Supporting Information for

Biochemical Studies of a β-1,4-Rhamnoslytransferase from *Streptococcus pneumonia* Serotype 23F

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I. Materials and Methods

Bacterial strains, plasmids, and other materials. *S. pneumonia* serotype 23F was purchased from ATCC (700669); *E. coli* BL21(DE3) competent cells from Tiangen Biotech (Beijing) Co. Ltd.; expression plasmid pET-22b from Novagen (Carlsbad); LA DNA polymerase from Takara; DNA extraction, BCA protein quantitation and bacterial genomic DNA extraction kit; from Cwbiotech; T4 DNA ligase, restriction endonuclease and Q5 high-fidelity DNA polymerase from New England Biolabs; MES and inorganic pyrophosphatease YIPP from Sigma; Immobilon Western Chemiluminescent horseradish peroxidase (HRP) substrate from Millipore; Mueller-Hinton Broth and Todd Hewitt Broth from Becton-Dickinson; yeast extract, agar and tryptone from OXOID. All kits and enzymes were used according to manufacturers' instructions. dTDP-Rha was synthesized by a one-pot four enzyme method reported in the literature.[36] All other reagents and solvents were obtained from Sangonbiotech unless otherwise noted.

Cloning, expression, and purification of Cps23FT. The DNA sequence of complete *cps23FT* gene was derived from the Gene Bank (*cps23FT*: AAC38749.1) and employed to design primers (Table S1) used for polymerase chain reaction (PCR) amplifications. The primers were synthesized by Sangonbiotech. Chromosomal DNA (1 mg/mL) of *S. pneumonia* 23F was extracted from the bacterial strain ATCC 700669 using a bacterial genomic DNA extraction kit and utilized for PCR. PCRs were performed with a S1000^{TM} Thermal Cycler using 2.5 unit of LA Taq DNA polymerase, 10 μg/mL template DNA, 0.4 mM of each deoxynucleotide triphosphate (dNTP), and 0.4 μM of the corresponding synthetic nucleotide primer. Amplified DNA fragments were digested with appropriate restriction endonucleases, inserted in pET-22b vector linearized by the same restriction enzymes to form pET-22b-cps23FT recombinant plasmids, and subsequently transformed into the *E. coli* BL21(DE3) competent cell. The transformants were selected on Luria-Bertani/Ampicillin (LB/Amp) plates. Plasmid DNA sequencing was performed by Sangonbiotech Shanghai Co. Ltd.

E. coli BL21(DE3) cells harboring recombinant plasmids were incubated in 1 L of LB/Amp at 37 °C until to the midlog phase ($OD_{600} \approx$ 0.6), at which point isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added to reach the final concentration of 0.5 mM. Cell growth was continued for an additional 10 h (25 °C, 200 r/min), and then the cells were collected by centrifugation (10 min, 8000 g/min) and suspended in 100 mL of 50 mM Tris-HCl (containing 300 mM NaCl) at pH 8.0 on ice. The cells were lysed via sonication (VCX800, Sonics) on ice for 15 min (5 s each time with 10 s pause interval) using a numerical control ultrasonicator at 70% power.

The resulting supernatant was loaded onto a Ni^{+} affinity column (GE Healthcare) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 0.3 M NaCl. The His-tagged Cps23FT was eluted with the same buffer containing 0.5 M imidazole. After concentration, the elution was loaded on a Q anion exchange column (GE Q Sepharose Fast Flow) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) for further purification. The target protein was eluted with the same buffer containing 200 mM NaCl. Finally, Cps23FT-containing fractions were combined, condensed, and desalted using an Amicon Ultra 10-kDa centrifugal filter (Millipore). The retained protein was washed twice with 50 mM Tris-HCl buffer (pH 7.5, 2 × 10 mL) containing 10% glycerol (v/v), suspended in 2 mL of the same buffer, and stored at -80 °C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (anti-His-tag rabbit polyclonal antibody staining) were performed by standard protocols, and enzyme concentrations were assessed with a BCA protein quantitation kit following the manufacturer's instruction.

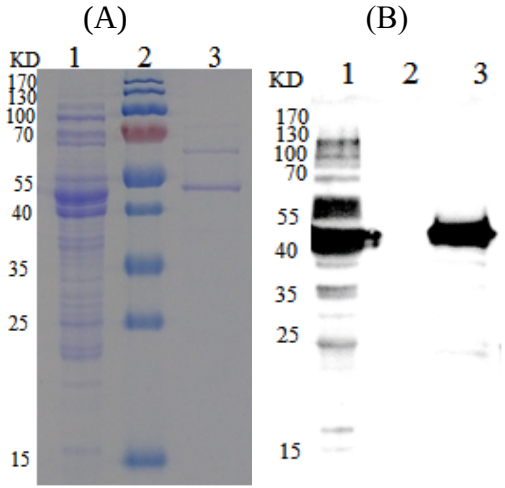


Figure S1. SDS-PAGE (A) and western blot stained with anti-His_6 tag rabbit polyclonal antibody (B) results of Cps23FT-His_6 after purification with a Ni-column (lane 1) or with both a Q-column and a Ni-column (lane 3), as well as molecular markers (lane 2).

Activity assays of Cps23FT. To a MES solution (50 mM, pH 7.5, 50 μL) of 10 mM $MnCl_2$, 0.5 mM dTDP-Rha and 1 mM **3** was added 400 μg/mL of purified Cps23FT. The reaction was allowed to continue at 16 °C for 1 h and then analyzed with LC-ESI-HRMS using a Shimadzu LCMS-IT-TOF mass spectrometer (HPLC condition: 4.6 × 250 mm C18 column; 10–100% methanol in water containing 10 mM NH_4HCO_3 as gradient eluent).

Investigating the influences of pH, temperature and metal cations on the activity of Cps23FT. The reactions were carried out in MES buffer (50 mM, 50 μL) containing varied concentration of $MnCl_2$ and 0.5 mM dTDP-Rha, 1 mM **3**, and 50 μg/mL of Cps23FT at varied pH values (3～8). The reaction mixture was incubated at varied temperature (5 °C, 10 °C, 16 °C, 25 °C, 37 °C and 42 °C) for 10 min and terminated by boiling for 30 s. After centrifugation at 15,000 g for 30 min, the reaction was analyzed with HPLC (DionexCarboPac^{TM} PA-100 4 × 250 mm column, 0～1 M ammonium acetate buffer eluent). Negative controls were performed in parallel under the same conditions using heat-deactivated Cps23FT. The reactions were monitored through the generation of dTDP by HPLC, and the conversion was calculated by the ratio between dTDP peak area and the total peak areas of dTDP-Rha, dTDP and dTMP (Figure 20S).

In the study of pH influence on the enzymatic activity of Cps23FT, the concentrations of Mn^{2+}, dTDP-Rha, **3** and Cps23FT were 10, 0.5 and 1 mM and 50 μg/mL, respectively, and the reaction was carried out at 37 °C for 10 min. In the study of temperature influence on the enzymatic activity, the buffer pH value was 7.5 and the concentrations of Mn^{2+}, dTDP-Rha, **3** and Cps23FT were 10, 0.5 and 1 mM and 50 μg/mL, respectively. To evaluate the influence of metal ions on the activity of Cps23FT, the reaction was carried out in MES buffer (pH 7.5) containing 0.5 mM dTDP-Rha, 1 mM **3**, 50 μg/mL of Cps23FT, and 5 mM of ethylenediamine tetraacetic acid (EDTA), Na^+, K^+, Zn^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+} or Ni^{2+}, and the reaction mixture was incubated at 37 °C for 10 min before quenching. To optimize the Mn^{2+} concentration for Cps23FT, the reaction was carried out under the same conditions using varied concentrations of Mn^{2+} (0.125～64 mM).

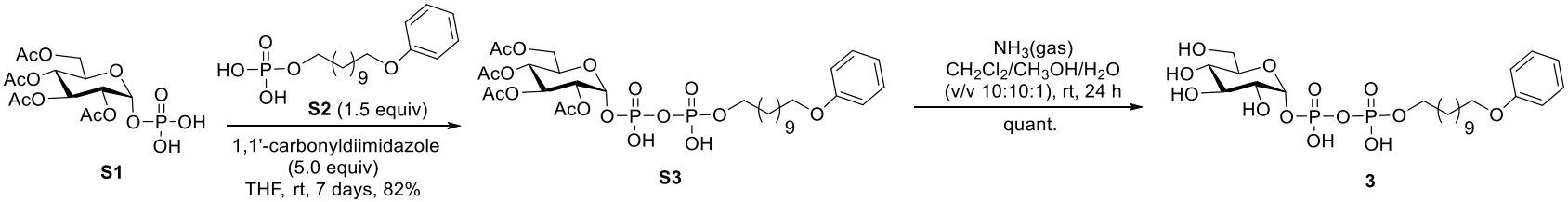
Enzyme kinetics of Cps23FT. The enzymatic reactions were carried out under above-optimized conditions, i.e., in MES buffer (50 mM, pH 7.5) containing 8 mM $MnCl_2$ and varied concentrations dTDP-Rha and **3** at 16 °C. The reaction was kept at these conditions for 10

min before quenching by boiling for 30 s. After centrifugation at 15,000 g for 30 min, the reaction was analyzed by HPLC using above-mentioned column and eluent. Negative controls were performed in parallel under the same conditions using heat-deactivated Cps23FT. Proper enzyme concentrations and reaction time were determined first using 0.5 mM dTDP-Rha, 1 mM **3**, and 25, 50, 100, 200 and 400 μg/mL of Cps23FT for incubation at 16 °C for 5, 10, 20, 40 and 80 min, respectively. Then, reactions using saturated dTDP-Rha (0.5 mM) and varied concentrations of **3** (0.1～6.4 mM) or saturated **3** (2 mM) and varied concentrations of dTDP-Rha (0.125～2.0 mM) for 10 min were performed. The results obtained were used to calculate the initial reaction velocities and determine the Michaelis constant (K_m) and maximal velocity (V_{max}) values using the GraphPad Prism 6.04 program.

Site-directed mutagenesis of Cps23FT. PCR reaction mixtures containing 2.5 unit of Q5 high-fidelity DNA polymerase, 10 μg/mL of pET-22b-Cps23FT recombinant plasmid, 0.4 mM dNTP, and 0.4 μM of the corresponding synthetic nucleotide primer were incubated using a S1000^{TM} Thermal Cycler. PCR products were digested by DpnI (New England BioLabs) and transformed into *E. coli* BL21(DE3) competent cell. Individual progeny plasmids were confirmed by DNA sequencing to carry the desired mutations. The Cps23FT mutants were expressed, purified, and applied to in vitro activity assays as described above for Cps23FT.

