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Supporting Information

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Semisynthesis of a Glycosylphosphatidylinositol-Anchored Prion Protein

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General Information.

All chemicals used were reagent grade and used as supplied except where noted. All reactions were performed in oven-dried glassware under an inert atmosphere (nitrogen or argon) unless noted otherwise. Reagent grade dichloromethane (CH_2Cl_2), tetrahydrofuran (THF), diethyl ether (Et_2O) and toluene (PhMe) were passed through activated neutral alumina column prior to use. Reagent grade *N*,*N*-dimethylformamide (DMF) and methanol (MeOH) were dried over activated molecular sieves prior to use. Pyridine, triethylamine and acetonitrile were distilled over CaH_2 prior to use. Analytical thin layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} plates (0.25mm). Compounds were visualized by UV irradiation or dipping the plate in a cerium sulfate-ammonium molybdate (CAM) solution or phosphomolybdic acid (PMA) or sulforic acid ethanol solution, or spraying with Bial's reagent (orcinol in acidic ethanol). Flash column chromatography was carried out using forced flow of the indicated solvent on Fluka Kieselgel 60 (230-400 mesh).

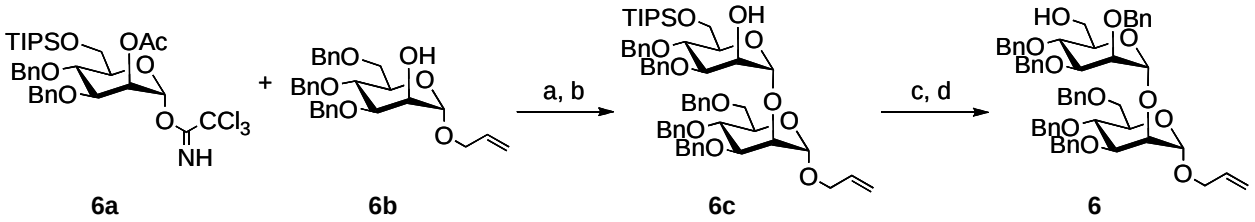
^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Varian Mercury 300 (300 MHz), Varian Gemini 300 (300 MHz), Bruker DRX400 (400 MHz), Bruker DRX500 (500 MHz), in $CDCl_3$ with chemical shifts referenced to internal standards $CDCl_3$ (7.26 ppm ^1H, 77.0 ppm ^{13}C) unless otherwise stated. ^{31}P spectra are reported in δ value relative to H_3PO_4 (0.0 ppm) as an external reference. Splitting patterns are indicated as s, singlet; d, doublet; t, triplet; q, quartet; brs, broad singlet for ^1H NMR data. Signals were assigned by means of DEPT, ^1H-^1H COSY and ^1H-^{13}C

HSQC spectra. High-resolution mass spectral (HRMS) analyses were performed by the MS-service at the Laboratorium für Organische Chemie (LOC) at ETH Zürich. High-resolution MALDI and ESI mass spectra were run on an IonSpec Ultra and a Bruker BioAPEXII instruments respectively. In case of MALDI-MS, 2,5-dihydroxybenzoic acid (DHB) or 3-hydroxypyridine 2-carboxylic acid (3-HPA) or 2-(4-hydroxyphenylazo) benzoic acid (HABA) served as the matrix. IR spectra were recorded on a Perkin-Elmer 1600 FTIR spectrometer. Optical rotations were measured using a Perkin-Elmer 241 polarimeter. Elemental analyses were performed by the Microanalytical Laboratory of the LOC, ETH Zürich.

2-(*N*-(*tert*-butoxycarbonyl)-*S*-(*tert*-butyl)-L-cysteinyl)amino ethanol (4a): To a solution of *N*-(*tert*-butoxycarbonyl)-*S*-(*tert*-butyl)-L-cysteine (2.0 g, 7.2 mmol) and *N*-hydroxyl succinimide (0.96 g, 8.7 mmol) in THF (20 mL) was added diisopropyl carbodiimide (DIPC) (1.35 mL, 7.8 mmol) at 0 °C. The reaction mixture was warmed up to room temperature within 1 h and stirred for an additional 24 h. The precipitate formed was filtered off through a pad of Celite to give the crude succinimide activated cysteine derivative. To this material in THF (20 mL) and DMF (4 mL) was added ethanolamine (2.17 mL, 36.0 mmol) at room temperature. Large amounts of precipitation formed within 1 hour. Filtration through a pad of Celite gave the crude product. Further purification over silica gel column gave the target compound **4a** (2.57 g, 90%) as colorless oil. R_f 0.54 (CH_2Cl_2/MeOH = 10 : 1); $[\alpha]_D^{r.t.}$ = -0.96 (*c* = 7.6, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.31 (s, 9H), 1.43 (s, 9H), 2.83 (dd, *J* = 12.6, 6.6 Hz, 1H), 2.96 (dd, *J* = 12.6, 6.6 Hz, 1H), 3.05 (t, *J* = 6.0 Hz, 1H), 3.41 (dd, *J* = 7.8, 5.1 Hz, 1H), 3.69 (dd, *J* = 7.8, 5.1 Hz, 1H), 4.24 (dd, *J* = 13.2, 6.9 Hz, 1H), 5.46 (d, *J* = 7.2 Hz, 1H), 6.83 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.5, 31.0, 42.4, 43.0, 54.6, 61.5, 80.5, 155.8, 171.7; Anal. Calcd for $C_{14}H_{29}N_2O_5S$: C, 52.47; H, 8.81. Found: C, 52.70; H, 8.63.

Triethylammonium 2-(*N*-(*tert*-butoxycarbonyl)-*S*-(*tert*-butyl)-L-cysteinyl)aminoethyl H-phosphonate (5): Ethanolamine derivative **4a** (400 mg, 1.25 mmol) and phosphonic acid (113 mg, 1.37 mmol) were combined and coevaporated with pyridine three times and dried under high vacuum for 2 h before placed under nitrogen and dissolved in pyridine (6.5 mL). To this mixture was added a solution of pivaloyl chloride (0.17 mL, 1.37 mmol) in pyridine (2.5 mL) at room temperature. The reaction completed within 1 h as indicated by TLC analysis. Excess pyridine

was removed *in vacuo* and the residue was directly subjected to silica gel column chromatography to give the target H-phosphonate **5** (447 mg, 75%) as white foam after lyophilization over dixoane. R_f 0.50 (CH_2Cl_2/MeOH = 4 : 1); ^{1}H NMR (300 MHz, $CDCl_3$:CD_3OD = 1:1) δ 1.17 (s, 9H), 1.29 (s, 9H), 1.32 (t, *J* = 7.5 Hz, 9H), 2.82 (dd, *J* = 12.6, 6.9 Hz, 1H), 2.89 (dd, *J* = 12.6, 6.9 Hz, 1H), 3.14 (q, *J* = 7.2 Hz, 6H), 3.41 (br, 2H), 3.87 (br, 2H), 4.23 (br, 1H), 6.25 (d, *J* = 7.8 Hz, 1H), 6.73 (d, *J* = 629.1 Hz, 1H) ^{13}C NMR (100 MHz, $CDCl_3$:CD_3OD = 1:1) δ 7.54, 26.0, 27.2, 29.7, 30.1, 39.5, 41.5, 45.7, 53.7, 61.1, 79.2, 155.2, 171.1; ^{31}P NMR (162 MHz, $CDCl_3$:CD_3OD = 1:1) δ 4.51; HRMS-MALDI (*m*/*z*): $[M]^-$ Calcd for $C_{14}H_{28}N_2O_6PS$, 383.1411; Found, 383.1413.



Reagents and conditions: (a) TMSOTf, DCM, 0 °C; (b) DCM/MeOH = 1:1, NaOMe cat. r.t.; (c) NaH, BnBr, DMF, 0 °C to r.t.; (d) TBAF, THF, r.t.

