"Click" Chemistry for the Assembly of Entirely Carbohydrate-based Vaccines

Supporting Information

Geraud Valentin, Andhina Satriani, Matthew Lohman and Peter R. Andreana*

Department of Chemistry and Biochemistry and School of Green Chemistry and Engineering, University of Toledo, 2801 W. Bancroft Street, Toledo, OH 43606, United States

**Corresponding Author:*
Peter R. Andreana, PhD
peter.andreana@utoledo.edu

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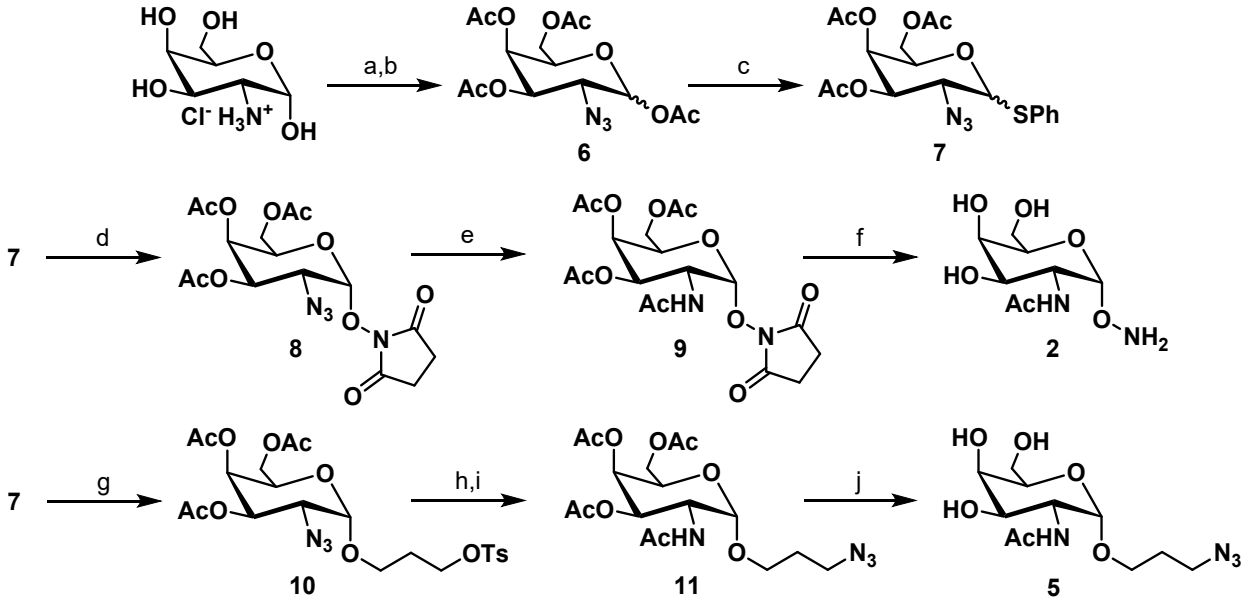
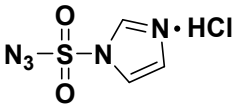
Chemical Synthesis

Thomsen nouveau (Tn) Derivatives

All solvents and reagents were purchased from common commercial sources and used without further purification unless specified otherwise. Products were purified by column chromatography using Silicycle SiliaFlash® F60 40-63 µm as a sorbent. Thin layer chromatography was performed on Silicycle 250 µm with F-254, catalog number TLG-R10014BK-323 and stained with anisaldehyde or ninhydrin. Analysis was performed by NMR on a Bruker Avance III 600 or Varian 400 spectrometers. NMRs in D_2O were performed using presaturation to eliminate the solvent peak. Mass spectrometry was performed on Finnigan LCQDeca. Tn antigen derivatives were synthesized as shown in **Scheme S1**.

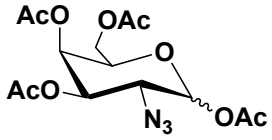
Scheme S1. Synthesis of aminooxy and azido Tn derivatives. (a) Stick's reagent, K_2CO_3, $CuSO_4$, methanol, rt, 3 h, not isolated (b) Pyridine, Ac_2O, DMAP, rt, 16 h, 51% (c) DCM, $BF_3{\cdot}Et_2O$, SPh, reflux, 3 h (d) NIS, TMSOTf, DCM, 0°C to rt, 30 min, 79%, α:β = 12:1, (e) Zn, 3:2:1 Ac_2O:THF:AcOH, rt, 3 h, 56% (f) $NH_2NH_2{\cdot}H_2O$, MeOH, rt, 16 h, 85% (g) NIS, TMSOTf, DCM, 0 °C, 90 min, 57%, α:β = 12:1 (h) Zn, 3:2:1 Ac_2O:THF:AcOH, rt, 3 h, 82% (i) NaN_3, TBHS, DCM, H_2O, 16 h, rt, 77% (j) $NH_2NH_2{\cdot}H_2O$, MeOH, rt, 16 h, 82%.



