Supporting Information

Regioselective Chitosan End-Group Activation: The Triskelion Approach

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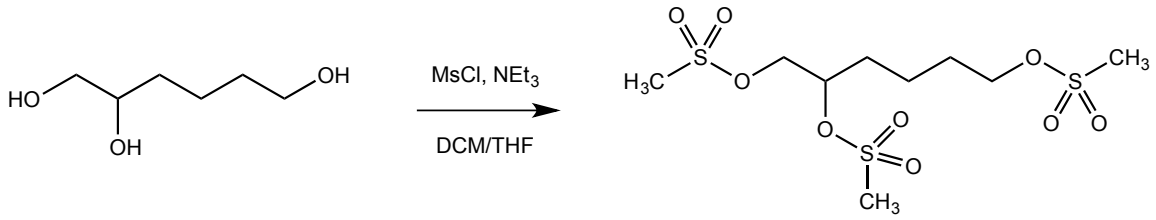
Each chemical reaction described in the following sections was performed on at least 3 independent occasions (N ≥ 3). Fresh reactants, as well as Ar degassed double deionized water and solvents, were used to minimize disulfide bond formation.

1. Triskelion linker synthesis: Hexane-1,2,6-trithiol

1.1. 2-[(methylsulfonyl)oxy]hexane-1,6-diyl dimethanesulfonate synthesis



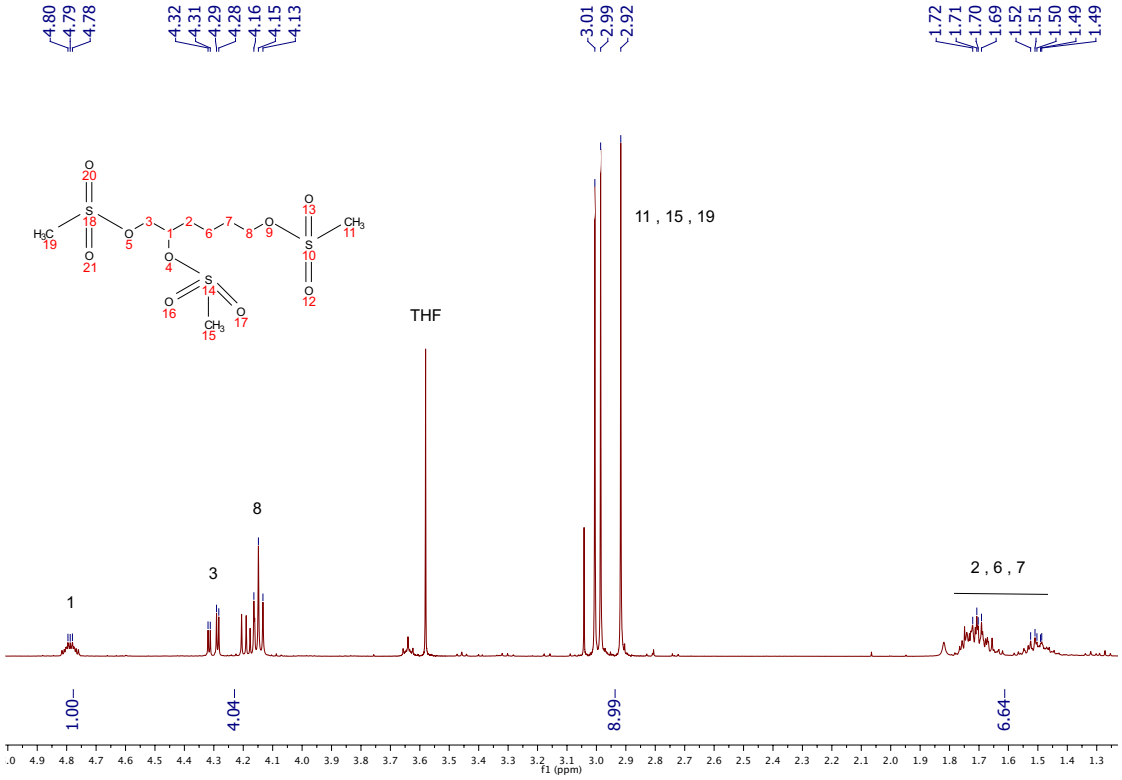
The triol starting material (Hexane-1,2,6-triol, m = 1.74 g, n_{OH} = 3.89 10^{-2} mol) was dissolved in 40 mL anhydrous dichloromethane (DCM) + 20 mL anhydrous Tetrahydrofurane (THF) (c_{OH} = 650 mM). Mesylate chloride (MsCl, 3 equivalents per hydroxyl group, n = 1.71 10^{-1} mol; v = 9.04 mL) was added stepwise to the stirring reaction medium. While stirring, the clear and homogeneous reaction medium was cooled down to 0-5°C within an ice bath and v = 16.6 mL triethylamine (NEt_3, 3 equivalents per hydroxyl group, n = 1.71 10^{-1} mol) was added. The reaction mixture was gently warmed up to room temperature and stirred for 24h under inert atmosphere. Heterogeneous and dark-orange reaction mixture was dissolved in v = 3 x 100 mL DCM and the organic bottom layer was successively extracted with 2 x 100 mL cold ddH_2O, 2 x 100 mL 10% v/v HCl solution, 2 x 100 mL saturated Na_2CO_3 solution and v = 100 mL saturated NaCl solution. Remaining organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give an orange oil with 93% yield that was analyzed by TLC, NMR and MS-ESI. This compound was engaged without further purification in the second step of the synthesis.