Allyl (3,4-di-*O*-benzyl-6-*O*-triisopropylsilyl-α-D-mannopyranosyl)-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (6c): Mannosyl trichloroacetimidate **6a** [1] (0.82 g, 1.17 mmol) and allyl mannoside **6b** [2] (0.48 g, 0.98 mmol) were co-evaporated with toluene (3 mL x 3) and dried under high vacuum for 2 h. The substrates were then dissolved in CH_2Cl_2 (10 mL), cooled to 0 °C and TMSOTf (21 μL, 0.12 mmol) was added. The reaction mixture was stirred for 30 min before quenched with Et_3N. The solvents were removed *in vacuo*. The residue was then dissolved in CH_2Cl_2 (5 mL) and MeOH (5 mL) at room temperature and added NaOMe (0.5 mL, 0.25 mmol, 0.5 M solution in MeOH) at room temperature. The reaction mixture was stirred for additional 12 h and the solvents were evaporated. The crude product was purified by silica gel column chromatography to give dimmanoside **6c** (899 mg, 91%) as a white foam. R_f 0.48 (Hexanes/EtOAc = 4 : 1); $[\alpha]_D^{r.t.}$ = +26.7 (*c* = 1.4, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.06-1.07 (m, 21H), 2.31 (d, *J* = 2.8 Hz, 1H), 3.69-3.81 (m, 4H), 3.84-3.97 (m, 4H), 4.10-4.16 (m, 2H), 4.52-4.73 (m, 4H), 4.84 (dd, *J* = 11.0, 3.5 Hz, 1H), 4.88 (d, *J* = 1.8 Hz, 1H), 5.12-5.24 (m, 2H), 5.25 (d, *J* = 1.8 Hz, 1H), 5.82-5.89 (m, 1H), 7.17-7.22 (m, 2H), 7.24-7.48 (m, 23H); ^{13}C

NMR (125 MHz, $CDCl_3$) δ 12.0, 18.1, 18.1, 58.5, 62.9, 67.8, 68.5, 69.3, 72.0, 72.1, 72.1, 72.7, 73.3, 73.3, 74.2, 74.8, 75.1, 75.2, 80.1, 80.3, 98.3, 100.2, 117.1, 127.4-128.5, 133.8, 138.1, 138.2, 138.4, 138.4; HRMS-MALDI (*m*/*z*): $[M+Na]^+$ Calcd for $C_{59}H_{76}O_{11}SiNa$, 1011.5049; Found: 1011.503.

Allyl (2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (6): To a solution of dimannoside **6c** (800 mg, 0.81 mmol) and BnBr (0.15 mL, 1.25 mmol) in DMF (5 mL) at 0 °C was added NaH (97 mg, 2.43 mmol, 60% in mineral oil). The reaction mixture was allowed to warm up to room temperature within 1 h and stirred for additional 12 h. MeOH was cautiously added at 0 °C to quench the reaction before water (10 mL) was added. The aqueous layer was then extracted four times with Et_2O (20 mL each) and the combined organic layers washed with additional water, brine, then dried over Na_2SO_4. Evaporation of the solvents under reduced pressure gave the crude residue. To a solution of the latter in THF (3 mL) at room temperature was added TBAF (5 mL, 5 mmol, 1M solution in THF). The reaction mixture was stirred for 12 h and diluted with saturated NH_4Cl aqueous solution. The aqueous layer was extracted with EtOAc and combined organic layers were dried over Na_2SO_4. Removal of the solvents *in vacuo* gave the crude product that was purified by silica gel column chromatography to give dimannoside **6** (620 mg, 83%) as a white foam. R_f 0.23 (Hexanes/EtOAc = 2 : 1); $[\alpha]_D^{r.t.}$ = +22.1 (*c* = 0.8, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.95 (t, *J* = 7.5 Hz, 1H), 3.67-3.81 (m, 8H), 3.88-3.97 (m, 4H), 4.01-4.03 (m, 1H), 4.11-4.16 (m 1H), 4.48-4.56 (m, 6H), 4.60-4.69 (m, 4H), 4.83 (d, *J* = 13.5 Hz, 1H), 4.87 (d, *J* = 1.8 Hz, 1H), 4.90 (d, *J* = 13.5 Hz, 1H), 5.16 (d, *J* = 1.8 Hz, 1H), 5.14-5.18 (m, 1H), 5.21-5.26 (m, 1H), 5.81-5.90 (m, 1H), 7.15-7.50 (m, 30H); ^{13}C NMR (125 MHz, $CDCl_3$) δ , 62.5, 67.8, 69.2, 72.0, 72.3, 72.4, 72.6, 72.7, 73.4, 74.2, 74.9, 75.0, 75.0, 75.1, 75.1, 79.7, 80.1, 98.0, 99.5, 117.4, 127.4-128.5, 133.7, 138.3, 138.3, 138.4, 138.5, 138.5, 138.6; HRMS-MALDI (*m*/*z*): $[M+Na]^+$ Calcd for $C_{57}H_{62}O_{11}$, 945.4184; Found: 945.4192.

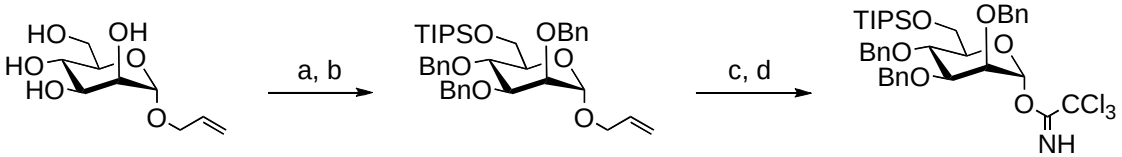
Allyl (2,3,4-tri-*O*-benzyl-6-*O*-(2-(*N*-(*tert*-butoxycarbonyl)-*S*-(*tert*-butyl)-L-cysteinyl)aminoethyl phosphonato)-α-D-mannopyranosyl)-(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (7): Dimannoside **6** (150 mg, 0.16 mmol) and H-phosphonate **5** (170 mg, 0.35 mmol) were coevaporated with pyridine three times and dried under high vacuum for 2 h

before placed under nitrogen and dissolved in pyridine (4.0 mL). To this solution was added pivaloyl chloride (0.86 mL, 0.70 mmol) at room temperature. Phosphonylation completed within 4 h, as indicated by TLC analysis. The reaction mixture was then cooled to 0 °C and added a solution of iodine (89 mg, 0.35 mmol) in pyridine and water (0.8 mL, v/v 10:1). Oxidation was completed within 1 h and the reaction was quenched by the addition of saturated $Na_2S_2O_3$ aqueous solution. The aqueous layer was extracted with chloroform three times and combined organic layers were dried over Na_2SO_4. Evaporation of the solvents under the reduced pressure led to the crude residue that was further purified by silica gel column chromatography. The phosphorylated dimannoside **7** (213 mg, 94%) was isolated as a white powder after lyophilization from dioxane. R_f 0.41 (CH_2Cl_2/MeOH = 10 : 1); $[\alpha]_D^{r.t.}$ = +23.5 (*c* = 1.2, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (s, 9H), 1.34 (t, *J* = 7.3 Hz, 9H), 1.42 (s, 9H), 3.30-3.39 (m, 1H), 3.45-3.53 (m, 1H), 3.65-3.83 (m, 8H), 3.97-4.02 (m, 7H), 4.05-4.35 (t, *J* = 14.4 Hz, 2H), 4.88 (d, *J* = 2.1 Hz, 1H), 5.14 (brs, 1H), 5.14-5.34 (m, 2H), 5.57 (d, *J* = 7.7 Hz, 1H), 5.82-5.92 (m, 1H), 7.15-7.35 (m, 30H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 8.59, 28.4, 30.9, 40.6, 42.5, 45.7, 54.2, 64.7, 64.7, 65.1, 68.0, 69.3, 71.8, 71.9, 72.3, 72.5, 72.8, 73.4, 74.3, 74.5, 74.9, 75.0, 75.1, 75.6, 79.7, 80.1, 98.2, 99.7, 117.3, 127.4-128.5, 133.8, 138.3-138.6, 138.6, 155.4, 170.7; ^{31}P NMR (161 MHz, $CDCl_3$) δ 0.79; HRMS-MALDI (*m*/*z*): $[M]^-$ Calcd for $C_{71}H_{88}N_2O_{17}PS$, 1303.556; Found: 1303.553.