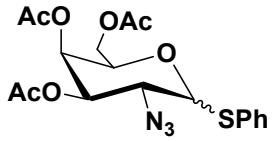


Imidazole-1-sulfonyl azide hydrochloride: (based on Stick *et al.*[1]) Warning: The procedure for synthesizing the reagent must be followed exactly or else it can be explosive. Refer to corrections to Stick's article for

up-to-date safety procedures.[1] Do not concentrate this reagent. Use plastic spatulas to prevent sparks. Sodium azide (13.0 g, 0.20 mol) was suspended in 150 mL acetonitrile. Sulfuryl chloride (16.1 mL, 0.20 mol) was added dropwise, and the reaction was stirred at room temperature overnight. The reaction was cooled on ice, stirred vigorously, and imidazole (25.9 g, 380 mmol) was added portion-wise under high stirring. The reaction must stay white to pale pink. Red indicates high formation of explosive by-products. After 3 hours at room temperature, 400 mL of ethyl acetate was added, and the reaction extracted with water and saturated ammonium carbonate. The reaction was dried with sodium sulfate and filtered. Finally, a fresh solution of HCl in ethanol, made by adding acetyl chloride (300 mmol, 21.5 mL) dropwise to absolute ethanol (75 mL), was added slowly to the ice-cold filtrate. The filtrate was collected and washed with cold ethyl acetate, yielding Stick's reagent (28 g, 67%).

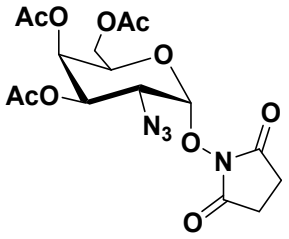


1,3,4,6-Tetra-*O*-acetyl-2-azido-2-deoxy-D-galactopyranoside (6): D-galactosamine hydrochloride (12.2 g, 56 mmol) was added to a suspension of Stick's reagent (14.6 g, 68 mmol, explosive: weigh with a plastic spatula), potassium carbonate (21 g, 152 mmol) and copper sulfate (140 mg, 0.56 mmol) in methanol (280 mL) to form a green clayish mixture that was stirred for 3 hours at room temperature. The reaction was then rotovaped and co-distilled three times with toluene to yield a brown powder. The flask was placed on ice, and the powder was resuspended in 200 mL pyridine. Acetic anhydride (43 mL, 553 mmol) and DMAP (0.867 g, 7 mmol) were slowly added. The reaction was stirred at room temperature overnight, extracted three times with 1 N HCl and saturated sodium bicarbonate, rotovaped and purified on a pad of silica using 3:1 hexanes:ethyl acetate, yielding **6** (10.8 g, 51%). Alpha (α) and beta (β) products were not separated. LRMS (ESI) m/z: [M + Na] + calculated for $C_{14}H_{19}N_3O_9Na$ = 396.1; Found 395.8

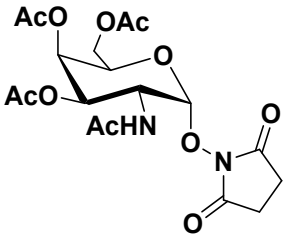


Phenyl 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-1-thiol-D-galactopyranoside (7): Compound **6** (10.8 g, 28.9 mmol) was placed under high vacuum overnight, then further dried by dissolution in a minimal amount of dichloromethane (DCM) and co-distilled with toluene three times. Dry DCM (150 mL), boron trifluoride etherate (17.7 mL, 144 mmol) and thiophenol (5.9 mL, 58 mmol) were added and the reaction was run at reflux for three hours. The reaction was diluted with 200 mL DCM and extracted with cold saturated sodium bicarbonate and water, rotovaped, and purified by flash silica gel column chromatography with a 1:9 to 1:1 ethyl acetate:hexanes ratio. Compound **7** was obtained as a pale-yellow powder (10.4 g, 86%). Alpha and beta products were not separated. LRMS (ESI) m/z: [M + Na] + calculated for $C_{18}H_{21}N_3O_7S$ = 446.1; Found 446.2

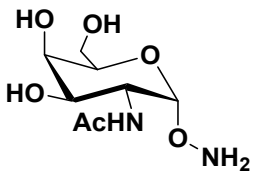
[1] E. D. Goddard-Borger and R. V. Stick, *Org. Lett.*, 2007, **9**, 3797–3800.



