# Development of an efficacious, semisynthetic glycoconjugate vaccine candidate against *Streptococcus pneumoniae* serotype 1

Supporting Information

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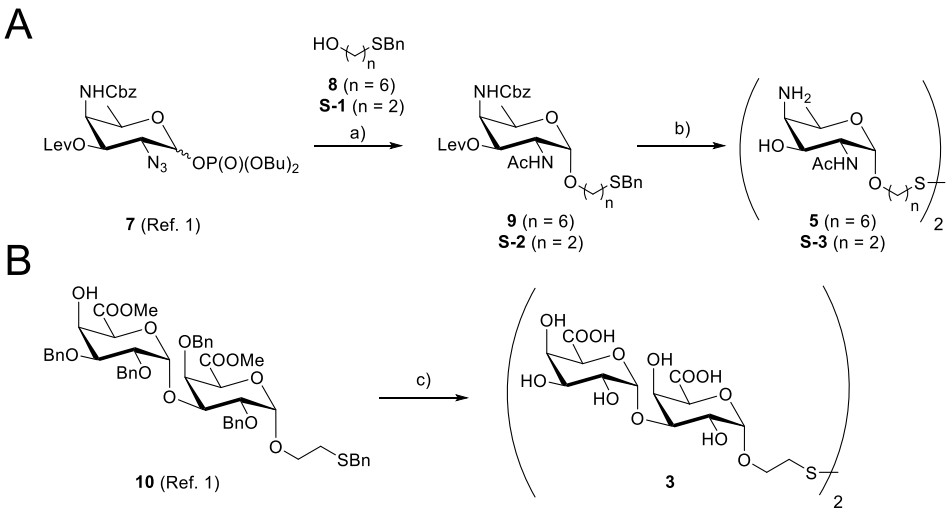
## Supporting Figures




**Scheme S1**. *A*, synthesis of D-AAT disulfides **5** and **S-3**. *B*, synthesis of di-GalA disulfide **3**. Reagents and conditions: a) For n = 6: i. **8**, TMSOTf, $CH_2Cl_2$, 0 °C (1:1 α:β); ii. AcSH, pyridine, r.t., 29% (two steps). For n = 2: i. **S-1**, TMSOTf, $Et_2O/CH_2Cl_2$ 5:2, -40 °C to 0 °C, 48% (5:2 α:β); ii. AcSH, pyridine, r.t., 76%; b) For n = 6: i. $N_2H_4{\cdot}H_2O$, AcOH, pyridine, $CH_2Cl_2$, r.t.; ii. Na, $NH_3$, *t*BuOH, THF, -78 °C, 34% (two steps). For n = 2: $N_2H_4{\cdot}H_2O$, AcOH, pyridine, $CH_2Cl_2$, r.t.; ii. Na, $NH_3$, *t*BuOH, THF, -78 °C, 70% (two steps); c) i. NaOH, $H_2O$, MeOH, THF, 0 °C to r.t.; ii. Na, $NH_3$, *t*BuOH, THF, -78 °C, 61% (two steps). TMSOTf = trimethylsilyl trifluoromethanesulfonate.

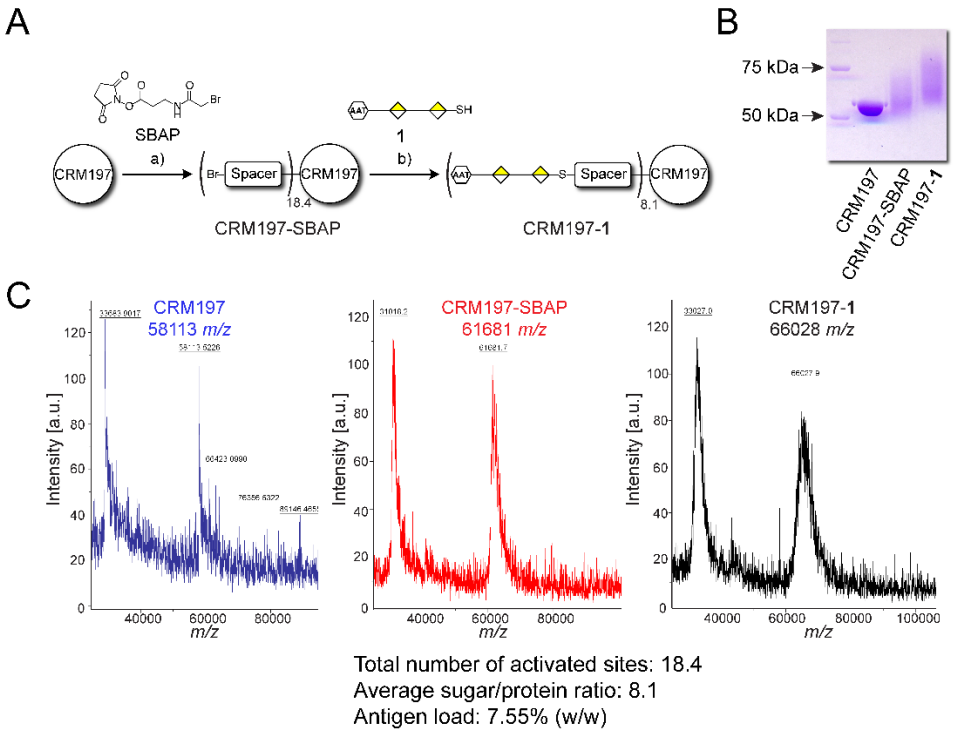



**Figure S1:** Conjugation of trisaccharide **1** to CRM197. *A*, Conjugation scheme. *B*, SDS-PAGE of CRM197 before reaction, after activation and after conjugation. *C*, MALDI-TOF analysis. Axes were re-drawn to enhance readability. Reagents and conditions: a) SBAP, 0.1 M NaPi pH 7.4, r.t.; b) i. **1**, TCEP, 0.1 M NaPi pH 8.0, r.t.; ii. L-cysteine, 0.1 M NaPi pH 7.4, r.t. NaPi = sodium phosphate buffer. SBAP = *N*-succinimidyl 3-(2-bromoacetamido)propanoate. TCEP = tris(2-carboxyethyl)phosphine.

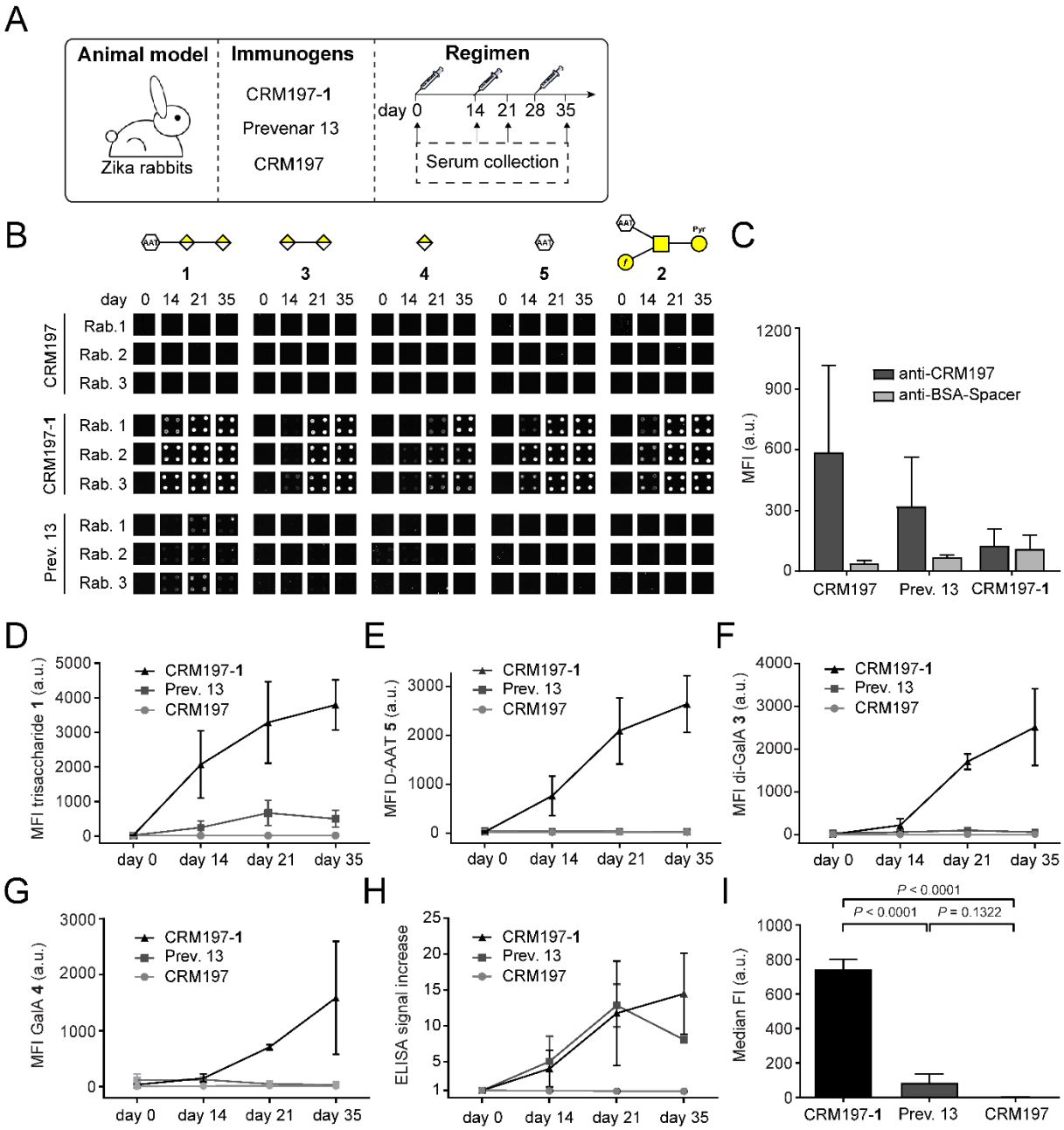



**Figure S2:** Immunogenicity of CRM197-**1**. *A*, immunization parameters and regimen. *B*, time course of glycan microarray binding of rabbit sera against CRM197-**1**, Prevenar 13 and CRM197 (raw data, 1:100 dilution). *C*, antibody responses of sera at day 35 against CRM197 and a BSA-spacer construct as measured by glycan microarray (1:100 dilution). Data are means ± SD of mean fluorescence intensities of three spots per serum sample from *n* = 3 rabbits. *D-G*, time course of binding to trisaccharide **1** (*D*), D-AAT **5** (*E*), di-GalA **3** (*F*) and GalA **4** (*G*). Data are means ± SD of mean fluorescence intensities of four spots per serum sample from *n* = 3 rabbits. *H*, time course of ELISA signal (1:400 dilution). Data are means ± SD of absorbance values relative to values on day 0 from *n* = 3 rabbits. *I*, binding of pooled immune sera to ST1 bacteria as assessed by flow cytometry. Data are median fluorescence intensity + SD of three independent experiments. Statistical analysis was performed by paired one-way ANOVA with Tukey's post-hoc test.

