Supporting information for

Photoaffinity Probes for the Identification of Sequence-Specific Glycosaminoglycan-Binding Proteins

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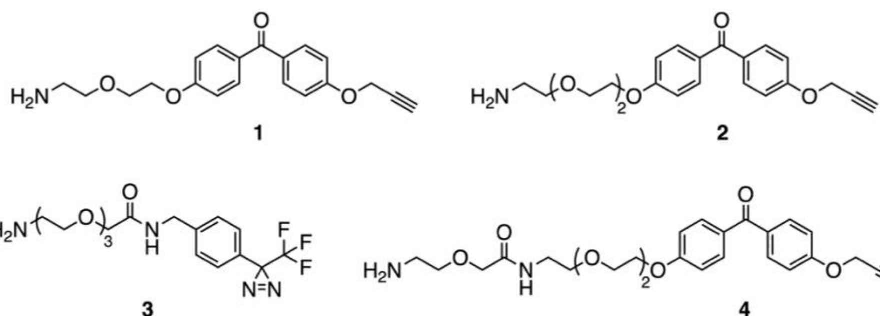
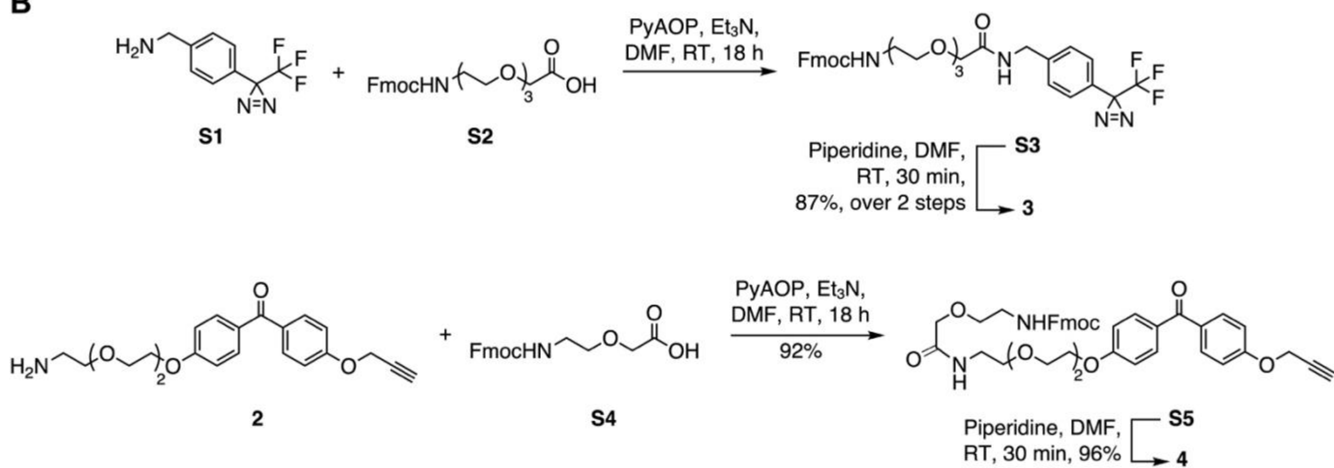
Table of Contents

1. Supporting Scheme
2. Supporting Figures
3. Supporting Tables
4. Experimental Methods
   4.1 Materials and Methods
   4.2 Synthetic Procedures
   4.3 Representative NMR spectra
5. References

1. Supporting Scheme





Scheme S1. Trifunctional photo-crosslinking reagents (TPRs) used in this study. (A) Structures of TPRs **1**–**4** (B) Synthesis of TPRs **3** and **4** from commercially available **S1** and **2**, respectively.

2. Supporting Figures

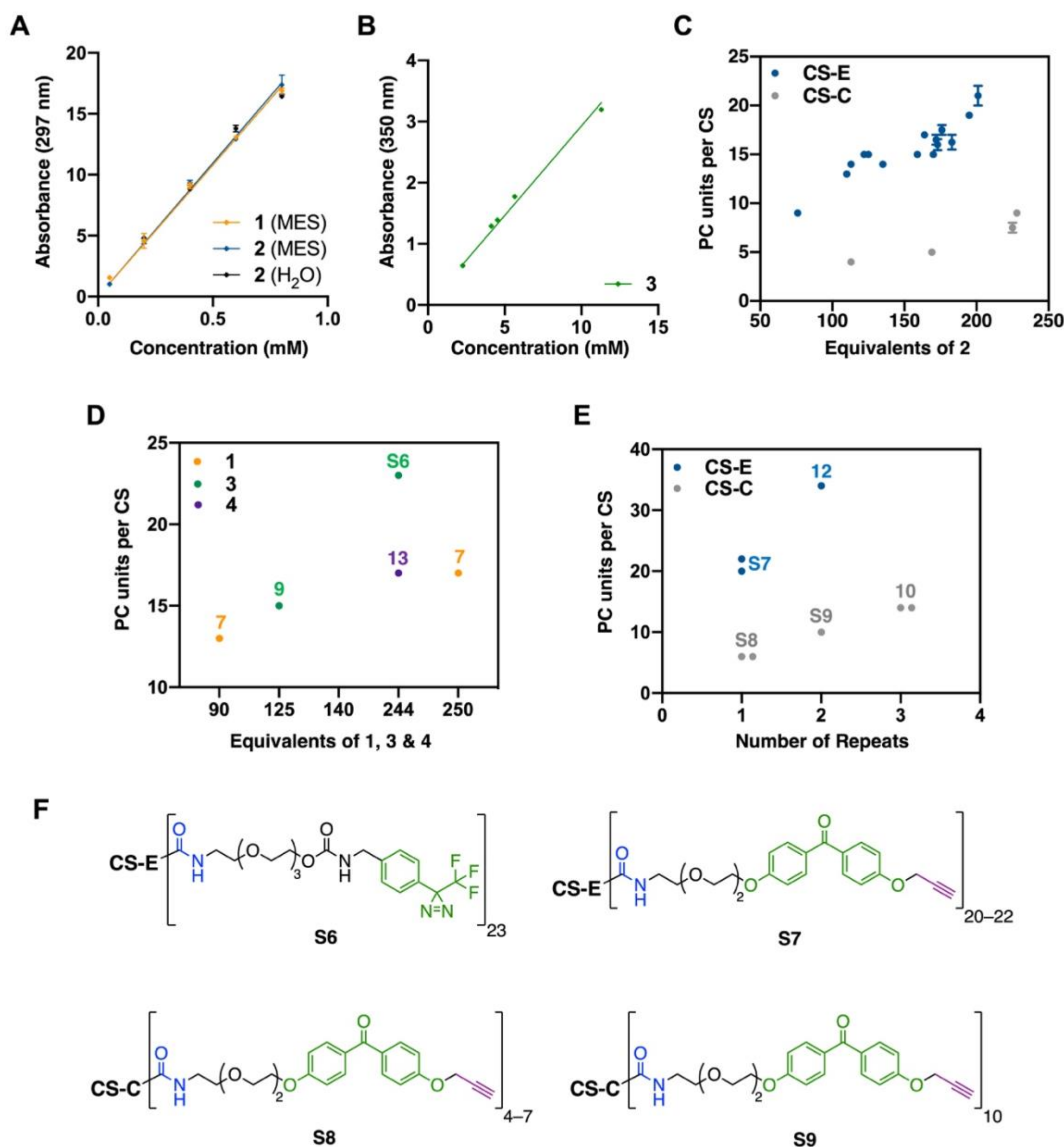


Figure S1. Determination of the number of photo-crosslinking (PC) units on each probe and optimization of the PC:CS ratio. (A) Calibration curve for **1** and **2** ($\varepsilon_{1,2\ \&\ 4}$ = 21,595 $M^{-1}cm^{-1}$, λ_{max} = 297 nm) used to calculate the number of PC units per CS polysaccharide (PC:CS ratio) in the benzophenone-containing probes. The same extinction coefficient was found in both H_2O and MES buffer (n = 2). Data represent the mean ± SEM. (B) Calibration curve for **3** (ε_3 = 293 $M^{-1}cm^{-1}$, λ_{max} = 350 nm) employed to calculate the PC:CS ratio in the diazirine-containing probes (n = 2). Data represent the mean ± SEM. (C) Optimization of the amide coupling reaction. Degree of substitution obtained for CS-E probes as a function of the number of equivalents of TPR **2** employed. (D) Degree of substitution obtained for CS-E probes as a function of the number of equivalents of TPR **1**, **3** and **4** employed (Probes **7**, **9**, **13** and **S6**; Figure S1F and Table S1). (E) The degree of substitution was further increased by submitting the probes to multiple rounds of coupling. For the CS-E probes, 201 equivalents of TPR **2** was used in 1 or 2 subsequent coupling reactions to give probes **S7** and **12**, respectively. For the CS-C probes, 272 equivalents of TPR **2** was used in 1, 2 or 3 subsequent coupling reactions to give probes **S8**, **S9** and **10**, respectively (Figure S1F and Table S1). (F) Structures of probes **S6–S9** (Table S1). CS: chondroitin sulfate; MES: 2-(*N*-morpholino) ethanesulfonic acid.

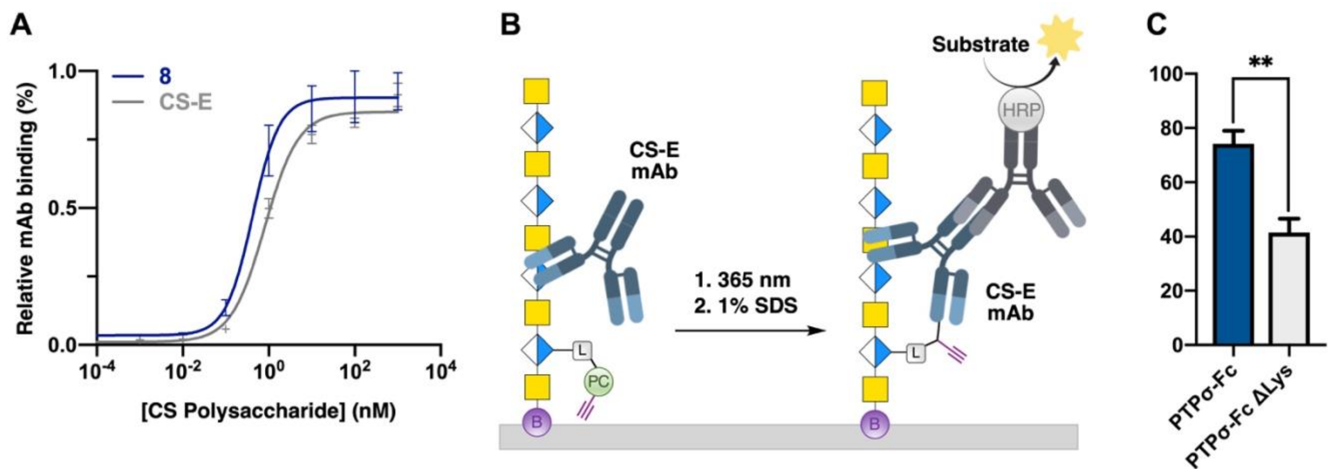


Figure S2. Probe optimization. (A) CS-E probe **8** and end-biotinylated CS-E bind to a CS-E-specific mAb[1,2] with comparable affinities. Purified PTPσ-Fc was immobilized in Protein A/G wells, and end-biotinylated CS-E probe **8** or CS-E polysaccharide was added. Binding was detected using streptavidin-HRP (n = 2). Data represent the mean ± SEM. (B) Photo-ELISA assay used to assess probe efficiency. The probes were adhered to streptavidin-coated plates, and the CS-E mAb was added. After irradiation at 365 nm and stringent washes with 1% SDS to remove non-covalent interactions, covalently crosslinked CS-E mAb was detected using an anti-mouse IgG HRP conjugate. (C) Mutation of Lys residues (K67–71) in the CS-E binding site reduced photo-crosslinking compared to PTPσ-Fc (n = 3). Data represent the mean ± SEM. **$P \leq 0.01$. HRP: horseradish peroxidase; SDS: Sodium dodecyl sulfate.