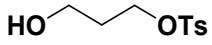
Succinimidyl 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranoside (8): Compound **7** (2.5 g, 5.8 mmol) and *N*-hydroxysuccinimide (2.7 g, 23.9 mmol) were dissolved in dry DCM, co-evaporated three times with toluene and dissolved in 60 mL dry DCM with 100 mg activated molecular sieves (4 Å) under argon. After 15 minutes, *N*-iodosuccinimide (2.0 g, 11.1 mmol) was added, the reaction was cooled with ice and, 15 minutes later, trimethylsilyl trifluoromethanesulfonate (100 µL, 0.55 mmol) was added. After 30 minutes at room temperature, the reaction was quenched with sodium thiosulphate until colorless then with sodium carbonate, filtered on celite, diluted to 250 mL with DCM and extracted three times with brine and saturated sodium carbonate. The solution was dried with sodium sulfate, filtered and purified by silica gel flash column chromatography, yielding **8** (1.995 g, 79%, α:β 12:1). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.60 – 5.58 (m, 1H), 5.55 (d, *J* = 3.8 Hz, 1H), 5.48 (dd, *J* = 11.4, 3.2 Hz, 1H), 5.12 (t, *J* = 6.4 Hz, 1H), 4.24 (dd, *J* = 11.3, 6.5 Hz, 1H), 3.98 – 3.91 (m, 2H), 2.80 (t, *J* = 5.6 Hz, 4H), 2.18 (s, 3H), 2.10 (s, 3H), 2.08 (s, 3H).^{13}C NMR (151 MHz, $CDCl_3$) δ 170.4, 170.4, 169.9, 169.6, 102.2, 69.0, 67.7, 67.3, 61.2, 56.6, 25.5, 20.8, 20.6. LRMS (ESI) m/z: [M + Na] + calculated for $C_{16}H_{20}N_4O_{10}Na$ = 451.1; Found 451.1



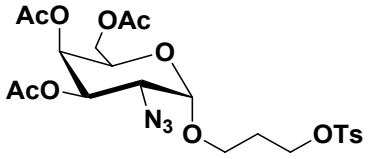
Succinimidyl 2-*N*-acetyl-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranoside (9): Compound **8** (0.70 g, 1.6 mmol) and zinc powder (0.5 g) were suspended in 18 mL of 3:2:1 acetic anhydride:tetrahydrofuran:acetic acid and placed on ice. The reaction reached room temperature and was left for 3 hours. The crude was diluted with 50 mL DCM, filtered on celite, concentrated and purified by silica gel flash column chromatography, yielding **9** (0.396 g, 56%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.03 (d, *J* = 9.6 Hz, 1H), 5.54 (d, *J* = 2.1 Hz, 1H), 5.36 – 5.26 (m, 2H), 5.01 (t, *J* = 6.3 Hz, 1H), 4.78 (ddd, *J* = 11.8, 9.7, 3.7 Hz, 1H), 4.30 (dd, *J* = 11.3, 6.0 Hz, 1H), 3.97 (dd, *J* = 11.3, 6.6 Hz, 1H), 2.78 (s, 3H), 2.19 (s, 3H), 2.10 (s, 3H), 2.08 (s, 3H), 2.05 (s, 3H).^{13}C NMR (151 MHz, $CDCl_3$) δ 170.9, 170.8, 170.6, 170.5, 170.2, 104.4, 77.2, 77.0, 76.8, 69.3, 67.3, 67.2, 61.6, 47.2, 25.4, 23.3, 20.8, 20.7. LRMS (ESI) m/z: [M + Na] + calculated for $C_{18}H_{24}N_2O_{11}Na$ = 467.1; Found 467.4



Aminooxy 2-*N*-acetyl-2-deoxy-α-D-galactopyranoside (2): Compound **9** (396 mg, 0.889 mmol) was dissolved in methanol (10 mL). Hydrazine hydrate (1 mL, 21 mmol) was then added, and the reaction was run for 16 hours at room temperature. Then, the reaction was placed at 4 °C and 113 mg of the tetrahydropyridazine-3,6-dione by-product was filtered out. The filtrate was rotovaped and dissolved in a minimal amount of hot methanol, then cooled and diluted with DCM to precipitate the product as a white solid. The resulting product was purified on a pad of silica (80:20 DCM: methanol), then on Bio-Rad® P2 gel. Fractions (1 mL) were individually lyophilized and taken for NMR, yielding pure **2** (180 mg, 85%). ^{1}H NMR (600 MHz, D_2O) δ 4.84 (d, *J* = 4.0 Hz, 1H), 4.10 (dd, *J* = 11.3, 4.0 Hz, 1H), 3.87 (dd, *J* = 8.0, 4.1 Hz, 2H), 3.76 – 3.61 (m, 3H), 1.93 (s, 3H). ^{13}C NMR (151 MHz, D_2O) δ 174.5, 173.5, 100.5, 70.9, 68.4, 67.5, 61.1, 49.1, 28.9, 21.9. LRMS (ESI) m/z: [M + H] + calculated for $C_8H_{17}N_2O_6$ = 237.1; Found 236.9; [M + H + Na] + calculated for $C_8H_{17}N_2O_6Na$ = 260.1; Found 260.0