TLC: Rf = 0.3, Ethyl acetate/Cyclohexane [7:3]

^{1}H NMR (400 MHz, $CDCl_3$, 22°C, ns=32, d1=2s, acquisition time=2s): δ 1.49-1.72 (m, *6H*, -$(CH_2)_3$-), 2.92-3.01 (3 s, 3x*3H*, -$(S\text{-}CH_3)_3$), 4.13-4.32 (m, 2x*2H*, -CH_2-O-), 4.78-4.80 (m, *1H*, -CH-O-)

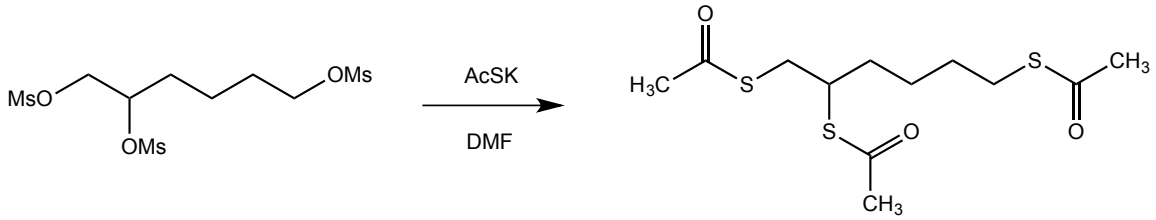
^{13}C NMR (400 MHz, $CDCl_3$, 22°C, ns=320, d1=1.5s, acquisition time=2s): δ 21.00, 28.61 (-CH_2-); 30.49 (-$\underline{C}H_2$-CH-); 37.53, 37.86, 38.89 (SO_2-CH_3); 69.40, 69.54 (-CH_2-O); 78.62 (-CH).

MS-ESI: $[M+NH_4]^+$ = 386.06226 (386.06077 expected); $[M+Na]^+$ = 391.01738 (391.01617 expected); $[M+K]^+$ = 406.99055 (406.99010 expected).



S 1. ^1H NMR, 400 MHz, $CDCl_3$, T=25°C, ns=32 scans, acquisition time=2s, d1=2s.

1.2. *S*,*S*',*S*''-hexane-1,2,6-triyl triethanethioate synthesis



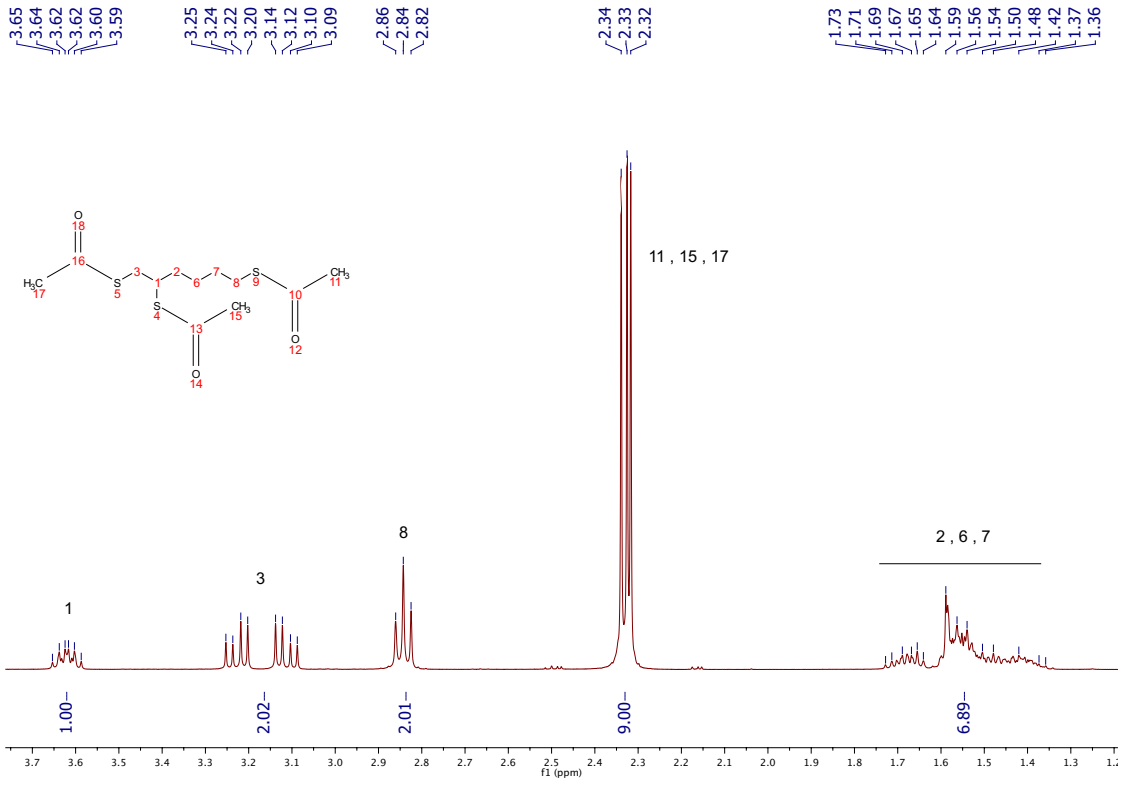
The mesylate intermediate (m = 4.78 g, n_{OMs} = 3.89 10^{-2} mol) was dissolved in v = 83 mL anhydrous DMF (c_{OMs} = 467 mM). The clear orange solution was degassed with 3 cycles vacuum/N_2 and was cooled down to 0-5°C within an ice-bath for 15 min. Potassium thioacetate (m = 22.2 g, 5 equivalents per –OMs group) was added stepwise to the stirring and cold reaction medium under inert atmosphere. The first 8h of reaction were done at 0-5°C and the mixture stirred at room temperature for an additional 16h. The red-brown reaction medium was concentrated to dryness and was solubilized in v = 3 x 200 mL cyclohexane. The organic layer was extracted with 5 x 200 mL ddH_2O, dried over $MgSO_4$ and concentrated under reduced pressure. The crude oil was purified by flash-chromatography using Cyclohexane/Ethyl acetate [95:5] as eluent, giving m = 2.25 g of acetylated Triskelion linker with 75% yield. The Triskelion linker is stored under its acetyl-protected form to avoid disulfide bonds formation and will be deprotected right before conjugation.