***n*-Propyl (6-*O*-(2-(*N*-(*tert*-butoxycarbonyl)-*S*-(*tert*-butyl)-L-cysteinyl)aminoethyl phosphonato)-α-D-mannopyranosyl)-(1→2)-α-D-mannopyranoside (8):** Phosphorylated dimannoside **7** (154 mg, 0.11 mol) was dissolved in a mixture of MeOH (15 mL) and formic acid (0.6 mL) at room temperature and Pd/C (256 mg, 0.24 mmol, 10% Pd content) was added. Hydrogen was then bubbled through the solution for 15 min and the reaction mixture was stirred under H_2 atmosphere for an additional 5 h. Non-soluble Pd/C was removed by filtration through a pad of Celite and the solvents were removed under reduced pressure to give the crude residue. Purification over C18 reverse phase column chromatography (H_2O:MeOH=10:1 to 5:1 with 0.1% TFA) gave target compound **8** (95 mg, quant) after lyophilization. R_f 0.40 (BuOH:AcOH:H_2O = 4 :1:1); ^{1}H NMR (500 MHz, CD_3OD) δ 0.88 (t, *J* = 7.4 Hz, 3H), 1.25 (s, 9H), 1.39 (s, 9H), 1.50-1.57 (m, 2H), 2.71 (dd, *J* = 12.6, 7.8 Hz, 1H), 2.89 (dd, *J* = 12.6, 5.8 Hz, 1H), 3.35-3.46 (m, 4H), 3.52 (t, *J* = 8.5 Hz, 1H), 3.59-3.67 (m, 4H), 3.74-3.83 (m, 4H), 3.90 (br, 1H), 4.00-4.15 (m, 4H),

4.21-4.24 (m, 1H), 4.91 (br, 1H), 4.95 (br, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 9.91, 22.8, 27.5, 30.1, 30.6, 39.7, 42.1, 55.2, 61.9, 65.2, 66.9, 67.1, 67.9, 67.9, 69.2, 70.6, 71.0, 71.1, 72.4, 73.4, 79.8, 98.8, 103.1, 156.4, 172.6; ^{31}P NMR (121 MHz, CD_3OD) δ 0.67; HRMS-MALDI (*m*/*z*): $[M]^-$ Calcd for $C_{29}H_{54}N_2O_{17}PS$, 765.2886; Found: 765.2899.

***n*-Propyl (6-*O*-(2-(*S*-(2-hydroxylethylthio)-L-cysteinyl)aminoethyl phosphonato)-α-D-mannopyranosyl)-(1→2)-α-D-mannopyranoside (9)**: Phosphorylated dimannoside **8** (21.0 mg, 26.1 μmol) was dissolved in a mixture of TFA and anisole (3 mL, 10:1 v/v) at 0 °C. After 5 min, $Hg(OTFA)_2$ (12.2 mg, 28.7 μmol) was added and stirred for 20 min. The solvents were removed under high vacuum at 0 °C. The residue was dried for 1 h at room temperature before redissolved in a mixture of AcOH and H_2O (3 mL, 7:3 v/v) and added mercaptoethanol (0.1 mL). The reaction mixture was further stirred for 12 h, concentrated *in vacuo* and filtered through a pad of Celite with a mixture of AcOH and H_2O (1:1 v/v) as washing solvents. Removal of the solvents gave the crude residue that was further purified by Sephadex G-25 column using a mixture of H_2O and MeOH (v/v 10:1) as eluent. The proper fractions were collected and the solvents were evaporated. Further lyophilizaiton gave the target molecule **9** (19.1 mg, 93%) as a white foam. R_f 0.60 (BuOH: EtOH: H_2O: 25%NH_3 in H_2O = 2:2:2:1); ^{1}H NMR (600 MHz, D_2O) δ 0.92 (t, *J* = 7.8 Hz, 3H), 1.59-1.63 (m, 2H), 3.11-3.29 (m, 1H), 3.30-3.39 (m, 1H), 3.41-3.79 (m, 9H), 3.81-3.93 (m, 4H), 3.95-4.03 (m, 4H), 4.04-4.14 (m, 4H), 5.03 (brs, 1H), 5.05 (brs, 1H); ^{13}C NMR (150 MHz, D_2O) δ 12.7, 24.8, 40.9, 43.1, 63.6, 66.5, 66.7, 67.4, 69.1, 69.7, 72.4, 72.6, 72.9, 73.0, 74.7, 74.8, 75.4, 81.5, 101.0, 105.1, 171.4; ^{31}P NMR (242 MHz, D_2O) δ 1.34.



12a **12b** **12**

Reagents and conditions: (a) TIPSCl, Imidazole DMF, r.t.; (b) NaH, BnBr, DMF, 0 °C to r.t.; (c) $PdCl_2$, AcONa, AcOH/H_2O = 10:1; (d) CCl_3CN, DBU cat., DCM, r.t.

Allyl 2,3,4-tri-*O*-benzyl-6-*O*-triisopropylsilyl-α-D-mannopyranoside (12b): To a solution of allyl mannoside **12a** (1.51 g, 6.86 mmol) and imidazole (2.34 g, 34.4 mmol) in DMF (10 mL)

was added TIPSCl (1.62 mL, 7.55 mmol) at 0 °C. The mixture was stirred for 2 h at 0 °C, followed by an additional 2 h at room temperature. Aqueous work-up then gave the crude 6-*O*-silylated allyl mannoside. The crude material was dissolved in DMF (45 mL) and BnBr (3.67 mL, 30.9 mmol) and NaH (1.37 g, 34.3 mmol, 60% in mineral oil) were added at 0 °C. The mixture was then stirred overnight and gradually warmed up to room temperature. Excess sodium hydride was then quenched carefully by addition of methanol. Following standard aqueous work-up and silica gel column chromatography purification gave mannoside **12b** (3 g, 68%) as an oil. R_f 0.51 (Hexanes/EtOAc = 10 : 1); $[\alpha]_D^{r.t.}$ = +27.7 (*c* = 4.7, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.06-1.21 (m, 21H), 3.70-3.73 (m, 1H), 3.84 (dd, *J* = 2.7, 1.8 Hz, 1H), 3.92-4.03 (m, 5H), 4.17-4.24 (m, 1H), 4.66-4.81 (m, 5H), 4.91 (d, *J* = 1.8 Hz, 1H), 4.97 (d, *J* = 13.8 Hz, 1H), 5.15-5.29 (m, 2H), 5.82-5.95 (m, 1H), 7.26-7.42 (m, 15H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.1, 18.2, 18.2, 63.3, 67.5, 72.2, 72.6, 73.6, 75.0, 75.1, 75.3, 80.4, 96.6, 117.1, 127.5-128.3, 133.9, 138.4, 138.6; HRMS-MALDI (*m*/*z*): $[M+Na]^+$ Calcd for $C_{39}H_{54}O_6SiNa$, 669.3582; Found: 669.3576.