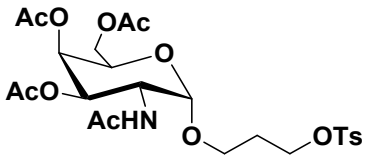
3-Hydroxypropyl 4-methylbenzenesulfonate (3H4MBS): (based on Steinbacher et al.[2]) 1,3-Propanediol (3.63 mL, 50.0 mmol) and triethylamine (1.53 mL, 11 mmol) were dissolved in 20 mL DCM. The flask was flushed with argon, placed on ice, and equipped with a dripping funnel containing tosyl chloride (1.91 g, 10.0 mmol) suspension in DCM (30 mL). The solution was added dropwise over 30 minutes and the reaction was stirred overnight at room temperature. The mixture was rotovaped, resuspended in a minimal amount of cold diethyl ether and the resulting white triethylammonium chloride salt was filtered out. Solvent was removed and the reaction was purified by silica gel flash column chromatography in 2:3 hexanes:ethyl acetate. **3H4MBS** was obtained as a viscous colorless oil (1.82 g, 75%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.90 – 7.71 (m, 2H), 7.37 (d, *J* = 8.0 Hz, 2H), 4.20 (t, *J* = 6.1 Hz, 2H), 3.73 (t, *J* = 5.9 Hz, 2H), 2.47 (s, 3H), 1.96 – 1.84 (m, 2H), 1.81 (s, 1H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 144.9, 133.0, 129.9, 127.9, 67.5, 58.3, 31.7, 21.7. LRMS (ESI) m/z: [M + Na] + calculated for $C_{11}H_{13}O_4SNa$ = 253.1; Found 251.9



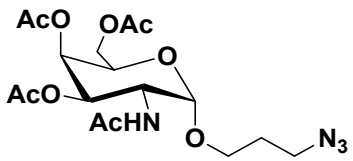
3-*O*-Tosylpropyl 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-1-α-D-galactopyranoside (10): Compound **7** (0.80 g, 1.89 mmol) and **3H4MBS** (0.87 g, 3.78 mmol) were dissolved in dry DCM and co-evaporated three times with toluene, then placed under high vacuum for thirty minutes. The reagents were dissolved in 25 mL dry DCM in the presence of activated 4 Å molecular sieves and placed on ice under argon. Then *N*-iodosuccinimide (0.85 g, 3.78 mmol) and trimethylsilyl trifluoromethanesulfonate (100 µL, 0.55 mmol) were added. The reaction was run on ice for 90 minutes, quenched with sodium thiosulphate and sodium bicarbonate, diluted to 100 mL with DCM, washed with brine and saturated sodium bicarbonate, rotovaped and purified by silica gel flash column chromatography (rf 0.6 in 3:2 ethyl acetate:hexanes), yielding **10** (α:β 2:1, 383 mg purified α, 38%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.82 (d, *J* = 8.3 Hz, 5H), 7.37 (d, *J* = 8.0 Hz, 5H), 5.34 (dd, *J* = 3.3, 0.8 Hz, 2H), 4.76 (dd, *J* = 10.9, 3.4 Hz, 2H), 4.29 (d, *J* = 8.0 Hz, 2H), 4.25 –

[2] J. J. Kasper, J. E. Hitro, S. R. Fitzgerald, J. M. Schnitter, J. J. Rutowski, J. A. Heck and J. L. Steinbacher, *J. Org. Chem.*, 2016, **81**, 8095–8103.

4.06 (m, 11H), 4.03 – 3.99 (m, 2H), 3.84 (td, *J* = 6.6, 0.9 Hz, 2H), 3.70 – 3.59 (m, 4H), 2.47 (s, 8H), 2.17 (s, 6H), 2.07 (s, 14H), 2.05 – 2.01 (m, 4H). LRMS (ESI) m/z: [M + Na] + calculated for $C_{22}H_{29}N_3O_{11}SNa$ = 566.2; Found 566.2