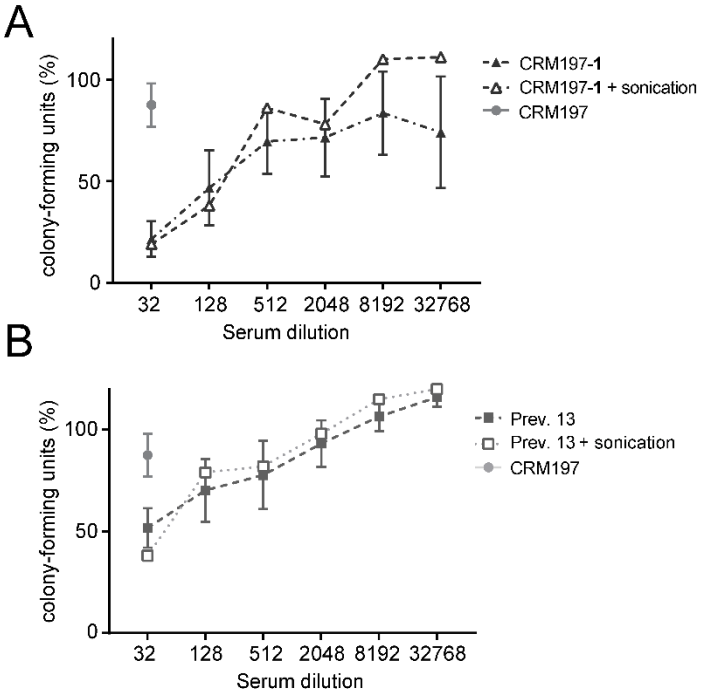



**Figure S3:** CRM197-**1** antisera cause bacterial agglutination at high serum dilutions. ST1 bacteria were subjected to opsonophagocytic killing as described in Figure 2F. One sample per serum dilution was subjected to sonication prior to plating on blood agar. *A*, CRM197-**1** antisera. *B*, Prevenar 13 antisera. Data are mean ± SD of three technical replicates (non-sonicated samples) or a single value (sonicated samples) of one out of two independent experiments (*A*) or a single experiment (*B*).

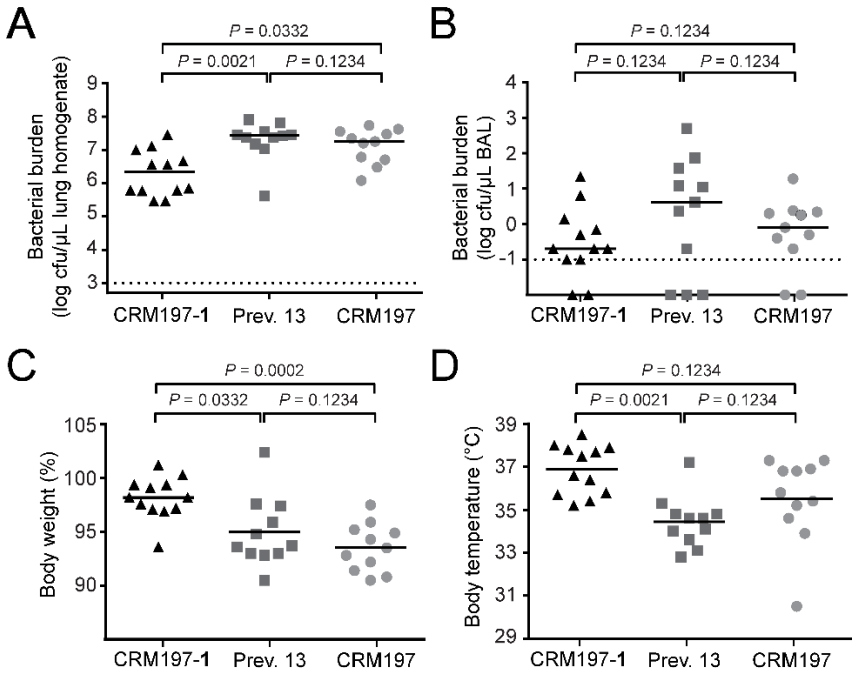



**Figure S4:** Bacterial burden and clinical parameters of mice (*n* = 11-12) passively immunized with pooled sera (day 35) from rabbits immunized with CRM197-**1**, Prevenar 13 or CRM197 and transnasally infected with live ST1. *A*, bacterial burden in lung homogenates. *B*, bacterial burden in bronchoalveolar lavage fluid. *C*, body weight. *D*, body temperature. Data are individual values and medians. Statistical analysis was performed by one-way ANOVA with Dunn's post-hoc test. Dotted lines depict detection limits.

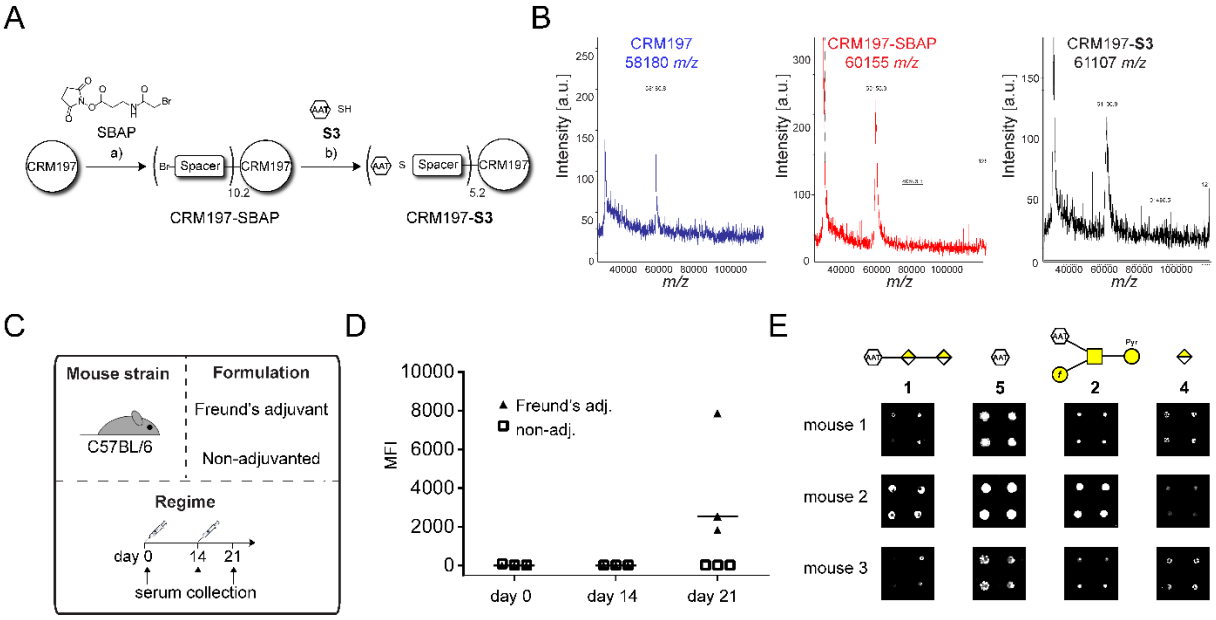



**Figure S5:** Conjugation of D-AAT **S3** to CRM197. *A*, Conjugation scheme. *B*, MALDI-TOF analysis. Axes were re-drawn to enhance readability. *C*, immunization regimen. *D* and *E*, comparison of immune responses against D-AAT between groups (*D*) or against synthetic glycans of mice immunized with Freund's adjuvant (*E*) by glycan microarray (1:100 dilution). Values in *D* are given as individual data and median. Reagents and conditions: a) SBAP, 0.1 M NaPi pH 7.4, r.t.; b) i. **S3**, TCEP, 0.1 M NaPi pH 7.4, r.t.; ii. L-cysteine, 0.1 M NaPi pH 7.4, r.t.

# Experimentals

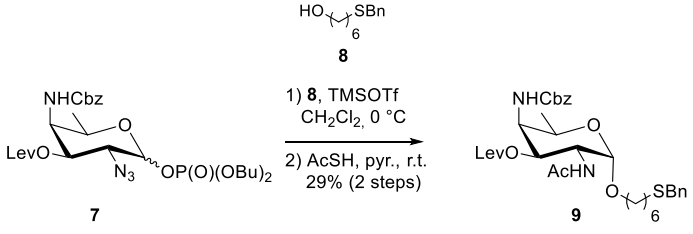
## General Chemical Experimentals

Commercial grade solvents and reagents were used unless stated otherwise. Anhydrous solvents were obtained from a Waters Dry Solvent System. Solvents for chromatography were of technical grade and distilled under reduced pressure prior to use. Sensitive reactions were carried out in heat-dried glassware and under an argon atmosphere. Analytical thin layer chromatography (t.l.c.) was performed on Kieselgel 60 F254 glass plates pre-coated with silica gel (0.25 mm thickness). Spots were visualized with sugar stain (0.1% (v/v) 3-methoxyphenol, 2.5% (v/v) sulfuric acid in EtOH) or CAM stain (5% (w/v) ammonium molybdate, 1% (w/v) cerium(II) sulfate and 10% (v/v) sulfuric acid in water) dipping solutions. Flash column chromatography was performed on Fluka Kieselgel 60 (230-400 mesh). Solvents were removed under reduced pressure using a rotary evaporator and high vacuum (<1 mbar). Freeze-drying of aqueous solutions was performed using a Christ Alpha 2-4 LD Lyophilizer.

$^{1}H$, $^{13}C$ and two-dimensional NMR spectra were measured with a Varian 400-MR spectrometer or a Varian 600 spectrometer at 296 K. Chemical shifts (δ) are reported in parts per million (ppm) relative to the respective residual solvent peaks ($CDCl_3$: δ 7.26 in $^{1}H$ and 77.16 in $^{13}C$ NMR; $D_2O$: δ 4.79 in $^{1}H$ NMR). Two-dimensional NMR experiments (HH-COSY, CH-HSQC, CH-HMBC) were performed to assign peaks in $^{1}H$ spectra. In addition, peaks in compound **3** were annotated by cross-referencing with compound **1**.[1] The following abbreviations are used to indicate peak multiplicities: *s* singlet; *d* doublet; *dd* doublet of doublets; *dt* doublet of triplets; *m* multiplet. Coupling constants (*J*) are reported in Hertz (Hz). Optical rotation (OR) measurements were carried out with a Schmidt&Haensch UniPol L1000 polarimeter at λ= 589 nm and a concentration (c) expressed in g/100 mL in the solvent noted in parentheses. High resolution mass spectrometry by electrospray ionization (ESI-HRMS) was performed at Freie Universität Berlin, Mass Spectrometry Core Facility, with an Agilent 6210 ESI-TOF mass spectrometer. Matrix-assisted laser desorption ionization-time of flight (MALDI/TOF-MS) high resolution mass spectra were recorded on a Bruker Daltonics Autoflex Speed spectrometer.

The synthesis of compounds **1**, **2**, **4**, **6**, **7** and **10** has been reported before.[1]

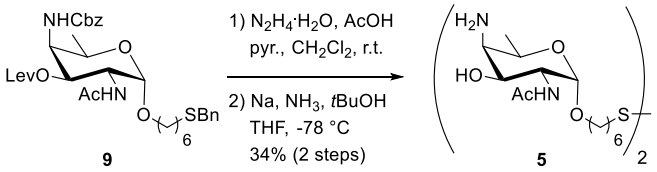
### 2-Acetamido-4-(benzyloxycarbonyl)amino-3-*O*-levulinoyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→1)-6-(benzylthio)hexanol (9)



Alcohol **8**[2] (29 mg, 0.171 mmol) and glycosyl phosphate **7**[1] (70 mg, 0.114 mmol) were co-evaporated with anhydrous toluene (3x10 mL) and kept under high vacuum for 30 min. The mixture was dissolved in $CH_2Cl_2$ (1.8 mL) and stirred over activated molecular sieves (4 Å-AW) for 1 h at room temperature. The solution was cooled to 0 °C and treated with TMSOTf (31 µL, 0.171 mmol in 0.2 mL anhydrous $CH_2Cl_2$). The mixture was stirred for 3 h at that temperature, quenched with a 1:1 (v/v) mixture of MeOH and $Et_3N$ (0.5 mL), diluted with $CH_2Cl_2$ (20 mL) and filtered through Celite. The residue was purified by flash column chromatography (EtOAc/hexanes 2:3 to 3:2) to give the corresponding glycosides (57 mg) as an inseparable α/β mixture.