TLC: Rf=0.3, Cyclohexane/Ethyl acetate [9:1].

^{1}H NMR (400 MHz, $CDCl_3$, 22°C, ns=32, d1=2s, acquisition time=2s): δ 1.36-1.73 (m, *6H*, -$(CH_2)_3$-), 2.32-2.34 (3 s, 3x*3H*, -$(CO\text{-}CH_3)_3$), 2.82-2.86 (t, *2H*, -CH_2-C$\underline{H_2}$-S-), 3.09-3.25 (m, 2H, -CH-C$\underline{H_2}$-S), 3.59-3.65 (m, *1H*, -CH-S-).

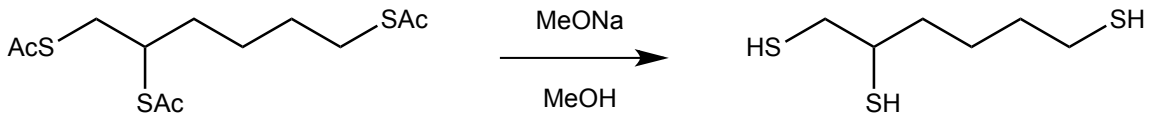
^{13}C NMR (400 MHz, $CDCl_3$, 22°C, ns=320, d1=1.5s, acquisition time=2s): δ 25.80, 28.62, 28.96 (-CH_2-); 30.36, 30.46, 30.55 (-CH_3-); 32.51 (-CH-$\underline{C}H_2$-S-); 33.84 (-$\underline{C}H_2$-CH-); 43.91 (-CH-); 194.67, 194.79, 195.67 (CO).

MS-ESI: $[M+H]^+$ = 309.06526 (309.06473 expected); $[M+NH_4]^+$ = 326.09217 (326.09128 expected); $[M+Na]^+$ = 331.04745 (331.04668 expected); $[M+K]^+$ = 347.02041 (347.02062 expected).



S 2. ^{1}H NMR, 400 MHz, $CDCl_3$, T=25°C, ns=32 scans, acquisition time=2s, d1=2s.

1.3. *Hexane-1,2,6-trithiol synthesis*



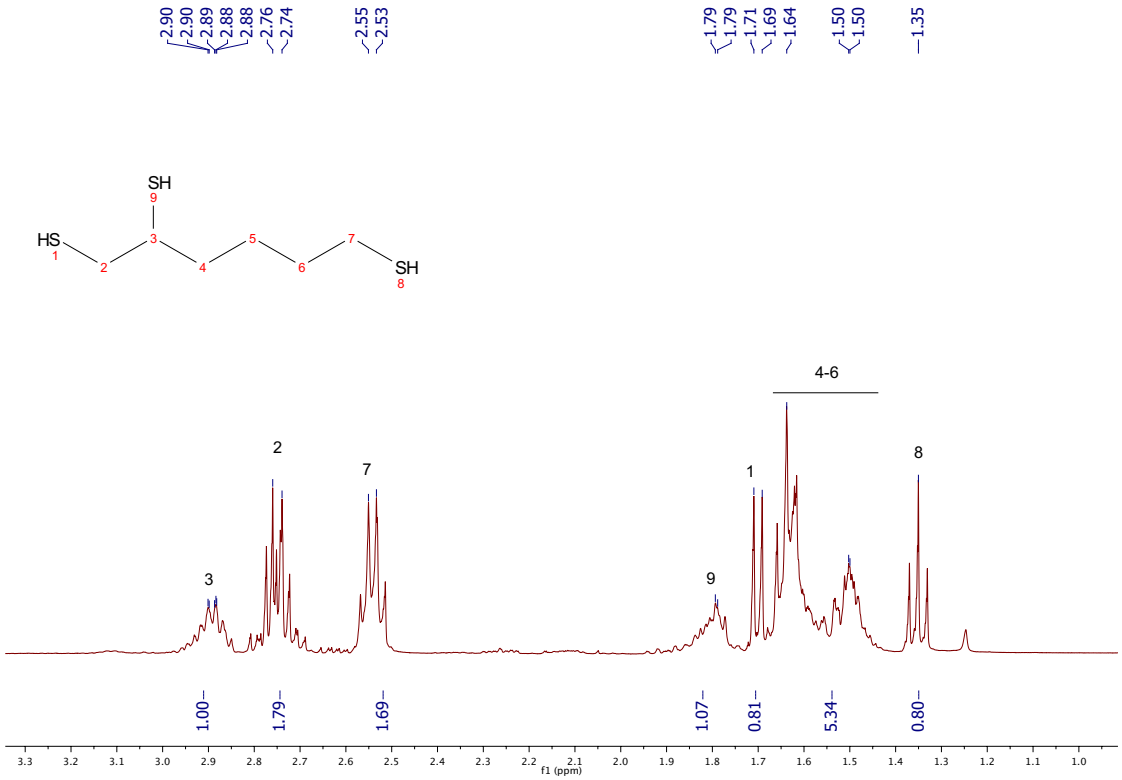
The acetyl-protected Triskelion linker (m = 100.0 mg, n_{SAc} = 9.73 10^{-4} mol) was dissolved in v = 2.9 mL of degassed 0.5M sodium methoxide solution (MeONa in MeOH, 1.5 equivalents per acetyl group to cleave). The reaction medium stirred for 10 min at room temperature and under inert atmosphere. Reaction medium was quenched with v = 241 μL of degassed HCl 37% (2 equivalents per MeONa) and the heterogeneous solution was then extracted with v = 4 mL of degassed ddH_2O. The isolated organic layer was carefully concentrated under reduced pressure to give the pure product as a clear yellowish oil with 95% yield.

