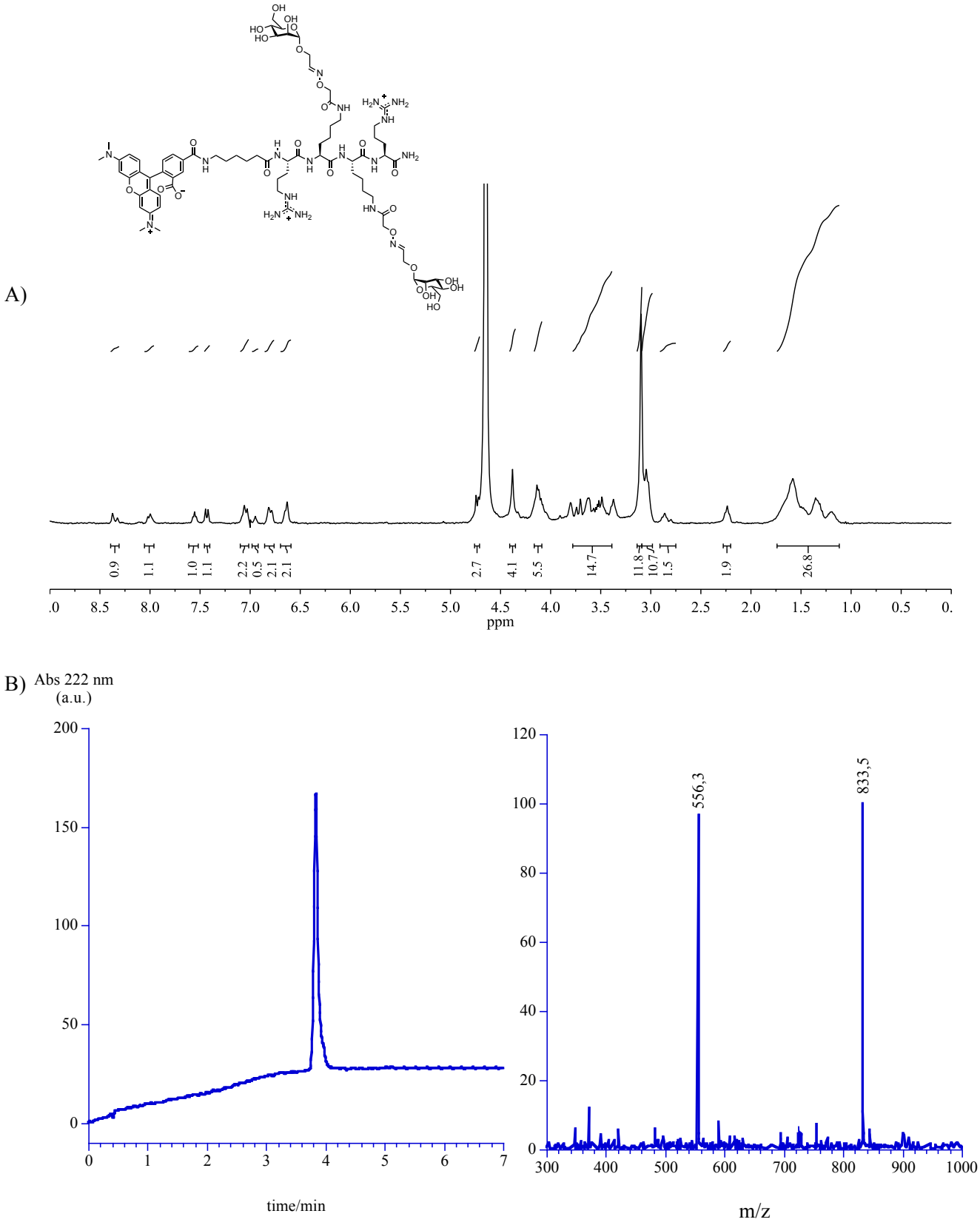


Figure S23. A) ^{1}H-NMR spectra in D_2O of $TmArg_2(Man)_2$. B) RP-HPLC [Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)] (R_t 3.8 min) and ESI-MS for $TmArg_2(Man)_2$.