3-*O*-Tosylpropyl 3,4,6-tri-*O*-acetyl-2-*N*-acetyl-2-deoxy-α-D-galactopyranoside (13): (based on Steinbacher et al.[3]) Compound **10** (93 mg, 0.174 mmol) and zinc powder (0.1 g) were suspended in 9 mL of 3:2:1 acetic anhydride:tetrahydrofuran:acetic acid and placed on ice. The reaction reached room temperature and was left for 3 hours. The reaction mixture was diluted with 50 mL DCM, filtered on celite, concentrated and purified by silica gel flash column chromatography with a gradient from 50% to 100% ethyl acetate in hexanes, yielding **13** (79 mg, 82%, rf 0.1 in 3:1 ethyl acetate:hexanes). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.80 (d, *J* = 8.3 Hz, 2H), 7.39 (d, *J* = 8.0 Hz, 2H), 5.93 (d, *J* = 9.7 Hz, 1H), 5.36 (d, *J* = 3.1 Hz, 1H), 5.13 (dd, *J* = 11.4, 3.3 Hz, 1H), 4.87 (d, *J* = 3.6 Hz, 1H), 4.64 (ddd, *J* = 11.3, 9.8, 3.6 Hz, 1H), 4.31 (ddd, *J* = 9.9, 7.5, 5.0 Hz, 1H), 4.15 – 4.06 (m, 4H), 3.85 – 3.79 (m, 1H), 3.58 (ddd, *J* = 9.8, 7.6, 5.2 Hz, 1H), 2.47 (s, 3H), 2.18 (s, 3H), 2.06 (s, 4H), 2.02 (s, 3H), 2.00 (s, 3H), 1.99 – 1.95 (m, 2H). LRMS (ESI) m/z: [M + Na] + calculated for $C_{11}H_{13}O_4SNa$ = 559.2; Found 560.1

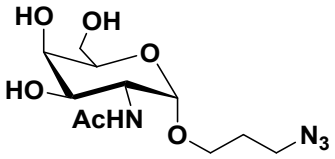


3-Azidopropyl 3,4,6-tri-*O*-acetyl-2-*N*-acetyl-2-deoxy-α-D-galactopyranoside (11): (based on Steinbacher et al.[4]) 3-*O*-tosylpropyl 3,4,6-tri-*O*-acetyl-2-*N*-acetyl-2-deoxy-α-D-galactopyranoside (**13**) (75 mg, 0.135 mmol) and tetrabutylammonium hydrogen sulphate (TBHS) (68 mg, 0.20 mmol) were dissolved in 5 mL DCM. A solution of sodium azide (85 mg, 1.31 mmol) in 5 mL water was added, and the biphasic mixture was vigorously stirred overnight at room temperature. 200 mg of TBHS and 100 mg of sodium azide were added, and the reaction was stirred for an extra five hours. The layers were separated, and the aqueous one was extracted twice with 25 mL DCM. Organic layers were dried with magnesium sulphate, combined, rotovaped and purified by silica gel flash column chromatography, yielding **11** (44 mg, 77%, rf 0.25 in 3:1 ethyl acetate:hexanes). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.81 (d, *J* = 9.5 Hz, 1H), 5.33 (d, *J* = 2.6 Hz, 1H), 5.10 (dd, *J* = 11.4, 3.3 Hz, 1H), 4.85 (d, *J* = 3.6 Hz, 1H), 4.52 (ddd, *J* = 11.4, 9.6, 3.6 Hz, 1H), 4.13 – 4.02 (m, 3H), 3.77 (dt, *J* = 10.1, 6.1 Hz, 1H), 3.49 (dt, *J* = 10.1, 6.1 Hz, 1H), 3.38 (dq, *J* = 18.8, 6.1 Hz, 2H), 2.11 (s, 3H), 2.00 (s, 3H), 1.93 (d, *J* = 15.7 Hz, 6H), 1.90 – 1.82 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.9, 170.4, 170.3,

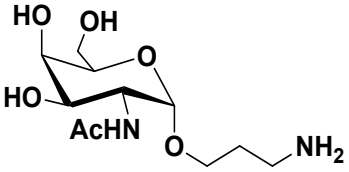
[3] J. J. Kasper, J. E. Hitro, S. R. Fitzgerald, J. M. Schnitter, J. J. Rutowski, J. A. Heck and J. L. Steinbacher, *J. Org. Chem.*, 2016, **81**, 8095–8103.

[4] J. J. Kasper, J. E. Hitro, S. R. Fitzgerald, J. M. Schnitter, J. J. Rutowski, J. A. Heck and J. L. Steinbacher, *J. Org. Chem.*, 2016, **81**, 8095–8103.

170.0, 97.8, 77.4, 77.2, 76.9, 68.3, 67.3, 66.8, 65.3, 62.0, 48.4, 47.7, 28.5, 23.2, 20.7, 20.7, 20.6. LRMS (ESI) m/z: [M + Na] + calculated for $C_{11}H_{13}O_4SNa$ = 430.2; Found 430.0