II. Synthesis of Glc*α*-PP-$(CH_2)_{11}$-OPh (3) and Rha*β*-Glc*α*-PP-C11-OPh (4)

Scheme S1. Synthetic procedure for compound **3**



Chemical synthesis of compound 3: A mixture of 11-phenoxyundecyl dihydrogen phosphate **S2**[1] (150 mg, 436 μmol) and 1,10-carbonyldiimidazole (282 mg, 1.74 mol) in 5 mL of dry THF was stirred at rt for 4 h, and then dry methanol (0.5 mL) was added. The resulting solution was stirred for another 2 h, and then concentrated under reduced pressure. After the residue was dried on a high vacuum

line for 1 h (no bubbling), a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-1-phosphate **S1**[2] (124 mg, 291 μmol) in dry THF (1 mL) was added. The reaction mixture was stirred at rt for one week, and then evaporated. The crude product was purified by column chromatography (CH_2Cl_2:CH_3OH = 4:1) to give $Ac_4Glc\alpha$-PP-$CH_2)_{11}$-OPh (**S3**) (180 mg, 82%) as white solid. ^{1}H NMR (600 MHz, CD_3OD): δ 7.23 (t, 2H, J = 7.8 Hz, Ph), 6.90-6.84 (m, 3H, Ph), 5.78 (dd, 1H, J = 7.8, 3.6 Hz, H-1^{Glc}), 5.51 (t, 1H, J = 9.6 Hz, H-3^{Glc}), 5.10 (t, 1H, J = 9.6 Hz, H-4^{Glc}), 4.85 (H-2^{Glc} overlapped by D_2O), 4.45 (td, 1H, J = 10.2, 3.0 Hz, H-5^{Glc}), 4.35 (dd, 1H, J = 12.6, 2.4 Hz, H-6a^{Glc}), 4.19 (dd, 1H, J = 12.6, 2.4 Hz, H-6b^{Glc}), 3.99-3.92 (m, 4H, -OC*H*$_2$CH$_2$-), 2.07, 2.03, 1.98, 1.96 (4 s, 4 × 3H, 4 × C*H*$_3$CO), 1.75 (m, 2H, -C*H*$_2$CH$_2$-), 1.64 (m, 2H, -C*H*$_2$CH$_2$-), 1.46 (m, 2H, -C*H*$_2$CH$_2$-), 1.41-1.28 (m, 12H, -C*H*$_2$CH$_2$-); ^{13}C NMR (150 MHz, CD_3OD): δ 171.02, 170.49, 170.10, 169.92 (4 C, 4 × CH_3*C*O), 159.15, 128.93 (2C), 119.99, 114.04 (2C), 92.06 (C-1^{Glc}), 70.43 (C-2^{Glc}), 70.16 (C-3^{Glc}), 68.04 (2C, C-4,5^{Glc}), 67.41 (-O*C*H$_2$CH$_2$-), 65.79 (-O*C*H$_2$CH$_2$-), 61.29 (C-6^{Glc}), 30.44, 30.39, 29.33, 29.28, 29.16, 29.13, 29.02, 25.77, 25.49 (9 C, -*C*H$_2$CH$_2$-), 19.33, 19.26, 19.18, 19.16 (4 C, 4 × *C*H$_3$CO). Ammonia was bubbled into a solution of **S3** (20 mg, 2.7 μmol) in CH_2Cl_2-CH_3OH-H_2O (5.2 mL, v/v/v 10:10:1) at rt until saturation. The mixture was then kept at rt for 24 h and then evaporated to dryness, giving **3** without further purification. ^{1}H NMR (600 MHz, CD_3OD): δ 7.22 (t, 2H, J = 7.8 Hz, Ph), 6.90-6.84 (m, 3H, Ph), 5.67 (dd, 1H, J = 7.8, 3.6 Hz, H-1^{Glc}), 4.01-3.91 (m, 4H, -OC*H*$_2$CH$_2$-), 3.90-3.85 (m, 1H, H-5^{Glc}), 3.81 (dd, 1H, J = 12.0, 2.4 Hz, H-6a^{Glc}), 3.69 (t, 1H, J = 9.0 Hz, H-3^{Glc}), 3.64 (dd, 1H, J = 12.0, 5.4 Hz, H-6b^{Glc}), 4.85 (td, 1H, J = 10.2, 2.4 Hz, H-2^{Glc}), 3.28 (t, 1H, J = 9.6 Hz, H-4^{Glc}), 1.74 (m, 2H, -C*H*$_2$CH$_2$-), 1.61 (m, 2H, -C*H*$_2$CH$_2$-), 1.46 (m, 2H, -C*H*$_2$CH$_2$-), 1.41-1.28 (m, 12H, -C*H*$_2$CH$_2$-); ^{13}C NMR (150 MHz, CD_3OD): δ 159.14, 128.94 (2C), 120.01, 114.04 (2C), 95.80 (d, J = 6.0 Hz, C-1^{Glc}), 73.60, 73.34, 72.62 (d, J = 7.5 Hz), 70.10, 67.40, 65.87 (d, J = 6.0 Hz), 61.32, 30.37, 30.31, 29.31, 29.26, 29.11 (2C), 29.02, 25.76, 25.46; ^{31}P NMR (243 MHz, CD_3OD) δ -10.19, -12.19; ESI-(-)-TOF HRMS m/z: $[M-H]^-$ Calcd for $C_{29}H_{49}O_{17}P_2$ 585.1871; Found 585.1858.

Enzymatic synthesis of Rha*β*-Glc*α*-PP-C11-OPh 4. To a MES solution (50 mM, pH 7.5, 10 mL) of 10 mM $MnCl_2$, 0.5 mM dTDP-Rha, and 1 mM **3** was added Cps23FT (400 μg/mL). The solution was incubated at 16 °C for 1 h and then quenched by adding a mixture of methanol and dichloromethane (1:1, 20 mL). The precipitated Cps23FT was removed via centrifugation, and the product in the supernatant was purified by reversed phase HPLC using a C18 column (4.6 × 250 mm) and gradient eluent (10-100% methanol in water containing 10 mM NH_4HCO_3). The fractions containing **4** (retention time = 26.5 min, Figure S10) were pooled and concentrated to

afford **4** as a white solid (1.8 mg, 67%). ^{1}H NMR (600 MHz, CD_3OD): 7.23 (d, 2H, *J* = 7.8 Hz, Ph), 6.89-6.85 (m, 3H, Ph), 5.66 (br s, 1H, H-1^{Glc}), 4.82 (s, 1H, H-1^{Rha}), 4.02-3.92 (m, 6H, H-5^{Glc}, H-2^{Rha}, 2 × -OC*H*$_2$CH_2-), 3.88 (m, 2H, H-3, 6a^{Glc}), 3.73 (dd, 1H, *J* = 12.0, 4.8 Hz, H-6b^{Glc}), 3.51 (t, 1H, *J* = 9.6 Hz, H-4^{Glc}), 3.39-3.34 (m, 2H, H-2^{Glc}, H-3^{Rha}), 3.32 (1H, H-4^{Rha} overlapped by CD_3OD), 3.21 (m, 1H, H-5^{Rha}), 1.75 (p, 2H, *J* = 6.6 Hz, -C*H*$_2$CH_2-), 1.67-1.59 (m, 2H, -C*H*$_2$CH_2-), 1.46 (p, 2H, *J* = 7.2 Hz, -C*H*$_2$CH_2-), 1.42-1.27 (m, 15H, -C*H*$_2$CH_2-, H-6^{Rha}); ^{13}C NMR (150 MHz, CD_3OD): δ 159.15, 128.93 (2C), 120.00, 114.05 (2C), 100.74, 95.61, 76.13, 73.55, 73.51, 72.76, 72.37, 72.26, 71.87, 70.84, 67.42, 65.87, 61.63, 30.34, 29.35, 29.32, 29.28, 29.15, 29.12, 29.02, 25.76, 25.48, 16.52; ^{31}P NMR (243 MHz, CD_3OD) δ -10.40, -12.64; ESI-(-)-TOF HRMS m/z: $[M-H]^-$ Calcd for $C_{29}H_{49}O_{17}P_2$ 731.2450; Found 731.2412.

III. Characterization of compounds S3, 3, and 4.

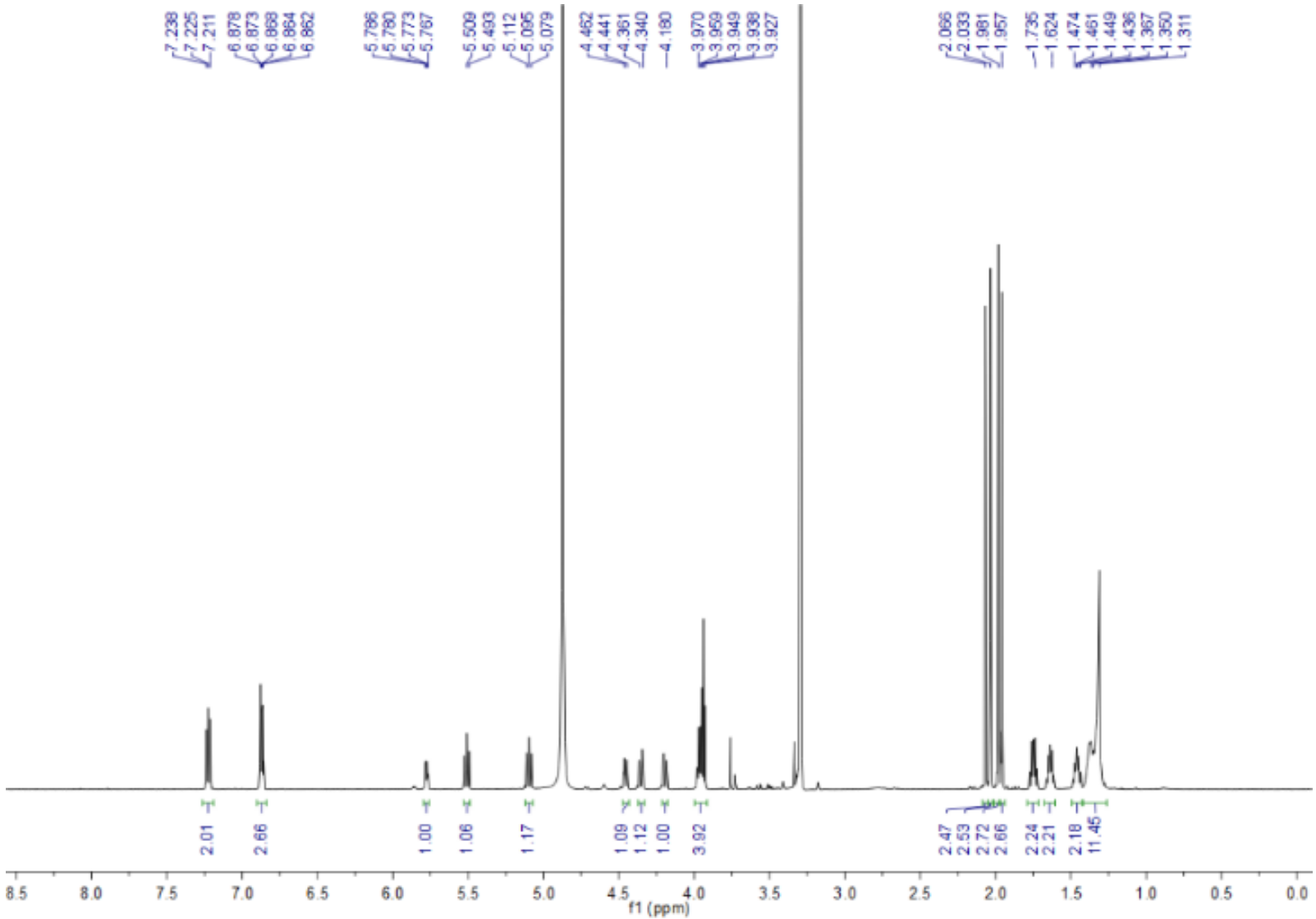


Figure S2. ^{1}H-NMR spectrum (600 MHz, CD_3OD) of $Ac_4Glc\alpha$-PP-$(CH_2)_{11}$-OPh **S3**.