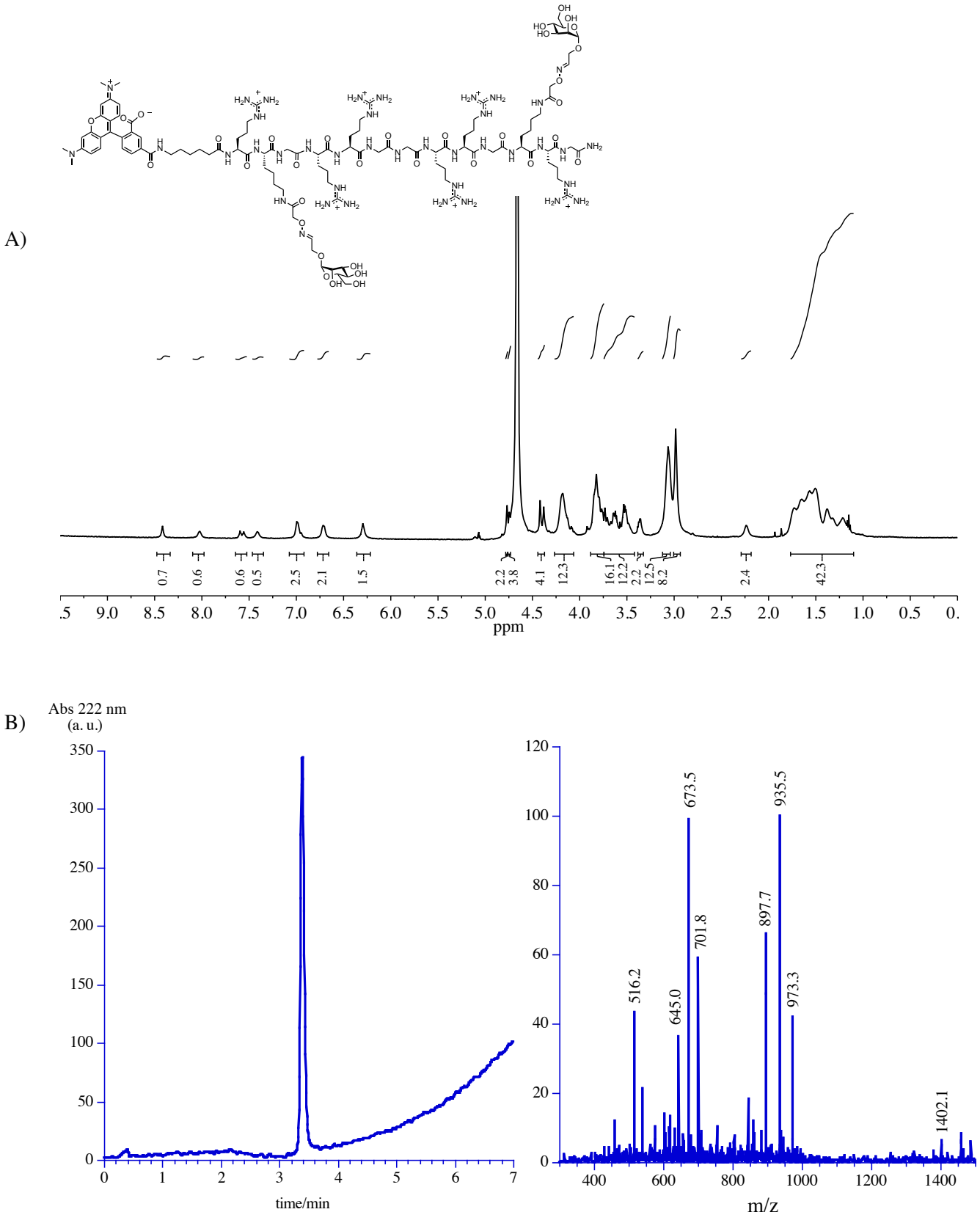


Figure S24. **A**) ^{1}H-NMR spectra in D_2O of $TmArg_6Gly_5(Man)_2$. B) RP-HPLC [Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)] (R_t 3.4 min) and ESI-MS for $TmArg_6Gly_5(Man)_2$.