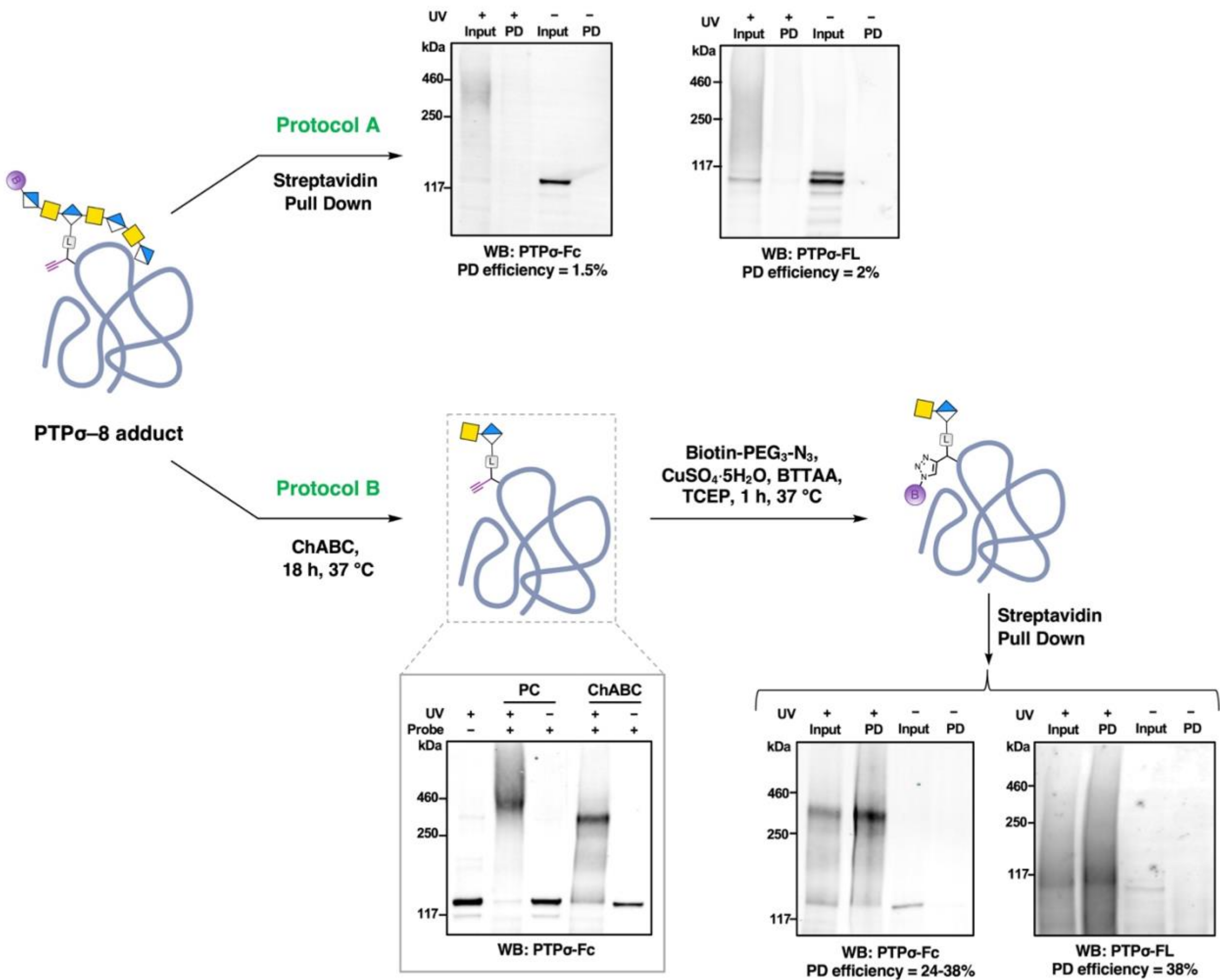


Figure S3. Pull down of the PTPσ-**8** adduct. In Protocol A, PTPσ-Fc or full-length PTPσ (PTPσ-FL) was incubated with probe **8** and irradiated at 365 nm. The resulting PTPσ-**8** adduct was pulled down via the biotin handle on the N-terminal peptide of probe **8**, resolved by SDS-PAGE, and detected by Western blotting (WB). Low pull-down (PD) efficiencies of ~1–2% were obtained. In Protocol B, the CS polysaccharides on the PTPσ-**8** adduct were trimmed using ChABC. Biotin groups were then appended via the alkynyl groups using CuAAC chemistry. Higher pull-down efficiencies of ~24–38% were obtained. BTTAA: 2-(4-((bis((1-*tert*-butyl-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)acetic acid; ChABC: chondroitinase ABC; CuACC: copper(I)-catalyzed azide-alkyne cycloaddition; PEG: polyethylene glycol; TCEP: tris(2-carboxyethyl) phosphine.

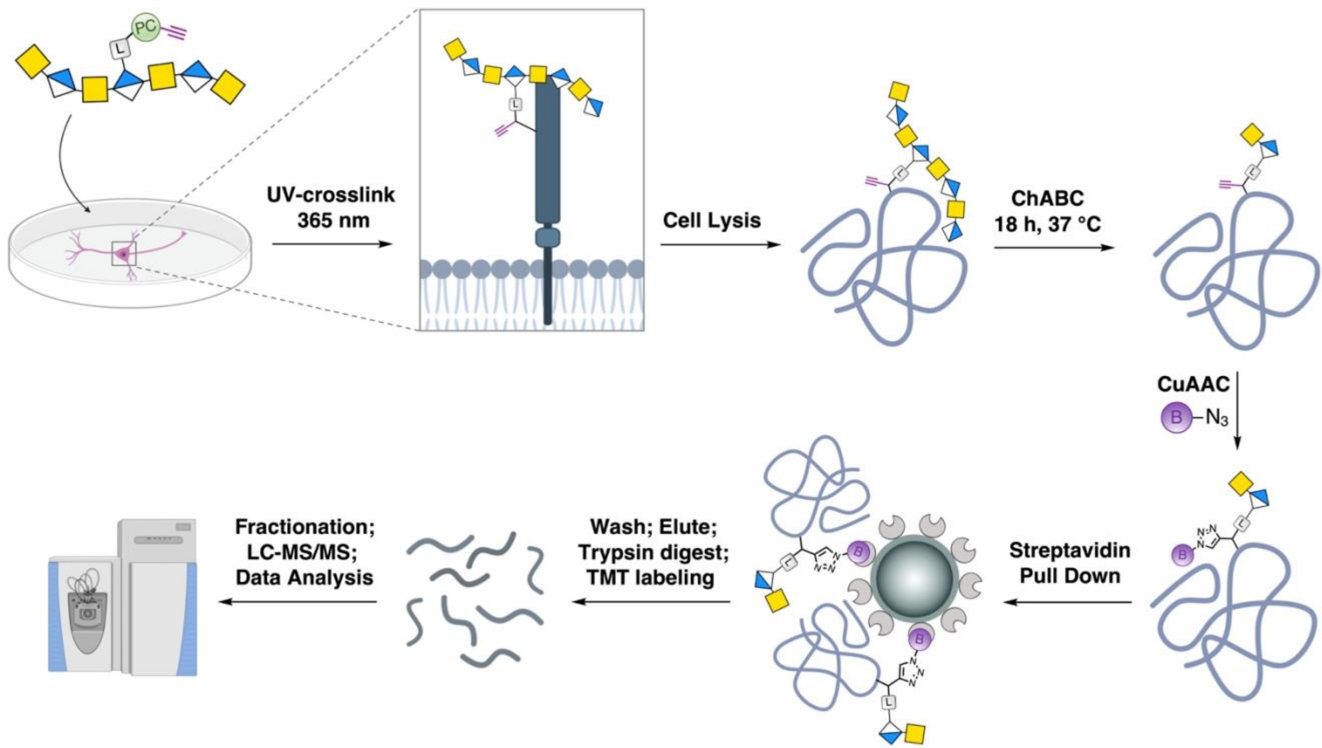


Figure S4. General workflow for protein photo-crosslinking in living cells using CS-E probe **8**. Cortical neurons (13 DIV) were incubated with either probe **8** or vehicle control (media) and irradiated at 365 nm. After cell lysis, CS polysaccharide chains were digested using ChABC (Protocol B). Biotin moieties were then appended to the crosslinked probe via CuAAC chemistry. The resulting biotinylated proteins were enriched by streptavidin pull down, resolved by SDS-PAGE, and subjected to in-gel proteolytic digestion. The peptides from each sample were TMT labeled, combined, fractionated, and analyzed by LC-MS/MS. Proteomics data analysis was carried out using the software Proteome Discover™. DIV: days in vitro; LC-MS/MS: liquid chromatography-tandem mass spectrometry; TMT: tandem mass tag.

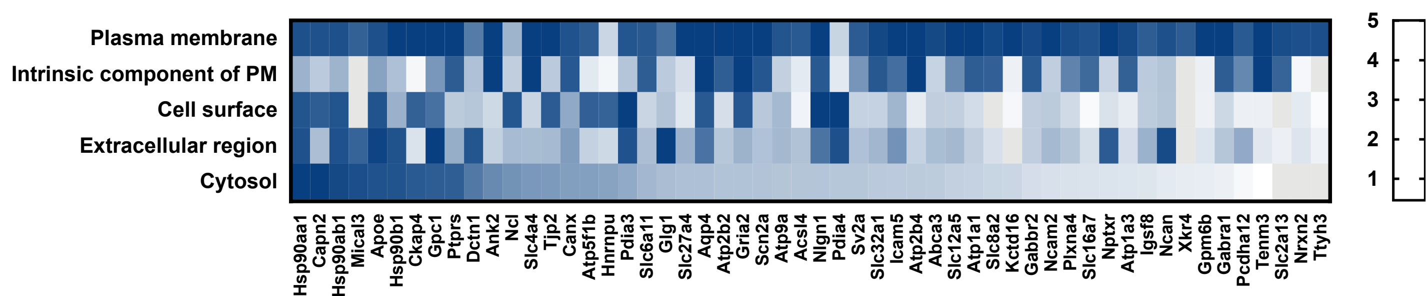


Figure S5. COMPARTMENTS database[3] subcellular localization scores (0–5) for the top 54 CS-E binding proteins identified using probe **8** (Table S2). Proteins with ‘cytosol’ scores ⩾2 also had a scores ⩾4 for ‘extracellular region’ or ‘cell surface’.