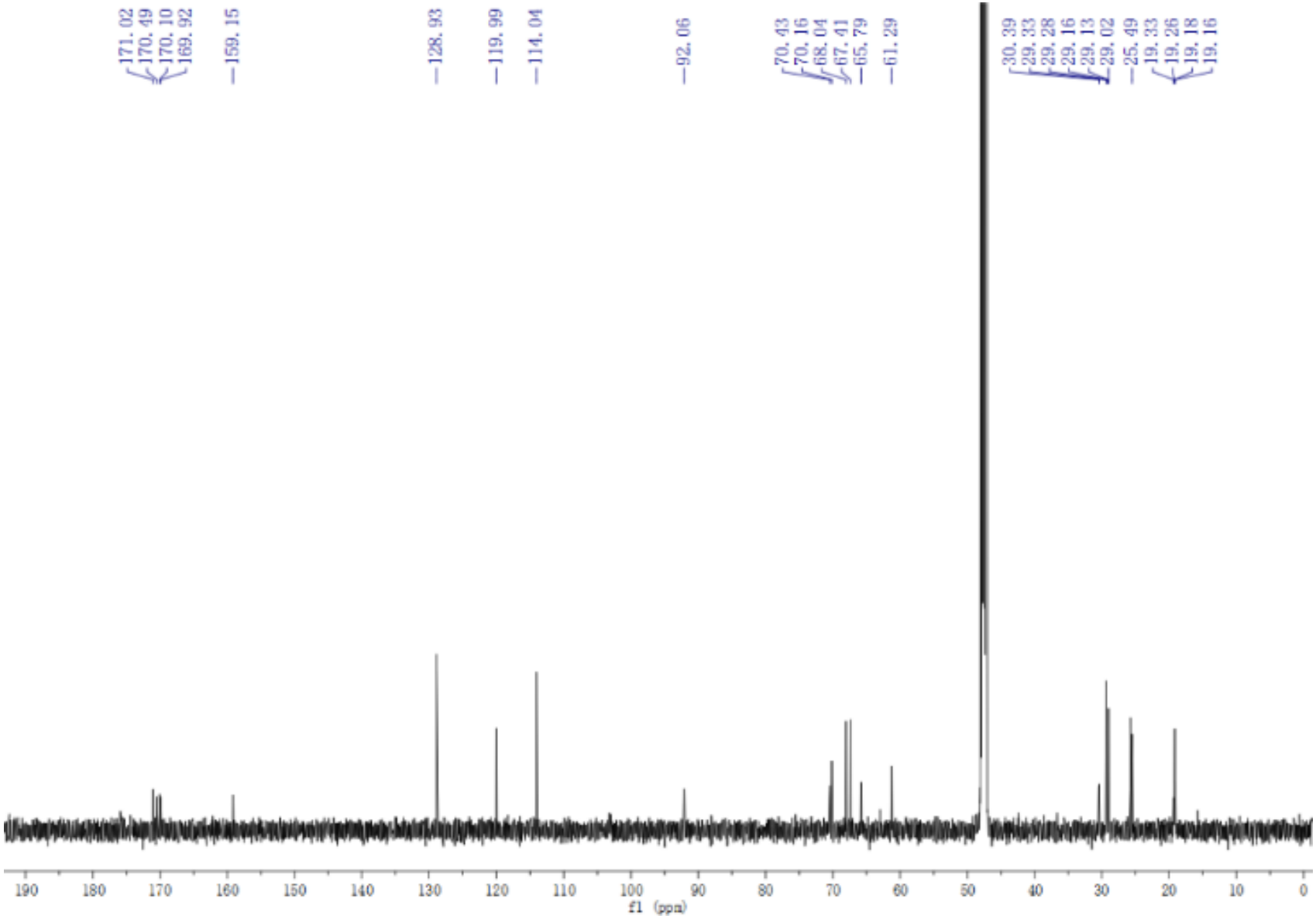


Figure S3. ^{13}C-NMR spectrum (150 MHz, CD_3OD) of $Ac_4Glc\alpha$-PP-$(CH_2)_{11}$-OPh **S3.**

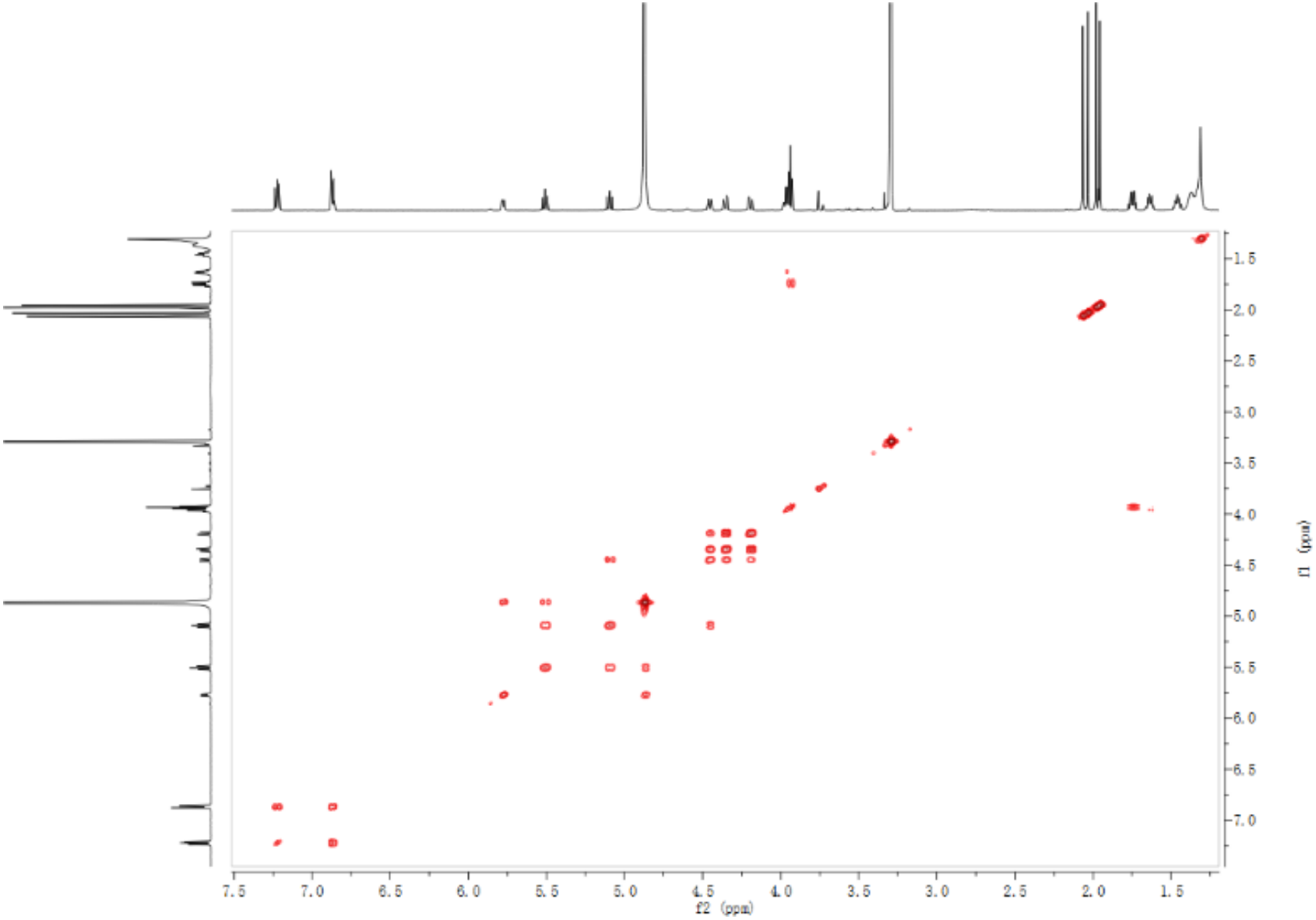


Figure S4. gCOSY spectrum (600/600 MHz, CD_3OD) of $Ac_4Glc\alpha$-PP-$(CH_2)_{11}$-OPh **S3**.

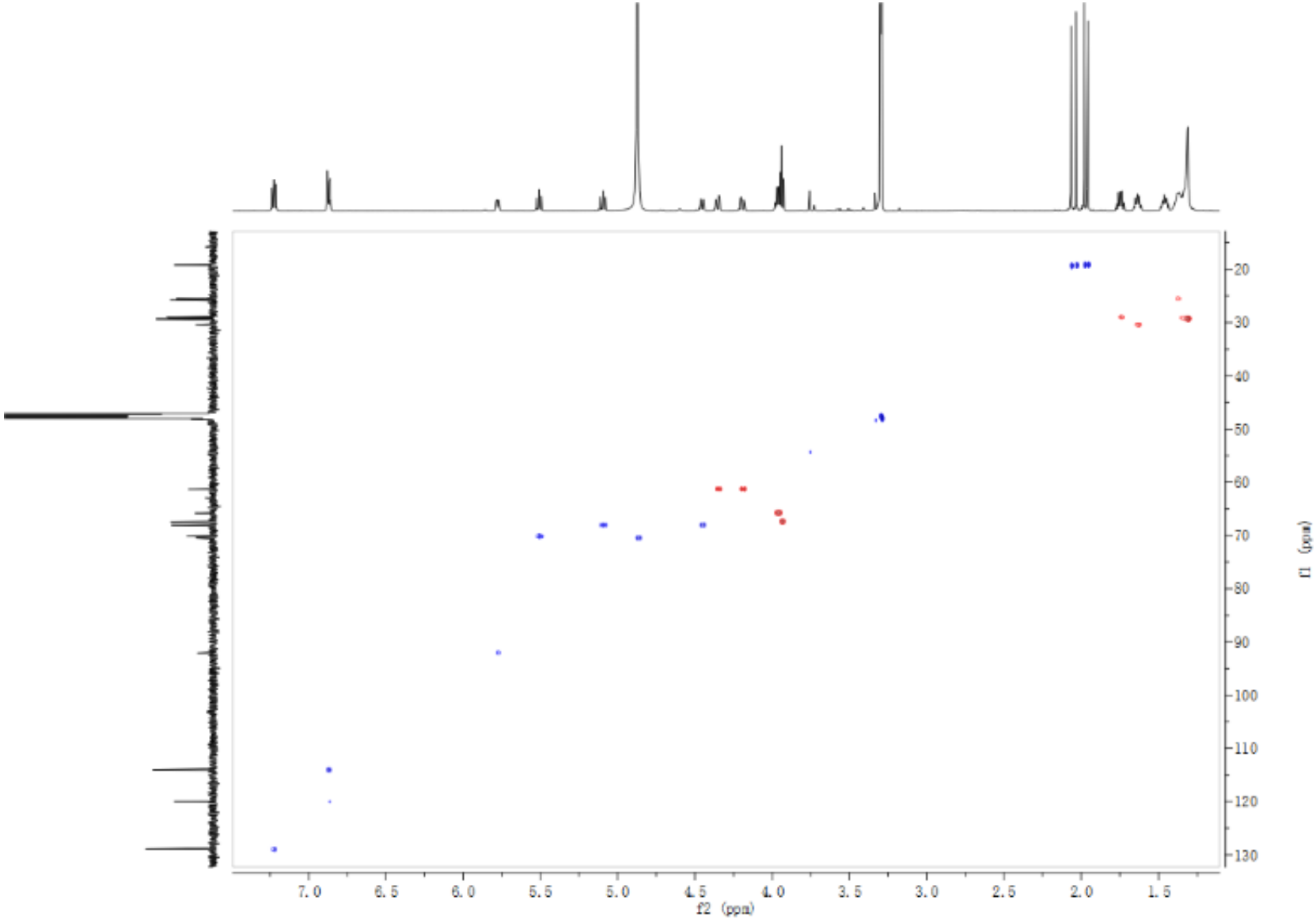


Figure S5. gHSQC spectrum (600/150 MHz, CD_3OD) of $Ac_4Glc\alpha$-PP-$(CH_2)_{11}$-OPh **S3**.