To a stirred solution of the glycoside mixture in dry pyridine (0.9 mL) was added at 0 °C thioacetic acid (0.9 mL). The mixture was warmed to room temperature and stirred for 24 h at that temperature. The solution was co-evaporated with toluene (2x5 mL) and the residue was purified by flash column chromatography (EtOAc/hexanes 1:2 to 2:1 to 6:1) to give acetamide **9** (22 mg, 0.034 mmol, 29% over two steps) as a white solid, along with the corresponding β-isomer (21.6 mg, 0.034 mmol, 29%). $R_f$ (EtOAc/hexanes 4:1) = 0.36; $[\alpha]_D^{20}$ = +24.7° (c = 0.50, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.28 (m, 8H, arom.), 7.28 – 7.20 (m, 2H), 5.57 (d, *J* = 9.3 Hz, 1H, NH), 5.09 (m, 4H, $PhCH_2$, H-3, NH), 4.74 (d, *J* = 3.9 Hz, 1H, H-1), 4.34 – 4.22 (m, 1H, H-2), 4.11 (dt, *J* = 13.0, 5.3 Hz, 2H, H-4, H-5), 3.71 (s, 2H, $PhCH_2$), 3.61 (m, 1H, A of AB, O-C*H*$_2$-$CH_2$), 3.35 (m, 1H, B of AB, O-C*H*$_2$-$CH_2$), 2.68 (m, 1H, Lev-$CH_2$), 2.61 – 2.30 (m, 5H, Lev-$CH_2$, $CH_2$-C*H*$_2$-S), 2.12 (s, 3H, Ac-$CH_3$ or Lev-$CH_3$), 1.98 (s, 3H, Ac-$CH_3$ or Lev-$CH_3$), 1.56 (dd, *J* = 15.6, 8.3 Hz, 4H, aliph.), 1.43 – 1.23 (m, 4H, aliph.), 1.15 (d, *J* = 6.4 Hz, 3H, H-6); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 206.7, 172.6, 170.2, 156.7, 138.6, 136.4, 128.8, 128.5, 128.4, 128.1, 128.0, 126.9, 97.4, 77.2, 70.2, 68.2, 66.9, 64.4, 52.8, 47.7, 37.7, 36.3, 31.3, 29.7, 29.2, 29.1, 28.5, 28.1, 25.8, 23.3, 16.5; HRMS (ESI) calcd for $C_{34}H_{46}N_2O_8S$ $(M+Na)^+$ 665.2872 found 665.2865 *m/z*.

**6,6'-Dithiobis[2-acetamido-4-amino-2,4,6-trideoxy-α-D-galactopyranosyl-(1→1)-1-hexanol] (5)**

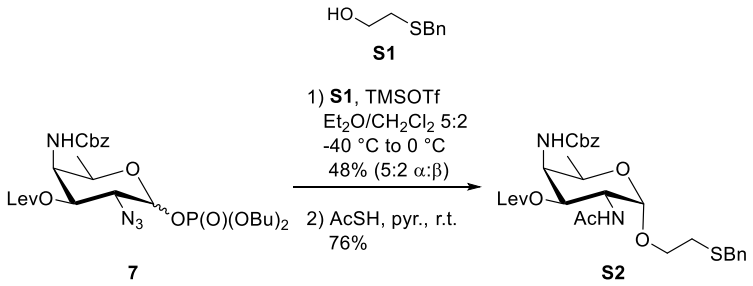



To a stirred solution of ester **9** (10 mg, 0.016 mmol) in anhydrous $CH_2Cl_2$ (1.0 mL) were added at room temperature first a mixture of pyridine (38 µL, 0.467 mmol) and acetic acid (24.9 µL, 0.436 mmol), and then hydrazine hydrate (1.0 µL, 0.020 mmol). The mixture was stirred for 2 h at that temperature, quenched with acetone (0.1 mL) and purified by size exclusion chromatography (Sephadex LH-20, $CH_2Cl_2$/MeOH 2:1) to give the corresponding alcohol as a clear oil.

To a stirred solution of liquid ammonia (5 mL) was added at -78 °C a solution of the intermediate alcohol in THF (1.2 mL). The mixture was treated with *t*BuOH (0.5 mL) and lumps of freshly cut sodium (80 mg) were added until a deeply blue color persisted. The reaction was stirred at -78 °C for 45 min and

quenched by addition of solid ammonium acetate (100 mg). The solution was warmed to room temperature under a stream of argon and co-evaporated with MeOH (2x10 mL) and water (2x5 mL). The residue was left under air for 16 h, purified by size exclusion chromatography (Sephadex G-25, 9:1 MeOH/5 mM aq. $NH_4OAc$) and lyophilized repeatedly to give thiol-linked monosaccharide **5** (acetate salt, 1.7 mg, 2.7 µmol, 34% over two steps) as a mixture of thiol and disulfide as a white solid. $[\alpha]_D^{20}$ = +19.9° (c = 0.02, $H_2O$); $^1$H NMR (400 MHz, $D_2O$) δ 4.91 (d, *J* = 3.1 Hz, 1H, H-1), 4.27 (m, 1H, H-5), 4.14 – 4.07 (m, 2H, H-2, H-3), 3.80 – 3.70 (m, 1H, A of AB, O-C*H*$_2$-$CH_2$), 3.59 – 3.49 (m, 1H, B of AB, O-C*H*$_2$-$CH_2$), 3.35 – 3.19 (m, 1H, H-4, free amine and acetate salt), 3.05 – 2.78 (m, 2H, $CH_2$-C*H*$_2$-S, thiol and disulfide), 2.11 (s, 3H, Ac-$CH_3$), 1.86 – 1.73 (m, 2H, aliph.), 1.72 – 1.58 (m, 2H, aliph.), 1.55 – 1.43 (m, 4H, aliph.), 1.34 – 1.28 (d, *J* = 6.6 Hz, 3H, H-6); $^{13}$C NMR (150 MHz, $D_2O$) δ 99.4, 70.7, 56.8, 53.5, 52.0, 40.8, 30.8, 30.0, 29.7, 27.4, 26.6, 25.9, 24.6, 18.5. HRMS (ESI) calcd. for $C_{28}H_{54}N_4O_8S_2$ $(M+Na)^+$ 661.3281 found 661.3306 *m/z*.

**2-Acetamido-4-(benzyloxycarbonyl)amino-3-*O*-levulinoyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→1)-2-(benzylthio)ethanol (S2)**

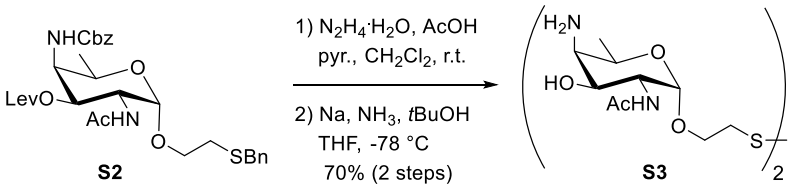



Alcohol **S1**[1] (71 mg, 0.421 mmol) and glycosyl phosphate **7** (171 mg, 0.281 mmol) were co-evaporated with anhydrous toluene (3x10 mL) and kept under high vacuum for 30 min. The mixture was dissolved in $CH_2Cl_2$ (1.8 mL) and stirred over activated molecular sieves (4 Å-AW) for 1 h at room temperature. The solution was then cooled to -40 °C and treated with TMSOTf (56 µL, 0.309 mmol in 0.2 mL dry $CH_2Cl_2$). The mixture was slowly warmed to 0 °C (2 h), quenched with a 1:1 (v/v) mixture of MeOH and $Et_3N$ (0.5 mL), diluted with $CH_2Cl_2$ (20 mL), filtered through Celite and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:3 to 1:1) to give the intermediate α-glycoside (55 mg, 0.096 mmol, 34%) along with the corresponding β-glycoside (22 mg, 0.039 mmol, 14%).

To a stirred solution of the intermediate α-glycoside (40 mg, 0.070 mmol) in anhydrous pyridine (0.4 mL) was added at 0 °C thioacetic acid (0.4 mL). The mixture was warmed to room temperature and stirred for 24 h at that temperature. The solution was co-evaporated with toluene (2x5 mL) and the residue was purified by flash chromatography (EtOAc/hexanes 1:3 to acetone/hexanes 1:2 to 2:3) to give acetamide **S2** (31 mg, 0.053 mmol, 76%) as a white solid. $R_f$ (acetone/hexanes 2:3) = 0.43; $[\alpha]_D^{20}$ = +109.5° (c = 0.50, acetone); $^1$H NMR (400 MHz, $CD_3OD$) δ 7.46 – 7.14 (m, 10H, arom.), 5.18 (d, *J* = 12.6 Hz, 1H, A of AB, $PhCH_2$), 5.07 – 4.99 (m, 2H, H-2, B of AB, $PhCH_2$), 4.75 (d, *J* = 3.8 Hz, 1H, H-1), 4.30 (dd, *J* = 11.7, 3.8 Hz, 1H, H-3), 4.23 – 4.16 (m, 1H, H-5), 4.11 – 4.04 (m, 1H, H-4), 3.77 (s,

2H, $PhCH_2$), 3.75 – 3.67 (m, 1H, A of AB, O-C*H*$_2$-$CH_2$), 3.53 (m, 1H, B of AB, O-C*H*$_2$-$CH_2$), 2.71 – 2.50 (m, 4H, Lev-$CH_2$, $CH_2$-C*H*$_2$-S), 2.42 – 2.18 (m, 2H, Lev-$CH_2$), 2.09 (s, 3H, Lev-$CH_3$ or Ac-$CH_3$), 1.93 (s, 3H, Lev-$CH_3$ or Ac-$CH_3$), 1.11 (d, *J* = 6.5 Hz, 3H, H-6); $^{13}$C NMR (100 MHz, $CD_3OD$) δ 209.3, 173.8, 173.5, 159.5, 140.0, 138.6, 1230.0, 129.52, 129.48, 129.0, 128.8, 128.1, 99.0, 71.5, 68.4, 67.5, 65.8, 54.0, 38.5, 37.2, 31.9, 29.7, 29.1, 22.7, 16.9; IR (thin film) 3322, 2930, 1718, 1667, 1533, 1423, 1244, 1158, 1122, 1050, 699 cm$^{-1}$; HRMS (ESI) calcd for $C_{30}H_{38}N_2O_8S$ $(M+Na)^+$ 609.2246 found 609.2256 *m/z*.

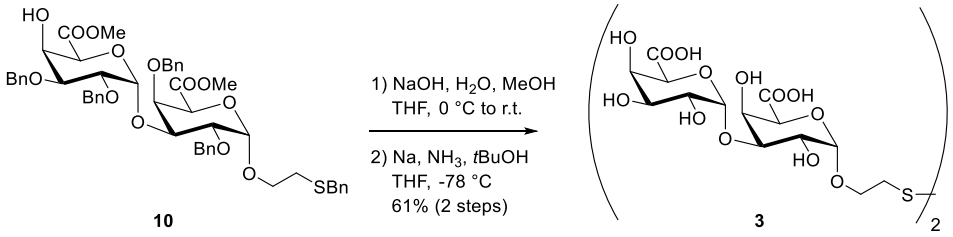
**2,2'-Dithiobis[2-acetamido-4-amino-2,4,6-trideoxy-α-D-galactopyranosyl-(1→1)-1-ethanol] (S3)**




To a stirred solution of ester **S2** (20.7 mg, 0.035 mmol) in anhydrous $CH_2Cl_2$ (3.0 mL) were added at room temperature first a mixture of pyridine (86 µL, 1.058 mmol) and acetic acid (57 µL, 0.988 mmol), and then hydrazine hydrate (3.4 µL, 0.071 mmol). The mixture was stirred for 5 h at that temperature, diluted with EtOAc (2 mL), quenched with acetone (0.1 mL) and poured into water (10 mL). The aqueous phase was extracted with EtOAc (4x5 mL), the combined organic fractions were dried over $Na_2SO_4$ and concentrated. The residue was purified by flash chromatography (acetone/hexanes 1:1) to give the intermediate alcohol as a white solid.