3. Supporting Tables

Table S1 – List of CS-E and CS-C photo-crosslinking probes synthesized and used in this work.

General Probe Information				Coupling Reaction Conditions			Probes PC:CS & Yields		
Probe #	**CS**	**Linkage**	**TPR #**	**TPR (eq)**	**CNBr[a] or EDC·HCl/NHS[b] (eq)**	**[CS] (μM)**	**PC:CS[c]**	**Yield (%)**	**Disaccharide labeled (%)**
5	CS-E	IU[a]	**1**	140	1000	11.6	12:1	42	8
6	CS-E	IU	**2**	280	1000	11.6	13:1	17	9
7	CS-E	Am[b]	**1**	90–250	1340/134	6.40–6.87	13–17:1[e]	41–52	9–12
8	CS-E	Am	**2**	110–250	1340/134	5.64–6.87	13–17:1[e]	46–73	9–13
9	CS-E	Am	**3**	125	1340/134	5.63	15:1	77	11
10	CS-C	Am	**2**	272 x 3[d]	1340/134	6.28	14:1	52–57	10–11
11	CS-E	Am	**2**	76	1340/134	5.63	9:1	74	7
12	CS-E	Am	**2**	201 x 2[d]	1340/134	6.40	34:1	74	25
13	CS-E	Am	**4**	244	1340/134	7.04	17:1	57	13
S6	CS-E	Am	**3**	244	1340/134	7.00	23:1	56	17
S7	CS-E	Am	**2**	183–201	2600/134	5.25–7.00	20–22:1[e]	17–53	15–16
S8	CS-C	Am	**2**	113–544	1340/134	6.28–7.56	4–7:1[e]	59–77	3–5
S9	CS-C	Am	**2**	272 x 2[d]	1340/134	-	10:1	55	8

[a]See General Procedure 1; [b]See General Procedure 2; [c]See General Procedure 3; [d]This probe was re-submitted to the reaction conditions 2 or 3 times (Figure S1E); [e]Represents multiple batches. See Experimental Methods. Am: amide; EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; eq: equivalent; IU: isourea; NHS: *N*-hydroxysuccinimide.

Table S2 – Top CS-E binding proteins identified in live cortical neurons using photoaffinity probe **8**.

Protein	Accession	Coverage [%]	# Unique Peptides	Exp. A Probe[a]	Exp. A Control[a]	Exp. B Probe[a]	Exp. B Control[a]	Ratio A	Ratio B	Function
Cell Adhesion, Recognition & Cell-Cell Interactions										
Gpm6b	P35803	9	4	95.5		785.2	52.6		15	Neuronal development, differentiation & myelination
Icam5[c,d]	Q60625	7	9	314.3	3.3	728.8	22.3	95	33	Neurite outgrowth, synapse maturation, neural development & immunity
Igsf8[c]	G3UYZ1	3	2	41.8		46.1	6.9		7	Neurite outgrowth & maintenance of neural network in the adult brain
Ncam2[b,c]	O35136	2	2	135.4	2	68.4	10.7	68	6	Neurite outgrowth & branching, axon guidance & synapse assembly
Ncan[c]	A0A0R4IZX5	4	6	57.3		48	3.6		13	Cell adhesion & migration
Nrxn2	E9Q7X7	5	7	85.9	2	88.6	7.2	43	12	Synaptic adhesion
Pcdha12	Q91Y18	2	2	33.9		36.6	5.3		7	Establishment & maintenance of neuronal connections
Plxna4[c,d]	Q80UG2	3	6	43.4		28.1	3.8		7	Axon guidance, Sema3A co-receptor
Ptprs[b,c]	B0V2N1	2	4	12.3		14.7				Neurite outgrowth
Tenm3	Q9WTS6-7	1	3	7.2		6.5				Neural development, axon guidance & cell adhesion
Tjp2	Q9Z0U1	6	8	51.4		114.7				Tight junctions & adherens junctions
Gpc1	Q9QZF2	5	3	96.9	5.1	87	4	19	22	Cell division & growth regulation
Cell Signaling										
Ckap4	Q8BMK4	11	7	92.2	2.2	94.5		42		High-affinity epithelial cell surface receptor for APF
Glg1	F8WHM5	3	4	118.4		35.5	2.6		14	Fibroblast growth factor binding
Kctd16	Q5DTY9	3	2	58.5		62.7	2.6		24	Subunit of GABA-B receptors, GPCR signaling pathway
Ncl	P09405	23	23	957.3	20.6	1425	30.3	46	47	Extracellular regulation of nuclear events
Nlgn1[d]	Q99K10	3	2	95.1		153.1	20.7		7	Synapse development, function & synaptic signal transmission
Nptxr[d]	F7BX42	7	4	119.1	5.6	146.6	22.2	21	7	Implicated in synapse remodeling and organization

Ion Channels										
Aqp4	P55088-2	5	2	19.2		165	17.1		10	Astrocyte migration & cell adhesion
Scn2a	B1AWN6	6	13	96		868	20.3		43	Voltage-dependent Na^+ permeability of excitable membranes
Ttyh3	Q6P5F7	9	6	77.5	2	220.1	18	39	12	Unknown
Enzymes										
Capn2	O08529	3	3	19.6		42.3	6.1		7	Protease, cytoskeletal remodeling & signal transduction
Mical3	Q8CJ19	3	6	15.4		31.2				Monooxygenase, exocytic vesicles tethering & fusion
Pdia4	A0A0R4J0Z1	14	11	524.8		375	14.7		26	Protein disulfide isomerase & molecular chaperone, modulates protein folding
Pdia3	P27773	18	10	318		124.8	12.5		10	Protein disulfide isomerase & molecular chaperone, modulates protein folding
Lipid Transport										
Abca3	Q8R420	3	4	15.3		59.3	4.4		13	Formation of pulmonary surfactant
Apoe[b]	P08226	38	13	230.6	5.1	1035	7.7	45	134	Neuron survival & sprouting
Protein Trafficking										
Sv2a	Q9JIS5	9	9	611.8		2188	166.5		13	Neurotransmitter release
Canx	P35564	6	4	99.8		77.4	3.6		22	Implicated in receptor-mediated endocytosis at the synapse
Hsp90aa1	P07901	5	5	118.4	4.3	120	4.5	28	27	Molecular chaperone, cell cycle control & signal transduction
Hsp90ab1	P11499	26	21	911.6	12.2	749	88.8	75	8	Molecular chaperone, cell cycle control & signal transduction
Hsp90b1	P08113	14	14	914.5	8.7	650.1	124.9	105	5	Molecular chaperone, cell cycle control & signal transduction
Dctn1	E9Q3M3	17	25	465.8	2.6	1083	171.5	179	6	Retrograde vesicle transport
Neurotransmitter Receptor										
Gabbr2	Q80T41	4	4	41.2	2.7	112.3	6.9	15	16	GABA-gated ion channel, synaptic inhibition
Gabra1	P62812	5	3	30.4		55	5.1		11	GABA-gated ion channel, synaptic inhibition
Gria2	E9QKC0	7	7	40.1	2.7	51.6	5.7	15	9	Glutamate receptor, excitatory synaptic transmission

Transporters										
Atp1a1	Q8VDN2	17	18	916.1	8	1085	174.6	115	6	Osmoregulation, Na^+-coupled transport of molecules & electrical excitability
Atp1a3	Q6PIC6	34	38	2948	23	5158	722.2	128	7	Osmoregulation, Na^+-coupled transport of molecules & electrical excitability
Atp2b2	Q3UHH0	11	15	268.3	14.1	554	74	19	7	Intracellular calcium homeostasis
Atp2b4	E9Q828	7	8	42.1		99	13.8		7	Intracellular calcium homeostasis
Atp5f1b	P56480	21	8	12.9		8.1				Respiratory electron transport & ATP synthesis
Atp9a	Q8C288	4	5	34.8		96.4	6.1		16	Unknown
Slc12a5	A0A076FR46	7	10	193.8		381.6	53.4		7	Dendritic spine formation and maturation
Slc16a7	O70451	4	3	85.9		202.2	19.7		10	Proton-coupled monocarboxylate transporter
Slc27a4	Q91VE0	8	6	212.5		350.3	20.2		17	Translocation of long-chain fatty acids across the plasma membrane
Slc2a13	Q3UHK1	12	7	17.4		84.2	5.3		16	Myo-inositol co-transporter
Slc32a1	O35633	11	5	40.9		147.1	6		25	Uptake of GABA & glycine into synaptic vesicles
Slc4a4	E9Q8N8	2	3	14.2		91.9				May regulate bicarbonate influx/efflux & intracellular pH
Slc6a11	P31650	6	4	42.3	2.2	140.8	25.4	19	6	Rapid removal of GABA, maintains low extracellular levels
Slc8a2	Q8K596	6	5	53.3		112.6	14.1		8	Synaptic plasticity, learning & memory
Others										
Ank2	Q8C8R3	9	37	1732	9.6	2148	137.8	180	16	Cell motility, activation, proliferation, contact & maintenance of membrane domains
Hnrnpu	Q8VEK3	16	15	1267	5	748.4	45.8	253	16	DNA- and RNA-binding protein
Xkr4	Q5GH67	2	2	26.2		110.1	13.2		8	Unknown
Acsl4	Q9QUJ7	9	7	107.1		130.6	23.6		6	Activation of fatty acids for synthesis of cell lipids & degradation via beta-oxidation

[a]Tandem Mass Tag (TMT) protein abundances for Experiments A & B; [b]Previously identified CS binders; [c]Ig domain-containing proteins; [d]Proteins validated by ELISA in this work (Figure 4D).

4. Experimental Methods

4.1 Materials and Methods

Chemicals and biochemical reagents. All chemicals and reagents were of analytical grade, purchased from commercial vendors, and used without further purification unless otherwise specified. Chondroitin sulfate-E (CS-E; average molecular weight (MW): 70,000; ~130 disaccharide units) and -C (CS-C; average MW: 63,000; ~132 disaccharide units) polysaccharides (Super Special Grade, Na salts) were purchased from Seikagaku Biobusiness Corporation and contain an estimated 60% of the CS-E motif and 90% of the CS-C motif, respectively. Trifunctional Photo-crosslinking Reagents (TPRs) **1** and **2** were purchased from Sigma-Aldrich (**1**: 900603, **2**: 900750), **S1** from Enamine (EN300-315164), and **S2** and **S5** from BroadPharm (**S2**: BP-22045, **S5**: BP-22043). Chondroitinase ABC (ChABC) was purchased from Sigma-Aldrich (C3667). All secondary antibody-Alexa Fluor (AF) conjugates, including goat anti-mouse AF680 and AF790, donkey anti-goat AF680 and AF790, were obtained from Life Technologies Corporation (Carlsbad, CA). All in vitro experiments were conducted in 96-well polyvinyl chloride flexible plates (BD Falcon), with gentle rocking. All experiments involving photo-crosslinking probes were carried out in the dark. All chemical reactions were carried out under an argon atmosphere unless otherwise specified. Reactions were monitored by analytical thin layer chromatography (TLC) on pre-coated silica plates (Merck 60 F254, 0.25 µm), and spots were visualized by UV (254 nm), heating with $KMnO_4$, or ninhydrin. Flash column chromatography was carried out using 230 or 400 mesh silica gel (0.040–0.063 mm, EMD Millipore). All NMR spectra (^1H and ^{13}C) were recorded on a Bruker 400 MHz NMR spectrometer with a Prodigy broadband cryoprobe. Chemical shifts are reported in parts per million (ppm) and are referenced with respect to appropriate residual solvent peaks (CD_3OD = 3.31 ppm, D_2O = 4.79 ppm).[4] Data for ^1H NMR are reported as follows: chemical shift (δ ppm) (integration, multiplicity, coupling constant (Hz), assignment). Multiplicities are reported as follows: s = singlet, d = doublet, t = triplet, m = multiplet. Data for ^{13}C NMR are reported in terms of chemical shifts (δ ppm). High-resolution mass spectra (HRMS) were obtained on a Waters LCT Premier XE time-of-flight mass spectrometer equipped with an electrospray ionization source (ESI).