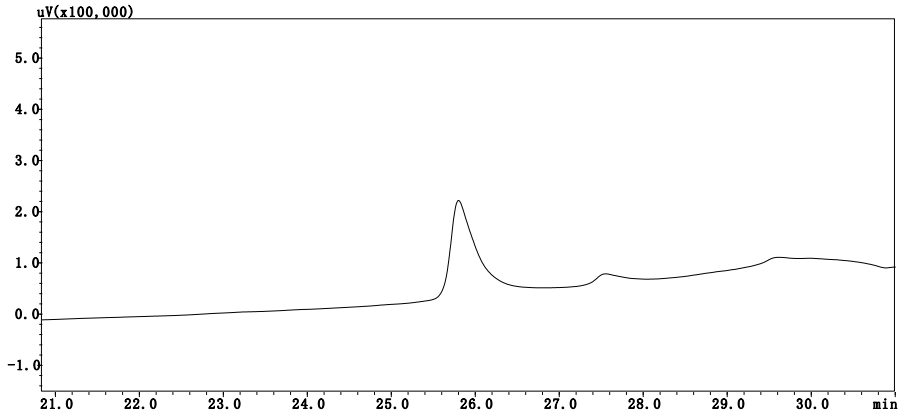


Figure S6. HPLC analysis of Glcα-PP-$(CH_2)_{11}$-OPh **3**. Column: C18 reverse phase column (4.6 mm × 250 mm, 5 μm, 100A pore size from XAqua); eluents: gradient elution with MeOH (10–100% MeOH) in water containing 10 mM NH_4HCO_3; retention time: 25.8 min.

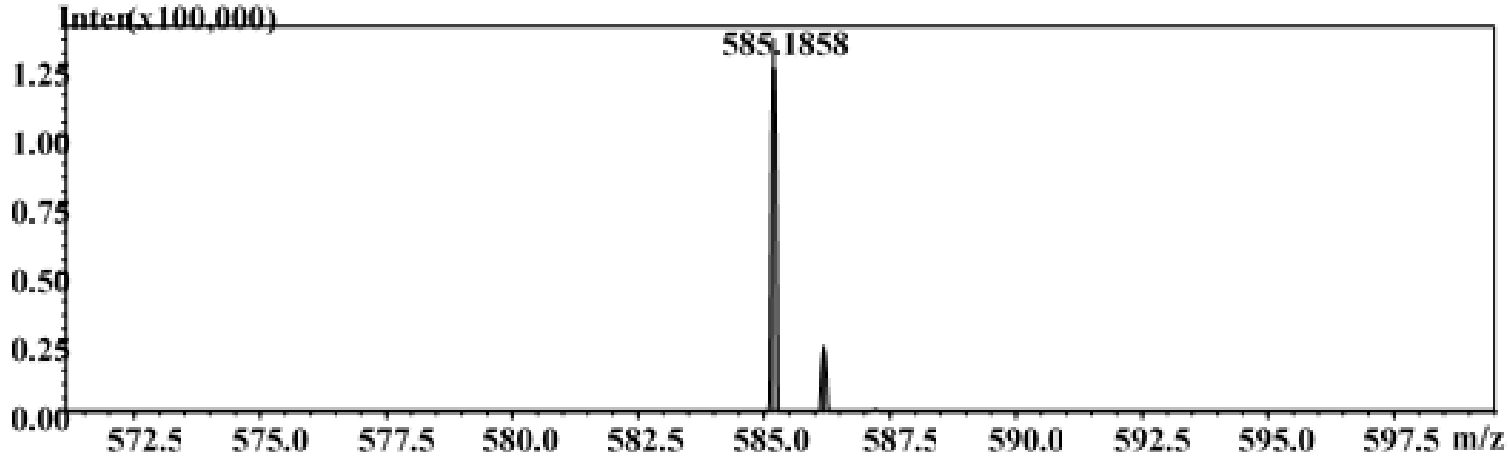


Figure S7. ESI-(-)-HRMS spectrum of Glcα-PP-$(CH_2)_{11}$-OPh **3**.

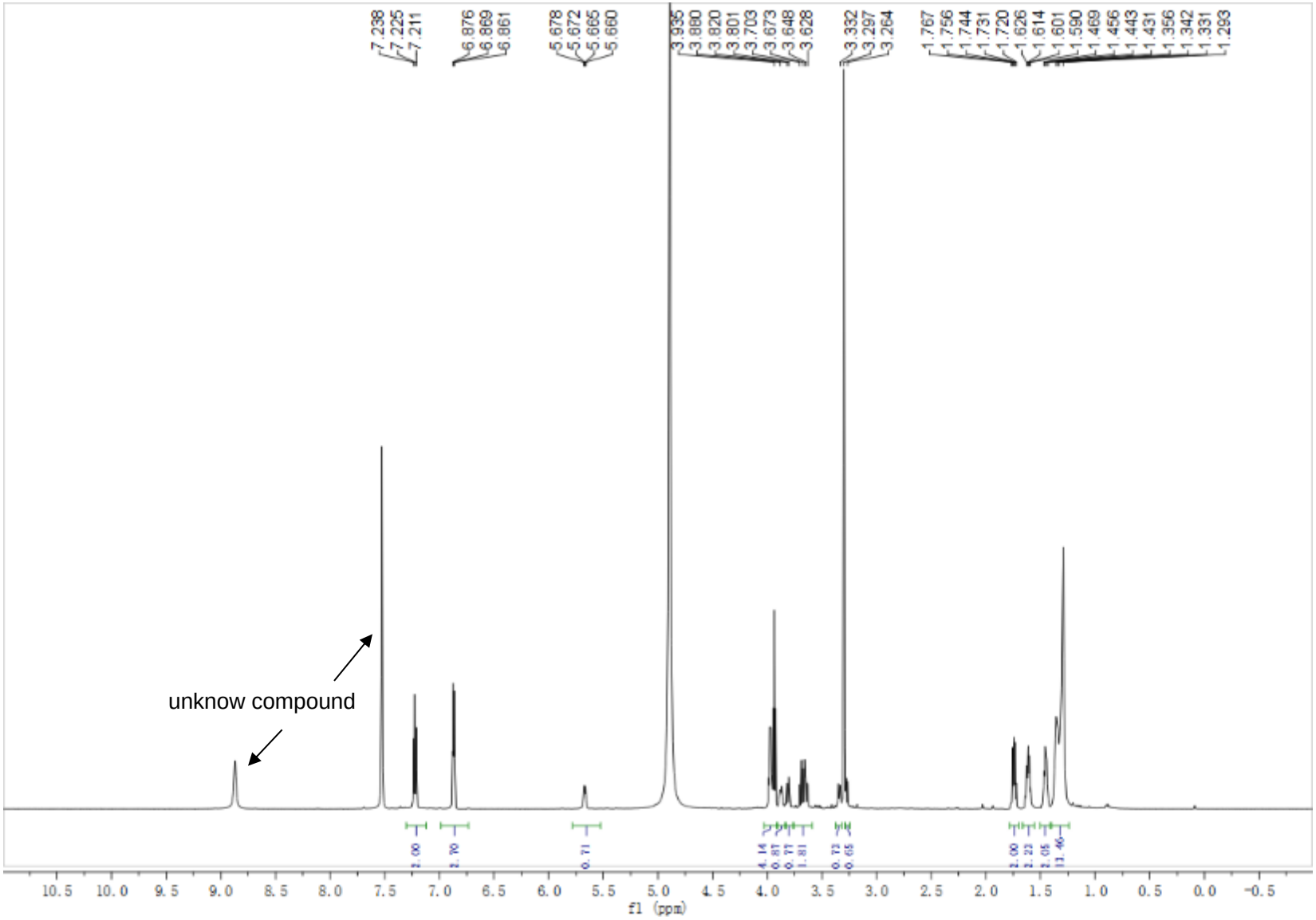


Figure S8. ^{1}H-NMR spectrum (600 MHz, CD_3OD) of Glcα-PP-$(CH_2)_{11}$-OPh **3**.