To a stirred solution of liquid ammonia (6 mL) was added at -78 °C a solution of the intermediate alcohol in THF (1.5 mL). The mixture was treated with *t*BuOH (0.5 mL) and lumps of freshly cut sodium (45 mg) were added until a deeply blue color persisted. The reaction was stirred at -78 °C for 45 min and quenched by addition of solid ammonium acetate (100 mg). The solution was warmed to room temperature under a stream of argon and co-evaporated with MeOH (2x10 mL) and water (2x5 mL). The residue was left under air for 16 h, purified by size exclusion chromatography (Sephadex G-25, 1:10 MeOH/5 mM aq. $NH_4OAc$) and lyophilized repeatedly to give disulfide **S3** (acetate salt, 7.91 mg, 12.3 µmol, 70% over two steps) as a white solid. $[\alpha]_D^{20}$ = +130.9° (c = 0.11, $H_2O$); $^1$H NMR (400 MHz, $D_2O$) δ 5.09 (d, *J* = 3.9 Hz, 1H, H-1), 4.56 (q, *J* = 6.6 Hz, 1H, H-5), 4.36 (dd, *J* = 11.2, 4.4 Hz, 1H, H-3), 4.13 (m, 2H, H-2, A of AB, O-C*H*$_2$-$CH_2$), 3.95 (m, 1H, B of AB, O-C*H*$_2$-$CH_2$), 3.75 (d, *J* = 4.1 Hz, 1H, H-4), 3.11 (t, *J* = 5.7 Hz, 2H, $CH_2$-C*H*$_2$-S), 2.19 (s, 3H, Ac-$CH_3$), 1.45 (d, *J* = 6.7 Hz, 3H, H-6); $^{13}$C NMR (100 MHz, $D_2O$) δ 181.3, 174.7, 96.9, 65.7, 64.5, 63.0, 55.1, 49.4, 37.1, 23.2, 22.0, 15.6; HRMS (ESI) calcd. for $C_{20}H_{38}N_4O_8S_2$ $(M+Na)^+$ 549.2029 found 549.2086 *m/z*.

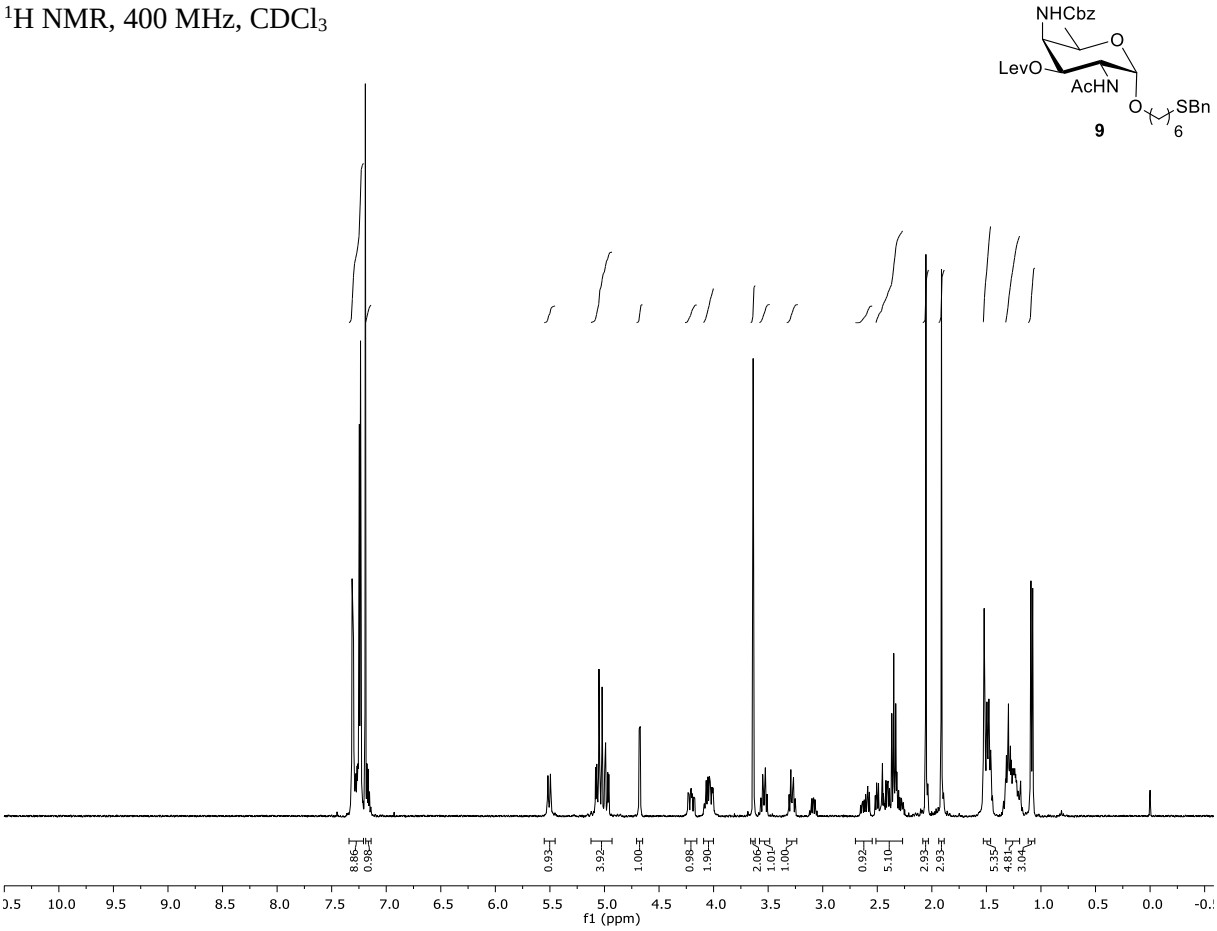
**2,2'-Dithiobis[α-D-galactopyranosyluronate-(1→3)-α-D-galactopyranosyluronate-(1→1)-1-ethanol] (3)**



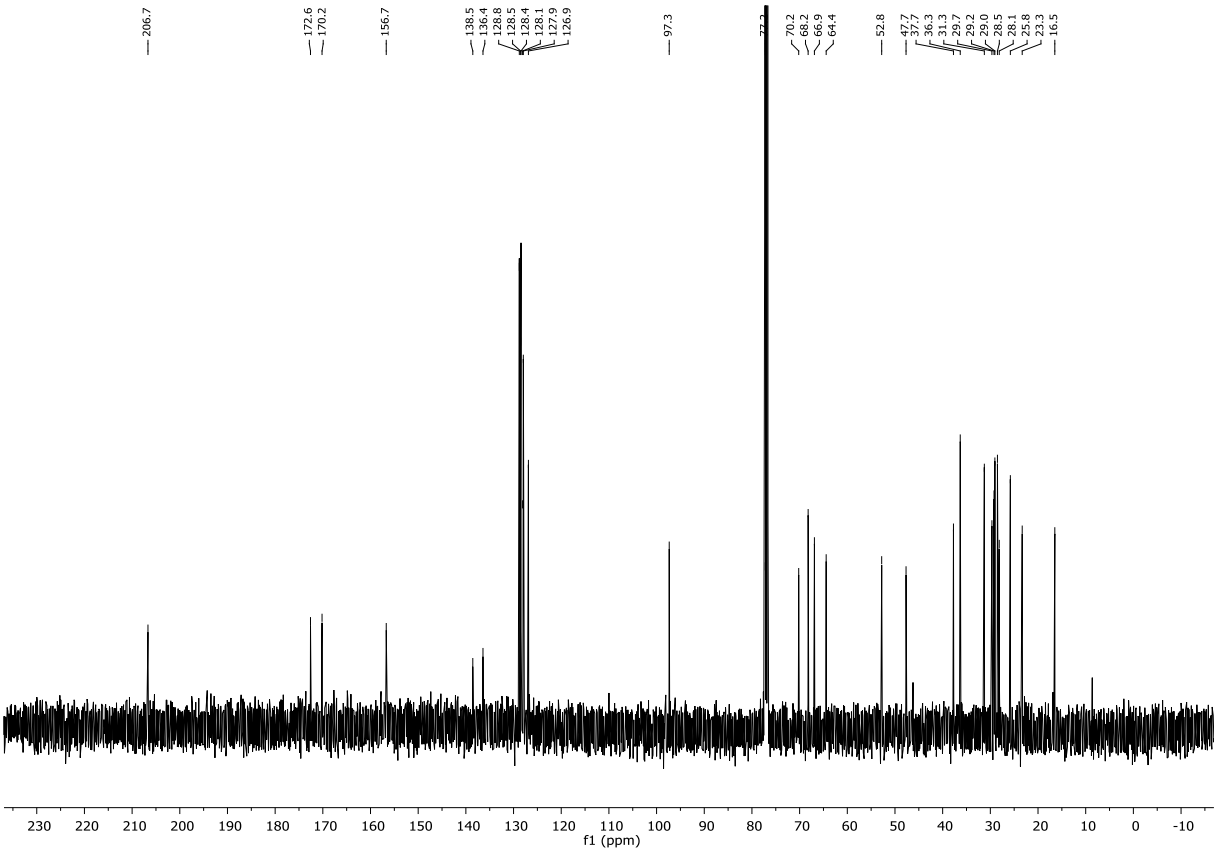

To a stirred solution of ester **10**[1] (8.6 mg, 9.5 µmol) in THF (0.6 mL) and MeOH (0.3 mL) was added at 0 °C a 1 M solution of NaOH in water (0.5 mL). The reaction was slowly warmed to room temperature and stirred for 16 h at that temperature. The reaction was diluted with EtOAc (5 mL) and water (5 mL) and acidified to pH 4 with 0.5 M aq. $NaHSO_4$. After separation, the aqueous fraction was extracted with EtOAc (8x5 mL), the combined organic fractions were dried over $Na_2SO_4$ and concentrated to give the intermediate diacid as a white solid.

To a stirred solution of liquid ammonia (6 mL) was added at -78 °C a solution of the crude diacid in THF (1.5 mL). The mixture was treated with *t*BuOH (0.4 mL) and lumps of freshly cut sodium (75 mg) were added until a deeply blue color persisted. The reaction was stirred at -78 °C for 45 min and quenched by addition of solid ammonium acetate (100 mg). The solution was warmed to room temperature under a stream of argon and co-evaporated with MeOH (2x10 mL) and water (2x5 mL). The residue was left under air for 16 h, purified by size exclusion chromatography (Sephadex G-25, 1:9 MeOH/5 mM aq. $NH_4OAc$) and lyophilized repeatedly to give disulfide **3** (2.5 mg, 2.9 µmol, 61% over two steps) as a white solid. $[\alpha]_D^{20}$ = +21.4° (c = 0.10, $H_2O$); $^1$H NMR (600 MHz, $D_2O$) δ 5.24 (s, 1H, H-1'), 5.05 (s, 1H, H-1), 4.63 (s, 1H, H-5'), 4.53 (s, 1H, H-4), 4.47 (s, 1H, H-4'), 4.33 (s, 1H, H-5), 4.13 – 3.80 (m, 6H, H-2, H-2', H-3, H-3', O-C*H*$_2$-CH$_2$), 3.05 – 2.92 (m, 2H, CH$_2$-C*H*$_2$-S); $^{13}$C NMR (150 MHz, $D_2O$) δ 113.0, 101.1, 98.7, 78.0, 74.4, 73.3, 71.9, 70.5, 70.2, 69.1, 68.7, 40.1; HRMS (MALDI) calcd for $C_{28}H_{42}O_{26}S_2$ (M-H$^+$) 901.0966 found 901.0981 *m*/*z*.