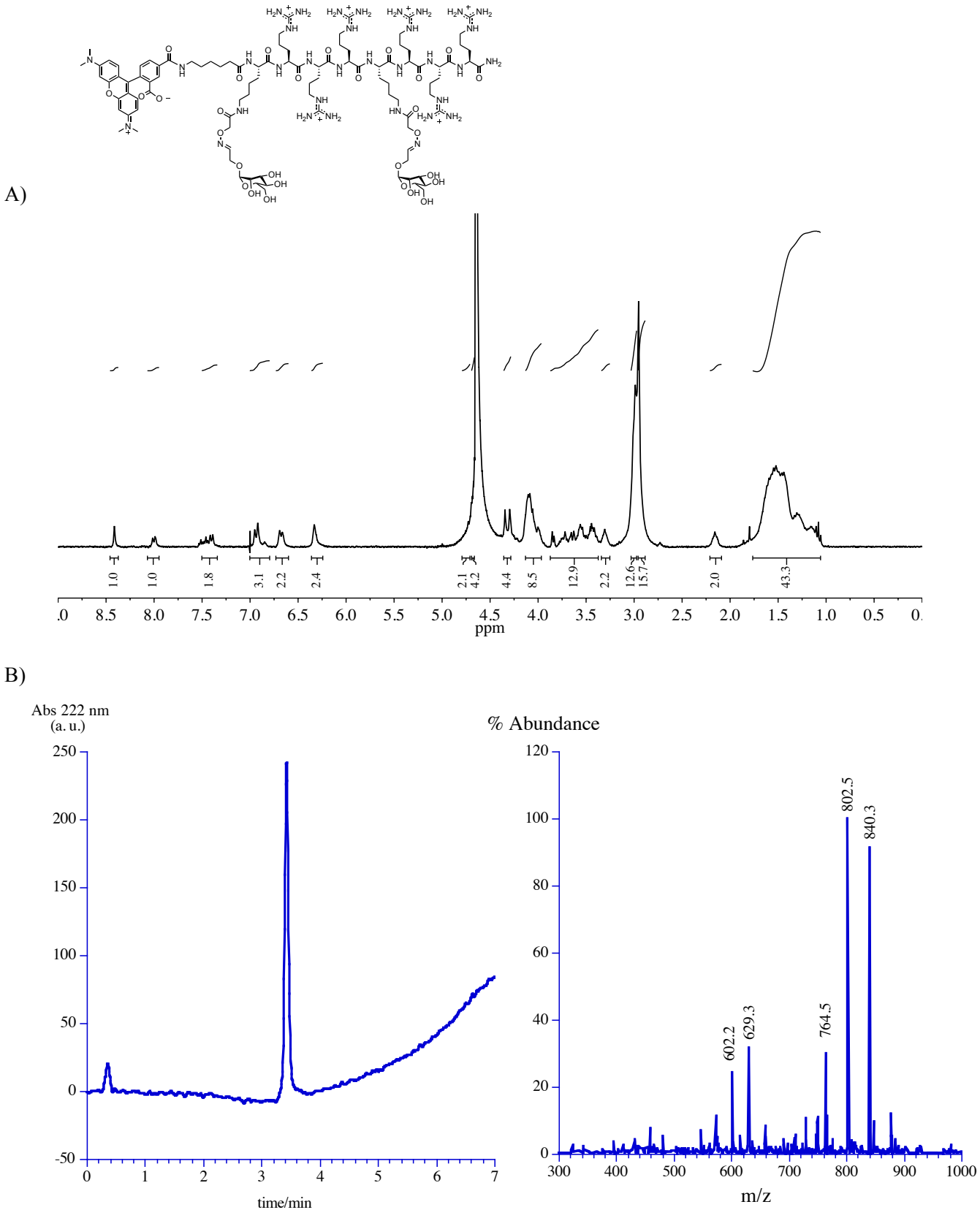


Figure S25. A) ^{1}H-NMR spectra in D_2O of $TmArg_6(Man)_2$. B) RP-HPLC [Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)] (R_t 3.4 min) and ESI-MS for $TmArg_6(Man)_2$.