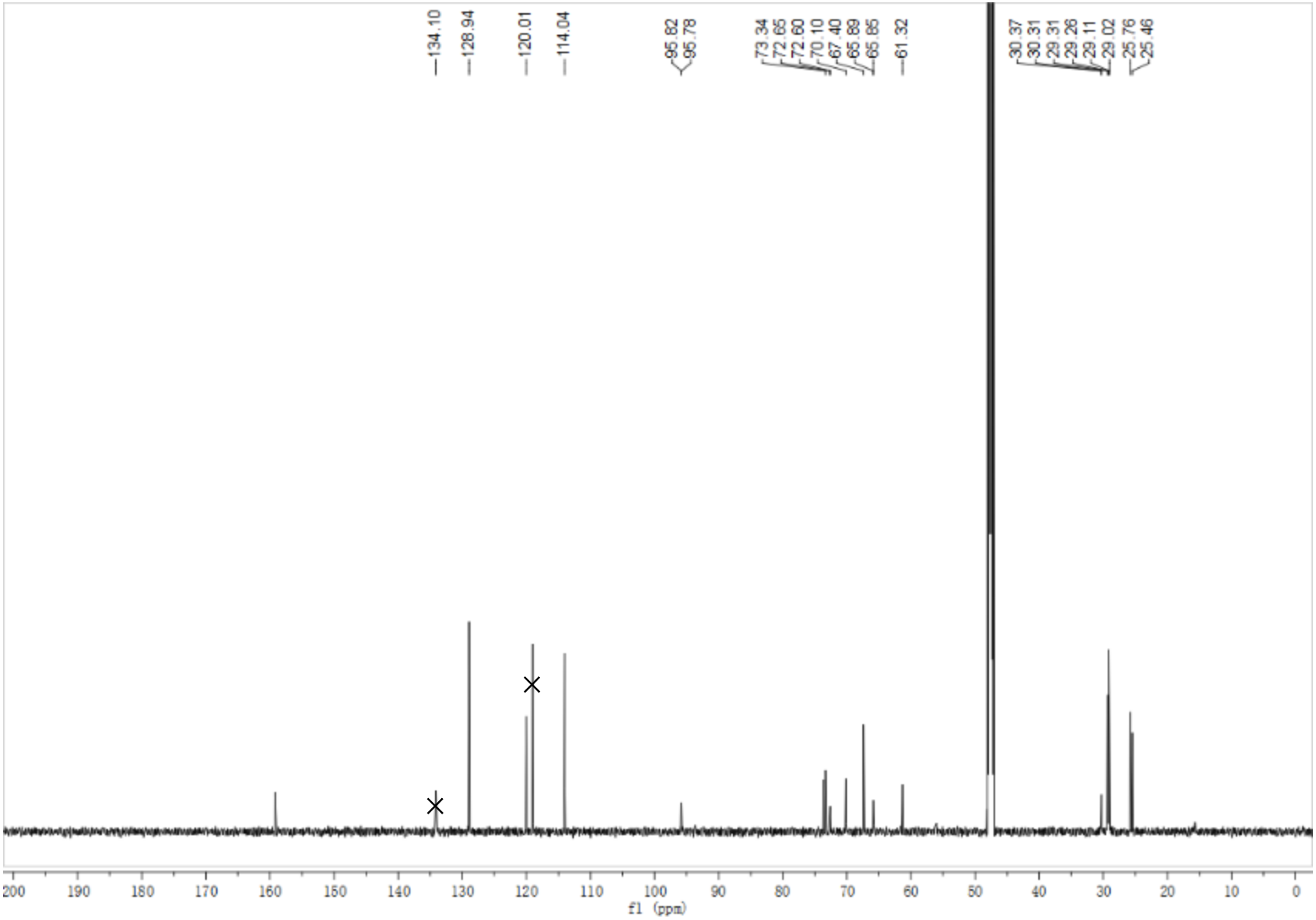


Figure S9. ^{13}C-NMR spectrum (150 MHz, CD_3OD) of Glcα-PP-$(CH_2)_{11}$-OPh **3.**

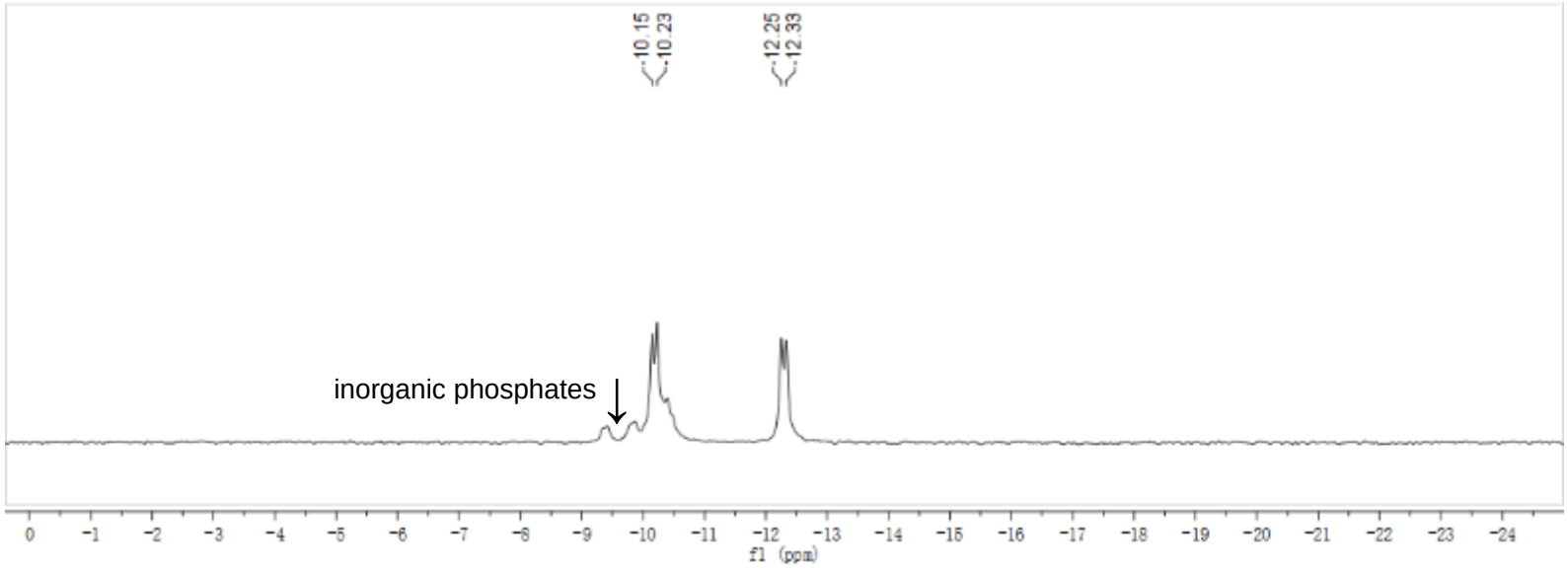


Figure S10. ^{31}P-NMR spectrum (243 MHz, CD_3OD) of Glcα-PP-$(CH_2)_{11}$-OPh **3**.

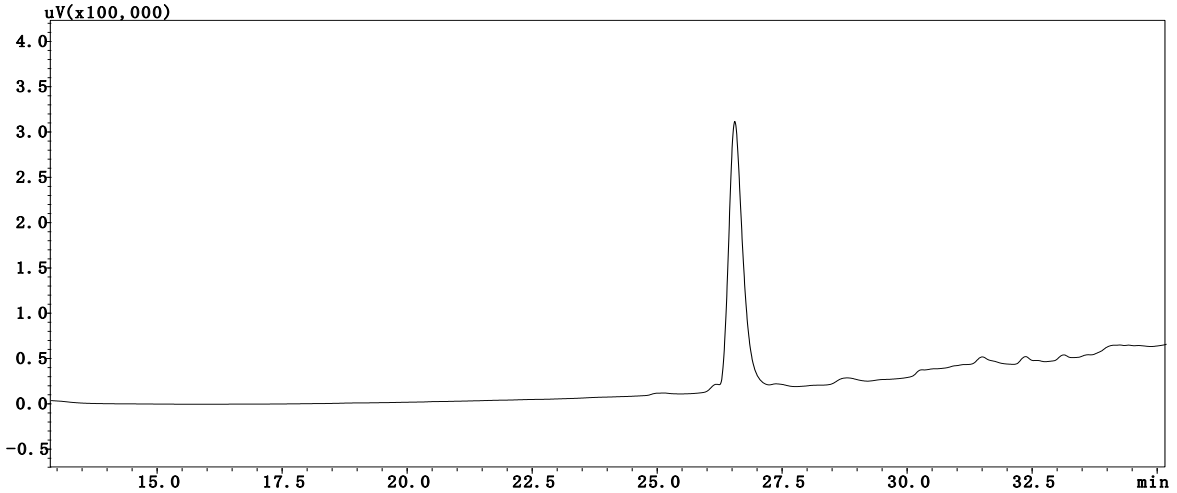


Figure S11. HPLC analysis of Rhaβ1,4-Glcα-PP-$(CH_2)_{11}$-OPh **4**. Column: C18 reversed phase column (4.6 mm × 250 mm, 5 μm, 100 A pore size); eluents: gradient elution with MeOH (10–100% MeOH) in water containing 10 mM NH_4HCO_3; retention time: 26.5 min.

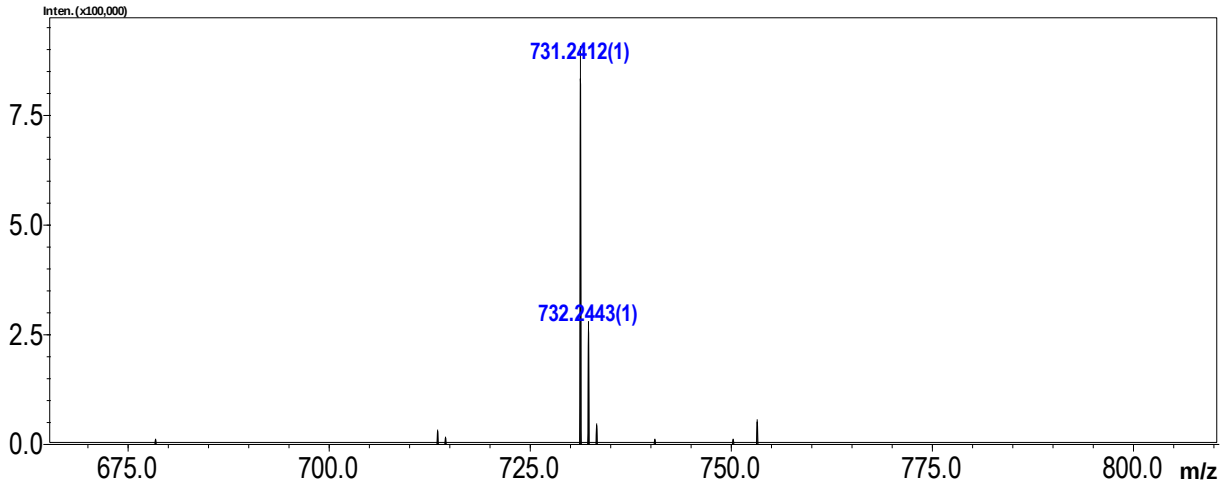


Figure S12. ESI (-)-TOF HRMS spectrum of Rhaβ1,4-Glcα-PP-$(CH_2)_{11}$-OPh **4**.