2,3,4-Tri-*O*-benzyl-6-*O*-triisopropylsilyl-α-D-mannopyranosyl trichloroacetimidate (12): Allyl mannoside **12b** (1.91 g, 2.94 mmol) was dissolved in AcOH (30 mL). Water (3 mL) was added, followed by NaOAc (2.0 g, 23.2 mmol) and $PdCl_2$ (1.0 g, 8.82 mmol), and the mixture was stirred for 12 h at room temperature. The reaction mixture was diluted with EtOAc and washed with water, saturated aqueous $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$, filtered, concentrated and chromatographed give an anomeric lactol. This lactol was dissolved in CH_2Cl_2 (10 mL), and tirchloroacetonitrile (3.8 mL) and DBU (38 μL) were added at 0 °C. The mixture was stirred for 1 h and concentrated. Silica gel column chromatography gave the target compound **12** (1.59 mg, 72% two steps). R_f 0.50 (Hexanes/EtOAc = 10 : 1); $[\alpha]_D^{r.t.}$ = +25.6 (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.05-1.09 (m, 21H), 3.80-3.85 (m, 2H), 3.91-4.05 (m, 3H), 4.20 (t, *J* = 9.6 Hz, 1H), 4.60-4.75 (m, 5H), 4.95 (d, *J* = 11.5 Hz, 1H), 6.33 (d, *J* = 1.8 Hz, 1H), 7.26-7.42 (m, 15H), 8.47 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.2, 18.2, 18.2, 62.7, 72.5, 72.6, 73.9, 74.0, 75.4, 75.8, 75.3, 79.0, 96.2, 127.5-128.5, 138.0, 138.2, 138.5, 160.3; HRMS-MALDI (*m*/*z*): $[M+Na]^+$ Calcd for $C_{38}H_{50}Cl_3NO_6SiNa$, 774.2365; Found: 774.2360.

Allyl (2,3,4-tri-*O*-benzyl-6-*O*-triisopropylsilyl-α-D-mannopyranosyl)-(1→2)-(2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→6)-(2-*O*-benzoyl-3,4-di-*O*-benzyl-α-D-mannopyranoside

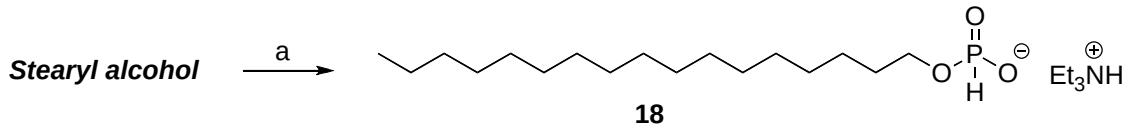
(13): Ally dimannoside **11** [3] (0.98 g, 1.09 mmol) and mannosyl trichloroacetimidate **12** (0.94 g, 1.26 mmol) were coevaporated three times with toluene and dried under high vacuum for 2 h. To the mixture was added freshly activated 4Å MS (1 g), dissolved in Et_2O (10 mL), and stirred for 30 min at room temperature. The solution was cooled to –10 °C, followed by addition of TMSOTf (19 μL, 0.11 mmol), and stirred for 30 min. The reaction was quenched by addition of Et_3N (0.1 mL) and the reaction mixture was concentrated. The crude product was purified by silica gel column chromatography to afford the target trimannoside **13** (1.52 g, 95%) as a white foam. R_f 0.50 (Hexanes/EtOAc = 4 : 1); $[\alpha]_D^{r.t.}$ = +15.5 (*c* = 1.7, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.03 (m, 21H), 3.52 (d, *J* = 10.2 Hz, 1H), 3.61-3.73 (m, 3H), 3.76-4.02 (m, 11H), 4.10-4.16 (m, 2H), 4.20 (m, 1H), 4.39-4.64 (m, 11H), 4.77-4.81 (m, 2H), 4.88-4.91 (m, 2H), 5.95 (d, *J* = 1.8 Hz, 1H), 5.15-5.28 (m, 2H), 5.28 (d, *J* = 1.8 Hz, 1H), 5.62 (dd, *J* = 3.2, 1.8 Hz, 1H), 5.83-5.89 (m, 1H), 7.11-7.44 (m, 43H), 8.05 (d, *J* = 7.1 Hz, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.1, 18.0, 18.1, 63.2, 66.4, 68.1, 69.1, 69.1, 70.8, 71.6, 71.9, 72.1, 72.2, 72.7, 73.2, 73.4, 73.9, 74.4, 74.7, 74.8, 75.0, 75.0, 75.1, 78.7, 79.8, 80.4, 96.7, 98.6, 99.1, 118.1, 127.3-128.8, 129.8, 129.9, 133.1, 133.3, 138.0-138.9, 165.7; HRMS-MALDI (*m*/*z*): $[M+Na]^+$ Calcd for $C_{93}H_{108}O_{17}SiNa$, 1547.7248; Found: 1547.727.

(2,3,4-Tri-*O*-benzyl-6-*O*-triisopropylsilyl-α-D-mannopyranosyl)-(1→2)-(2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→6)-2-*O*-benzoyl-3,4-di-*O*-benzyl-α-D-mannopyranosyl trichloroacetimidate (14): Trimannoside **13** (600 mg, 0.39 mmol) was dissolved in AcOH (10 mL). Water (0.5 mL) was added, followed by NaOAc (600 mg, 7.31 mmol) and $PdCl_2$ (348 mg, 1.97 mmol), and the mixture was stirred for 6 h at room temperature. The reaction mixture was diluted with EtOAc and washed with water, saturated aqueous $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$, filtered, concentrated and separated by column chromatography to give the anomeric hemiacetal. This hemiacetal was dissolved in CH_2Cl_2 (3 mL), before trichloroacetonitrile (1.5 mL) and DBU (15 μL) were added at 0 °C. The mixture was stirred for 2 h and concentrated. Silica gel column chromatography gave trimannosyl trichloroacetimidate **14** (640 mg, 68% from **13**). R_f 0.58 (Hexanes/EtOAc = 4 : 1); $[\alpha]_D^{r.t.}$ = +16.2 (*c* = 2.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.03-1.04 (m, 21H), 3.54 (dd, *J* = 11.2, 1.5 Hz, 1H), 3.64-3.82 (m, 6H), 3.84-4.04 (m, 7H), 4.13 (dd, *J* = 9.2, 3.2 Hz, 1H), 4.16 (t, *J* = 2.0 Hz, 1H), 4.39-4.65 (m, 12H), 4.78 (d, *J* = 9.8 Hz, 1H), 4.80 (d, *J* = 11.0 Hz, 1H), 4.86 (d, *J* = 1.8 Hz, 1H), 4.89 (d, *J* =

11.0 Hz, 1H), 4.90 (d, *J* = 11.0 Hz, 1H), 5.28 (d, *J* = 1.7 Hz, 1H), 5.73 (dd, *J* = 3.2, 2.1 Hz, 1H), 6.34 (d, *J* = 1.9 Hz, 1H), 7.11-7.31 (m, 40H), 7.38-74.7 (m, 3H), 8.07-8.09 (m, 2H), 8.70 (s, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.1, 18.3, 18.7, 63.2, 66.1, 67.8, 69.2, 71.9, 71.9, 72.1, 72.2, 72.2, 72.5, 73.2, 73.6, 73.7, 73.9, 74.7, 74.7, 75.0, 75.0, 75.1, 75.2, 78.0, 79.8, 80.5, 90.8, 95.1, 98.6, 99.0, 127.3-128.6, 129.6, 129.9, 133.4, 137.4, 138.0-139.0, 159.6, 165.4; HRMS-MALDI (*m*/z): $[M+Na]^+$ Calcd for $C_{92}H_{104}Cl_3NO_{17}SiNa$, 1650.6031; Found: 1650.606.