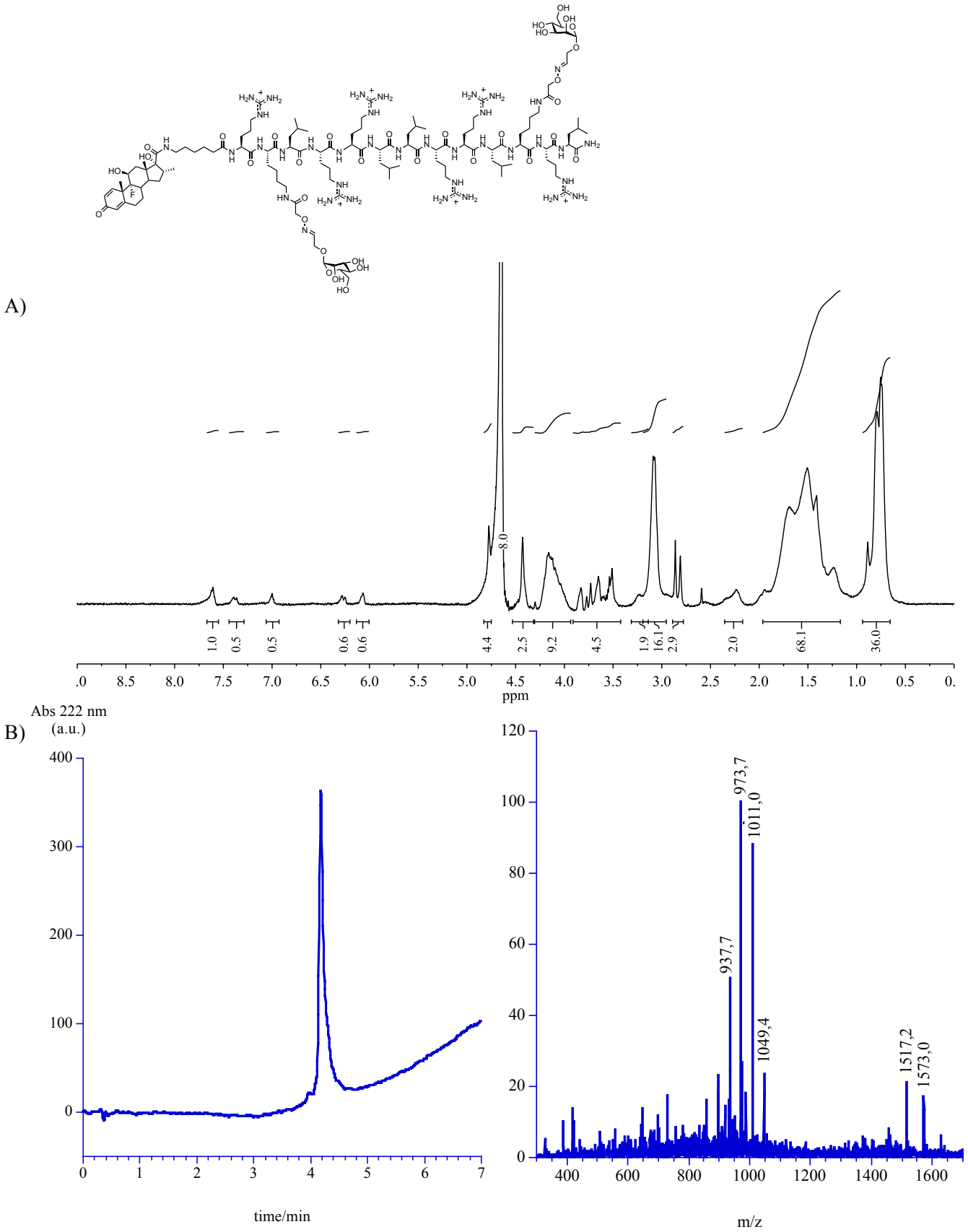


Figure S26. A) ^{1}H-NMR spectra in D_2O of DexP(Man)$_2$. B) RP-HPLC [Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)] (R_t 4.2 min) and ESI-MS for DexP(Man)$_2$.