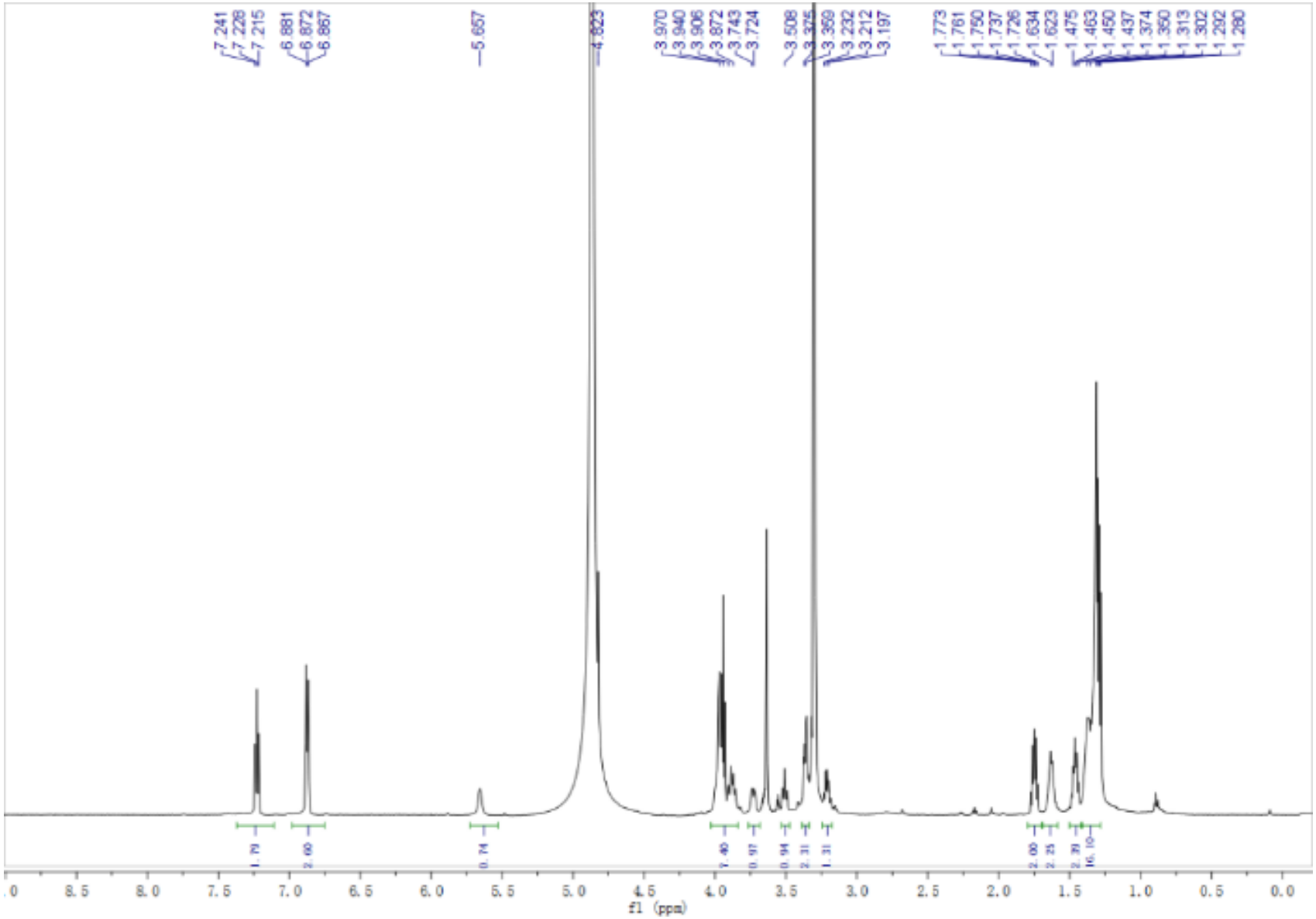


Figure S13. ^{1}H-NMR spectrum (600 MHz, CD_3OD) of Rhaβ1,4-Glcα-PP-$(CH_2)_{11}$-OPh **4**.

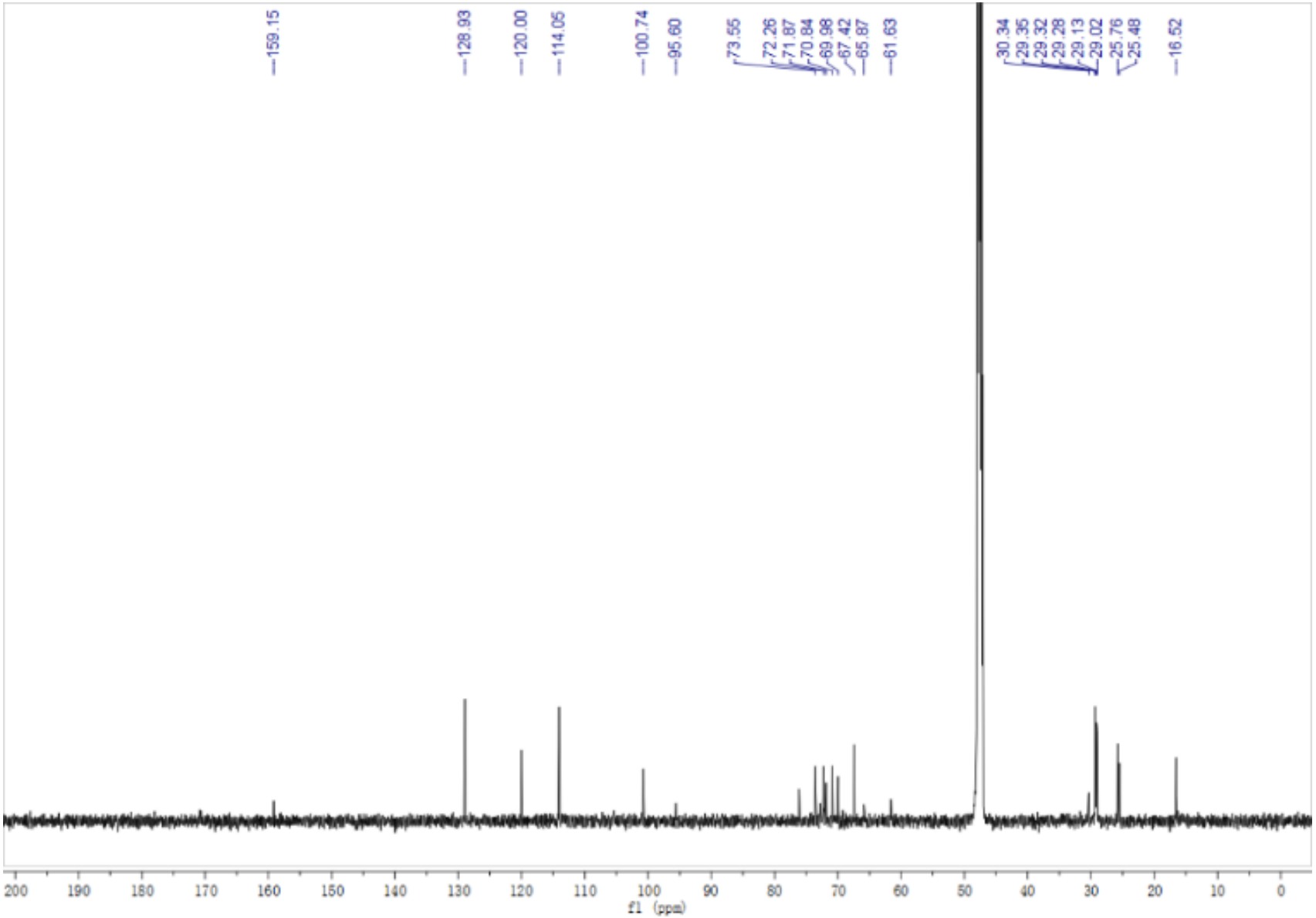


Figure S14. ^{13}C-NMR spectrum (150 MHz, CD_3OD) of Rhaβ1,4-Glcα-PP-$(CH_2)_{11}$-OPh **4**.

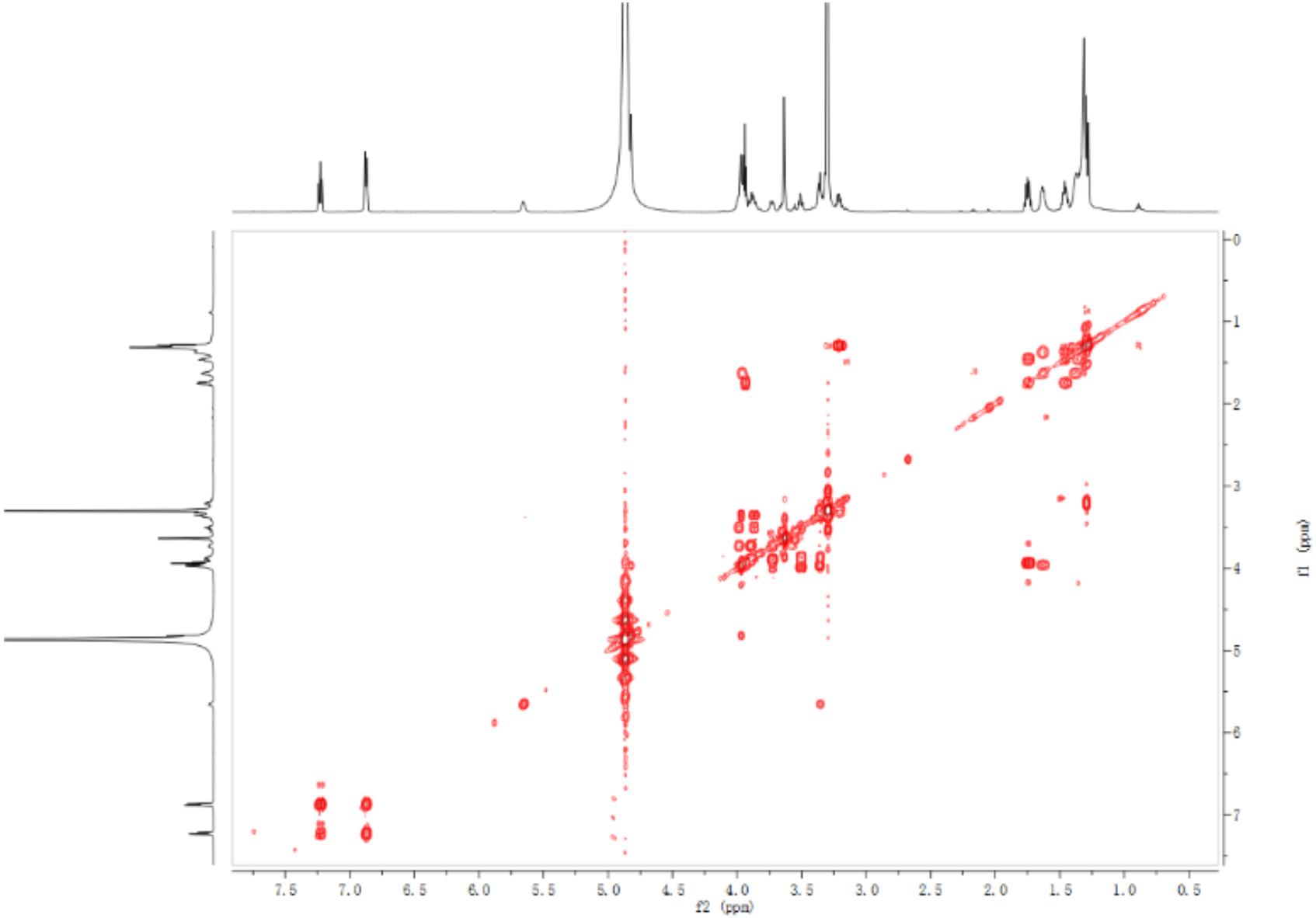


Figure S15. gCOSY spectrum (600/600 MHz, CD_3OD) of Rhaβ1,4-Glcα-PP-$(CH_2)_{11}$-OPh **4**.