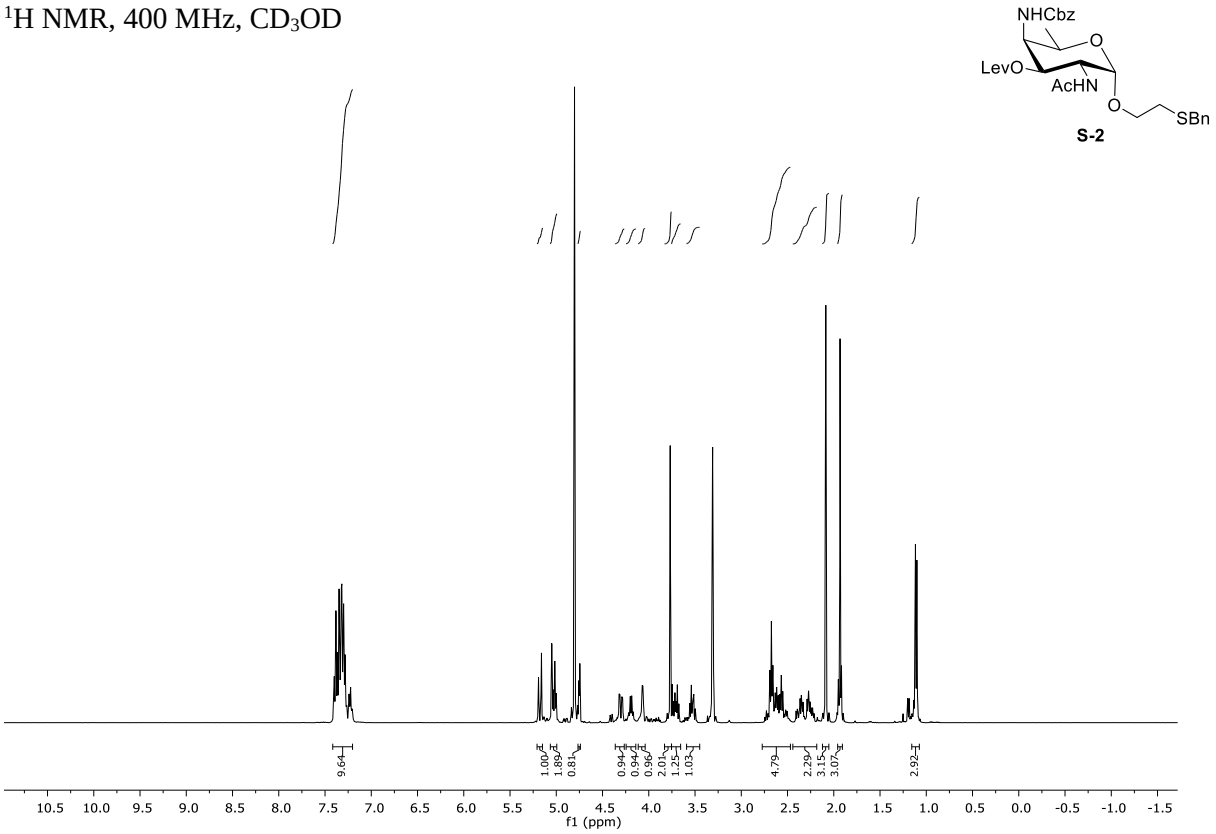
$^{1}$H NMR, 400 MHz, $CDCl_3$



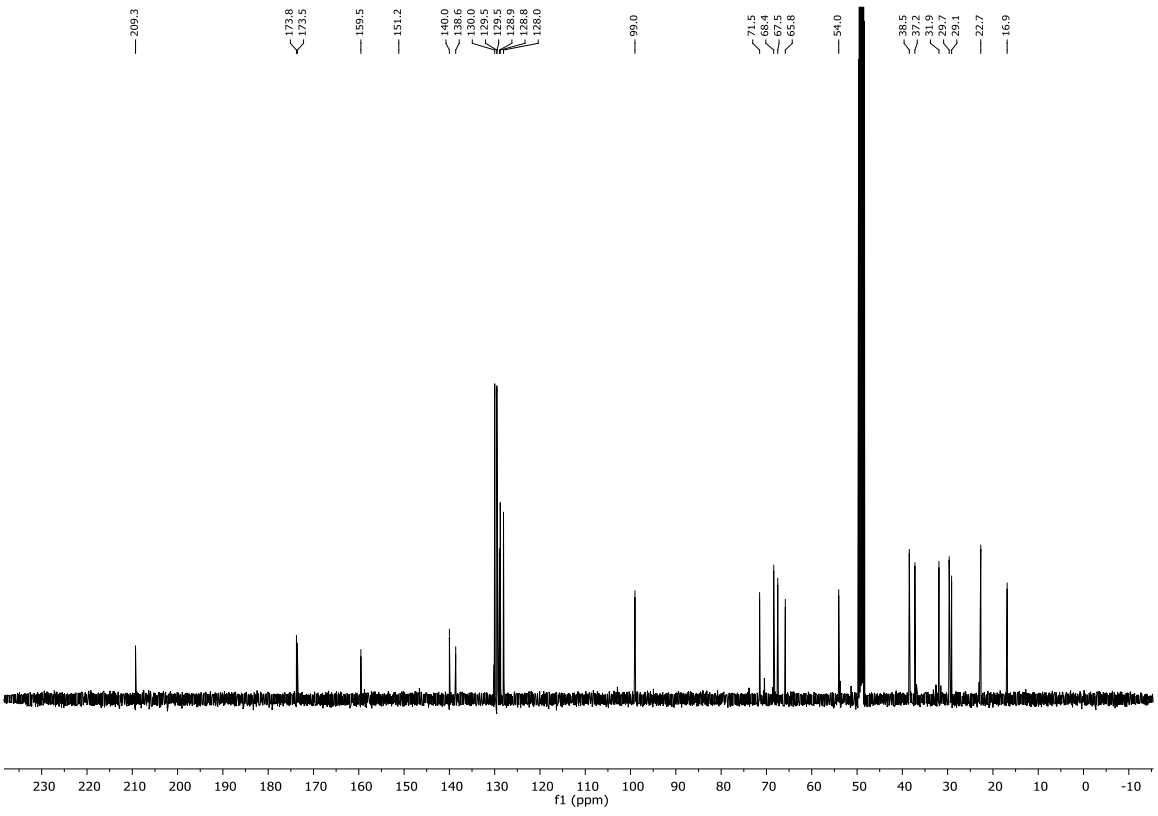

$^{13}$C NMR, 100 MHz, $CDCl_3$



$^{1}$H NMR, 400 MHz, $CD_3OD$




$^{13}$C NMR, 100 MHz, $CD_3O$



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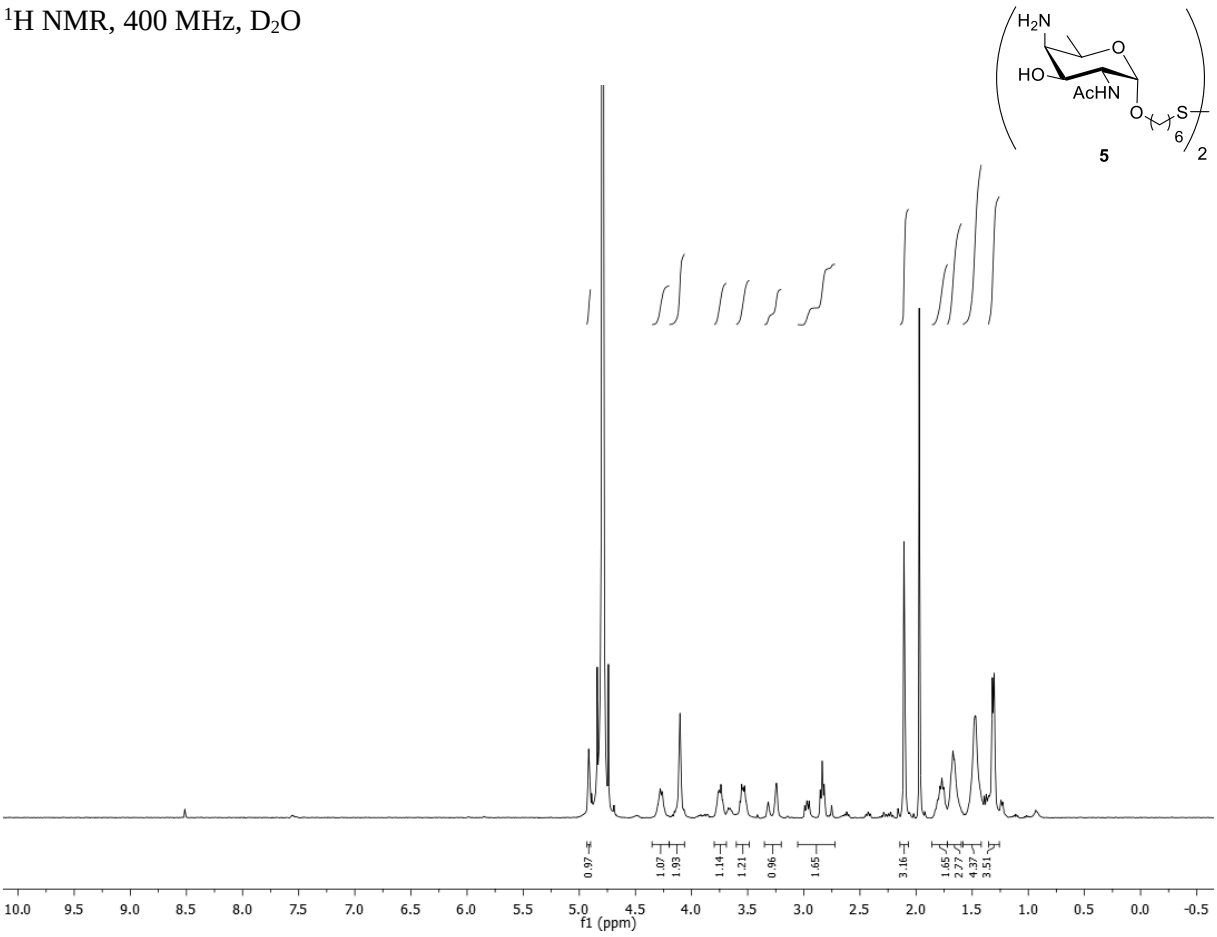
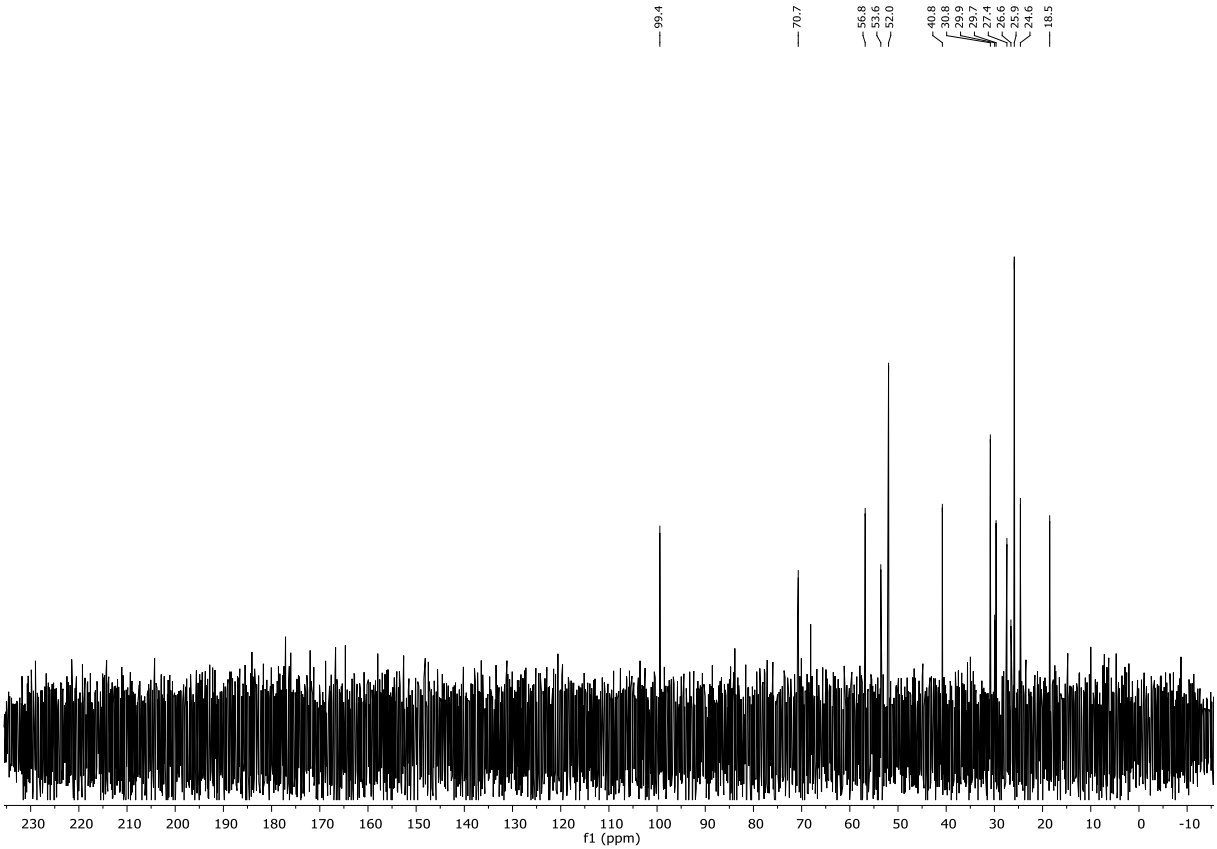
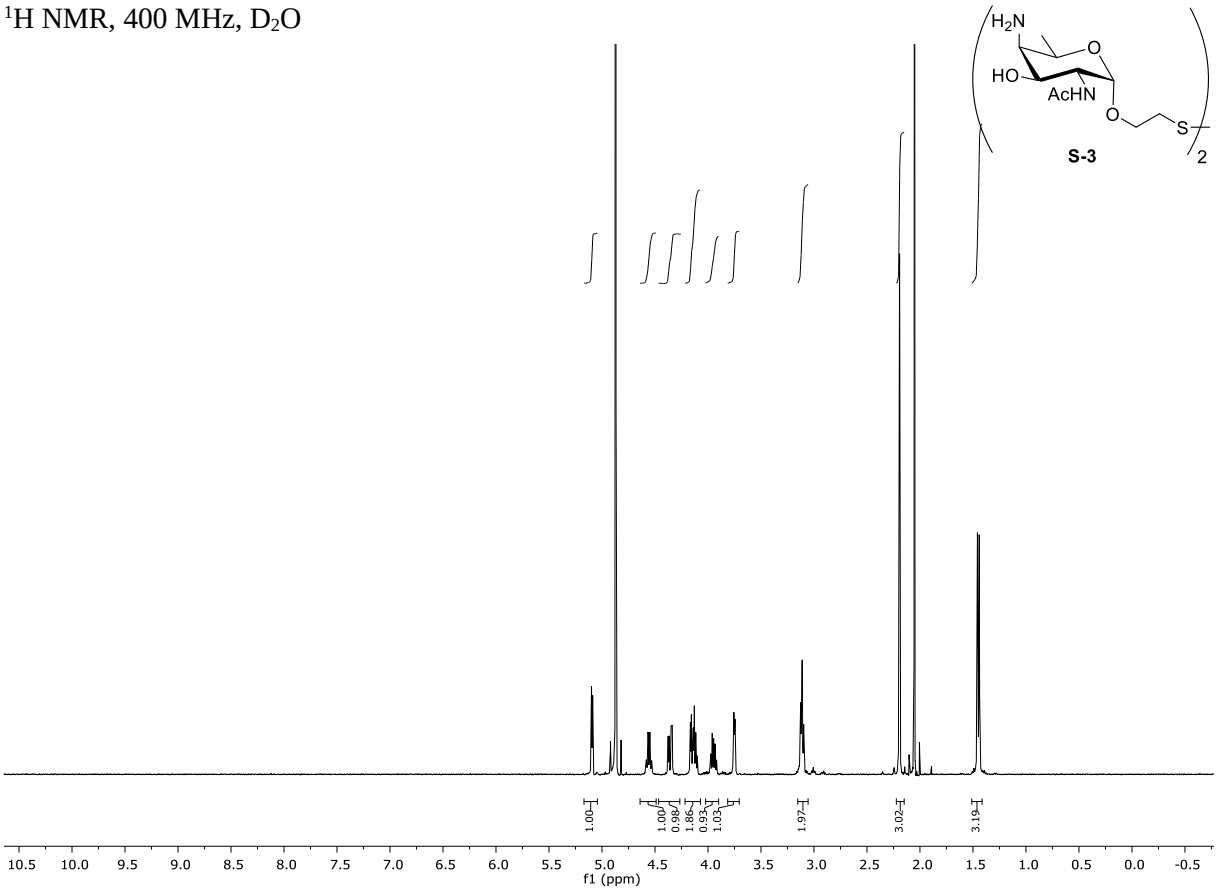