Cell culture. All reagents used for cell culture were obtained from Thermo Fisher Scientific unless otherwise noted. The HEK-293T cell line was obtained from the American Type Culture Collection. HEK-293T cells were cultured in DMEM (high glucose, GlutaMAX™ Supplement) supplemented with 10% fetal bovine serum (FBS) and 1x Penicillin-Streptomycin (P/S; complete DMEM). Cells were generally grown on plastic, tissue culture plates (Falcon™), and were passaged and dissociated with 0.25% trypsin (Life Technologies). Cells were incubated in a 5% CO_2, humidified chamber at 37 °C.

Animals and Tissue Isolation. C57BL/6 wild type mice were maintained in accordance with proper Institute of Animal Care and Use Committee (IACUC) procedures. E15–16 timed-pregnant mice were anesthetized with CO_2 and dissected to remove the cortex as previously described.[5]

Western blotting. Protein samples were first diluted with one-fourth volume of 4x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (200 mM Tris pH 6.8, 400 mM DTT, 8% SDS, 40% glycerol, 0.4% bromophenol blue) and heated to 95 °C for 10 min. Samples were loaded onto precast NuPAGE 4–12% Bis-Tris protein

gels (Invitrogen, NP0321BOX) or NuPAGE 3–8% Tris-Acetate protein gels (Invitrogen, EA0375BOX) and resolved at a constant voltage of 120 V, at RT for 1 h 10 min using MOPS (Invitrogen, NP0001) or Tris-Acetate (50 mM Tricine, 50 mM Tris, 0.1% SDS, pH 8.2–8.3) running buffers, respectively. The molecular weight of protein targets was estimated using Precision Plus Protein Dual Color Standards (Bio-Rad, 1610374) or HiMark™ Pre-stained Protein Standard (Thermo Fisher Scientific, LC5699). Gels were transferred to Immobilion-FL PVDF membranes (EMD Millipore, IPFL00010) using 350 mA for 1.5 h. Membranes were then blocked for 0.5–1 h at RT with 5% bovine serum albumin (BSA; Thermo Fisher Scientific) in TBST (50 mM Tris, 150 mM NaCl, pH 7.4, 0.1% Tween). Primary antibodies used for Western blotting, dilutions, and commercial sources are as follows: mouse anti-myc-tag (Cell Signaling Technology, 9B11, 1:1000), rabbit anti-His-tag (Cell Signaling Technology, D3I10, 1:1000), goat anti-PTPRS (R&D systems, AF3430, 1:1000), mouse anti-EphA4 (Life Technologies, 4C8H5, 1:500), and mouse anti-α-tubulin (Sigma-Aldrich, T9026, 1:1000). Primary antibodies were diluted in the same buffer as the blocking step and incubated at 4 °C overnight with gentle rocking. Blots were rinsed three times for 5 min with TBST prior to incubation with secondary antibodies. For all Western blots, highly cross-adsorbed secondary antibodies containing AF680 or AF790 were used at 1:10,000 (0.2 µg/mL) in the same buffer as the blocking step and incubated at RT for 1.5 h with gentle rocking. Blots were then washed three times for 5 min with TBST prior to imaging with the Li-COR Odyssey CLx. Quantification was performed using Image Studio Software (Li-COR). If necessary, blots were stripped using NewBlot PVDF Stripping Buffer (Li-COR) according to the manufacturer's protocol.

CS-E/CS-C Biotinylation.[2] CS-E or -C polysaccharides (2.0 mg) were dissolved in 1.0 mL of DPBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, pH 7.4) and 180 µL of a 10 mM solution of EZ-Link Sulfo-NHS-LC-Biotin (1 mg, Thermo Fisher Scientific, A39257) in DPBS was added. The resulting solution was rotated end-over-end for 3–4 hours at RT. Following biotinylation, excess reagent was removed using Amicon Ultra centrifugal filters with a 3K molecular weight cut-off (MWCO; Amicon Ultra, UFC500324), and the buffer was exchanged to ddH_2O by repeated centrifugation and concentration (4–5 times). Biotinylated CS preparations were then normalized for uronic acid content using the carbazole assay,[6] diluted to 1 mg/mL, and aliquoted. The aliquots were either lyophilized or flash frozen using MeOH/dry ice and stored at −80 °C.

*General Procedure 1 for the conjugation of **1–2** to CS-E via isourea linkages (See Supporting Table 1).* To a 200-µL solution of 15 µM (1.0 mg/mL) biotinylated CS-E (0.20 mg, 0.0029 µmol, 1.0 eq) in ddH_2O was added 12 µL of a 25 mg/mL solution of CNBr (0.31 mg, 2.9 µmol, 1000 eq) in ddH_2O. The pH of the reaction was adjusted to ~11 using a 200 mM solution of NaOH, and the reaction was rotated end-over-end at RT for 15 min, while keeping the pH at ~11. The resulting reaction mixture was buffer exchanged to borate buffer (200 mM, pH 8.0) using an Amicon Ultra centrifugal filter with a 3K MWCO by repeated centrifugation and concentration (4–5 times). To the recovered solution of activated CS polysaccharide was added a solution of **1** or **2** (140–280 eq, according to Table S1) in borate buffer. The reaction volume was adjusted to give a final CS concentration of ~12 µM, and the resulting solution was rotated end-over-end at RT in the dark. After 18 h, the reaction was centrifuged at 14,000 xg for 5 min. Excess borate buffer was then added to re-dissolve any remaining solid. The probe was separated from excess reagents, and the buffer was exchanged to ddH_2O using an Amicon Ultra centrifugal filter with a 3K MWCO, by repeated centrifugation and concentration (5 times). The CS-E probes obtained were normalized

for uronic acid content using the carbazole assay,[6] and the photo-crosslinker to CS polysaccharide ratio (PC:CS) was quantified by UV-Vis spectroscopy as described below.[7]

*General Procedure 2 for the conjugation of **1–4** to CS-E and -C via amide linkages (See Supporting Table 1).* A 250-µL aliquot of 15 µM biotinylated CS-E (0.25 mg, 0.0036 µmol, 1.0 eq) or CS-C (0.23 mg, 0.0037 µmol, 1.0 eq) in ddH$_2$O was lyophilized. A solution of **1–4** (according to Table S1) in MES buffer (250 mM, 150 mM NaCl, pH 6.5) was added, followed by 11 µL of a freshly prepared 5.0 mg/mL solution of NHS (0.056 mg, 0.49 µmol, 134 eq) in MES, and 31 µL of a freshly prepared 30 mg/mL solution of EDC·HCl (0.93 mg, 4.9 µmol, 1340 eq) in MES. The reaction volume was then adjusted with MES to give a final CS concentration of ~5.0–7.0 µM and the resulting solution was rotated end-over-end at RT in the dark. After 18 h, the reaction was centrifuged at 14,000 xg for 5 min. Excess MES was then added to re-dissolve any remaining solid. The probe was separated from excess reagents, and the buffer was exchanged to ddH$_2$O using an Amicon Ultra centrifugal filter with a 3K MWCO by repeated centrifugation and concentration (5 times). The CS probes obtained were normalized for uronic acid content using the carbazole assay,[6] and the PC:CS ratio was quantified by UV-Vis spectroscopy as described below. In cases when higher substitution was needed, the probe obtained was re-submitted to the same conditions 2 or 3 times (Figure S1E and Table S1).

General Procedure 3 for the determination of the PC:CS *ratios*. Following General Procedure 1 or 2, the probe solution obtained was re-adjusted to a volume of ~210 µL in ddH$_2$O. The concentration of the probes was calculated using the carbazole assay.[6] Briefly, to construct a calibration curve, 0, 2, 5, 10, or 15 µL of a 1.0 mg/mL solution of glucuronolactone in ddH$_2$O was diluted to 50 µL. Next, 20 µL and 10 µL of the probe solution, and a CS-E or CS-C standard solution, were diluted to 50 µL. To each sample was added 950 µL of ice cold Reagent A (25 mM sodium tetraborate in sulfuric acid). The mixture was heated to 100 °C for 10 min and then cooled rapidly on ice. After adding 50 µL of Reagent B (0.125% *wt/vol* carbazole in EtOH), the reaction was mixed thoroughly by vortexing and was reheated at 100 °C for 10 min. The reaction was cooled rapidly on ice, and the absorbance was determined at 525 nm using a Bio-tek Instruments Uvikon XS UV/Vis Spectrophotometer. The concentration of disaccharide (DS) was calculated using the glucuronolactone calibration curve and the CS polysaccharide (PS) concentration was determined accordingly:

$$[\text{CS Probe}] = [\text{DS}] \div (\#\text{DS per PS})$$

	CS-E	CS-C
Average CS MW	70,000	63,000
DS MW	520.52	475.38
#DS per PS	134.48	132.53

DS: disaccharide; PS: polysaccharide

The remaining 180 µL of probe solution was lyophilized and re-dissolved in 50 µL of ddH$_2$O for benzophenone (BP)-containing probes or 6–8 µL ddH$_2$O for diazirine (DA)-containing probes. Calibration curves for **1–4** were constructed in ddH$_2$O and MES (Figures S1A and 1B), and the absorption of each standard and probe was measured using a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific; Path Length = 0.1 cm). The concentration of BP or DA in the

samples was determined using the following molar extinction coefficients: ε_{BP} = 21,595 $M^{-1}cm^{-1}$ (λ_{max} = 297 nm, H_2O) and ε_{DA} = 293 $M^{-1}cm^{-1}$ (λ_{max} = 350 nm, H_2O). These values are within the range previously reported.[8,9]