TLC: Rf=0.7, Cyclohexane/Ethyl acetate [4:1].

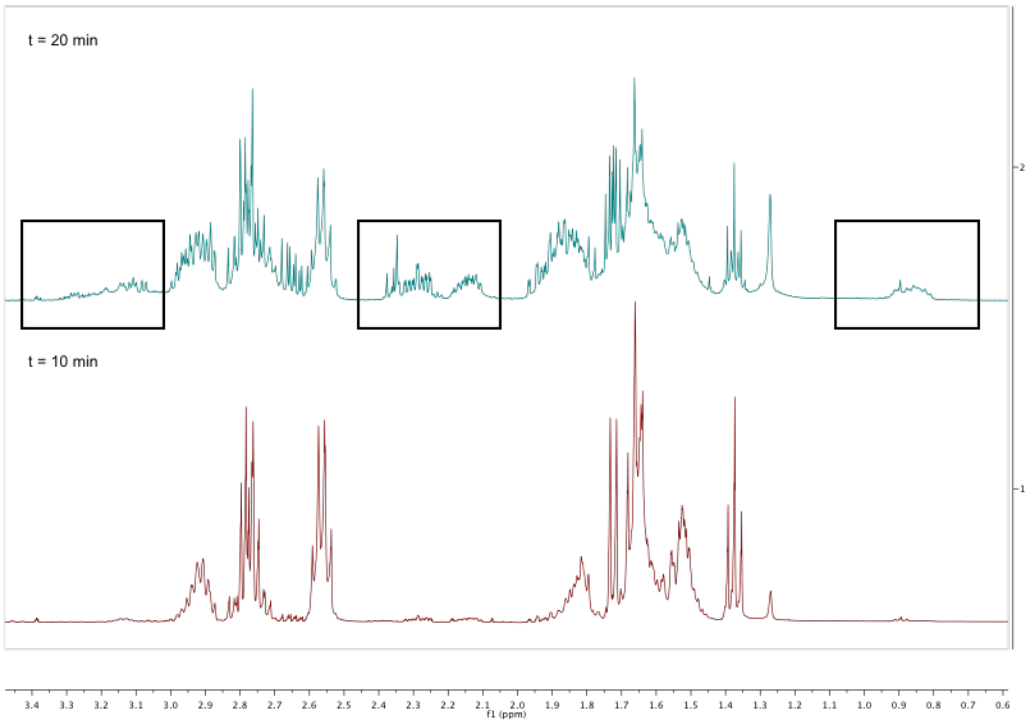
^{1}H NMR (400 MHz, $CDCl_3$, 22°C, ns=32, d1=2s, acquisition time=2s): δ 1.35 (m, *1H*, -SH-), 1.50-1.69 (br., *6H*, -$(CH_2)_3$-), 1.79-1.86 (m, *2H*, -SH-), 2.53-2.55 (m, 2H, -C$\underline{H_2}$-S), 2.68-2.83 (m, 2H, -CH-C$\underline{H_2}$-SH), 2.88-2.90 (m, *1H*, -CH-S-).

^{13}C NMR (400 MHz, $CDCl_3$, 22°C, ns=320, d1=1.5s, acquisition time=2s): δ 24.06 (-CH_2-SH); 25.97, 30.08, 33.64 (-CH_2-); 34.06 (-CH-$\underline{C}H_2$-SH); 54.84 (-CH_2-$\underline{C}$H-CH_2-).

MS-ESI: $[M+H]^+$ = 183.0342 (183.0330 expected); $[M+Na]^+$ = 205.0175 (205.0149 expected).



S 3. ^{1}H NMR, 400 MHz, $CDCl_3$, T=25°C, ns=32 scans, acquisition time=2s, d1=2s.



S 4. Stacked ^{1}H NMR spectra representing the Triskelion linker decomposition which occurs upon longer sodium methoxide deprotection duration (t = 10 min vs. t = 20 min).