(2,3,4-Tri-*O*-benzyl-6-*O*-triisopropylsilyl-α-D-mannopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→6)-(3,4-di-*O*-benzyl-α-D-mannopyranosyl)-(1→4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→6)-1-*O*-allyl-2,3,4,5-tetra-*O*-benzyl-D-*myo*-inositol (16): Pseudodisaccharide **15** [3] (105 mg, 0.11 mmol) and trimannosyl trichloroacetimidate **14** (165 mg, 0.10 mmol) were azeotropically dried with toluene (3 × 3 mL), dried *in vacuo* and dissolved in CH_2Cl_2 (2 mL). This solution was cooled to 0 °C, followed by addition of TMSOTf (1.8 μL, 21 μmol), and stirred for 1 h. The reaction was quenched by addition of Et_3N (0.1 mL) and the reaction mixture was concentrated and dried under high vacuum. The crude product was then dissolved in CH_2Cl_2 (1. mL), and added freshly prepared NaOMe solution (3 mL, 0.35 M in MeOH). The mixture was heated at 50 °C for 12 h and the solvents were removed *in vacuo*. The residue was purified by silica gel column chromatography to give pseudopentasaccharide **16** (200 mg, 86% 2 steps) as a white foam. R_f 0.40 (Hexanes/EtOAc = 3 : 1); $[\alpha]_D^{r.t.}$ = +41.7 (*c* = 3.1, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.04-1.05 (m, 21H), 2.03 (d, *J* = 3.2 Hz, 1H), 3.18-3.22 (m, 2H), 3.27-3.28 (m, 1H), 3.33-3.52 (m, 8H), 3.56-3.89 (m, 14H), 3.96-4.15 (m, 8H), 4.26-4.35 (m, 4H), 4.37-4.46 (m, 7H), 4.51-4.55 (m, 4H), 4.58-4.73 (m, 7H), 4.79 (d, *J* = 11.0 Hz, 1H), 4.83 (s, 2H), 4.88-4.92 (m, 4H), 4.96 (d, *J* = 11.0 Hz, 1H), 5.16-5.19 (m, 1H), 5.22 (d, *J* = 1.6 Hz, 1H), 5.25 (d, *J* = 1.6 Hz, 1H) 5.25-5.29 (m, 1H), 5.74 (d, *J* = 3.7 Hz, 1H), 5.89-5.95 (m, 1H), 7.05-7.42 (m, 70H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.1, 18.1, 18.1, 62.8, 63.4, 65.9, 68.6, 69.1, 69.6, 70.8, 71.2, 71.6, 71.8, 71.9, 72.0, 72.1, 72.1, 72.8, 72.8, 73.2, 73.2, 73.7, 74.0, 74.1, 74.4, 74.6, 74.7, 75.0, 75.0, 75.2, 75.4, 75.8, 76.8, 79.7, 80.0, 80.2, 80.3, 80.9, 81.5, 81.9, 81.9, 97.4, 98.2, 99.3, 101.6, 117.1, 127.5-128.5, 134.2, 137.6-139.2; HRMS-MALDI (*m*/*z*): $[M+Na]^+$ Calcd for $C_{140}H_{159}N_3O_{25}SiNa$, 2333.0924; Found: 2333.086.

(2,3,4-Tri-*O*-benzyl-6-*O*-triisopropylsilyl-α-D-mannopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→6)-(2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→6)-2,3,4,5-tetra-*O*-benzyl-D-*myo*-inositol (17): To a solution of pseudopentasaccharide **16** (200 mg, 86.5 μmol) in DMF (10 mL) at 0 °C was added BnBr (13 μL, 109 μmol) and NaH (4.3 mg, 108 μmol, 60% in mineral oil). The reaction mixture was stirred over 12 h and allowed to warm up to room temperature gradually. Excess NaH was then quenched with MeOH and the product was extracted with Et_2O. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated. The crude material thus obtained was dissolved in AcOH (5 mL). Water (250 μL) was added, followed by NaOAc (137 mg, 1.67 mmol) and $PdCl_2$ (77 mg, 0.43 mmol), and the mixture was stirred for 6 h at room temperature. The reaction mixture was diluted with EtOAc and washed with water, saturated aqueous $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$, filtered, concentrated and purified silica column chromatography to give compound **17** (155 mg, 76% over 2 steps). R_f (Hexanes/EtOAc = 3 : 1); $[\alpha]_D^{r.t.}$ = +38.0 (*c* = 4.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.03-1.08 (m, 21H), 2.98 (d, *J* = 7.4 Hz, 1H), 3.33-3.41 (m, 5H), 3.46-3.48 (m, 2H), 3.60-3.67 (m, 4H), 3.70-3.73 (m, 2H), 3.77-3.92 (m, 18H), 3.96-4.18 (m, 4H), 4.26-4.35 (m, 4H), 4.37-4.46 (m, 7H), 4.51-4.55 (m, 4H), 4.58-4.73 (m, 7H), 4.79 (d, *J* = 11.0 Hz, 1H), 4.83 (s, 2H), 4.88-4.92 (m, 4H), 4.96 (d, *J* = 11.0 Hz, 1H), 5.16-5.19 (m, 1H), 5.22 (d, *J* = 1.6 Hz, 1H), 5.25 (d, *J* = 1.6 Hz, 1H) 5.25-5.29 (m, 1H), 5.74 (d, *J* = 3.7 Hz, 1H), 5.89-5.95 (m, 1H), 7.05-7.42 (m, 70H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.1, 18.1, 18.1, 62.9, 64.1, 66.4, 68.8, 69.1, 70.5, 71.6, 71.9, 71.9, 72.0, 72.0, 72.1, 72.1, 72.3, 72.7, 73.1, 73.2, 73.3, 73.8, 74.2, 74.5, 74.6, 74.9, 75.0, 75.0, 75.0, 75.3, 75.8, 76.0, 76.8, 77.0, 77.2, 77.4, 79.7, 79.8, 80.3, 80.6, 81.0, 81.4, 81.8, 97.6, 98.5, 99.2, 100.1, 127.0-128.5, 137.7-139.1; HRMS-MALDI (*m*/*z*): $[M+Na]^+$ Calcd for $C_{144}H_{161}N_3O_{25}SiNa$, 2383.1086; Found: 2383.103



18

Reagents and conditions: (a) H_3PO_3, PivCl, Pyridine, then cation exchange.

Triethylammonium octadecyl H-phosphonate (18): Stearyl alcohol (400 mg, 1.25 mmol) and phosphonic acid (113 mg, 1.37 mmol) were combined and coevaporated with pyridine for three times and dried under high vacuum for 2 h before placed under nitrogen and dissolved in pyridine (6.5 mL). To this mixture was added a solution of pivaloyl chloride (170 μL, 1.37 mmol) in pyridine (2.5 mL) at room temperature. The reaction was completed within 1 h as indicated by TLC analysis. Excess pyridine was removed *in vacuo* and the residue was directly subjected to silica gel column chromatography (CH_2Cl_2/MeOH = 5 : 1, silica impregnated with 1.5 eq of triethylamine) to give the target H-phosphonate **18** (447 mg, 75%) as a white foam after lyophilization over dixoane. R_f 0.20 (CH_2Cl_2/MeOH = 4 : 1); ^{1}H NMR (300 MHz, $CDCl_3$:CD_3OD = 1:1) δ 0.85 (t, *J* = 6.7 Hz, 3H), 1.24 (bs, 30 H), 1.30 (t, *J* = 7.3 Hz, 9H), 1.62 (m, 2H), 3.11 (q, *J* = 7.3 Hz, 6H), 3.85 (m, 2H), 6.79 (d, *J* = 663 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, CD_3OD = 1:1) δ 9.6, 15.0, 23.9, 27.1, 28.2, 30.6, 30.7, 30.9, 31.0, 32.0, 33.2, 47.6, 65.6; ^{31}P NMR (162 MHz, CDCl3) δ 8.36.