Molecular Biology. All reagents used for molecular biology were obtained from New England Biolabs unless otherwise noted. All primers were obtained from Integrated DNA Technologies. All miniprep, maxiprep, and gel extraction steps were conducted using Zymogen kits (Genesee Scientific). The PTPσ-Fc fusion protein construct was generated by PCR amplification of nucleotides 250–2715 of a full-length mouse PTPσ clone (MGC: 63375, IMAGE: 6834684). The Icam5-Fc fusion protein construct was generated by PCR amplification of nucleotides 134–2539 of a full-length mouse Icam5 clone (MGC: 130520, IMAGE: 40048010). The ectodomains of PTPσ and Icam5 were subcloned into a pAPtag-5 vector (GenHunter Corporation), containing an N-terminal murine Ig kappa chain leader sequence, a C-terminal human IgG1 Fc sequence replacing the AP sequence, Myc, and 6xHis tags.[10] To generate the PTPσ ΔLys contruct, the lysine residues K68, K69, K71 and K72 in the N-terminal Ig domain were simultaneously changed to alanine residues using the Q5 site directed mutagenesis kit (New England Biolabs).[10,11]

Protein Expression and Purification. For each protein construct, five 15-cm plates of 90% confluent HEK-293T cells in 20 mL of complete DMEM were transfected with 20–30 µg of PTPσ-Fc or Icam5-Fc DNA construct using poly(ethyleneimine) (PEI; Polysciences, Inc., 23966-2). Briefly, DNA was diluted in 1 mL of pre-warmed, Opti-MEM® (Thermo Fisher Scientific, 31985070), and PEI in sterile ddH_2O (1 mg/mL) was added dropwise with vigorous shaking at a 3:1 *w/w* PEI/DNA ratio. DNA/PEI complexes were allowed to form for 15–20 min at RT and then added dropwise to each plate. After 6–8 h, the medium in each plate was replaced with DMEM containing 2% FBS and 1x P/S. At 3 d post-transfection, the medium was harvested, centrifuged and filtered through a 0.1 µm filter (Thermo Fisher Scientific, Nalgene™, 124-0045) to remove cell debris. A new 20-mL aliquot of DMEM, 2% FBS, 1x P/S was added, which was harvested 2 d later, centrifuged, filtered, and combined with the first aliquot. The pH of the solution was adjusted to 8.0 using 2 M Tris, and the medium was made up to 150 mM NaCl and 10 mM imidazole. The medium was then rotated end-over-end for 2 h at 4 °C with 500 µL of Ni-NTA resin (Qiagen, 30230) that had been prewashed twice with DPBS. The resin was separated from the medium by centrifugation and loaded onto a disposable Poly-Prep chromatography column (Bio-Rad). The resin was washed with 30 mL of wash buffer (50 mM $NaH_2PO_4 \cdot H_2O$, 300 mM NaCl, 20 mM imidazole, pH 8.0), and the protein was eluted with three or four 2-mL aliquots of elution buffer (50 mM $NaH_2PO_4 \cdot H_2O$, 300 mM NaCl, 250 mM imidazole, pH 8.0). Elution samples were combined and buffer exchanged by centrifugation with DPBS. Samples were stored with BSA as a carrier protein (1 mg/mL) at 4 °C for up to one week or flash frozen and stored at −80 °C. For all overexpressed, purified proteins, protein concentrations were normalized using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific) before use.

Carbohydrate enzyme-linked immunosorbent assay (ELISA). Biotinylated CS-E and CS-C were prepared as described above.[2] ELISAs were performed using PTPσ-Fc or Icam5-Fc (overexpressed and purified from mammalian cells as described above), an anti-CS-E mAb (generated and purified as previously described[1]), or commercially available PlxnA4-His_6 (R&D Systems, 5856-PA-050), Nptxr-His_6 (R&D Systems, 4414-NP-050), Nlgn1-His_6 (R&D Systems, 7066-NL-050), and IgG1 Fc (R&D Systems, 110-HG-100).

Wells coated with Protein A/G (8-well strips; Thermo Fisher Scientific) were washed three times with 200 μL of PBST (DPBS, 0.05% Tween-20) and then incubated with 50 μL of PTPRS-Fc or CS-E mAb (10 μg/mL in PBST containing 1% BSA) for 2 h at RT, with gentle rocking. Wells were washed three times with PBST and incubated with 100 μL of 3% BSA in PBST for 1 h at RT. During this incubation step, a dilution series of biotinylated CS-E, CS-C, probe **8** (PC:CS 14:1) or probe **10** was produced in PBST. Wells were washed three times with PBST and incubated with 50 μL of each GAG solution for 2–3 h at RT with gentle rocking. Wells were washed three times with PBST, then incubated with 100 μL of HRP-conjugated streptavidin (1:15,000 dilution in 1% BSA/PBST, Thermo Fisher Scientific, 21130) for 1 h at RT with gentle rocking. After washing five times with PBST, wells were incubated with 100 μL of development 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (R&D Systems) for 10–15 min at RT in the dark. Reactions were quenched by the addition of 100 μL of 2 N H_2SO_4, and the absorbances were read at 450 nm using a Flexstation 3 microplate reader.

Alternatively, Ni^{2+}-coated (Nlgn1-His_6) wells (Thermo Fisher Scientific) were washed three times with 200 μL of PBST (DPBS, 0.05% Tween-20) and then incubated with 50 μL of Nlgn1-His_6 (10 μg/mL in PBST containing 1% BSA) for 2 h at RT with gentle rocking. During this incubation step, a dilution series of biotinylated CS-E or CS-C was produced in PBST. Wells were washed three times with PBST and incubated with 50 μL of each GAG solution for 2–3 h at RT with gentle rocking. Wells were washed four times with PBST, then incubated with 3% BSA in PBST for 1 h at RT. Wells were washed three times with PBST, then incubated with 100 μL of HRP-conjugated streptavidin (1:15,000 dilution in 1% BSA/PBST) for 1 h at RT with gentle rocking. After washing five times with PBST, wells were incubated with 100 μL of development TMB substrate solution for 10–15 min at RT in the dark. Reactions were quenched by the addition of 100 μL of 2 N H_2SO_4, and the absorbances were read at 450 nm using a Flexstation 3 microplate reader.

Alternatively, streptavidin-coated wells (8-well strips; Thermo Fisher Scientific) were washed three times with 200 μL of PBST and then incubated with 50 μL of biotinylated CS-E, CS-C or CS probes (20 μg/mL in PBST) for 2–3 h at RT, with gentle rocking. Wells were washed three times with PBST and incubated with 200 μL of 3% BSA in PBST for 1 h at RT. During this incubation step, a dilution series of PTPσ-Fc, Icam5-Fc, Nptxr-His_6, PlxnA4-His_6, IgG1 Fc, or anti-CS-E mAb was produced in 1% BSA/PBST. Wells were washed three times with PBST and incubated with 50 μL of each protein-containing solution for 2–3 h at RT with gentle rocking. Wells were washed three times with PBST, then incubated with 100 μL of goat anti-human, goat anti-mouse or goat anti-His IgG Fc-HRP conjugate (1:2,000 dilution in 1% BSA/PBST, Life Technologies, 628420; Invitrogen, A16078; R&D Systems, MAB050H-SP) for 1 h at RT with gentle rocking. After washing five times with PBST, wells were incubated with 100 μL of development TMB substrate solution for 10–15 min at RT in the dark. Reactions were quenched by the addition of 100 μL of 2 N H_2SO_4, and the absorbances were read at 450 nm using a Flexstation 3 microplate reader.

Assays were performed two independent times, and values are reported as the average ± SEM. Curves were fitted and $K_{D,app}$ values calculated with Prism 8 (GraphPad) using the nonlinear curve fitting of specific binding with Hill slope function.

Photo-ELISA assay. Wells coated with streptavidin (Nunc® Immobilizer™ Streptavidin strips; Thermo Fisher Scientific, 436020) were washed three times with 200 μL of PBST and then incubated with biotinylated CS-E polysaccharides, CS-C polysaccharides, or CS photoaffinity probes (20 μg/mL in PBST; probe **7**, PC:CS 13:1; probe **8**, PC:CS 14:1) for 2 h at RT

in the dark with gentle rocking. Wells were washed three times with PBST and incubated with 100 µL of 5% BSA in PBST for 30 min to 1 h at RT. Wells were washed three times with PBST, then incubated with 50 µL of an anti-CS-E mAb[1] solution (10 nM in 1% BSA/PBST) for 2 h at RT, with gentle rocking. Relevant wells were then irradiated at 365 nm (Black-Ray® XX-20BLB UV Bench Lamp, 20 Watt, P/N 95-0045-05, 10 cm distance) at 4 °C for 40 min. Control wells were kept in the dark at 4 °C. All wells were washed four times for 5 min with 200 µL of a 1% SDS solution in DPBS. Control wells were kept in PBST during that time. All wells were washed 5 times with PBST and then incubated with 100 µL of goat anti-mouse IgG Fc-HRP conjugate (1:2000 dilution in 1% BSA/PBST) for 1 h at RT with gentle rocking. Wells were washed five times with PBST, and then incubated with 100 µL of TMB substrate solution for 10 min at RT in the dark. Reactions were quenched by the addition of 100 µL of 2 N H_2SO_4, and the absorbances were read at 450 nm. Assays were performed two independent times, and values were reported as the average ± SEM. Relative photo-crosslinking was assessed by comparing the SDS washed, irradiated wells, and control dark wells for each CS photo-crosslinking probe.

General protocol for photo-crosslinking CS-E binding proteins in vitro. To a solution of PTPσ-Fc or PTPσ-Fc-ΔLys$_{10}$ (100–330 ng) and BSA (6 µg) in DPBS was added a solution of CS photo-crosslinking probe (40 µM in ddH_2O; probe **7**, PC:CS 16:1; probe **8**, PC:CS 15:1) or ddH_2O as a control (final concentrations: 20–65 nM protein, 0.15–6.5 µM probe, and 0.15 mg/mL BSA in 40 µL). After 2 h of incubation at RT with gentle rocking, the samples were irradiated at 365 nm for 10–40 min at 4 °C. Following irradiation, a 12-µL sample of each well was used to assess and quantify photo-crosslinking by Western blotting using a mouse anti-myc-tag antibody.

General protocol for photo-crosslinking CS-E binding proteins in vitro – Competition experiment. To a solution of PTPσ-Fc (100 ng) and BSA (6 µg) in 30 µL of DPBS was added 8 µL of a 200 µM solution of CS-E, CS-A or CS-C polysaccharides in DPBS (for competition experiments) or DPBS (without competition). After 1 h of incubation at RT with gentle rocking, 2 µL of 20 µM CS-E probe **8** (PC:CS 16–17:1) CS-C probe **10**, or ddH_2O as a control was added (final concentrations: 20 nM protein, 1.0 µM probe, 40 µM CS-E, CS-A or CS-C polysaccharides, and 0.15 mg/mL BSA in 40 µL). After 2 h of incubation at RT with gentle rocking, the samples were irradiated at 365 nm for 40 min at 4 °C. Following irradiation, a 12-µL sample of each well was used to assess and quantify photo-crosslinking by Western blotting using a mouse anti-myc-tag antibody as described above.