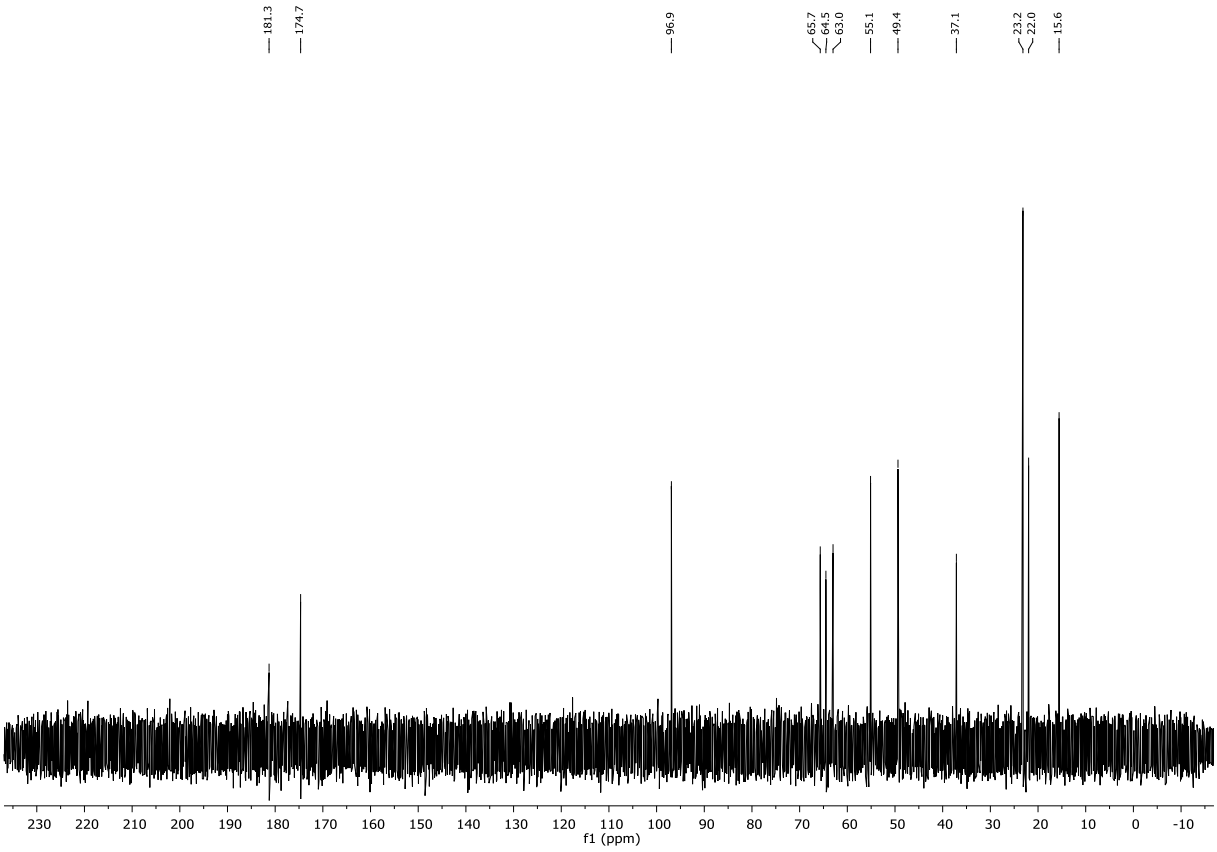

$^{13}$C NMR, 100 MHz, $D_2O$



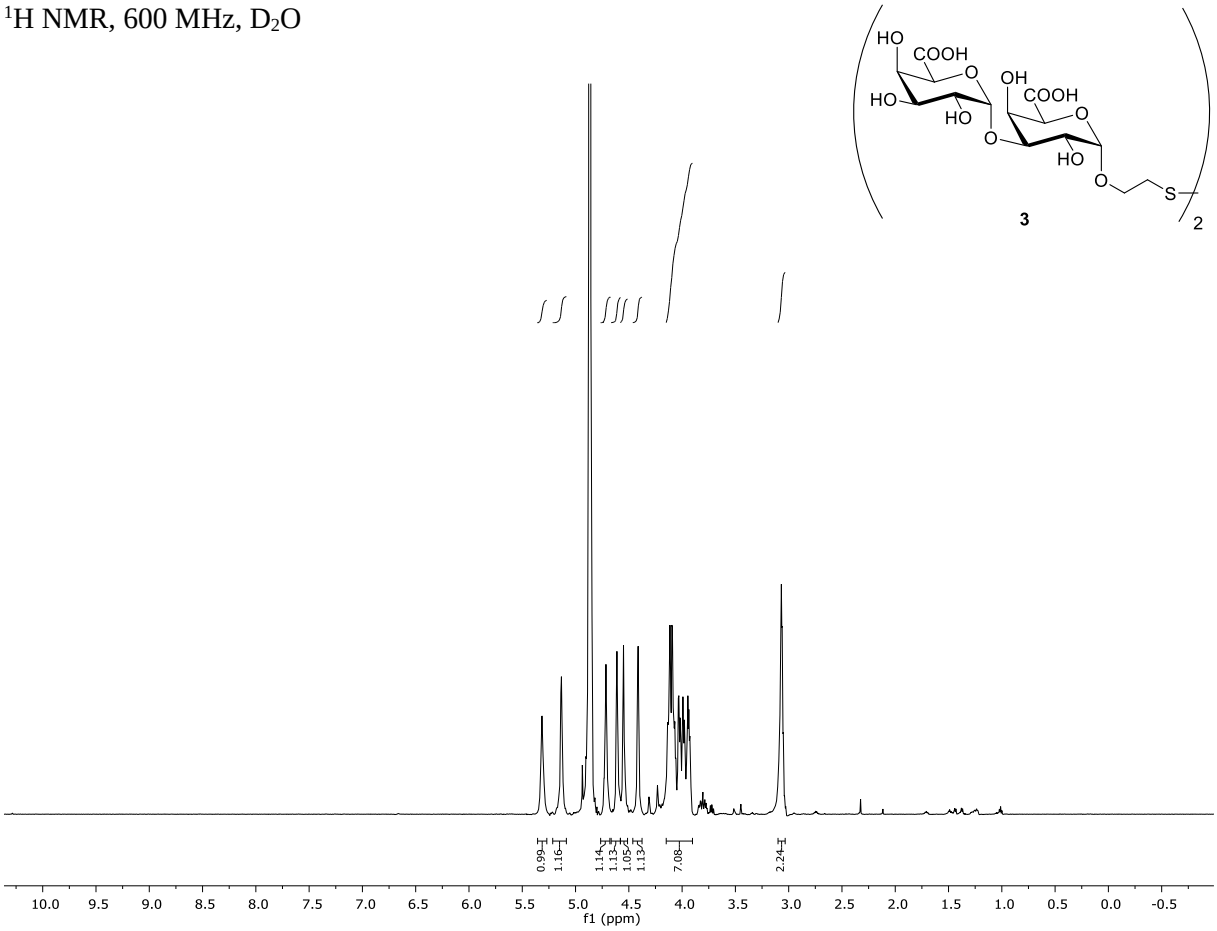
$^{1}$H NMR, 400 MHz, $D_2O$



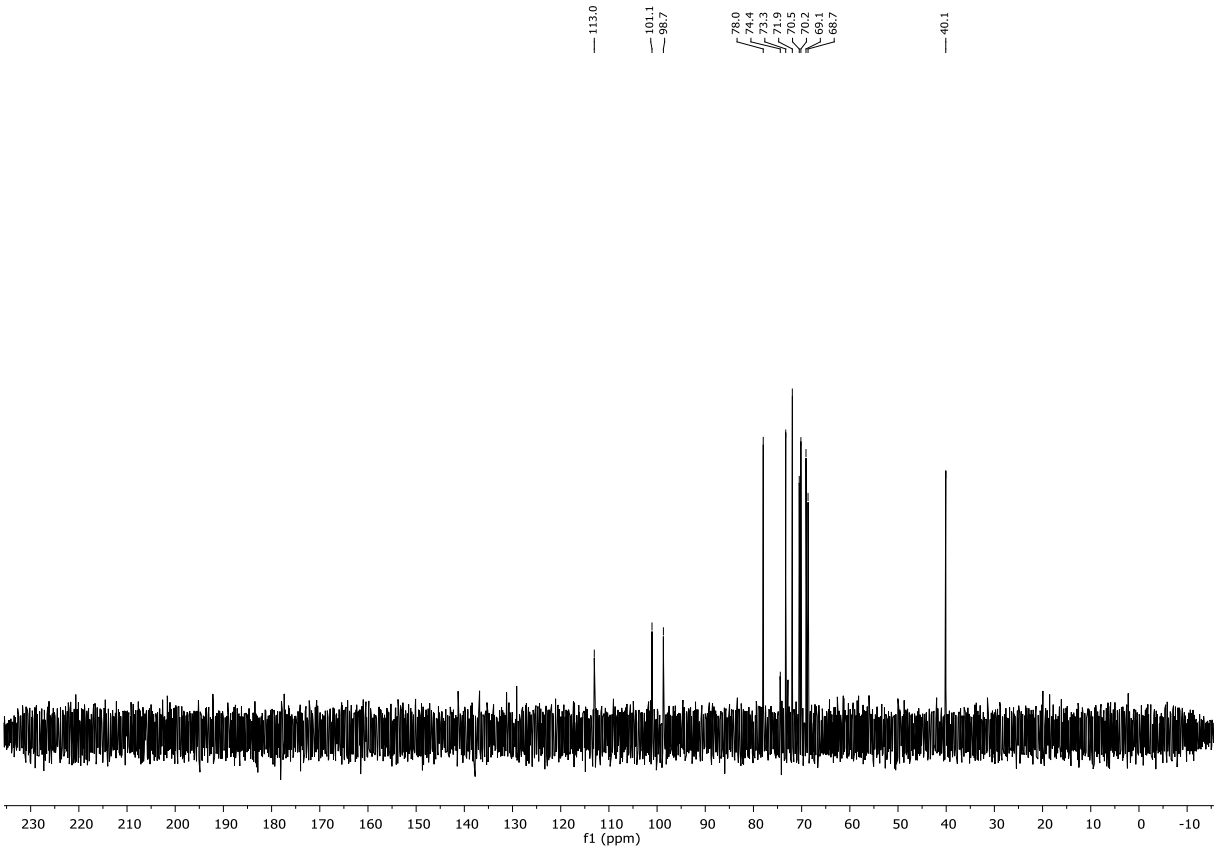

$^{13}$C NMR, 100 MHz, $D_2O$



$^{1}$H NMR, 600 MHz, $D_2O$




$^{13}$C NMR, 150 MHz, $D_2O$



**Polysaccharides, Sera, Carrier Protein and Bacteria**

Pneumococcal ST1 CPS, serotype 22F (ST22F) CPS, pneumococcal cell wall polysaccharide (CWPS), and ST1 typing serum (Type 1 Neufeld antiserum) were purchased from SSI Diagnostica (Hillerod, Denmark). A rabbit anti-*B. fragilis* serum was a kind gift from Dennis Kasper (Harvard Medical School, United States). CRM197 was purchased from Pfenex (San Diego, United States).

*S. pneumoniae* serotype 1 (ATCC 6301) was a gift from Sven Hammerschmidt (Universität Greifswald), maintained in growth medium (Todd Hewitt Broth with 0.5% (w/v) yeast extract and 10% FCS) at 37 °C/5% $CO_2$ and preserved in freezing medium (growth medium with 20% (v/v) glycerol) at -80 °C.

**Glycan Microarray Binding Experiments**

Glycan microarray slides (3D-Maleimide) were purchased from Poly-An (Berlin, Germany). Glycan disulfides (1 mM) or proteins (1 µM) in phosphate-buffered saline (PBS) were reduced by adding 1 mM tris(2-carboxyethyl)phosphine (from a 100 mM aq. stock solution pH 7.5-8) and spotted onto the microarray slides using an automatic piezoelectric arraying robot (Scienion, Berlin, Germany) at 0.2 nL per spot. Slides were incubated in a humidified chamber for 24 h at room temperature and quenched in a 0.2% (v/v) solution of 2-mercaptoethanol in PBS for 1 h at room temperature. Slides were washed with water (3x) and MeOH (3x), dried and stored under argon until use. Immediately before use, slides were blocked with a 1% (w/v) bovine serum albumin (BSA) solution in PBS (blocking buffer) for 1 h at room temperature, washed with water (3x) and MeOH (3x) and dried. A 64-well gasket (FlexWell 64, Grace Bio-Labs, Bend, United States) was appended and antisera were applied in the depicted dilutions. Dilutions of ST1 typing serum contained 10 µg/mL pneumococcal cell wall polysaccharide (CWPS), and dilutions of sera from rabbits immunized herein contained a mixture of 5 µg/mL CWPS and 5 µg/mL ST22F CPS to reduce non-specific binding.[3] Samples were incubated for 30 min at room temperature prior to application. Microarray slides were incubated with serum samples overnight at 4 °C, washed with washing buffer (0.1% (v/v) Tween 20 in PBS, 3x) and incubated with secondary antibody (goat anti-rabbit IgG-fluorescein isothiocyanate (FITC) conjugate ab6717 (abcam, Cambridge, UK), 1:200 in blocking solution, or goat anti-mouse IgG-FITC conjugate F9137 (Sigma-Aldrich, St. Louis, United States), 1:400 in blocking solution) for 2 h at room temperature. The slides were washed with washing buffer (3x) and water (3x) and dried. Fluorescence readout was performed using an Axon GenePix 4300A microarray scanner and GenePix Pro 7 software (both MDS, Sunnyvale, US). Samples to be compared (e.g. all sera from immunized rabbits) were measured on one slide, and brightness and contrast were adjusted equally for all samples on the same slide using Photoshop CS5 (Adobe, San Jose, United States).