Triethylammonium (2,3,4-Tri-*O*-benzyl-6-*O*-triisopropylsilyl-α-D-mannopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→6)-(2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→6)-2,3,4,5-tetra-*O*-benzyl-1-*O*-octadecyl-phosphonato-D-*myo*-inositol (19): Triethylammonium octadecyl H-phosphonate 18 (48.0 mg, 0.11 mmol) and pseudopentasaccharide **17** (65 mg, 0.028 mmol) were coevaporated three times with pyridine and dried under high vacuum for 1 h. They were then dissolved in pyridine (4 mL) and pivaloyl chloride (23 μL, 0.19 mmol) was added at room temperature. The mixture was stirred for 12 h, when phopshonylation was complete as indicated by TLC analysis. The reaction mixture was then cooled to 0 °C, and a solution of iodine (300 μL, 0.36 M) in pyridine and water (2.2 mL, v/v 10:1). The reaction was stirred at room temperature for 3 h and then quenched by the addition of a saturated aqueous solution of $Na_2S_2O_3$. The aqueous layer was extracted three times with chloroform and the combined organic layers were dried over Na_2SO_4. Evaporation of the solvents under reduced pressure led to the crude residue that was further purified by gel permeation size exclusion chromatography. R_f 0.42 (CH_2Cl_2/MeOH = 10 : 1); $[\alpha]_D^{r.t.}$ = +37.2 (*c* = 2.1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.89 (t, *J* = 6.9 Hz, 3H) 1.06 (bs, 21H), 1.24-1.313 (m, 39 H), 1.613 (m, 2H), 2.22 (t, *J* = 7.8 Hz, 2H), 2.97 (q, *J* = 7.2 Hz), 3.13 (dd, *J* = 3.6 Hz, *J* = 10.2 Hz, 1H), 3.31-5.06 (m, 56 H), 5.22 (s, 2H), 5.35 (m, 1H), 5.98 (d, J =

3.9 Hz, 1H), 7.04-7.40 (m, 75 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 8.5, 12.1, 14.1, 18.1, 22.7, 25.5, 25.9, 27.2, 29.1, 29.3, 29.5, 29.7, 31.1, 31.2, 31.9, 35.9, 45.5, 62.9, 63.2, 65.9, 66.5, 69.1, 69.4, 70.6, 71.6, 71.9, 72.0, 72.1, 72.3, 72.8, 73.0, 73.2, 73.7, 73.8, 74.4, 74.7, 75.0, 75.7, 76.0, 76.3, 76.7, 77.1, 77.3, 77.5, 78.2, 79.7, 79.9, 80.1, 81.1, 81.8, 82.0, 96.4, 98.5, 99.3, 100.9, 126.9-128.9, 129.8, 130.0, 138.0, 138.5-139.2, 139.9; ^{31}P NMR (161 MHz, $CDCl_3$) δ –0.45; HRMS-MALDI (*m*/*z*): $[M+Na]^+$ Calcd for $C_{162}H_{198}N_3O_{28}PSiNa$, 2715.356; Found: 2715.35.

Triethylammonium (2,3,4-Tri-*O*-benzyl-α-D-mannopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→6)-(2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→6)-2,3,4,5-tetra-*O*-benzyl-1-*O*-octadecyl-phosphonato-D-*myo*-inositol (20): Phosphorylated pseudopentasaccharide **19** (64 mg, 0.023 mmol) was dissolved in a mixture of methanol and dichloromethane. The temperature was brought to 0 °C and acetyl chloride (80 μL, 1.12 mmol) was added. The mixture was stirred at room temperature for 5 h, then cooled to 0 °C and additional acetyl chloride (48 μL, 0.67 mmol) was added. After 2 h, the reaction was quenched by the addition of excess triethylamine. The mixture was then concentrated and directly purified by gel permeation size exclusion chromatography, to furnish the target compound **20** (53 mg, 88%) as R_f 0.34 (CH_2Cl_2/MeOH = 10 : 1); $[\alpha]_D^{r.t.}$ = +40.5 (*c* = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.86 (m, 3H), 1.20-1.26(m, 39 H), 1.61 (m, 2H), 2.05 (s, 1H), 2.88 (d, *J* = 6.3 Hz, 9H), 3.13 (dd, *J* = 3.6 Hz, *J* = 10.2 Hz, 1H), 3.37-5.05 (m, 56 H), 5.23 (s, 1H), 5.96 (d, J = 3.9 Hz, 1H), 7.02-7.43 (m, 75 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 8.5, 14.2, 22.7, 25.9, 29.4, 29.6, 29.8, 31.0, 31.1, 32.0, 45.4, 62.3, 63.1, 66.2, 68.9, 69.1, 69.6, 71.8, 72.0, 72.1, 72.2, 72.3, 72.4, 72.9, 73.1, 73.3, 73.8, 74.3, 74.4, 74.7, 75.0, 75.2, 75.7, 75.9, 76.4, 76.7, 77.1, 77.3, 77.5, 79.4, 79.9, 81.0, 81.8, 96.5, 99.5, 99.7, 100.8, 127.0-128.6, 138.0, 138.1, 138.5, 138.5, 138.7, 138.8, 139.0, 139.8; ^{31}P NMR (161 MHz, $CDCl_3$) δ –0.83; HRMS-MALDI (*m*/*z*): $[M+Na]^+$ Calcd for $C_{153}H_{178}N_3O_{28}PNa$, 2559.223; Found: 2559.22.

Bis-Triethylammonium (2,3,4-Tri-*O*-benzyl-6-*O*-(2-(*N*-(*tert*-butoxycarbonyl)-*S*-(*tert*-butyl)-L-cysteinyl)aminoethyl phosphonato)-α-D-mannopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→6)-(2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→4)-(2-azido-3,6-di-

***O*-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→6)-2,3,4,5-tetra-*O*-benzyl-1-*O*-octadecyl-phosphonato-D-*myo*-inositol (21):** Compound **20** (65 mg, 0.024 mmol) and H-phosphonate **5** (60 mg, 0.12 mmol) were coevaporated three times with pyridine and dried under high vacuum for 2 h before placed under nitrogen and dissolved in pyridine (2.0 mL). To this solution was added pivaloyl chloride (30 μL, 0.24 mmol) at room temperature. The reaction was judged to be complete after 1 h, as indicated by TLC analysis. The reaction mixture was then cooled to 0 °C, and a solution of iodine (420 μL, 0.29 M) in pyridine and water (1.1 mL, v/v 10:1). The reaction was stirred at room temperature for 2 h and then quenched by the addition of a saturated aqueous solution of $Na_2S_2O_3$. The aqueous layer was extracted with $CHCl_3$ for three times and combined organic layers were dried over Na_2SO_4. Evaporation of the solvents under the reduced pressure led to the crude residue that was further purified by size exclusion gel permeation chromatography. The target bis-phosphorylated fully protected pseudopentasaccharide **21** (70 mg, quant) was isolated as a white powder after lyophilization from dioxane. R_f 0.34 (CH_2Cl_2/MeOH = 10 : 1); $[\alpha]_D^{r.t.}$ = +34.3 (*c* = 1.2, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.86 (m, 3H), 1.10-1.5(m, 57 H), 1.61 (m, 2H), 2.62-2.90 (m, 10H), 3.13-5.10 (m, 58 H), 5.24 (s, 1H), 5.53 (d, J = 7.1Hz, 1H), 6.00 (d, J = 3.7 Hz, 1H), 7.03-7.43 (m, 75 H), 8.00 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) d 8.5, 14.2, 22.7, 25.9, 28.4, 29.4, 29.6, 29.7, 29.7, 31.0, 31.1, 31.2, 31.8, 32.0, 41.3, 42.2, 45.3, 54.2, 63.2, 63.9, 64.1, 65.8, 65.9, 69.0, 70.1, 70.6, 71.8, 71.9, 72.1, 72.3, 72.8, 73.0, 73.3, 73.8, 74.0, 74.3, 74.5, 74.6, 74.7, 74.9, 75.7, 76.16, 76.7, 79.4, 80.0, 81.1, 81.8, 82.0, 96.2, 99.2, 99.6, 100.7, 127.0-128.6, 138.1-139.0, 155.2, 170.4; ^{31}P NMR (161 MHz, $CDCl_3$) δ –0.51, 2.17; HRMS-MALDI (*m*/*z*): $[M+Na]^+$ Calcd for $C_{167}H_{205}N_5O_{34}P_2SNa$, 2941.355; Found: 2941.363.