General protocol for photo-crosslinking & pull down of CS-E binding proteins in vitro. To a solution of PTPσ-Fc or full-length PTPσ (R&D Systems, 3430-PR-050; 300 ng) and BSA (4 µg) in ChABC buffer (50 mM Tris, 60 mM NaOAc, pH 8.0) was added probe **8** (PC:CS 17:1) in ddH_2O or ddH_2O as a control (final concentrations: 60 nM protein, 3.5 µM probe, and 0.10 mg/mL BSA in 40 µL). After 2 h of incubation at RT with gentle rocking, the samples were irradiated at 365 nm for 40 min at 4 °C. The samples were diluted to 56 µL and adjusted to 0.5% Triton and 1.0 mg/mL BSA in ChABC buffer. Two protocols were optimized for the pull down of photo-crosslinked proteins: via the end-terminal biotin on the CS probe (Protocol A) or via biotin handles appended using CuACC chemistry (Protocol B). For Protocol A, samples were flash frozen and stored at −80 °C prior to pull down with Pierce™ streptavidin magnetic beads (Thermo Fisher Scientific, 8817), as described below. For Protocol B, 4 µL of a 10 U/mL solution of ChABC enzyme in 0.01% BSA solution (4 mU/µg

of CS) was added to the samples (final concentrations: ~1 mg/mL protein, 2.3 μM probe, and 0.5% Triton X-100 in 60 μL). The samples were incubated end-over-end for 19 h at 37–38 °C in the dark, then diluted to 0.1% Triton X-100. Digestion by-products were removed using Amicon Ultra centrifugal filters with a 10K MWCO (Amicon Ultra, UFC501024), followed by buffer exchange to DPBS by repeated centrifugation and concentration (4–5 times). Click reactions were subsequently performed similarly to previously described methods.[12] Briefly, the ChABC treated samples were diluted to 100 μL and a final concentration of 1% SDS. Separately, 2.5 μL of 10 mM BTTAA ligand in DMSO, 5 μL of freshly prepared 50 mM $CuSO_4·5H_2O$ in ddH_2O, followed by 82.4 μL of DPBS and 22.6 μL of 100 mM azide-PEG$_3$-biotin (Sigma-Aldrich, 762024) in DMSO were combined. To each protein sample was added 100 μL of the copper/ligand/alkyne solution, followed by 2 μL of 100-mM freshly prepared TCEP (final concentrations: 0.11 mM BTTAA, 1.11 mM $CuSO_4·5H_2O$, 10 mM azide-PEG$_3$-biotin, and 2 mM TCEP in 200 μL). The mixture was incubated end-over-end at 37 °C for 1 h before 1 mL of acetone was added. The reaction mixture was vortexed briefly and kept at −20 °C overnight. After centrifugation (5 min, 20,000 xg, 4 °C), the protein pellet was collected, washed twice with cold methanol (1 mL), and re-dissolved in 50 μL of 1% SDS in DPBS. To each protein sample from Protocol A was added 50 μL of 6% Triton X-100, followed by 650 μL of DPBS to give a final concentration of 0.4% Triton X-100. Samples from Protocol B were thawed and diluted to 750 μL and a final concentration of 0.4% Triton X-100. For both Protocols A and B, 30-μL aliquots of Pierce™ streptavidin magnetic beads were washed three times with DPBS and added to each sample. The samples were incubated end-over-end at 4 °C overnight. The beads were washed with 1 M NaCl (3 x 0.5 mL), 1% SDS (10 x 0.5 mL), 1 M NaCl (3 x 0.5 mL), and a freshly prepared solution of UltraPure 2 M urea (Thermo Fisher Scientific; 3 x 0.5 mL). Captured proteins were eluted from the beads using 40 μL of 1x SDS-PAGE loading buffer containing 2 mM biotin, by boiling at 95 °C for 15 min, with occasional vortexing. The elution volume was removed, and samples were flash-frozen and stored at −80 °C prior to analysis by SDS-PAGE.

Photo-crosslinking endogenous CS-E binding proteins and competition experiment in live cortical neurons. Tissue culture plates (6-well, Falcon™) were coated with 20 μg/mL of poly-D-lysine (PDL, Sigma-Aldrich) in sterile DPBS overnight at 37 °C and 5% CO_2. The plates were then rinsed three times with sterile ddH_2O and air-dried before use. Cortical neurons were dissected from E15–16 timed-pregnant C57BL/6 mice as previously described.[5] Briefly, dissected cortices were incubated with papain and L-cysteine for 15 min at 37 °C, triturated to dissociate to single-cell suspensions, filtered using a 40-μm cell strainer (Fisherbrand™) to remove non-dissociated cells, and seeded at approximately 500,000 cells per well. Cortical neurons were cultured in Neurobasal Plus medium (Thermo Fisher Scientific, A3653401) supplemented with B-27 Plus, 1.5 mM GlutaMAX™ (Life Technologies), and 1x P/S at 37 °C and 5% CO_2 for 13–14 days in vitro (DIV). The media was removed, and the neurons were treated with 430 μL of either CS-E polysaccharide (20 μM solution in warm Neurobasal Plus medium supplemented with 1.5 mM GlutaMAX™ and 1x P/S) or vehicle control (Neurobasal Plus medium supplemented with 1.5 mM GlutaMAX™ and 1x P/S). The neurons were incubated for 1 h at 37 °C and 5% CO_2. Subsequently, 36 μL of media was removed from each well and replaced with 36 μL of either probe **8** (24 μM in ddH_2O; PC:CS 16–17:1) or ddH_2O control (final concentrations: 2 μM probe **8** and 18 μM CS-E competitor). After 3 h of incubation at 37 ºC and 5% CO_2, the neurons were washed gently once with pre-warmed DPBS (1–2 mL) to remove the excess probe, then another volume of pre-warmed DPBS (1 mL) was added for irradiation. Cells were irradiated at 365 nm for 40 min at

4 °C. DPBS was gently removed, and the cells were lysed with 100 µL of cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 7.4) containing cOmplete™ protease inhibitor cocktail (Sigma-Aldrich, 11697498001) and universal nuclease (1:1500, Pierce™, 88700). After rotating end-over-end for 10 min at 4 °C in the dark, the lysates were centrifuged for 5 min at 20,000 xg, 4 °C. The protein samples were precipitated using chloroform/MeOH/H_2O (3:1:2.25), washed twice with cold MeOH, air dried, then re-dissolved in 30 µL of 1% SDS. Protein concentrations were measured using the BCA assay, and 20 µg of each sample was used to quantify the % photo-crosslinking relative to the no probe control by Western blotting using goat anti-PTPRS, mouse anti-EphA4, and mouse anti-α-tubulin antibodies.

Photo-crosslinking of CS-E binders in live cortical neuron and proteomics. Tissue culture plates (10 cm) were coated with 20 µg/mL of PDL in sterile DPBS overnight at 37 °C and 5% CO_2. The plates were then rinsed three times with sterile ddH_2O and air dried before use. Cortical neurons were dissected from E15–16 timed-pregnant C57BL/6 mice, seeded at approximately 4–4.5 million cells per plate, and cultured as described above.[5] After 14 DIV, the medium was removed and replaced with 3.4 mL of either probe **8** (1 µM solution in warm Neurobasal Plus medium supplemented with 1.5 mM GlutaMAX™ and 1x P/S; PC:CS 16–17:1) or vehicle control (Neurobasal Plus medium supplemented with 1.5 mM GlutaMAX™ and 1x P/S). After 3 h of incubation at 37 ºC and 5% CO_2, the neurons were washed gently once with pre-warmed DPBS (5 mL) to remove the excess probe, then another volume of pre-warmed DPBS was added for irradiation (5 mL). Cells were irradiated at 365 nm for 40 min at 4 ºC. DPBS was removed, and the cells were lysed with 200 µL of cold lysis buffer (1% Triton X-100, 60 mM NaOAc, 50 mM Tris·HCl, pH 8.0) containing cOmplete™ protease inhibitor cocktail without EDTA (Sigma-Aldrich, 11873580001) and universal nuclease (1:1500). The lysates were passed through a 26G x 1/2 needle 20 times, incubated for 10 min at 4 °C in the dark, and centrifuged (5 min at 20,000 xg, 4 °C). Protein concentrations were measured using the BCA assay (~2–3 mg/mL). The samples were diluted to 0.95 mL with ChABC buffer containing cOmplete™ protease inhibitor cocktail without EDTA, and 50 µL of a 20 U/mL solution of ChABC enzyme in 0.01% BSA solution (4 mU/µg of CS) was added (final concentrations: ~0.6 mg/mL protein, 3.4 µM CS-E probe, and 0.5% Triton X-100 in 1 mL). The samples were incubated end-over-end for 19 h at 37–38 °C in the dark. The samples were diluted to 0.1% Triton X-100, and the digestion by-products were removed using Amicon Ultra centrifugal filters with a 10K MWCO. The samples were buffer exchanged to DPBS by repeated centrifugation and concentration (4–5 times). Click reactions were subsequently performed similarly to previously described methods.[12] Briefly, the ChABC-treated lysates were diluted to 480 µL and 1% SDS in PBS to obtain a final concentration of ~1 mg/mL. In a separate tube, 12.5 µL of 10 mM BTTAA ligand in DMSO and 25 µL of freshly prepared 50 mM $CuSO_4 \cdot 5H_2O$ in ddH_2O were combined, followed by 412 µL of DPBS and 113 µL of 100 mM azide-PEG$_3$-biotin in DMSO. To each protein sample was added 500 µL of the copper/ligand/alkyne solution, followed by 20 µL of 100 mM freshly prepared TCEP (final concentrations: 0.11 mM BTTAA, 1.11 mM $CuSO_4 \cdot 5H_2O$, 10 mM azide-PEG$_3$-biotin, and 2 mM TCEP in 1 mL). The mixture was incubated end-over-end at 37 °C for 1 h before 5 mL of acetone was added. The reaction mixture was vortexed and kept at −20 °C overnight. After centrifugation (5 min, 500 xg, 4 °C), the protein pellet was collected, washed twice with cold methanol (1 mL), and re-dissolved in 150 µL of 1% SDS in DPBS. To each protein sample was added 150 µL of 6% Triton X-100, followed by 1.75 mL of DPBS to give a final concentration of 0.4% Triton-X100. Prewashed streptavidin magnetic beads (100 µL) were

added, and the mixture was incubated end-over-end at 4 °C overnight. The beads were washed with 1 M NaCl (3 x 0.5 mL), 1% SDS (10 x 0.5 mL), 1 M NaCl (3 x 0.5 mL), and a freshly prepared solution of UltraPure 2 M urea (3 x 0.5 mL). Captured proteins were eluted twice from the beads with 160 µL of elution buffer (2% SDS, 100 mM DTT, 50 mM Tris·HCl, 2 mM biotin, pH 6.8) by boiling at 95 °C for 15 min, with occasional vortexing. The proteins were precipitated with cold acetone and kept at −20 °C overnight. After centrifugation (10 min, 20,000 xg, 4°C), the protein pellet was re-dissolved in 27 µL of 1% SDS in DPBS, and 9 µL of 4x SDS-PAGE loading buffer was added. After heating to 95 °C for 10 min, the protein samples were loaded on a 3–8% NuPAGE Tris-Acetate protein gel and run with a Tris-Acetate running buffer for 15 min at 120 V to remove detergent. In-gel digestion was performed on each gel lane as previously described.[13] This complete protocol was repeated two independent times (Experiments A & B; Table S2).