3-Azidopropyl 2-*N*-acetyl-2-deoxy-α-D-galactopyranoside (5): Compound **11** (40 mg, 131 µmol) was mixed with hydrazine hydrate (300 µL) and methanol (2 mL) and stirred at room temperature for 12 hours. The reaction mixture was evaporated and resuspended in water, then purified by size exclusion chromatography on Bio-Rad® P2-gel. All fractions (1 mL/fraction) were individually lyophilized and assessed for purity by NMR. Pure samples were pooled and lyophilized, yielding **3** (23 mg, 82%). ^{1}H NMR (600 MHz, D_2O) δ 4.35 (d, *J* = 8.5 Hz, 1H), 3.92 – 3.82 (m, 2H), 3.79 (dd, *J* = 10.4, 8.9 Hz, 1H), 3.75 – 3.53 (m, 5H), 3.29 (dd, *J* = 10.2, 6.5 Hz, 2H), 1.96 (s, 3H), 1.89 – 1.61 (m, 2H). ^{13}C NMR (151 MHz, D_2O) δ 174.6, 97.0, 70.9, 68.5, 67.6, 64.9, 61.2, 50.0, 48.1, 27.9, 21.9. LRMS (ESI) m/z: [M + H] + calculated for $C_{11}H_{21}N_4O_6$ = 305.1; Found 246.4; [M + Na] + calculated for $C_{11}H_{21}N_4O_6Na$ = 327.1; Found 326.9



3-Aminopropyl 2-*N*-acetyl-2-deoxy-α-D-galactopyranoside (12): Tn-azido **3** (7.9 mg, 28 µmol) was dissolved in 0.6 mL methanol. Zinc powder (20 mg, 307 µmol) and saturated ammonium chloride (60 µL) were added and the solution was vigorously mixed until gas stopped forming. After 15 minutes, the reaction was filtered on celite and dried. The crude was resuspended in 0.3 mL water and purified by size exclusion chromatography on Bio-Rad® P2-gel. All fractions (1 mL/fraction) were individually lyophilized and assessed for purity by ^{1}H NMR. Pure samples were pooled and lyophilized, yielding Tn-amine **12** (6.2 mg, 86%). ^{1}H NMR (600 MHz, D_2O) δ 4.83 (d, *J* = 3.6 Hz, 7H), 4.08 (dd, *J* = 11.1, 3.7 Hz, 10H), 3.98 – 3.81 (m, 20H), 3.76 – 3.47 (m, 29H), 3.47 – 3.44 (m, 2H), 3.04 (t, *J* = 7.6 Hz, 15H), 2.00 – -57.98 (m, 198H). ^{13}C NMR (151 MHz, D_2O) δ 174.6, 97.1, 71.1, 68.5, 67.5, 65.0, 61.3, 49.9, 37.2, 26.7, 21.9. Tn-amine was fully converted back into Tn-azido by adding it to a suspension of Stick's reagent (6.0 mg, 24 µmol), potassium carbonate (9 mg, 48 µmol) and copper sulfate (70 µg, 0.28 µmol, diluted from a stock solution) in methanol (100 µL). The reaction was run for three hours, evaporated, filtered and passed through a P2 column for purification, yielding Tn-N_3 (72%, 4.9 mg).

PS A1 Preparation

PS A1 was extracted as previously described.[5] 1 L septum-equipped culture bottles were filled with PYG broth medium made with 20 g proteose peptone (Sigma 107229), 5 g sodium chloride, and 5 g yeast

[5] R. A. De Silva, Q. Wang, T. Chidley, D. K. Appulage and P. R. Andreana, *J. Am. Chem. Soc.*, 2009, **131**, 9622–9623.

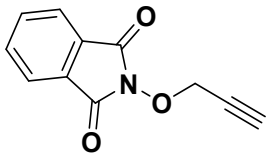
extract (MP Biomedicals 3065-522), dissolved in one Liter of ultrapure water and autoclaved. Once cooled, the bottles were purged with nitrogen gas for 15 minutes and the medium was supplemented with 100 µL of hemin (0.5% in 1.0 M NaOH), 50 µL vitamin K1 (0.5% in absolute ethanol) and 5 mL of saline solution (10% glucose, 10% dibasic potassium phosphate, 1% cysteine). All solutions were syringe-filtered for sterility. Finally, the bottle was inoculated with a stock of *Bacteroides fragilis* (ATCC 25285/NTCT 8343, stored in 50% glycerol at -78 °C) and placed in an incubator at 37 °C for 2 days. The culture was processed by centrifugation (Eppendorf 5810R, 3000 rpm/2000 rcf, 25 min, 4 °C). The supernatant was neutralized with diluted bleach and discarded, and the bacteria pellets were resuspended in a minimal amount of PBS and stored at -78 °C until 40 L of culture was processed.