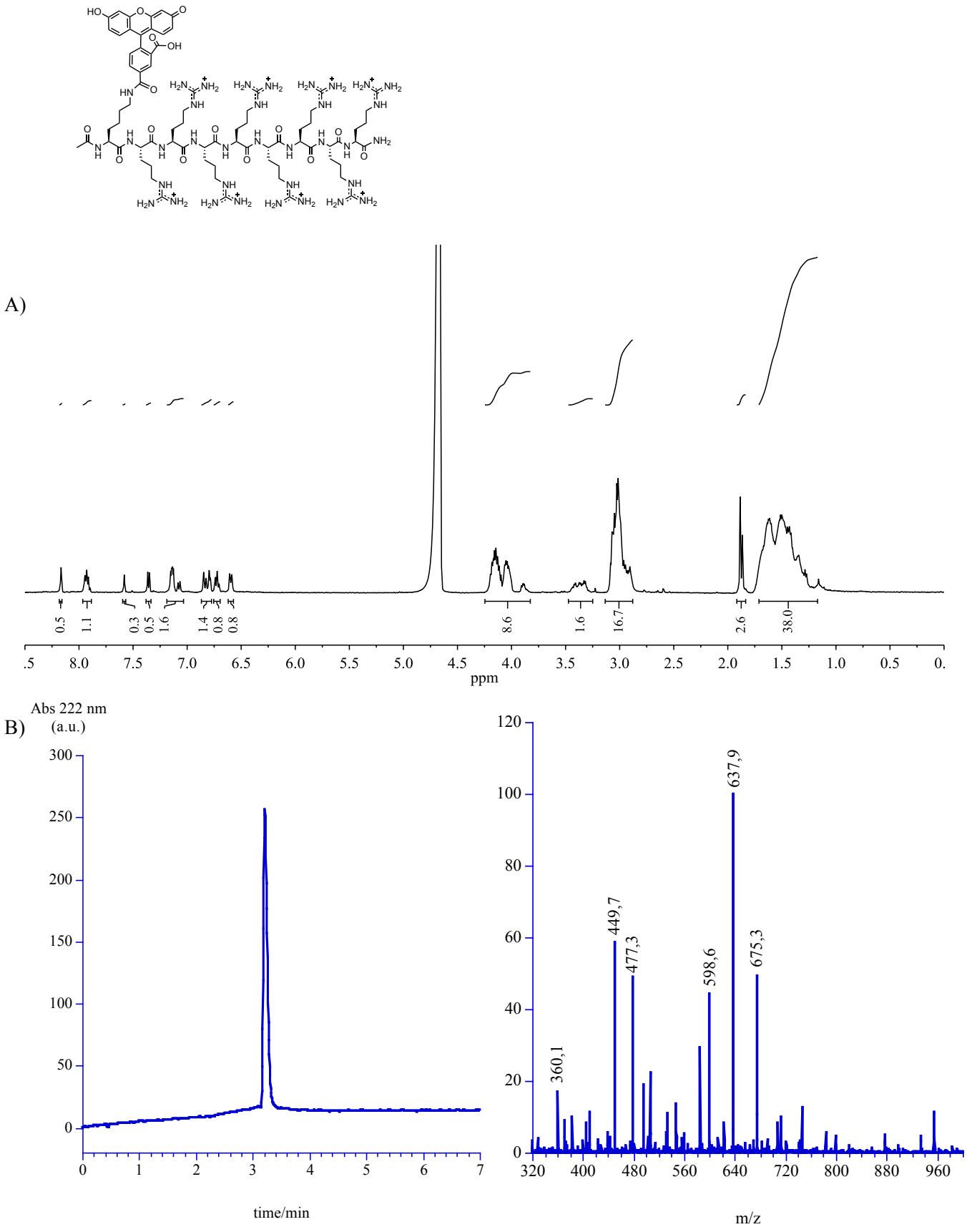


Figure S27. A) ^{1}H-NMR spectra in D_2O of $CFArg_8$. B) RP-HPLC [Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)] (R_t 3.2 min) and ESI-MS for $CFArg_8$.