2. 2,5-anhydro-D-mannose (M-Unit) / Thiol-hook molecules conjugation

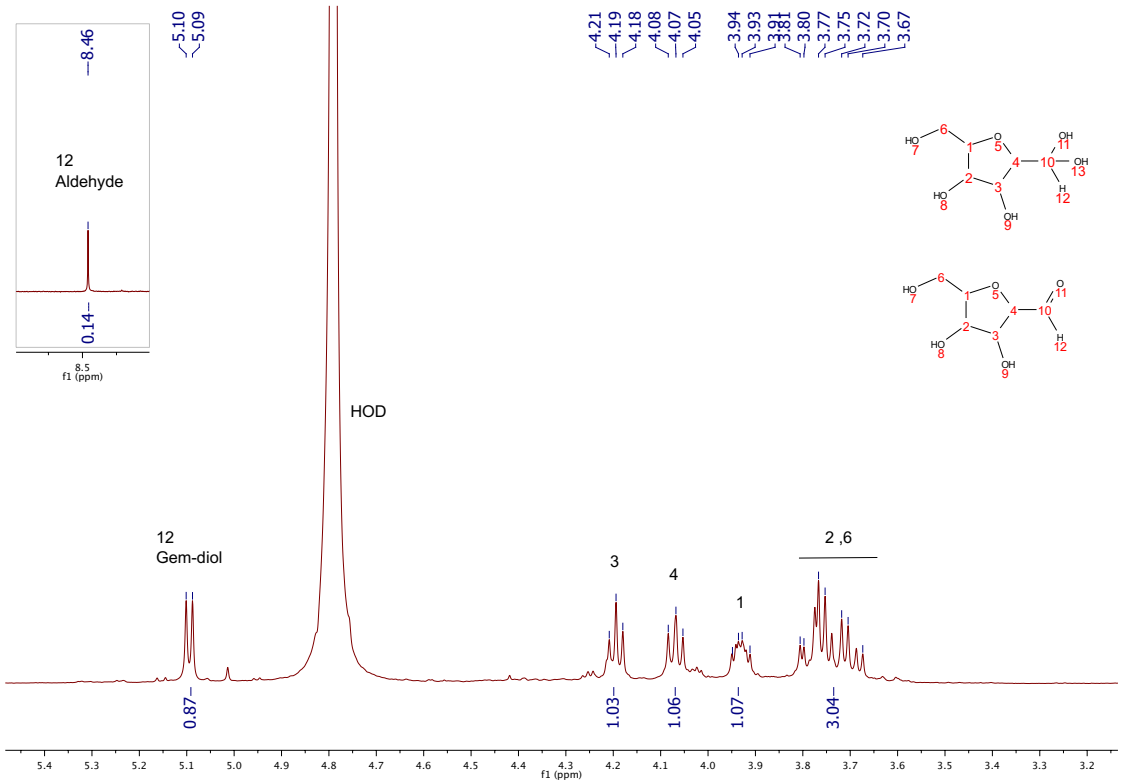
2.1. 2,5-anhydro-D-mannose synthesis

The 2,5-anhydro-D-mannose synthesis described therein was inspired from the work of Claustre *et al.* [34], with some slight modifications. Briefly, D-(+)-glucosamine hydrochloride ($GlcNH_3^+Cl^-$, 1185 mg, 5.50 10^{-3} mol) was dissolved in 25 mL double deionized water (ddH_2O). Glucosamine solution was acidified with 10 g Dowex [H^+] beads and the heterogeneous reaction medium was cooled down to 5°C. After 15 min stirring, sodium nitrite ($NaNO_2$, 1780 mg, 2.75 10^{-2} mol, 5 equivalents/glucosamine) was added by portions and the reaction medium stirred overnight at 50°C. TLC (EtOAc/MeOH [4:1]) of the crude reaction medium confirmed the complete conversion of $GlcNH_3^+Cl^-$ into the desired product (Rf=0.4). Heterogeneous medium was filtered through glass filter and the beads were rinsed with 10 mL ddH_2O. The filtrate was carefully treated with 12 g Dowex [HCO_3^-] in order to increase the pH up to 6.5. This solution was filtered on glass filter and the filtrate was flash-frozen and freeze-dried.

^{1}H NMR (400 MHz, D_2O, 25°C, ns=32, d1=6s, acquisition time=2s) δ 3.67-3.81 (m, *3H*, H4 & H6), 3.91-3.94 (dd, J=9.0, 5.9 Hz, *1H*, H5), 4.05-4.08 (t, J=5.6 Hz, *1H*, H2), 4.18-4.21 (t, J=5.7 Hz, *1H,* H3), 5.09-5.10 (d, J=5.4Hz, *0.87H*, H1 Gem-diol), 8.46 (s, *0.14H*, H1 Aldehyde).

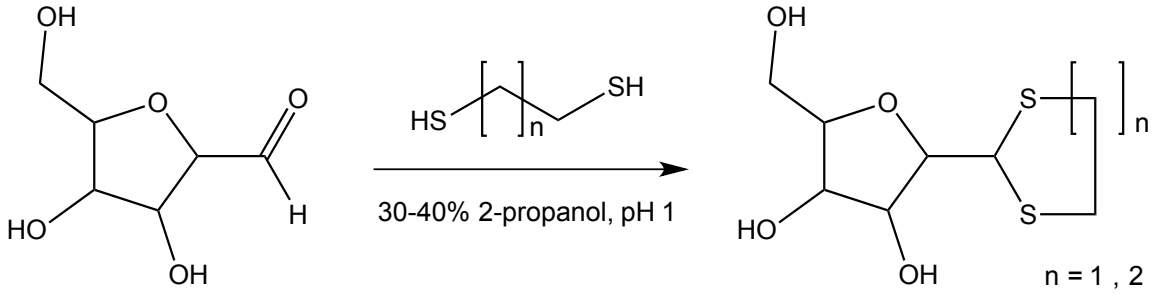
^{13}C NMR (400 MHz, D_2O, 25°C, ns=320, d1=1.5s, acquisition time=2s) δ 60.86 (C6), 76.47 (C4), 77.39 (C3), 82.85 (C2), 84.21 (C5), 89.62 (C1).

MS(ESI+): Aldehyde form [$M+H^+$] = 163.0601; [$M+Na^+$] = 185.0428 (Expected: [$M+H^+$] = 163.0601; [$M+Na^+$] = 185.0420); *Gem*-diol-form [$M+Na^+$] = 203.0538 (Expected: 203.0526).



S 5. ^{1}H NMR, 400 MHz, D_2O, T=25°C, ns=32 scans, acquisition time=2s, d1=6s.

2.2. 2,5-anhydro-D-mannose / Thiol-hook molecules conjugation: LCMS Mechanistic studies



Molecules bearing a thiol-hook (Ethanedithiol, EDT and Propanedithiol, PDT) were used to assess the intramolecular thioacetylation process, where both thiol attacks occur simultaneously on the M-Unit aldehyde forming instantaneously the stable thioacetal conjugate. Briefly, the synthesized 2,5-anhydro-D-mannose M-Unit (0.1 mmol, 16.2 mg) was dissolved in 5mL degassed 30 or 40 % v/v 2-propanol in ddH_2O for EDT or PDT coupling, respectively. The pH of the solution was adjusted to 1 with 3M HCl solution prior to the addition of the thiol-bearing molecule (0.5 mmol, 41.9 µL for EDT and 50.2 µL for PDT). The reaction mixture was stirred for 72h at 50°C, under Ar atmosphere and covered with aluminum foil. The reaction mixture turned clear pink-orange after 72h and was split into 2 parts (Methods I and II): the first Method (Method I) was dedicated to the direct LC-MS analysis of the reaction medium in order to determine the thioacetal proportion in resulting conjugates that was formed *in situ*; whereas the second one (Method II) was immediately flash-frozen and then freeze-dried prior to LC-MS analyses to assess the effect of drying on the thioacetal proportion in resulting conjugates and to ascertain that no by-products appear post concentration.