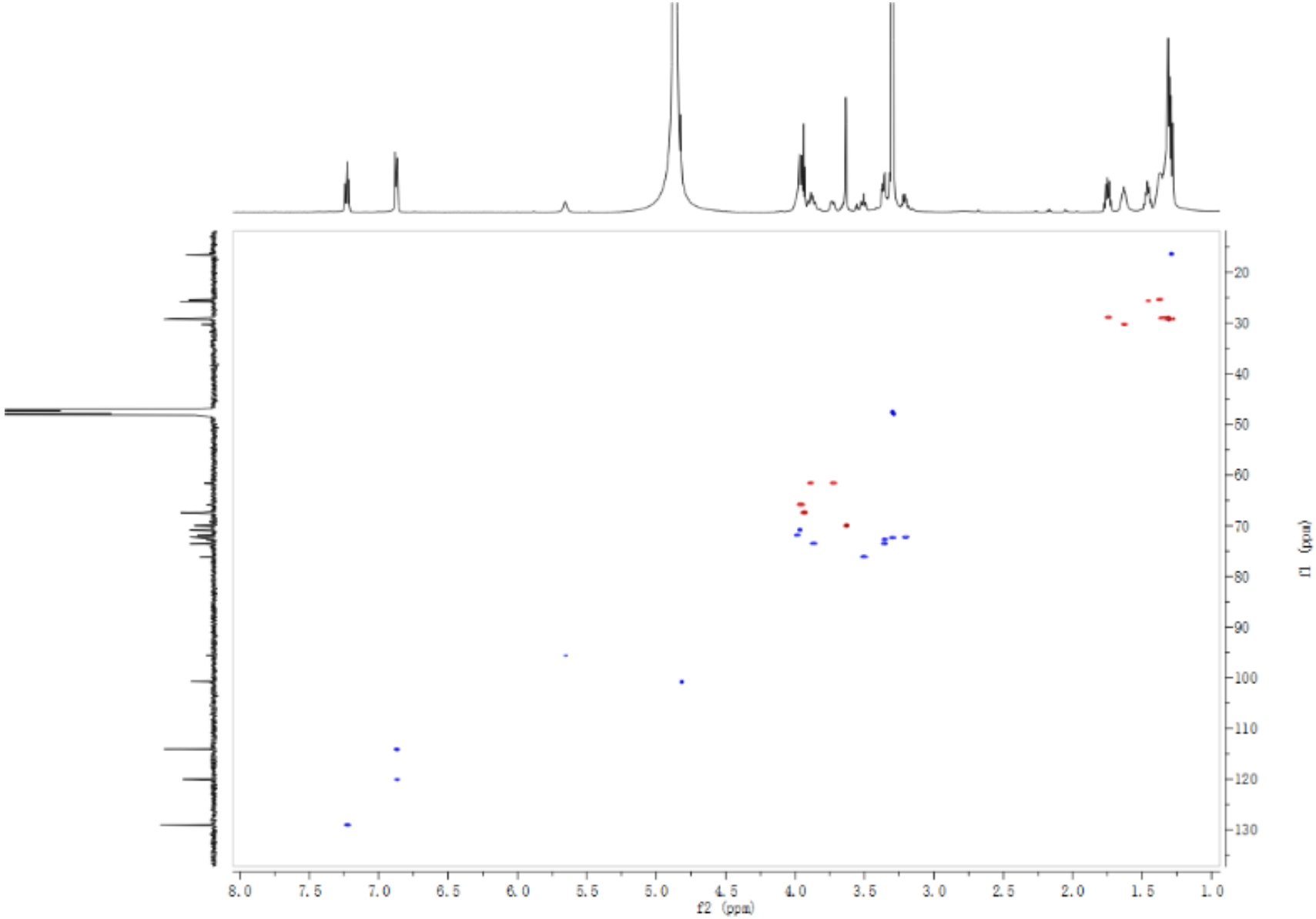


Figure S16. gHSQC spectrum (600/150 MHz, CD_3OD) of Rhaβ1,4-Glcα-PP-$(CH_2)_{11}$-OPh **4**.

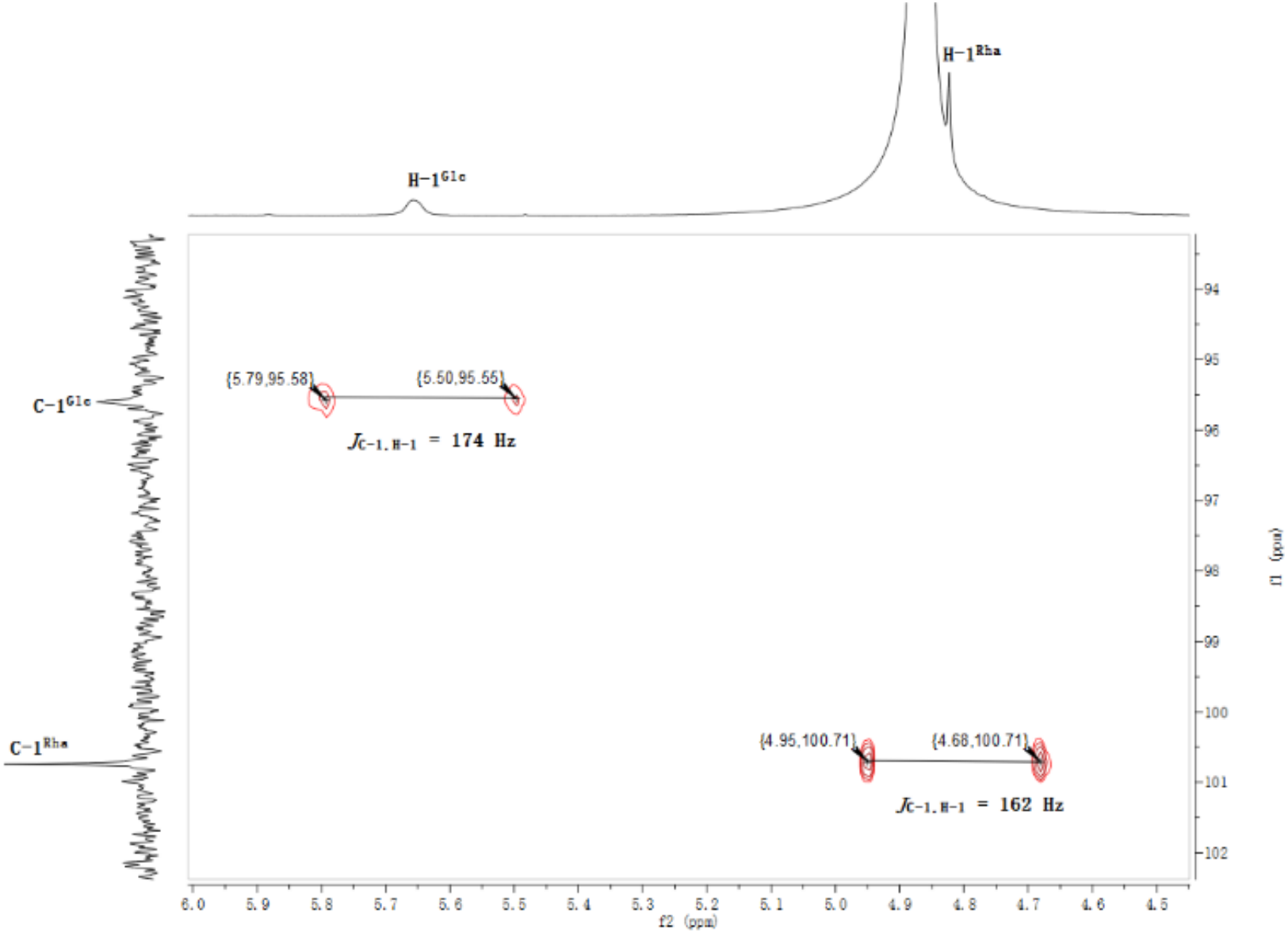


Figure S17. ^{1}H-coupled gHSQC spectrum (600/150 MHz, CD_3OD) of Rhaβ1,4-Glcα-PP-$(CH_2)_{11}$-OPh **4**.

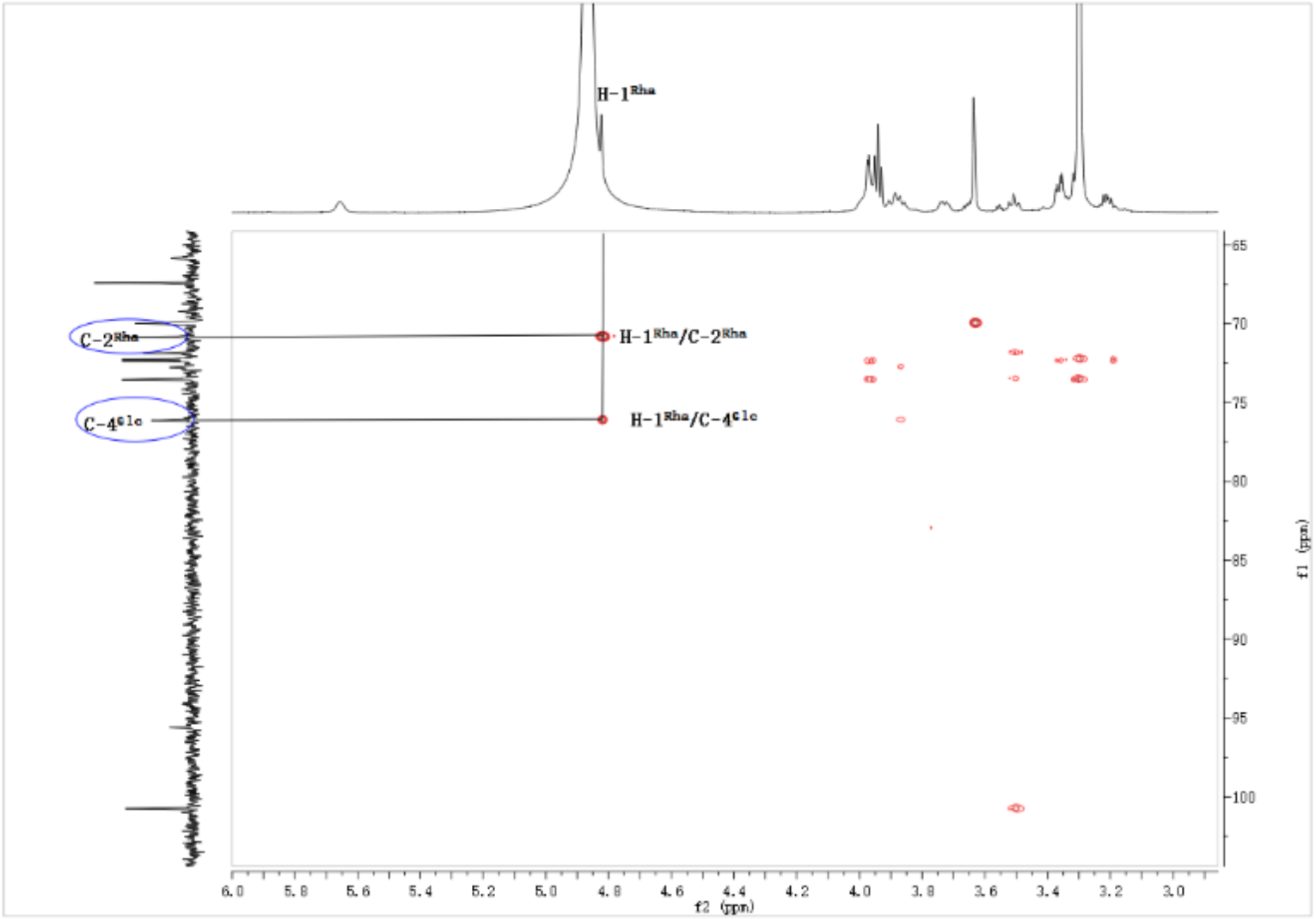


Figure S18. gHMBC spectrum (600/150 MHz, CD_3OD) of Rhaβ1,4-Glcα-PP-$(CH_2)_{11}$-OPh **4**.