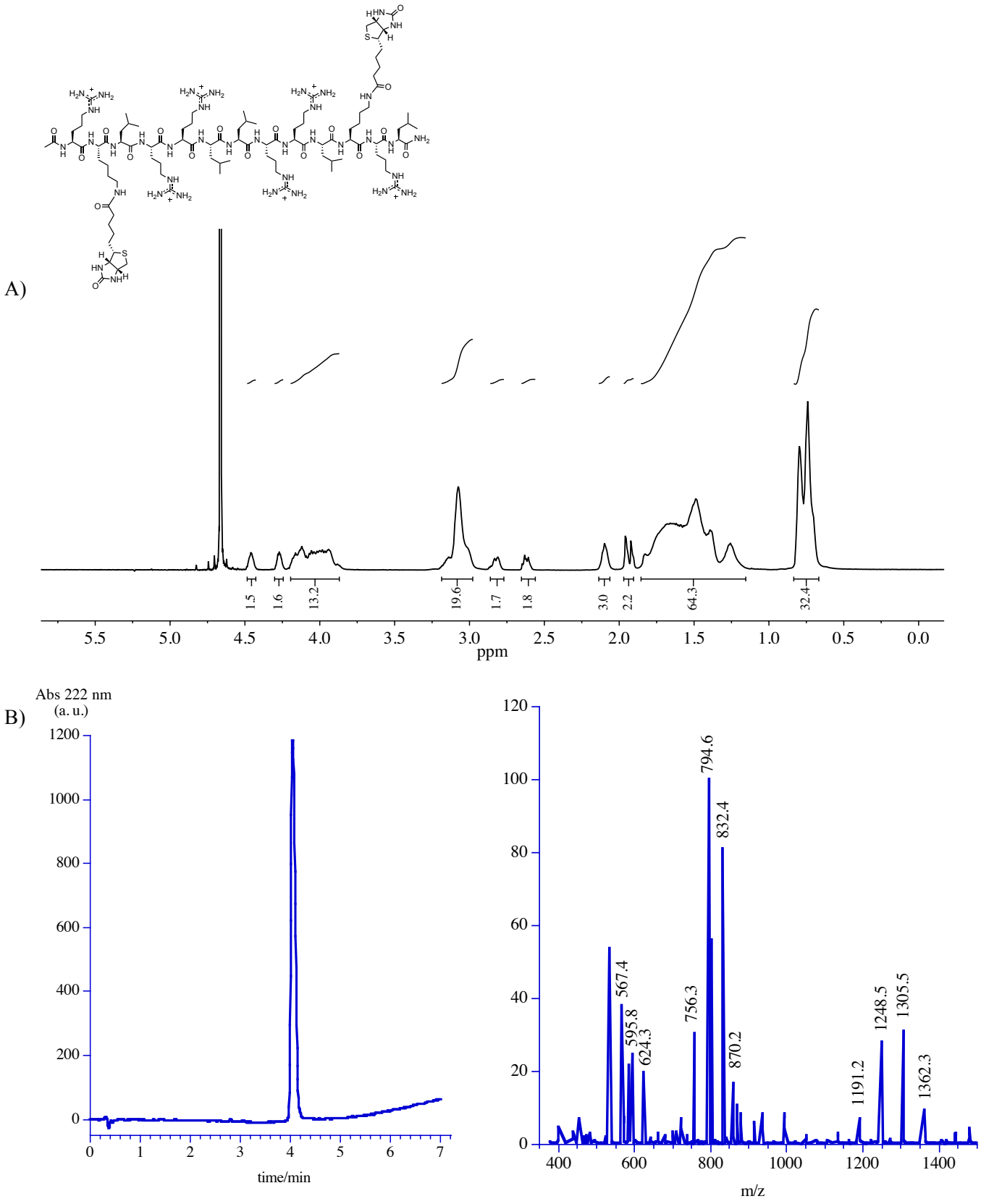


Figure S28. A) ^{1}H-NMR spectra in D_2O of Ac**P**$(Biot)_2$ B) RP-HPLC [Agilent SB-C18 column, H_2O (0.1% TFA)/ CH_3CN (0.1% TFA) 95:5→5:95 (0→5 min)] (R_t 4.1 min) and ESI-MS for Ac**P**$(Biot)_2$.