Only one product was detected by LC-MS, namely the corresponding stable thioacetal formed by intramolecular cyclization, independently from both the thiol-hook molecule engaged and the Method used post-reaction. No linear thioacetals were observed using the thiol-hook molecules and no hemithioacetal intermediates were detected for all experiments performed ($N \geq 3$ for each condition tested).

MS(ESI+): EDT conjugates $[M+Na]^+$ = 261.0215 (Expected: 261.0226); PDT conjugates $[M+Na]^+$ = 275.0374 (Expected: 275.0382).

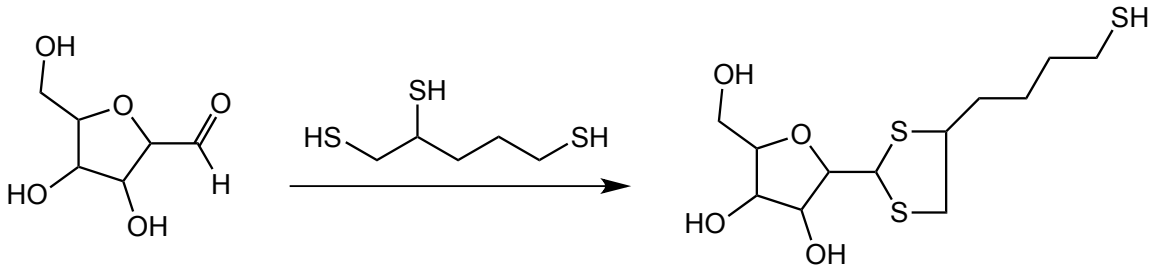
2.3. 2,5-anhydro-D-mannose / Triskelion linker conjugation: Mechanistic studies by LCMS

The conjugation of the acetyl-protected Triskelion linker to the 2,5-anhydro-D-mannose (M-Unit) was performed in a 2-steps process. Briefly, the acetyl-protected Triskelion was first deprotected using sodium methoxide (MeONa) treatment right before conjugation in order to minimize Triskelion linker disulfide linkage formation. In a second step, M-Unit that was synthesized by HONO treatment of glucosamine was dissolved in an appropriate mixture of solvents (30% THF v/v in ddH_2O or 90% methanol v/v in ddH_2O to reach 20 or 10 mM aldehyde concentration at pH 1, respectively). This solution was added to the deprotected Triskelion and the reaction medium was allowed to stir for 72h under inert atmosphere at T=50°C. Reaction medium was treated according to the following methods prior to LCMS analysis: Method I refers to a direct LC-MS analysis of the reaction medium whereas Method II stands for a concentration to dryness step prior LC-MS.

2.3.1. Triskelion linker deprotection

The amount of Triskelion linker was set to 20 equivalents per 2,5-anhydro-D-mannose aldehyde engaged in the conjugation process. The acetyl-protected Triskelion linker was deprotected as described in section 1.3.

2.3.2. M-Unit / Triskelion linker conjugation



Triskelion linker conjugation (30% THF): The following description corresponds to the conjugation performed in a degassed mixture of 30% v/v THF in ddH_2O.The 2,5-anhydro-D-mannose (m = 5 mg, n = 2.78 10^{-5} mol) was dissolved in 960 μL of degassed ddH_2O and then v = 416 μL of degassed THF was added. The pH of the reaction medium was adjusted to 1 with v = 11 μL of degassed HCl 37%, reaching a final aldehyde concentration of 20 mM. This solution was added to the deprotected Triskelion and the reaction medium was stirred for 72h at T=50°C under inert atmosphere.

Triskelion linker conjugation (90% MeOH): The following description corresponds to the conjugation performed in a degassed mixture of 90% v/v Methanol in ddH_2O. The 2,5-

anhydro-D-mannose (m = 5 mg, n = 2.78 10^{-5} mol) was dissolved in 255 μL of degassed ddH_2O and then v = 2498 μL of degassed methanol was added. The pH of the reaction medium was adjusted to 1 with v = 23 μL of degassed HCl 37%, reaching a final aldehyde concentration of 10 mM. This solution was added to the deprotected Triskelion and the reaction medium was stirred for 72h at T=50°C under inert atmosphere.

Reaction media were treated according to the methods described above prior to LCMS analysis.