Mass spectrometry (MS) analysis. The eluted peptides from both independent live cell photo-crosslinking experiments were dried down, resuspended in 50 µL of 100 mM triethylammonium bicarbonate (TEAB), and TMT labeled using the TMTsixplex™ isobaric label reagent set (Thermo Fisher Scientific 90061) according to the manufacturer's protocol. The samples were combined and desalted using an HP Agilent 1100 HPLC with an Optimized Technologies C8 peptide Macrotrap (3 x 8 mm; 200 µg maximum capacity). To enhance peptide separation efficiency and subsequent MS analysis, initial offline peptide fractionation was conducted with alkaline C4 reversed-phase chromatography (Kromasil 150 × 2.1 mm, 3.5 µm particle, 100 Å pore size, Merck KGaA, Darmstadt, Germany) using gradient mobile phase conditions as previously reported.[14] Fractionated peptides were concatenated, then dried down and stored at −80 °C until MS analysis. Liquid chromatography-mass spectrometry (LC-MS) analysis was carried out on an EASY-nLC 1000 system (Thermo Fisher Scientific) coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptide samples were resuspended in 0.2% formic acid, and 5 µg of peptides per concatenated sample were loaded onto a monolithic column (Capillary EX-Nano MonoCap C18 HighResolution 2000, 0.1 x 2000 mm, Merck, Darmstadt Germany) and separated over 180 min at a flow rate of 0.5 µL/min with the following gradient: 2–6% Solvent B (10 min), 6-40% B (135 min), 40-100% B (1 min), and 100% B (34 min). Solvent A consisted of 99.8% ddH_2O, and 0.2% formic acid, and solvent B consisted of 19.8% ddH_2O, 80% ACN, and 0.2% formic acid. Raw data were acquired in a data-dependent manner using SPS-MS3 with a target cycle time of 3 sec. MS1 scans were acquired in the Orbitrap with 120,000 resolution, 60% RF lens, *m/z* range of 400–1500, AGC target of 400,000 and max injection time of 50 ms. MIPS "peptide" mode was enabled, and restrictions are relaxed when too few precursors are found. The minimum intensity threshold was 5,000, and only charge states 2–5 were allowed. Dynamic exclusion was enabled with mass tolerance of 10 ppm, duration of 60 sec, exclusion of isotopes, and single scan per charge state enabled. MS2 scans were acquired in the ion trap in rapid mode with 0.7 Da quad isolation, CID fragmentation at 35% NCE, AGC target of 10,000 and max injection time of 250 ms. The top ten fragment ions were simultaneously selected for further HCD fragmentation at 55% NCE for SPS-MS3 scans, which were acquired in the Orbitrap with 50,000 resolution, AGC target of 150,000, max injection time of 500 ms.

Proteomics data analysis. The MS raw files were searched using a target-decoy approach with Proteome Discoverer (v 2.4 Thermo Scientific, San Jose, CA). Spectra were searched using Byonic against the UniProt *mus muculus* protein database comprised of 61,681 entries. The algorithm considered tryptic peptides with up to two missed cleavages, oxidation (M) and

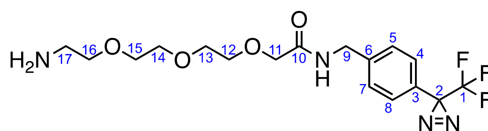
protein N-terminal acetylation as variable modifications, as well as carbamidomethylation (C) and TMTsixplex (K, N-term) as fixed modifications. Precursor mass tolerance was 10 ppm, and fragment mass tolerance was 0.5 Da. Protein, peptide, and PSM (peptide spectrum match) were identified at a 1% false discovery rate. Quantitative MS3 spectra only included those with co-isolation less than 25% and SPS matches greater than or equal to 40%. The following stringent filtering criteria were used to identify putative protein hits based on quantitative results: (1) two or more identified peptides per protein; (2) two or more abundance counts for each probe sample; (3) protein abundance of 5 or more for both probe samples; (3) if a signal was detected for the control samples, the ratio of abundances for the probe to control must be $\geqslant 5$ for both biological replicates. These criteria resulted in 346 putative CS-E binding proteins (Figure 4C). The list was further refined for plasma membrane and extracellular proteins of interest using the COMPARTMENTS mouse database (all channels integrated, downloaded on 12.19.2019; https://compartments. jensenlab.org/Downloads).[3] Overall, 126 proteins mapped to the GO-terms: plasma membrane (GO:0005886), extracellular region (GO:0005576), extracellular matrix (GO:0031012), cell surface (GO:0009986) and/or intrinsic component of plasma membrane (GO:0031226) with confidence scores $\geqslant 4$ (out of 5). Potential peripheral or cytosolic proteins that mapped to the GO-term plasma membrane (GO:0005886) with confidence scores $\geqslant 4$ and cytosol (GO:000XX) with confidence scores $\geqslant 2$, as well as proteins with confidence scores $\geqslant 3$ for the GO-term cytosolic side of plasma membrane (GO:0009898), were removed. This analysis resulted in 54 putative, plasma membrane and extracellular CS-E binding proteins (Figures 4D, S5, and Table S2).

Statistical Analysis. All data were expressed as mean ± SEM. The unpaired Student's *t* test (two-tailed) was used to evaluate the significance of differences between two experimental conditions. The one-way ANOVA followed by the Tukey's multiple comparisons test was used to evaluate the significance of three or more experimental conditions. All of the experiments were performed at least in duplicate unless otherwise indicated. The level of significance was set at $P < 0.05$.

Figures were created using BioRender.com.

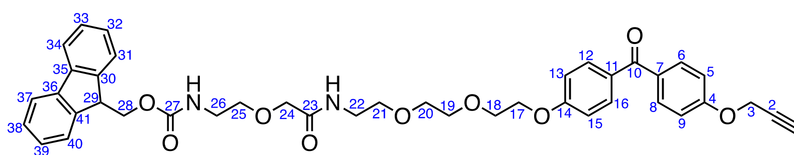
4.2 Synthetic Procedures

2-(2-(2-(2-Aminoethoxy)ethoxy)ethoxy)-*N*-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzyl)acetamide, 3



A solution of **S1** (38 mg, 0.15 mmol, 1.3 eq), **S2** (50 mg, 0.12 mmol, 1.0 eq), 7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) (70 mg, 0.14 mmol, 1.2 eq), and triethylamine (33 µL, 0.23 mmol, 2.0 eq) in DMF (360 µL) was stirred at RT under an Ar atmosphere. After 18 h, the solvent was evaporated *in vacuo,* and the resulting crude material was purified by silica gel chromatography (EtOAc/petroleum ether (PE) 5:5 to 10:0) to give **S4** as a colorless foam: R_f 0.26 (EtOAc); HRMS *m/z* (ESI^+) found 649.2246 $[M+Na]^+$ ($C_{32}H_{33}N_4O_6F_3Na$ requires 649.2250 $[M+Na]^+$). The resulting product **S3** was used in the subsequent step without further purification or characterization: **S3** was dissolved in DMF (2.1 mL), and piperidine (900 µL) was added. The reaction was stirred at RT for 30 min. The solvent was evaporated *in vacuo*, and the resulting crude material was purified by silica gel chromatography (H_2O/IPA/EtOAc 1:1:1) to give **3** (41 mg, 87% over 2 steps) as a colorless solid: ^1H NMR (D_2O; 500 MHz) δ_H 7.39–7.35 (2H, m, H-5, H-7), 7.29–7.25 (2H, m, H-4, H-8), 4.45 (s, 2H, H-9), 4.13 (s, 2H, H-11), 3.76–3.59 (10H, m, H-12 to H-16), 3.16–3.11 (2H, m, H-17); ^{13}C NMR (101 MHz, D_2O) δ_C 172.7 (C-10), 139.6 (C-3), 127.70 (C-5, C-7), 127.67 (C-4, C-8), 126.84, 126.82, 122.0 (C-1, q, $J_{C\text{-}F}$ 277.8), 70.3 (C-12), 69.6, 69.5, 69.5, 69.4 (C-11, C-13, C-14, C-15), 66.4 (C-16), 42.0 (C-9), 39.0 (C-17), 28.3 (C-2, q, $^2J_{C\text{-}F}$ 40.7); HRMS *m/z* (ESI^+) found 427.1578 $[M+Na]^+$ ($C_{17}H_{23}N_4O_4F_3Na$ requires 427.1569 $[M+Na]^+$).

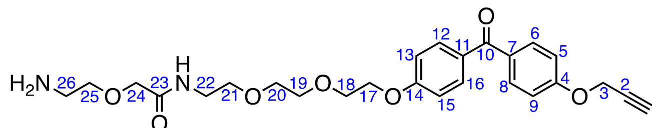
(9*H*-Fluoren-9-yl)methyl(5-oxo-14-(4-(4-(prop-2-yn-1-yloxy)benzoyl)phenoxy)-3,9,12-trioxa-6-azatetradecyl) carbamate, S5



A solution of **2** (32 mg, 0.076 mmol, 1.3 eq), Fmoc-5-amino-3-oxapentanoic acid (20 mg, 0.059 mmol, 1.0 eq), PyAOP (37 mg, 0.070 mmol, 1.2 eq), and triethylamine (16 µL, 0.12 mmol, 2.0 eq) in DMF (200 µL) was stirred at RT under an Ar atmosphere. After 18 h, the solvent was evaporated *in vacuo,* and the resulting crude material was purified by silica gel chromatography (EtOAc/PE 5:5 to 10:0) to give **S5** (38 mg, 92%) as a colorless foam: R_f 0.43 (EtOAc); ^1H NMR (CD_3OD; 500 MHz) δ_H 7.77 (2H, d, *J* 7.6, H-34 and H-37), 7.73–7.68 (4H, m, H-6, H-8, H-12 and H-16), 7.61 (2H, d, *J* 7.5, H-31 and H-40), 7.36 (2H, t, *J* 7.5, H-33 and H-38), 7.28 (2H, t, *J* 7.4, H-32 and H-39), 7.10–7.06 (2H, m, H-5 and H-6), 7.00–6.95 (2H, m, H-13 and H-15), 4.84–4.81 (2H, m, H-3), 4.38 (2H, d, *J* 6.6, H-28), 4.21–4.11 (3H, m, H-29 and H-17), 3.92 (2H, s, H-24), 3.83–3.77 (2H, m, H-18), 3.69–3.48 (8H, m, H-19, H-20, H-21 and H-25), 3.40 (2H, t, *J* 5.4, H-22), 3.33–3.27 (2H, m, H-26), 3.02 (1H, t, *J* 2.4, H-1); ^{13}C NMR (101 MHz, CD_3OD) δ_C 196.6 (C-10), 172.4 (C-23), 163.9 (C-14),