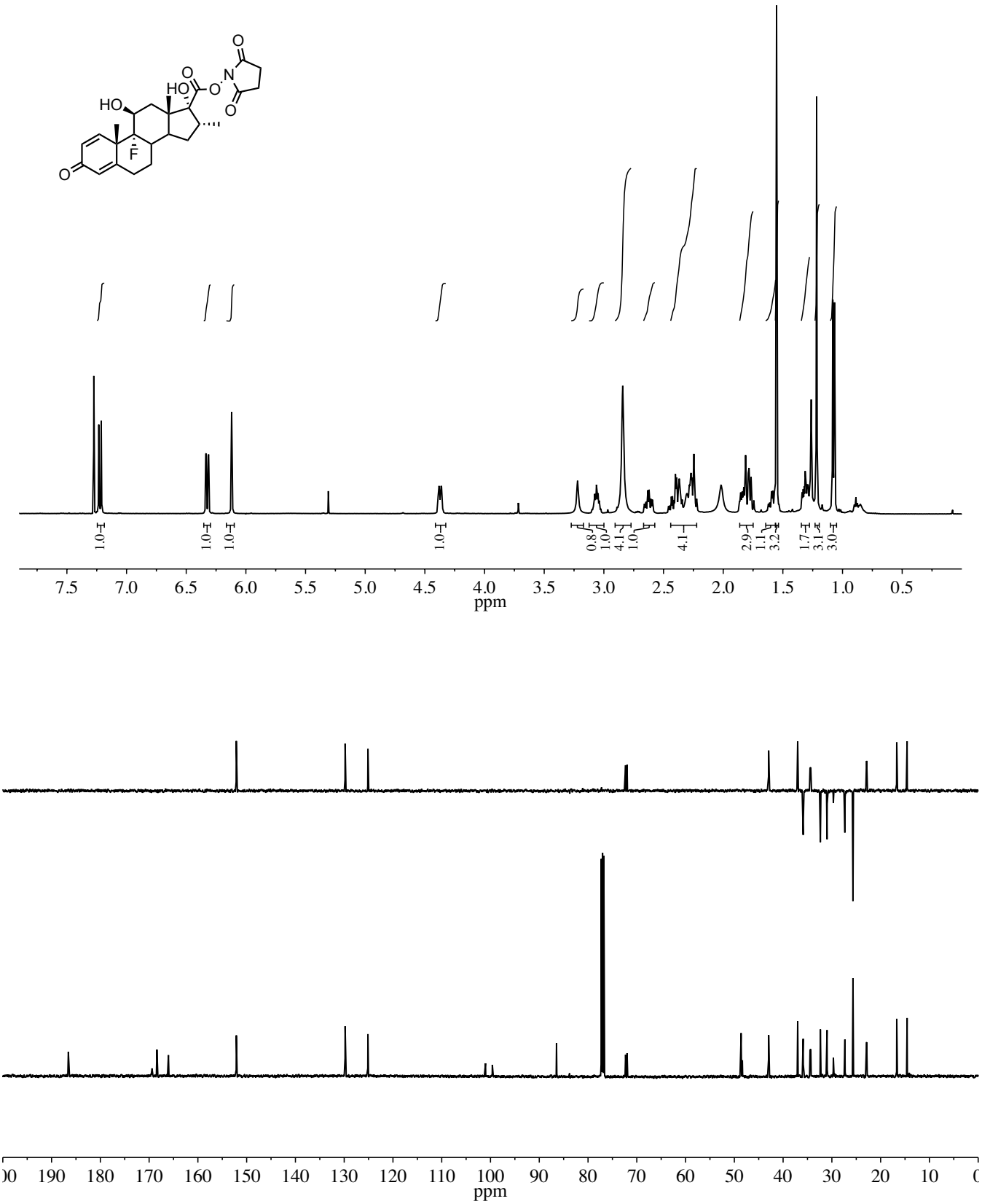


Figure S29. ^{1}H-NMR, DEPT and ^{13}C-NMR spectra in $CDCl_3$ of DexNHS.

Supporting References

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