(6-*O*-(2-(*N*-(*S*-*S*-mercaptoethanol-L-cysteinyl)aminoethyl phosphonato)-α-D-mannopyranosyl)-(1→2)-(α-D-mannopyranosyl)-(1→6)-(α-D-mannopyranosyl)-(1→4)-(2-azido-2-deoxy-α-D-glucopyranosyl)-(1→6)-1-*O*-octadecyl-phosphonato-D-*myo*-inositol (22): Phosphorylated pseudopentasaccharide **21** (59.0 mg, 0.019 mmol) was dissolved in mixture of THF/MeOH/H_2O (2:1:1, 5 mL) with formic acid (4% v/v, 200 μL) at room temperature and Pd/C (256 mg, 0.24 mmol, 10% Pd content) was added. Hydrogen was then bubbled through the solution for 15 min and the reaction mixture was stirred under an atmosphere of hydrogen for additional 5 h. Non-soluble Pd/C was removed by filtration through a pad of Celite and the

solvents were removed under reduced pressure to give 30 mg of crude product. The identity of the intermediate compound was established by HRMS. The crude material was then dissolved at 0 °C in a mixture of trifluoroacetic acid and anisole (11 ml, 10:1 v/v) and stirred for 5 min, when $Hg(TFA)_2$ (9.1 mg, 21 μmol) was added and the mixture was stirred for an additional 30 min. All volatile compounds were then removed under high vacuum at 0 °C. The resulting solid residue was dissolved in an AcOH/water mixture (15 mL, 7:3 v/v) and mercaptoethanol (400 μL, 5.7 mmol) was added at room temperature. The mixture was stirred at the same temperature for 10 h. After filtration of the solid residues over a pad of Celite, the solution was dried on a rotary evaporator, to give a pale yellow residue. The amphiphilic nature of GPI **22** hampered the further purification process and the efforts to characterize it by NMR. After extensive trials, it was found that the quality of the crude **22** was sufficient for the subsequent ligation event. And the resulting GPI-prion **23** conjugate could be more easily purified by HPLC to avoid the loss of **22** due to the repeated purification steps (see below).

Expression and purification of recombinant PrP

Expression of recombinant murine PrP(90-232) thioester was carried out as described before.[4]

Native chemical ligation

Native chemical ligations of rPrP with compounds **9** and **22** were carried out in ligation buffer (6 M GdnHCl, 300 mM NaP_i, 1% (v/v) thiophenol at pH 7.8) using concentrations of 5-6 mM of all components and 1.5 equivalents of **9** or **22**, respectively. Reactions were quenched by addition of 3 volume equivalents of ligation buffer and 20% (v/v) of β-mercaptoethanol. The ligation mixtures were purified by RP-HPLC on a C4-column from Vydac (Hesperia, CA) using linear gradients of buffer A (water, 0.1% trifluoroacetic acid) and buffer B (acetonitrile, 0.08% trifluoroacetic acid). Protein masses were determined by electrospray ionization mass spectrometry on an LCQ Advantage Max (Finnigan) operating in positive ion mode. The molecular masses were reconstructed from the charged ion spectra.

Folding of GPI anchored rPrP 23

The protein was incubated in a buffer of 6 M GdnHCl, 50 mM Tris-HCl at pH 7.5 containing 5mM GSH and 0.5 mM GSSG for 1 h. Complete folding was achieved by rapid 10fold dilution into 20 mM NaOAc at pH 5.5 (folding buffer) and subsequent dialysis against the same buffer.

Preparation of liposomes

DOPC lipids were solved in dichloromethane and a thin lipid film was formed by evaporation of the solvent under rotation in a Helium stream. The lipid film was hydrated in folding buffer for 1h. Formation of small unilamellar vesicles (SUVs) was achieved by sonication and subsequent centrifugation at 100,000g for 30 min at 22°C. The size of SUVs was checked by dynamic light scattering on a DynaPro system (Wyatt Technology).

Reconstitution of lipidated samples

Folded GPI anchored rPrP **23** was diluted (10 fold) into a 10 mg/ml SUV-solution in 20 mM NaOAc at pH 5.5.

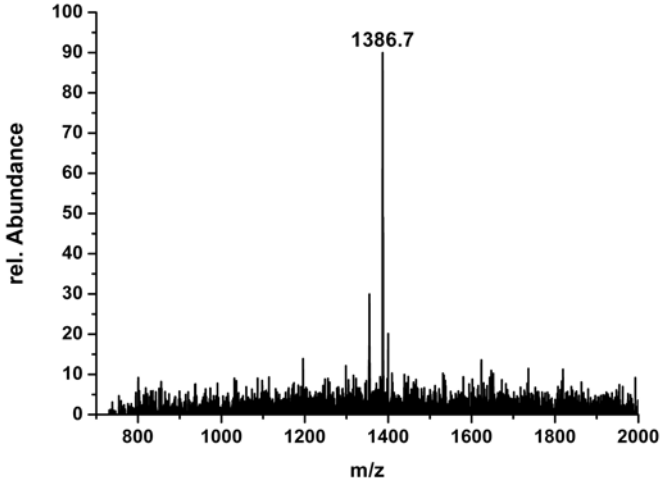
Pull down assay

GPI-anchored rPrP **23** and non-modified rPrP, as a control, were independently reconstituted in DOPC SUVs and centrifuged at low speed (5000 g) for 5 min to remove aggregates. The supernatant was subsequently centrifuged for 3 h at 200,000g to collect the SUVs. Pellets of both centrifugation steps and supernatant of the high speed centrifugation were analyzed by Western blotting with a PrP-specific antibody (A7).

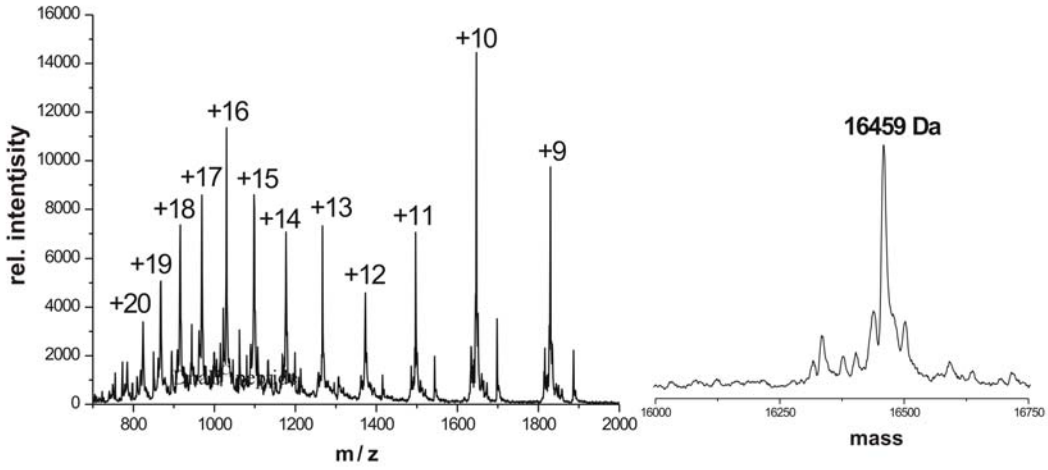
CD spectroscopy

Far-UV CD spectra were recorded on a Jasco J-715 spectropolarimeter. Protein concentrations were 0.2 mg/ml in 20 mM NaOAc at pH 5.5. All spectra were recorded in a 0.1 cm cuvette between 200-250nm.