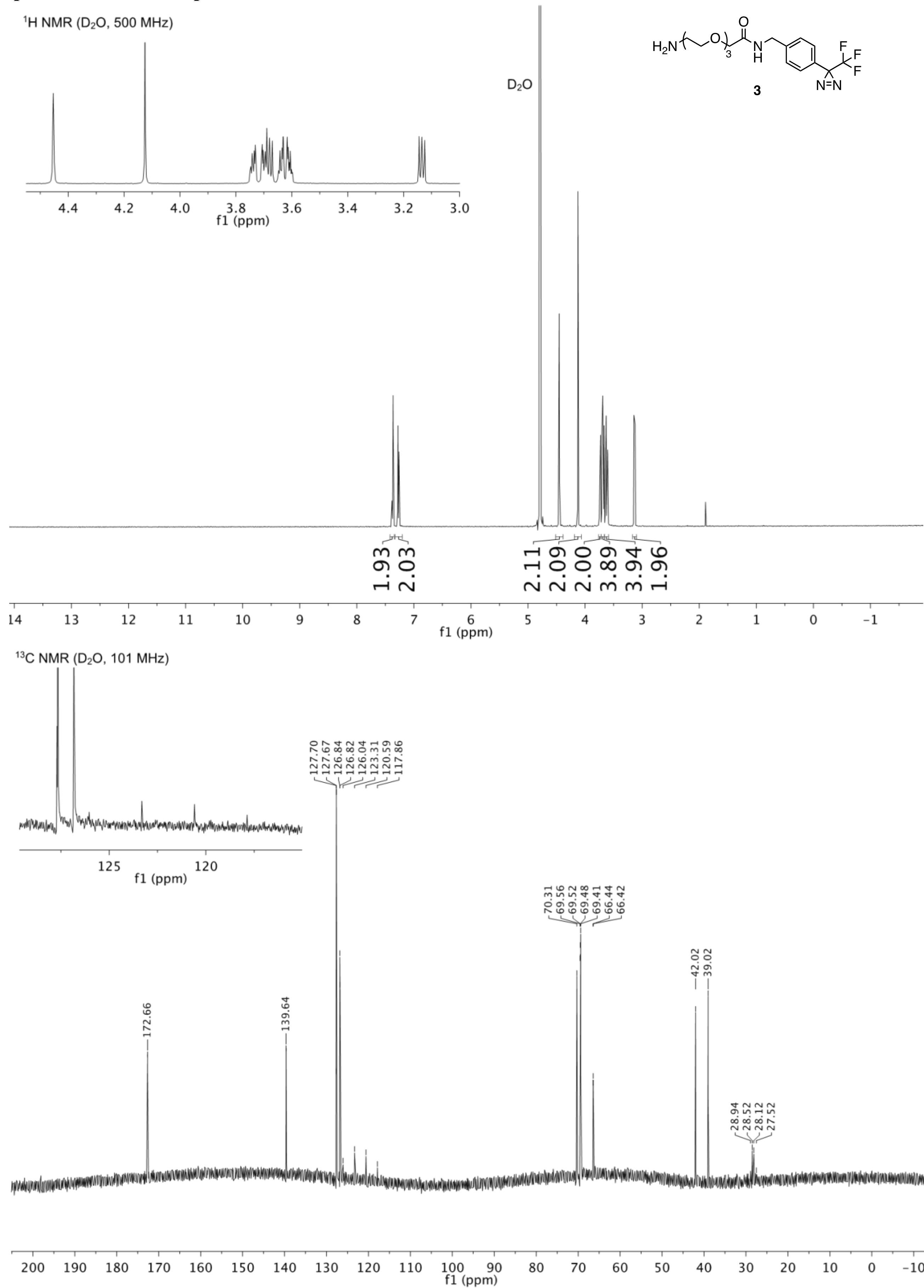
162.7 (C-4), 158.9 (C-27), 145.3 (C-30, C-41), 142.6 (C-35, C-36), 133.4, 133.2 (C6, C-8, C-12, C-16), 132.3 (C-7), 131.7 (C-11), 128.8 (C-33, C-38), 128.2 (C-32, C-39), 126.1 (C-31, C-40), 121.0 (C-34, C-37), 115.6 (C-5, C-9), 115.3 (C-13, C-15), 79.2 (C-2), 77.4 (C-1), 71.8, 71.7, 71.2, 71.1, 70.6, 70.5 (C-18, C-19, C-20, C-21, C-24 and C-25), 68.8 (C-17), 67.5 (C-28), 56.8 (C-3), 48.5 (C-29), 41.6 (C-26), 39.8 (C-22); HRMS *m/z* (ESI+) found 729.2770 [M+Na]+ ($C_{41}H_{42}N_2O_9Na$ requires 729.2788 [M+Na]+).

2-(2-Aminoethoxy)-*N*-(2-(2-(2-(4-(4-(prop-2-yn-1-yloxy)benzoyl)phenoxy)ethoxy)ethoxy)ethyl) acetamide, 4



Piperidine (600 μL) was added to a solution of **S5** (37 mg, 0.052 mmol, 1.0 eq) in DMF (1.4 mL). The reaction was stirred at RT for 30 min. The solvent was evaporated *in vacuo,* and the resulting crude material was purified by silica gel chromatography (H_2O/IPA/EtOAc 1:1:1) to give **4** (25 mg, 97%) as a colorless solid: ^{1}H NMR (CD_3OD; 500 MHz) δ_H 7.49–7.40 (4H, m, H-6, H-8, H-12, H-16), 6.85–6.73 (4H, m, H-13, H-15, H5 and H-9), 4.56 (2H, s, H-3), 4.03 (2H, s, H-24), 4.06–3.98 (2H, m, H-17), 3.78–3.70 (4H, m, H-25 and H-18), 3.63–3.50 (6H, m, H-19, H-20 and H-21), 3.37 (2H, t, *J* 3.4, H-22), 3.24–3.19 (2H, m, H-26), 2.90–2.86 (1H, m, H-1); ^{13}C NMR (101 MHz, D_2O) δ_C 195.6 (C-10), 171.9 (C-23), 162.0 (C-14), 160.6 (C-4), 132.3, 132.2 (C-6, C-8, C-12, C-16), 130.3, 129.7 (C-7, C-11), 114.3, 114.1 (C-5, C-9, C-13, C-15), 78.2, 77.3 (C-1, C-2), 69.8, 69.3, 68.8 (C-18, C-20, C-21, C-24), 67.3, 66.9 (C-25, C-17), 55.8 (C-3), 39.0 (C-26), 38.4 (C-22); HRMS *m/z* (ESI+) found 485.2270 [M+H]+, 507.2106 [M+Na]+ ($C_{26}H_{33}N_2O_7$ requires 485.2288 [M+H]+).

4.3 Representative NMR Spectra



^{1}H NMR (CD_3OD, 500 MHz)

FmocHN O N H O O 2 O O O

S5

H_2O

MeOD

2.01 4.16 2.00 2.08 2.02 2.07 2.03 2.05 2.02 3.14 2.05 2.09 2.06 2.05 3.97 2.07 1.59 0.99

f1 (ppm)

^{13}C NMR (CD_3OD, 101 MHz)

MeOD

196.55 172.44 163.92 162.58 158.92 145.31 142.63 133.39 133.18 132.31 131.66 128.80 128.17 126.12 120.98 115.60 115.25 79.20 77.41 71.76 71.23 70.63 70.49 56.77 48.49 41.64 39.80

f1 (ppm)

^{1}H NMR (D_2O, 500 MHz)

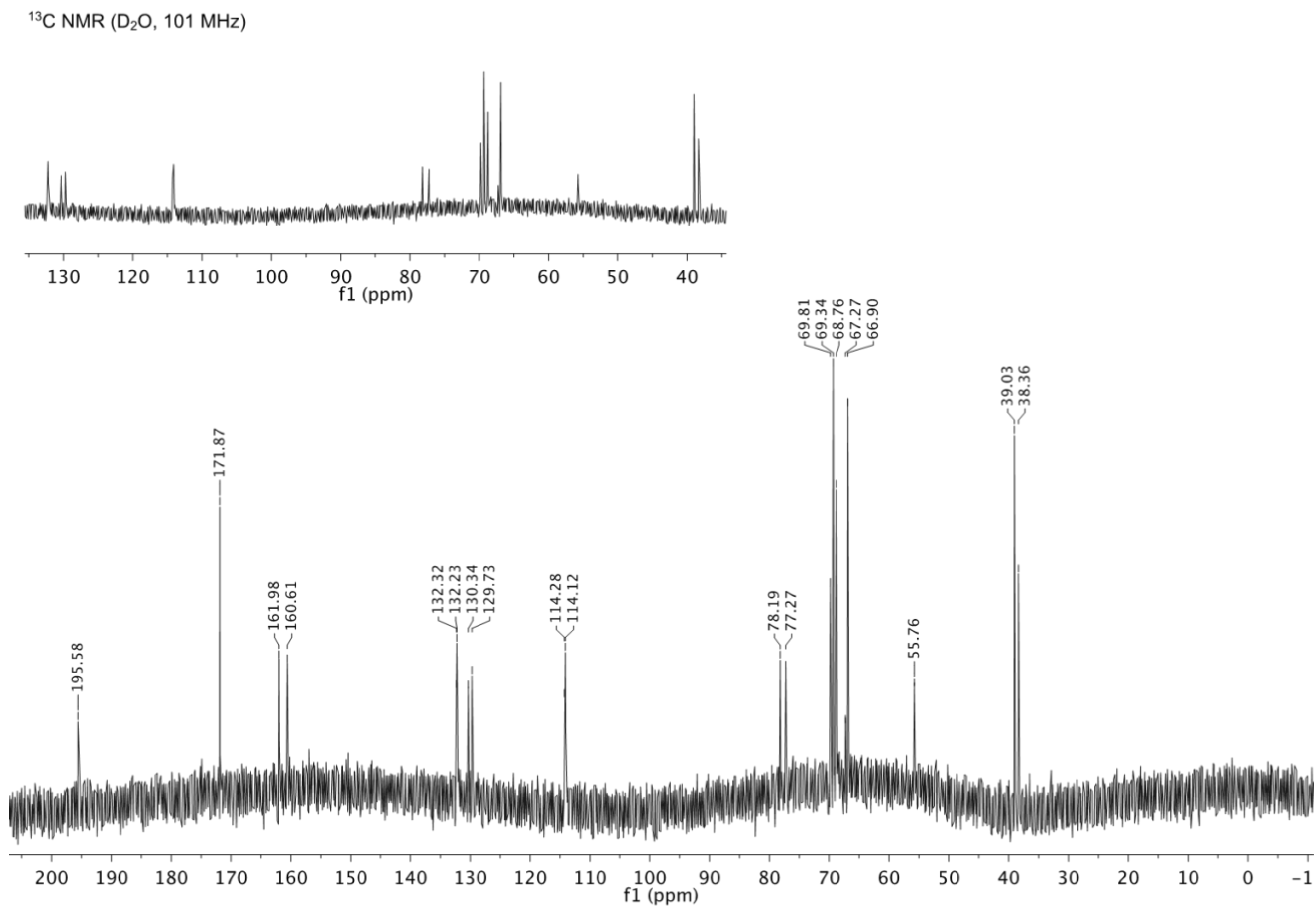
4.5 4.0 3.5 3.0
f1 (ppm)

D_2O

4

4.03 4.09 2.00 4.03 4.10 6.27 2.03 2.07 1.03

12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 −0.5 −1.0
f1 (ppm)



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