3. M-Unit chitosan (CS) salts / Thiol-hook molecules conjugation

3.1. M-Unit CS 92-4 HCl salt / Ethanedithiol conjugation

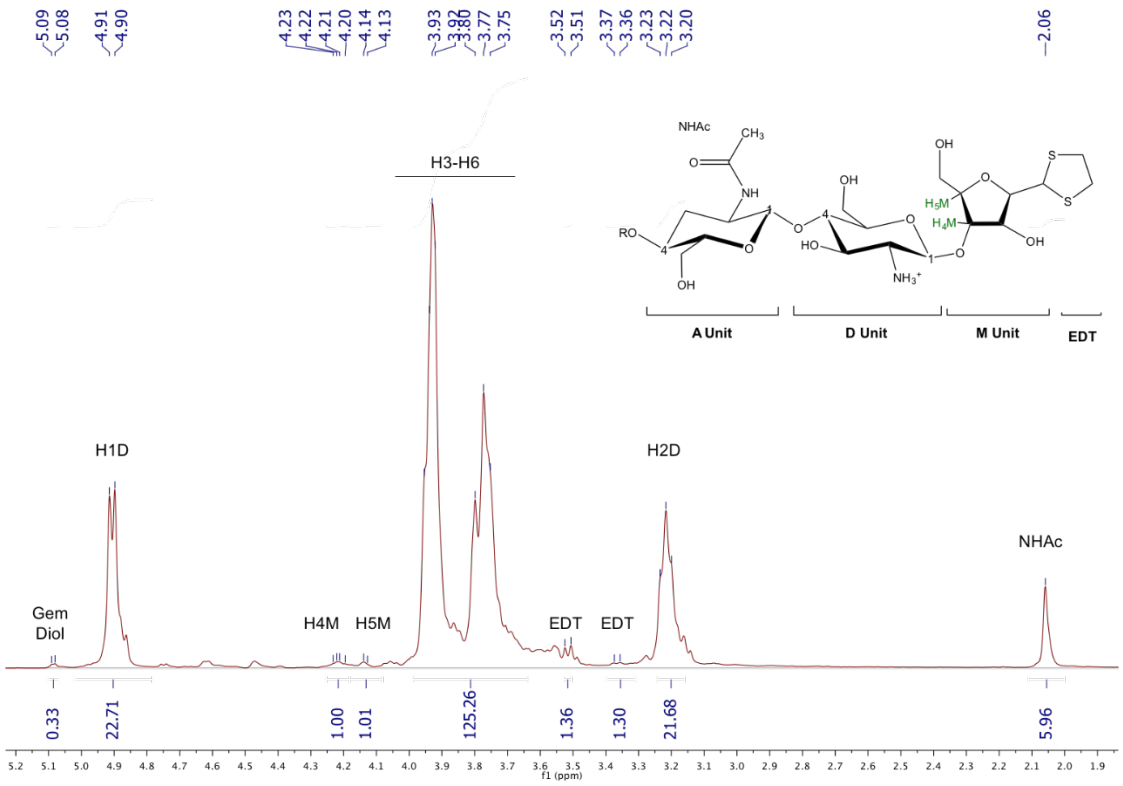
Reaction 1 (30% 2-propanol, EDT 5 equivalents): CS 92-4 HCl salt (Mn = 4030 g/mol, 356.7 mg, 0.089 mmol aldehyde) was dissolved in a degassed mixture of 4.43 mL 30% v/v 2-propanol in ddH_2O ($c_{aldehyde}$ = 20 mM). The pH of the homogeneous reaction medium was adjusted to 1 with degassed 3M HCl solution. This solution was divided into 3 equal volumes of v = 1477 µL (0.03 mmol aldehyde) and each of those was treated with v = 12.4 µL ethanedithiol (EDT; 5 equivalents per aldehyde, n = 0.148 mmol). Reaction media were stirred for 72h at T=50°C, under inert atmosphere.

Reaction 2 (30% 2-propanol, EDT 20 equivalents): CS 92-2 HCl salt (Mn = 4030 g/mol, 70.0 mg, 0.017 mmol aldehyde) was dissolved in a degassed mixture of 0.87 mL 30% v/v 2-propanol in ddH_2O ($c_{aldehyde}$ = 20 mM). The pH of the homogeneous reaction medium was adjusted to 1 with degassed 3M HCl solution. This homogeneous solution was treated with v = 9.7 µL ethanedithiol (EDT; 20 equivalents per aldehyde, n = 0.116 mmol) and was allowed to stir for 72h at T=50°C, under inert atmosphere.

Workups and purification: All reaction media were divided into 2 equal volumes dedicated to Workup I (no freeze-drying) and Workup II (direct freeze-drying) and were treated as follows for comparison purposes. Workup I samples were directly engaged in the purification process, whereas Workup II samples were flash-frozen and freeze-dried prior to removal of unreacted thiol-bearing molecule. All reaction media were treated with 1N sodium hydroxide solution (pH of the solutions was increased up to 9) in order to remove some potential hemithioacetal intermediates (even if they were not observed by LCMS in the conditions implemented therein), ensuring that only the stable thioacetals conjugates would be quantified by ^{1}H NMR. After acidification of the solutions for chitosan solubilization, unreacted thiol molecules (EDT) were discarded by 5 successive reprecipitations in fresh 2-propanol. The remaining precipitates were dissolved in 5 mL ddH_2O and these solutions were flash-frozen and freeze-dried to give a white solid with 60-75% massic yield. Conjugation efficiencies were determined by ^{1}H NMR, using both Equations 1&2.

^{1}H NMR (400 MHz, D_2O, 70°C, ns = 64, d1 = 10s, acquisition time = 2s, HOD presaturation) δ 2.06 (s, 5.96H, NHAc), 3.20–3.23 (br, 22H, H2D), 3.36–3.37 (d, J=9.0Hz, 1.30H, EDT), 3.51-3.52 (d, J=9.0Hz, 1.36H, EDT), 3.75–3.93 (m, 125H, H3–H6), 4.13 (br,

1H, H5M), 4.22 (br, 1H, H4M), 4.87–4.92 (m, 22H, H1D), 5.08 (br, 0.33H, H1M gem-diol).



S 6. ^{1}H NMR, 400 MHz, D_2O, T=70°C, ns=64 scans, acquisition time=2s, d1=10s. Functionalization degree were calculated with Equations 1 and 2, giving F = 67%.

3.2. M-Unit CS 92-2 HCl salt / Triskelion linker conjugation

Step 1: Triskelion linker deprotection was performed as described above. The amount of Triskelion used in the following examples corresponds to 20 equivalents per chitosan's M-Unit aldehyde.