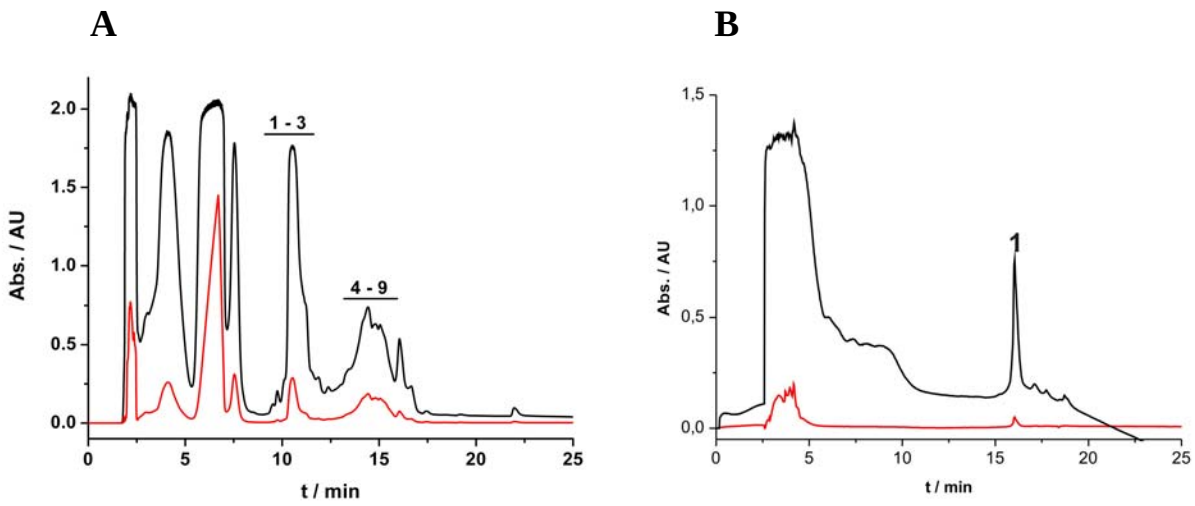
Supporting Figures



Supporting Figure S1: ESI-MS analysis of GPI anchor **22** as recovered from RP-HPLC purification after NCL with rPrP (calculated mass of **22** without β-mercaptoethanol protecting group: 1386 Da).



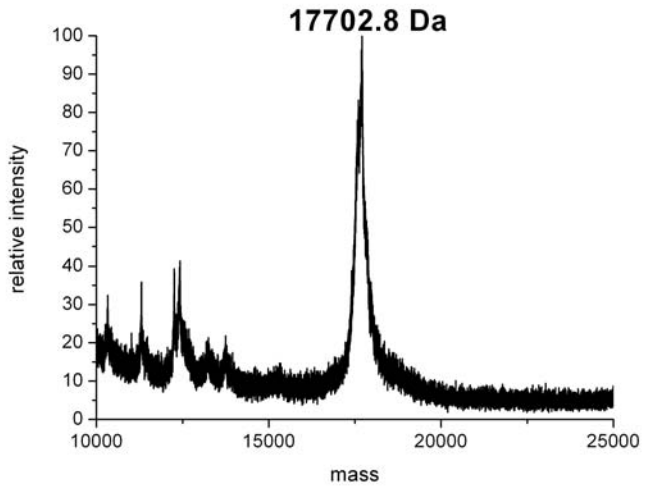
Supporting Figure S2: Left - ESI-MS spectrum of recombinant PrP (rPrP) with a C-terminal MesNa thioester. Right – Deconvoluted spectrum showing the molecular weight of rPrP (calculated mass 16459 Da).



Supporting Figure S3: A) RP-HPLC chromatogram of a purification run (10 mg of rPrP) of the ligation mixture of rPrP with GPI anchor **22** after 18 h at room temperature on a C4 column from Vydac (Hesperia, CA) using a linear gradient from 20 to 80 % buffer B in buffer A. The detector limit was exceeded for peaks eluting before 10 min retention time (salt and ligation mediator, e.g. thiophenol). All fractions were analyzed by ESI-MS in order to identify collected compounds and to assess puritiy.

Fractions 1-3 contained unreacted rPrP Cα-thioester and fractions 4-9 contained rPrP-GPI **23**. The broad peak corresponding to **23** is most likely caused by hydrophobic effects of the long alkyl chain. The peak eluting after fractions 4-9 contains the GPI anchor **22** without its β-mercaptoethanol protecting group.

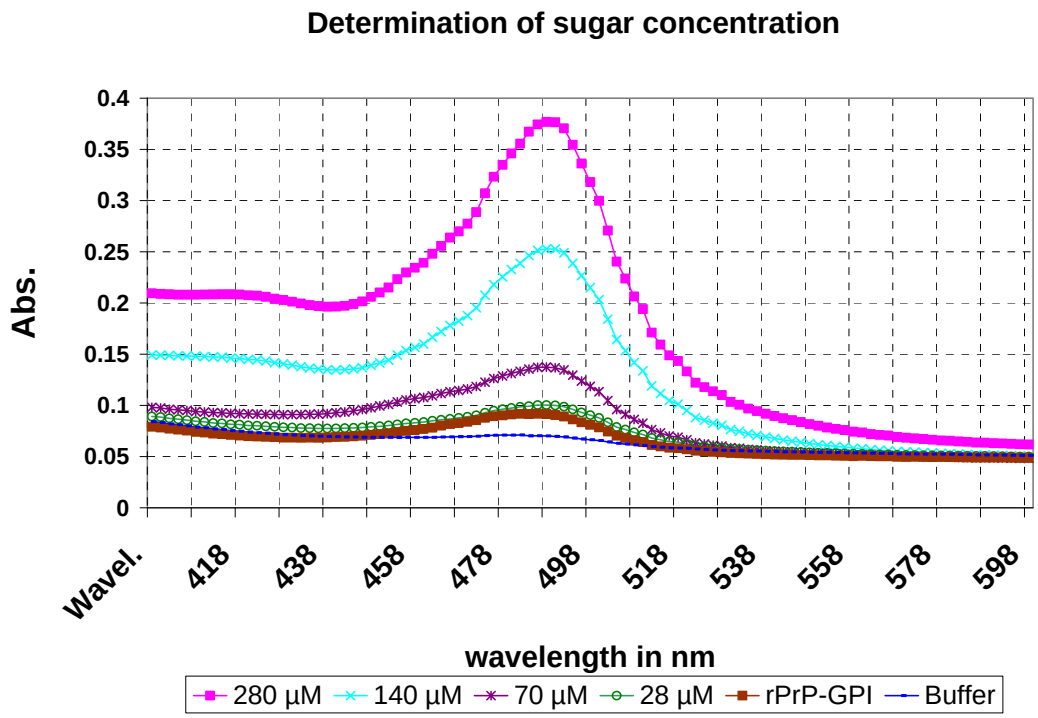
B) Re-purification of **23** (fractions 4-9). Peaks eluting before 10 min were caused by buffer components such as guanidine hydrochloride required to solubilize **23**. Peak 1 was collected and used for analysis by SDS-PAGE and mass spectrometry. The slightly shifted elution time is most likely due to the use of a different HPLC system. Similar conditions as described in A) were applied.



Supporting Figure S4: MALDI-TOF mass spectrum of RP-HPLC purified **23** using a Bruker Daltonics system and α-cyano-4-hydroxycinnamic acid as matrix. The obtained mass of 17702.8 Da is in good agreement with the calculated MW of 17705 Da.

Determination of carbohydrate content of 23

A colorimetric assay as described by Dubois *et al.* was used as a quick method to determine which protein fractions contain carbohydrates after NCL reaction of rPrP Cα-thioester with **22**.[5] The concentration of purified **23** was also determined by this method (S4).



Supporting Figure S5: Different concentrations of mannose dissolved in folding buffer were compared to rPrP-GPI **23** (brown line). The total amount of **23** found by this assay after folding was 0.1 mg/ml, which is in good agreement with the concentration determined by UV absorption at 280nm.

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