**Conjugation of Trisaccharide 1 to CRM197**

A solution of *N*-succinimidyl-3-(bromoacetamido)propionate (SBAP) (1.05 mg, 3.4 µmol) in DMF (40 µL) was added to a stirred solution of CRM197 (2 mg, 34.5 nmol) in 0.1 M sodium phosphate buffer (NaPi) pH 7.4 (1.33 mL) at room temperature. The mixture was stirred for 1 h and dialyzed using a

centrifugal filter (10 kDa MWCO, Millipore, Darmstadt, Germany). The protein solution was diluted to 4 mL with autoclaved water and concentrated again. Washing was repeated three times and the solution was diluted to 0.5 mL using autoclaved water. 20 µL were taken for analysis, and the protein solution was re-buffered to 0.1 M NaPi pH 8.0 (0.5 mL) using membrane filtration. Trisaccharide disulfide **1** (1.44 mg, 2.33 µmol resp. to the monomer) in 0.1 M NaPi pH 8.0 (0.2 mL) was treated at room temperature with tris(2-carboxyethyl)phosphine (TCEP, 25 µL of a 100 mM stock solution, pH 7.4), left for 1 h at that temperature under an argon atmosphere and added to the solution of the activated protein. The mixture was stirred at room temperature for 16 h, and washed with autoclaved water using membrane filtration. Another analytical sample was taken, and the solution was re-buffered to 0.1 M NaPi pH 7.4 (0.5 mL). The glycoconjugate was then treated at room temperature with L-cysteine (625 µg, 5.1 µmol) in 100 µL autoclaved water. The mixture was left for 2 h and purified by membrane filtration. Incorporation of glycan into the glycoconjugate was assessed by matrix-assisted laser desorption/ionization mass spectrometry with time-of-flight detection (MALDI/TOF-MS). Of note, no substantial mass shift was observed between glycoconjugates before and after quenching with L-cysteine (not shown).

**Conjugation of Monosaccharide S3 to CRM197**

A solution of *N*-succinimidyl-3-(bromoacetamido)propionate (SBAP) (530 µg, 1.7 µmol) in DMF (40 µL) was added to a stirred solution of CRM197 (2 mg, 34.5 nmol) in 0.1 M NaPi pH 7.4 (1.33 mL) at room temperature. The mixture was stirred for 1 h at that temperature, and dialyzed using a centrifugal filter (10 kDa MWCO). The protein solution was diluted to 4 mL with sterile water and concentrated again. This process was repeated three times and the solution was diluted to 0.5 mL using autoclaved water. 20 µL were taken for analysis, and the protein solution was re-buffered to 0.1 M NaPi pH 7.4 (2 mL) using membrane filtration. Disulfide-containing D-AAT **S3** (336 µg acetate salt, 1.035 µmol resp. to the monomer) in 0.1 M NaPi pH 7.4 (15 µL) was treated at room temperature with TCEP (25 µL of a 100 mM stock solution), left for 1 h at that temperature under an argon atmosphere and added to the solution of the activated protein. The mixture was stirred at 4 °C for 16 h, and washed with autoclaved water using membrane filtration (see above). The solution was re-buffered to 0.1 M NaPi pH 7.4 (0.5 mL). The glycoconjugate was then treated at room temperature with L-cysteine (420 µg, 3.47 µmol) in 100 µl autoclaved water. The mixture was left for 1 h at that temperature and purified by membrane filtration. Incorporation of glycan into the glycoconjugate was assessed by MALDI/TOF-MS.