The solution (1 L) was mixed 1:1 with a 3:1 phenol:water solution and vigorously stirred at 70 °C for 30 minutes. The mixture was cooled and separated by centrifugation and the phenol phase was discarded. The aqueous phase was concentrated overnight *via* airflow and washed 1:1 with THF three times. The aqueous phase was dialyzed against deionized water for 48 hours (10 kDa MWCO Snakeskin® membrane, ThermoFisher Scientific 88245). The obtained powder was resuspended in 50 mL of 50 mM Tris buffer with 15 mM NaCl (pH 7.5) and incubated overnight with 10 mg RNAse A (Roche 10109142001). The solution was dialyzed, lyophilized and resuspended in 50 mL 0.1 M acetate buffer at pH 5, in the presence of 10 mg of DNAse 1 (Alfa Aesar J62229). The solution was incubated overnight at 37 °C, dialyzed, lyophilized and resuspended in 50 mL of 0.1 M Tris buffer with 10 mM $CaCl_2$ and 10 mg Pronase (Roche 10165921001). Finally, the solution was dialyzed, lyophilized and examined by ^{1}H NMR. If any aromatic peaks were left, the enzymatic treatment was repeated.

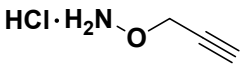
After removal of lipids, proteins, DNA, RNA and small molecules, only large polysaccharides were left. To isolate PS A1, the crude mixture was first passed through an ion exchange column (Sephacryl S-400) with 0.5% sodium deoxycholate, 50 mM glycine and 10 mM EDTA as the eluent corrected to pH 9.5 with 5 M NaOH. 10 mL fractions were collected, and separately dialyzed, lyophilized and analysed by ^{1}H NMR. Fractions containing PS A1 were pooled and lyophilized. Finally, PS A1 was separated from the other zwitterionic capsular polysaccharide, PS B, by ion exchange chromatography. The lyophilized, cloudy solid was dissolved in 5 mL 5% acetic acid at 80 °C for 30 minutes, neutralized with 2 M NaOH and immediately passed through a DEAE Sephacel GE column using 50 mM Tris buffer (pH 7.3) as the eluent with increasing NaCl concentrations (0, 0.1 M, 0.2 M, 0.5 M, 1 M, 2 M). 10-12 mL fractions were individually dialyzed, lyophilized and assessed by ^{1}H NMR. Impure fractions were repurified through S-400 and DEAE.

Preparation of Tn-PS A1 **A** and Tn-click-PS A1 **B**

Synthesis of the Alkyne Linker



***N*-(Propargyloxy)phthalimide:** 80 wt. % progargyl bromide in toluene (12 g, 80 mmol), *N*-hydroxyphthalimide (5 g, 30.6 mmol) and triethylamine (11 mL, 82 mmol) were mixed in 150 mL *N,N*-dimethylformamide and the reaction was stirred overnight at room temperature, then poured in 500 mL ice-cold water. The product was filtered out as a crystalline solid and dried (5.1 g, 83%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.88 (dd, *J* = 5.4, 3.1 Hz, 2H), 7.79 (dd, *J* = 5.5, 3.1 Hz, 2H), 4.90 (d, *J* = 2.4 Hz, 2H), 2.62 (s, 1H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 163.4, 134.7, 128.8, 123.7, 78.2, 77.3, 77.1, 76.8, 76.4, 65.0.



***O*-2-Propynylhydroxylamine hydrochloride (3):** *N*-(Propargyloxy)phthalimide (300 mg, 1.5 mmol) and hydrazine hydrate (0.5 mL, 10 mmol) were mixed in 5 mL diethyl ether and stirred overnight under argon. The byproduct was filtered out and the filtrate was treated with 2 M HCl in diethyl ether until precipitate stopped forming. The suspension was placed in the freezer for 2 hours, then the product was filtered out as a crystalline solid (102 mg, 94%). ^{1}H NMR (600 MHz, D_2O) δ 4.75 (d, *J* = 2.4 Hz, 2H), 3.18 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (151 MHz, D_2O) δ 80.0, 75.2, 62.6.

Vaccine Assembly

PS A1 Oxidation: PS A1 (1 mg, 9 nmol) was first dissolved in 1 mL sodium acetate buffer (0.1 M, pH 5.0) and oxidized with 55 µL of a fresh sodium periodate solution (10 mM, 2.1 mg/mL). The reaction was stirred for 90 minutes in the dark at room temperature, then KCl (2 mg) was added to precipitate excess periodate as KIO4, yielding crude oxidized PS A1 1, which was not isolated.

Tn-PS A1 (A): To the previous oxidized PS A1 solution, Aminooxy Tn **2** (10 mg, 75 µmol) was added, and the reaction was stirred overnight in the dark at room temperature. Diafiltration on a centrifuge (10 kDa MWCO, 5000 rcf, 8 washes with DI water) and lyophilization yielded conjugate **A** (0.8 mg, 80% recovery).

PS A1-alkyne (4): To the previous oxidized PS A1 solution, alkyne linker **3** (10 mg, 34 µmol) was added, and the reaction was stirred overnight in the dark at room temperature. Diafiltration on a centrifuge (10 kDa MWCO, 5000 rcf, 8 washes with DI water) and lyophilization yielded conjugate **A** (0.8 mg, 80% recovery).