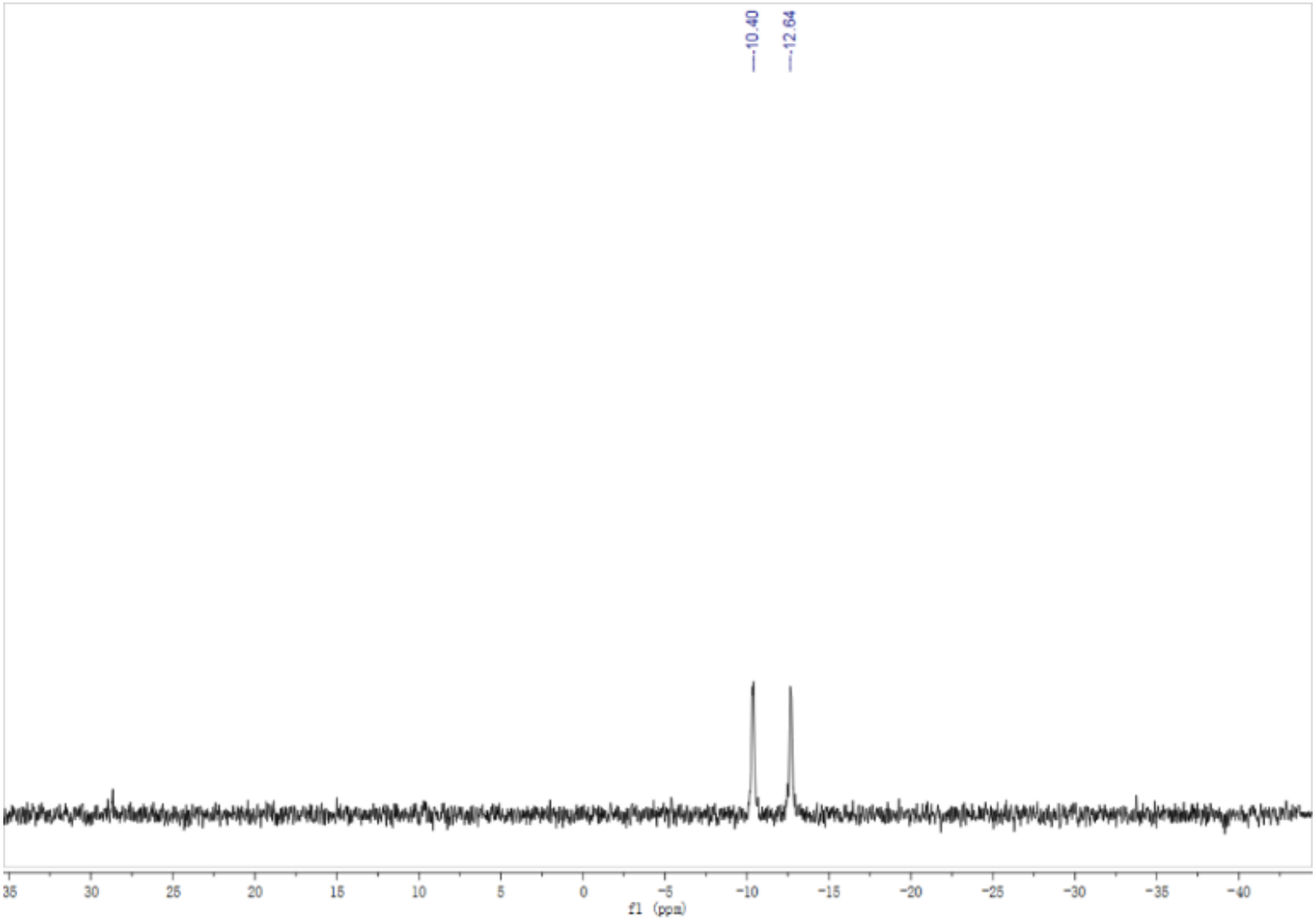


Figure S19. ^{31}P-NMR spectrum (243 MHz, CD_3OD) of Rhaβ1,4-Glcα-PP-$(CH_2)_{11}$-OPh **4**.

IV. The biochemical studies of Cps23FT by HPLC using dTDP detection

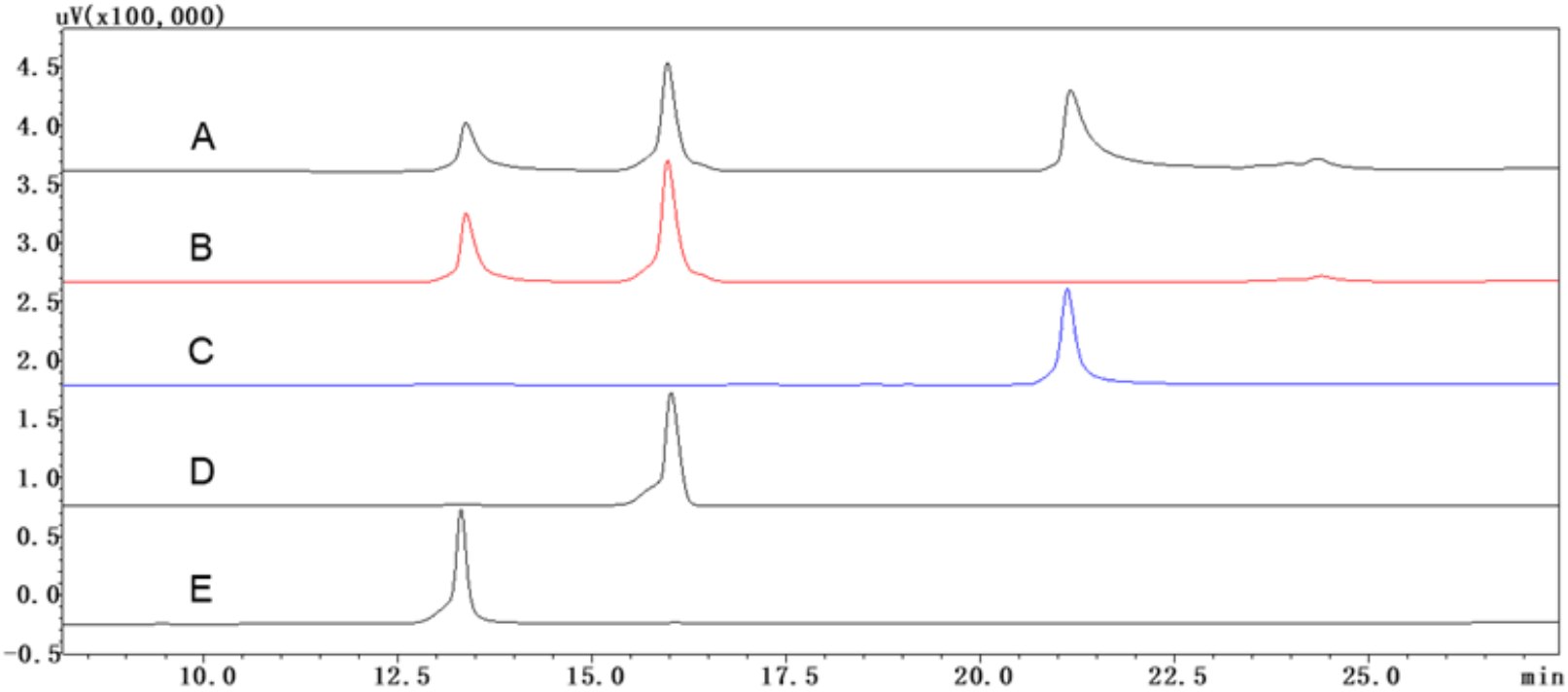


Figure S20. HPLC analysis of enzymatic reaction between glycolipid **3** and dTDP-Rha catalyzed Cps23FT. (A) reaction mixture of Cps23FT; (B) reaction mixture of heat-deactivated Cps23FT; (C) dTDP; (D) dTDP-Rha; (E) dTMP. Column: DionexCarboPacTM PA-100 (4×250 mm); eluent: 0～1 M ammonium acetate.

V. The amino acid sequence of Cps23FT

MKKSVYIIGSKGIPAKYGGFETFVEKLTAFQQDKAIQYYVACMRENSAKSGTTEDVFEHNGAICYNVDVPNIGPARAIAYD
IAAINRAIEIAKENK[97]**DED**[99]PIFYILACRIGPFIHGIKKKIQEIGGTLLVNPDGHEWLRAKWSAPVRRYWKISEGLMVKHADL
LVCDSKNIEKYIQEDYKQYQPKTTYIAYGTDTTRSVLKSSDEKVRSWFKEKNVSENEYYLVVGRFVPENNYESMIRGFLAS
NSKKDFVLITNVEQNKFYNQLLAKTGF[271]**DKD**[273]PRVKFVGTVYEQELLKYIRENAFAYFHGHEVGGTNPSLLEALASTKL
NLLLDVGFNREVAEDGAIYWKKDNLHEIIETSEQKTQKEIDEKDILSIKQVTERFSWELIVNEYEKLFLCEK

Figure S21. Sequence alignment of *S. pneumonia* Cps23FT. The amino acid sequence is translated by *cps23FT* gene (derived from the Gene Bank (*cps23FT*: AAC38749.1) and can be searched on NCBI (GenBank: CAR68180.1). It contains two Asp-X-Asp sequences (bold font) as DXD motif candidates.

VI. Experimental data related to site-directed mutagenesis

Table S1. The primers designed for site-directed mutagenesis

Enzyme	Direction	Primer sequence (5'→3')
P23FT05	Forward	CG GGATCC CAT ATG AAAAAGTCAGTTTATATCATTGG
P23FT03	Reverse	CCGCTCGAGTTATTTCTCACATAAAAAAAGTTTCTCA
D97A05	Forward	GCCAAAGAAAATAAGGCTGAAGATCCA
D97A03	Reverse	AGCCTTATTTTCTTTGGCAATCTCAAT
D99A05	Forward	GAAAATAAGGATGAAGCTCCAATCTTC
D99A03	Reverse	AGCTTCATCCTTATTTTCTTTGGCAAT
D271A05	Forward	GCAAAAACTGGTTTTGCTAAGGACCCA
D271A03	Reverse	AGCAAAACCAGTTTTTGCCAACAACTG
D273A05	Forward	ACTGGTTTTGATAAGGCTCCACGAGTA
D273A03	Forward	AGCCTTATCAAAACCAGTTTTTGCCAA

References

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2. Z. Dinev, A. Z. Wardak, R. T. Brownlee and S. J. Williams, *Carbohyd. Res.*, 2006, **341**, 1743-1747.