**Ethics Statement**

Rabbit immunization experiments were carried out by BioGenes GmbH (Berlin, Germany). Rabbits were handled and housed according to international animal regulations (EU Directive 2010/63/EU) and sanctioned by governmental authorities (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern).

Mouse experiments were approved by local institutional (Charité-Universitätsmedizin Berlin) and governmental authorities (Landesamt für Gesundheit und Soziales Berlin, approval ID A0305/12 and ID G0128/12). Animal housing and experiments were in strict accordance with the regulations of the Federation of European Laboratory Animal Science Associations (FELASA) and recommendations for the care and use of laboratory animals. Mice were housed under specific pathogen-free conditions in cages of 3 mice. During passive immunization and infection, cages contained one mouse of each group.

**Statistics**

Sample sizes were calculated using G*Power 3.1.9.2.[4] For active immunization experiments, a signal fold increase of 10 ± 2.5 in glycoconjugate-treated vs. 1 ± 2.5 in CRM197-treated animals in polysaccharide ELISA or glycan microarray was targeted,[5] resulting in an effect size of d = 3.6 with a = 0.05 and a power of 1 – β = 0.8. Thus, *n* = 3 (both one-tailed t test and one-tailed Mann-Whitney U test). Although little variation was observed, the sample size was too small to assess normal distribution. Thus, both non-parametric and parametric tests were conducted for statistical analyses of rabbit immunizations and the corresponding *P* values are given in the figures. For passive immunization experiments, the bacterial burden ($log_{10}$ cfu/µL blood) after infection was determined as 2 ± 1.2.[6] A reduction of 70% of this value was targeted by antisera treatments, resulting in an effect size of $f = 0.55$ with $\alpha = 0.05$ and a power of $1 - \beta = 0.8$, hence $n = 12$ (one-way ANOVA). Non-parametric tests were used since samples were generally not normal distributed (D'Agostino-Pearson omnibus normality test). Mice were kept in cages of three animals, and distribution of each mouse into one the three groups was performed at random. No blinding was performed. One mouse was excluded from the CRM197 control group (see below).

**Rabbit Immunization**

Rabbits (female Zika rabbits, 10-12 weeks, 2.5-3 kg, *n* = 3 per group) were immunized subcutaneously at days 0, 14 and 28 at four different sites with one human dose of Prevenar 13 or CRM197-**1** at an equivalent (2.2 µg) glycan dose in 200 µL PBS adsorbed to Alum adjuvant (Alhydrogel, Brenntag, 200 µL). Sera from rabbits treated with 100 µg CRM197 per dose under the same regimen were taken from a previous experiment.[5] Sera were collected at days 0, 14, 21 and 35.

**Mouse Immunization**

Mice (6-8 week old female C57BL/6J mice, Charles River, Sulzfeld, Germany) were immunized subcutaneously with the CRM197-**S3** glycoconjugate corresponding to 1.7 µg D-AAT saccharide **S3** and formulated either as a 1:1 (v/v) emulsion with Complete Freund's Adjuvant (CFA, Sigma-Aldrich) or without adjuvant at a total volume of 100 µL. Booster doses were given at days 14 and 28 using the same strategy. Mice primed with CFA received booster doses with Incomplete Freund's Adjuvant (Sigma-Aldrich). Blood (50 µL) was withdrawn once a week from the tail vein or the facial vein and centrifuged (5000 g, 10 min, room temperature) to retrieve serum.

**Enzyme-linked Immunosorbent Assay (ELISA)**

ELISA was performed on Costar® high-binding polystyrene 96-well plates (Corning, Corning, United States). Plates were coated using native ST1 CPS at a concentration of 10 µg/mL in PBS for 20 h at 4 °C. Plates were blocked with 10% (v/v) fetal calf serum in PBS for 2 h at 37 °C and washed once with washing buffer. Rabbit sera were serially diluted in blocking buffer containing 5 µg/mL CWPS and 5 µg/mL ST22F CPS as described above, and applied (30-50 µL) on the ELISA plates. Plates were incubated for 1 h at 37 °C, washed with PBS-T (3x) and treated with a goat anti-rabbit IgG (whole molecule)-peroxidase conjugate A6154 (Sigma-Aldrich) according to the manufacturer's specifications. Plates were washed with PBS-T (3x) and HRP activity was measured with TMB substrate (BD Biosciences, Franklin Lakes, United States) according to the manufacturer's instructions. Titers were determined as the highest dilution with an absorbance of 0.1 arbitrary units or higher.

**Flow Cytometry**

Bacteria were grown to log phase, harvested by centrifugation and washed once with PBS. Bacteria were harvested, suspended in PBS and inactivated by irradiation at $\lambda$ = 254 nm for 10 min. Cells were harvested, washed once with PBS and frozen in freezing medium at $10^8$-$10^9$ inactivated cfu/mL. Approx. $3x10^7$ inactivated cfu were harvested, washed with blocking buffer (300 µL) and incubated with pooled rabbit sera (1:100 dilution in blocking buffer, 300 µL) for 30 min at room temperature with agitation. Bacteria were harvested, washed once with blocking buffer and incubated with secondary antibody (goat anti-rabbit-fluorescein isothiocyanate (FITC) conjugate ab6717 (abcam), 1:100 in 200 µL blocking buffer) for 30 min at room temperature. Bacteria were harvested, washed once and analyzed by flow cytometry by counting 10,000 events using a FACSCanto II flow cytometer (BD Biosciences), and analyzed using FlowJo software (Flowjo LLC, Ashland, Uinted States).

**Opsonophagocytic Killing Assay**

Opsonophagocytic killing was assessed as described.[7] All antisera were incubated at 56 °C for 30 min to inactivate endogenous complement before use. HL-60 cells were a gift from Dr. Leif Sander (Charité Berlin, Germany) and tested for mycoplasma before use. These cells had been used for a different project,[5] where results were reproduced using a fresh HL-60 cell line from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (ACC-3) without any differences in opsonophagocytic capacity. HL-60 cells were differentiated with *N*,*N*-dimethylformamide for 6-7 days, washed twice with Hank's balanced sterile saline with 0.1% (w/v) gelatin (OPKA buffer) and diluted to a density of $10^7$ cells/mL in OPKA buffer directly prior to use. *Streptococcus pneumoniae* ST1 bacteria were grown to log phase in growth medium, diluted in freezing medium to a density of $10^6$ cfu/mL and frozen in 0.5 mL aliquots at -80 °C. Bacteria were thawed on ice, diluted with OPKA buffer and aliquoted (1000 cfu in 20 µL per well) in a 96 well-plate. Wells were treated with serial antiserum dilutions in triplicates and incubated for 15 min at 37 °C. Rabbit complement (10% (v/v) of the total volume, CedarLane, Burlington, Canada)

and differentiated HL-60 cells (40 µL cell suspension, phagocyte/bacteria ratio 400:1) were added and the plates were incubated for 45 min at 37 °C with shaking. Plates were placed on ice and an aliquot of each well was transferred to Columbia Agar plates with 5% (v/v) sheep blood (BD Biosciences). Colonies were counted after overnight incubation at 37 °C/5% $CO_2$. Control wells lacked either antibody or complement. Opsonophagocytic killing was calculated relative to wells lacking antiserum or both antiserum and complement (to assess agglutination). Samples were sonicated after opsonophagocytic killing for 2 min at 37 °C in a water bath sonicator (Grant Instruments, Royston, UK).

**Passive Immunization and Infection**

Rabbit sera were incubated at 56 °C for 30 min to inactivate complement before use, and sera of rabbits from the same group were pooled. ST1 bacteria were grown to log phase, harvested and suspended in sterile PBS.

Female NMRI mice (*n* = 11-12, 11 weeks, 31-37 g, Charles River) were treated i.p. with rabbit sera (100 µL) 2 h prior to infection. Mice were kept in cages of three animals, and distribution of each mouse into one the three groups was performed at random. Mice were anaesthetized by i.p. injection of ketamine (80 mg/kg, Ketavet, Pfizer, New York City, United States) and xylazine (25 mg/kg, Rompun, Bayer, Leverkusen, Germany) and subsequently t.n. inoculated with $4x10^6$ cfu ST1 in 20 µL PBS. Body weight and temperature were assessed at 12 h intervals. After 24 h, mice were anesthetized with ketamine (160 mg/kg body weight (BW)) and xylazine (75 mg/kg BW). After heparinization, blood was drawn from the *Vena cava caudalis*. Bronchoalveolar lavage (BAL) was performed twice using 800 µL PBS containing protease inhibitor each time (Roche, Mannheim, Germany). Lungs were flushed with sterile 0.9% saline *via* the pulmonary artery and were removed.

Serial dilutions of blood, lung and BAL fluid (BALF) samples were plated on Columbia agar plates with 5% (v/v) sheep blood and incubated at 37 °C under 5% $CO_2$ overnight to count cfu. One mouse in the CRM197 group was excluded from analysis as no bacterial load was observed in blood, lung or BALF, and no leukocyte recruitment was observed in BALF (not shown), indicating that infection was not successful. One mouse in the Prevenar 13 group deceased during anaesthesia.


1 Schumann, B., Pragani, R., Anish, C., Pereira, C. L. & Seeberger, P. H. Synthesis of conjugation-ready zwitterionic oligosaccharides by chemoselective thioglycoside activation. *Chem. Sci.* **5**, 1992-2002 (2014).

2 Salerni, O. L., Clark, R. N. & Smart, B. E. Synthesis of N-monosubstituted S-aminoethyl hydrogen thiosulphates with sulphone substituents. *J. Chem. Soc. C* **6**, 645-648 (1966).

3 Poolman, J. T., Frasch, C. E., Käythy, H., Lestrate, P., Madhi, S. A. & Henckaerts, I. Evaluation of Pneumococcal Polysaccharide Immunoassays Using a 22F Adsorption Step with Serum Samples from Infants Vaccinated with Conjugate Vaccines. *Clin. Vaccine Immunol.* **17**, 134-142 (2010).

4 Faul, F., Erdfelder, E., Lang, A. G. & Buchner, A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods* **39**, 175-191 (2007).

5 Schumann, B., Hahm, H. S., Parameswarapa, S. G., Reppe, K., Wahlbrink, A., Govindan, S., Kaplonek, P., Pirofski, L., Witzenrath, M., Anish, C., Pereira, C. L. & Seeberger, P. H. A Semisynthetic Streptococcus pneumoniae Serotype 8 Glycoconjugate Vaccine. *Sci. Transl. Med.* **9**, online (2017).

6 Parameswarappa, S. G., Reppe, K., Geissner, A., Menova, P., Govindan, S., Calow, A. D. J., Wahlbrink, A., Weishaupt, M. W., Monnanda, B. P., Bell, R. L., Pirofski, L., Suttorp, N., Sander, L. E., Witzenrath, M., Pereira, C. L., Anish, C. & Seeberger, P. H. A Semi-synthetic Oligosaccharide Conjugate Vaccine Candidate Confers Protection against Streptococcus pneumoniae Serotype 3 Infection. *Cell Chem. Biol.* **23**, 1407-1416 (2016).

7 Romero-Steiner, S., Libutti, D., Pais, L. B., Dykes, J., Anderson, P., Whitin, J. C., Keyserling, H. L. & Carlone, G. M. Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against Streptococcus pneumoniae using differentiated HL-60 cells. *Clin. Diagn. Lab. Immunol.* **4**, 415-422 (1997).