Step 2: Conjugation (2 kDa CS, 85% MeOH, pH 1, 24, 48 and 72h, Workup I&II: CS 92-2 HCl salt (Mn = 3244 g/mol, 76.7 mg, 2.36 10^{-5} mol aldehyde) was dissolved in v = 315 µL degassed ddH_2O. The pH of the homogeneous reaction medium was adjusted to 1 with v = 39.4 µL degassed HCl 37%. Degassed methanol (v = 2010 µL) was added to the chitosan acidic solution giving an 85% v/v methanol in ddH_2O mixture and reaching a chitosan end-group concentration of 10 mM. The protected Triskelion linker (20 equivalents per aldehyde, n = 4.73 10^{-4} mol, m = 145.9 mg) was deprotected and purified

according to the protocol described above. Chitosan solution was added to the Triskelion oil and the reaction medium stirred for 24h, 48h and 72h at T=50°C, under inert atmosphere. The reaction media corresponding to the time-points (24h, 48h and 72h) were treated with both Workups I&II described above giving a white solid with 65-75% massic yield. Conjugation efficiencies were determined by ^{1}H NMR, using both Equation 1 and Equation 2.

^{1}H NMR (400 MHz, D_2O, 70°C, ns = 64, d1 = 10s, acquisition time = 2s, HOD presaturation) δ 1.53-1.87 (br, 5.50H, Trisk 1-3), 2.06 (s, 6.40H, NHAc), 2.74-2.80 (br, 0.92H, Trisk 4), 3.14–3.18 (br, 16H, H2D), 3.66–3.96 (m, 81H, H3–H6), 4.13 (br, 1H, H5M), 4.22 (br, 1H, H4M), 5.08 (br, 0.08H, H1M gem-diol).

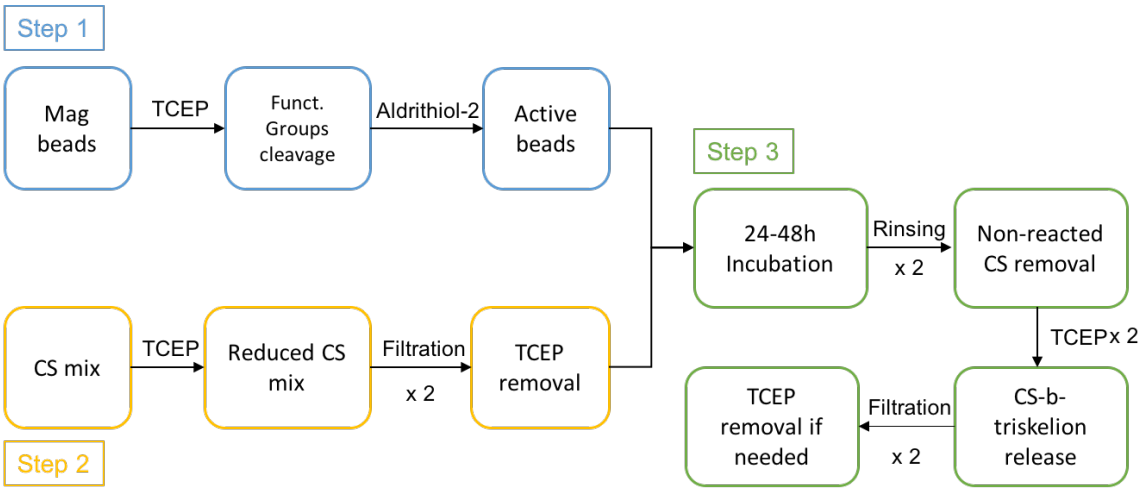
4. PureCube Thiol-activated Magbeads / Cs-b-Triskelion conjugation

The Thiol-activated Magnetic beads "brush-like" coating with CS-b-Triskelion 3-steps procedure is summarized in Figure S 7.

Step 1: Since Thiol-activated Magbeads (v = 1 mL, 1.5 μmol.mL^{-1} functional group density) needs to be freshly activated prior use, they were treated with a degassed solution of 50 mM Tris(2-carboxyethyl)phosphine (TCEP) in Acetate buffer pH 4 + 200 mM NaCl for 20 min at room temperature, under inert atmosphere. Beads were rinsed twice with degassed Acetate buffer pH 4 + 200 mM NaCl and were incubated overnight with a [1:4] degassed 50 mM sodium borate pH solution + 20 mM Aldrithiol-2 in degassed ethanol, at room temperature and under inert atmosphere. Beads were washed twice with degassed ethanol and twice with Acetate buffer pH 4 + 200 mM NaCl. Supernatants were discarded but the beads were not allowed to dry until conjugation.

Step 2: After M-Unit CS aldehyde / Triskelion linker conjugation, CS-b-Triskelion within the crude CS mixture may have formed some disulfide linkages. A degassed 50 mM TCEP solution was prepared and CS mix (m = 10 mg, F = 70%, n_{SH} = 3 μmol, c = 10 mg.mL^{-1}) was reduced for 20 min at room temperature, under inert atmosphere. After complete reduction, reduced CS mix was filtered on Macrosep ultracentrifugal filter (Omega membrane, MWCO = 1 kDa), rinsed twice with a 0.1M degassed HCl solution and finally with Acetate buffer pH 4 + 200 mM NaCl.

Step 3: Reduced CS mix solution (1 mL, 10 mg.mL^{-1}) from Step 2 was added on the freshly activated Magbeads (1 mL) and both species were allowed to react for 24-48h at room temperature, under inert atmosphere. Beads were rinsed twice with Acetate buffer pH 4 + 200 mM NaCl to discard unreacted CS (supernatant was discarded) and then twice with 50 mM TCEP in Acetate buffer pH 4 + 200 mM NaCl. Combined supernatants were filtered on Macrosep ultracentrifugal filter (Omega membrane, MWCO = 1 kDa), rinsed twice with a 0.1M degassed HCl solution and finally with degassed ddH_2O. Clear colorless solution was flash-frozen and freeze-dried to afford the pure CS-b-Triskelion conjugate with 51% yield.



S 7. Schematic representation of the Thiol-activated surface modification with CS-b-Triskelion 3-steps procedure. Step 1 refers to Thiol-activated Magbeads preparation, whereas Steps 2 and 3 refer to CS-b-Triskelion reduction and its conjugation onto beads surface, respectively.