Tn-click-PS A1 (B): Tn-N_3 **3** and PS A1-alkyne **4** were dissolved in 0.5 mL phosphate buffer (pH 7.0, 0.1 M) in the presence of copper sulfate (12.5 µg, 2 µmol, 0.10 mM), THPTA (100 µg, 0.50 µmol) and sodium

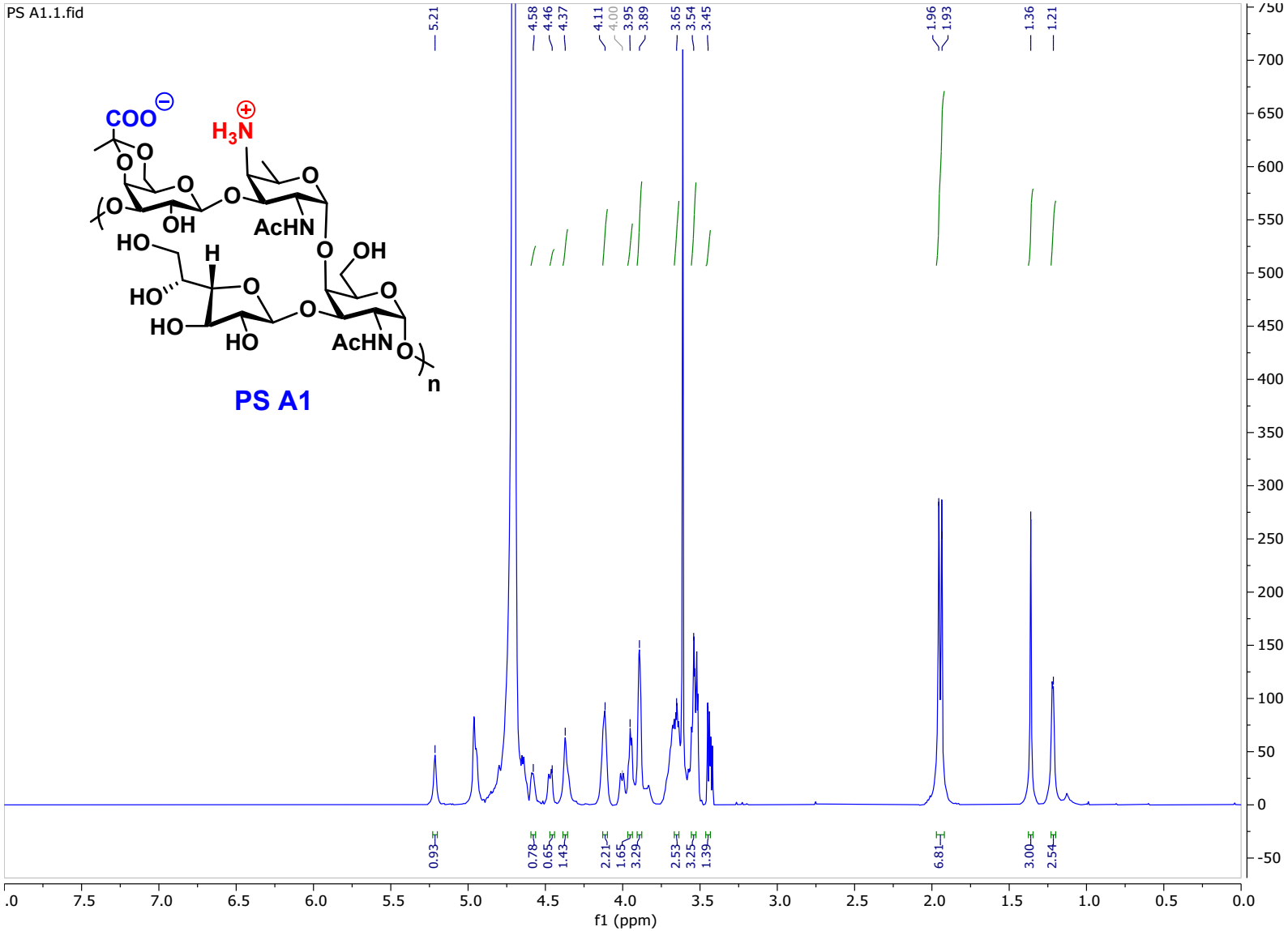
ascorbate (0.500 mg, 5.0 mM). After 16 hours, the reaction was purified by diafiltration on a centrifuge (4 °C, 14,000 rcf, 10-30 kDa MWCO, 8*25 min).

Characterization: Vaccines **A** and **B** were characterized by ^{1}H NMR by comparing the integration of the newly formed E/Z oxime peaks (7-8 ppm) with a reference acetate CH_3 of PS A1. Loading was calculated as:

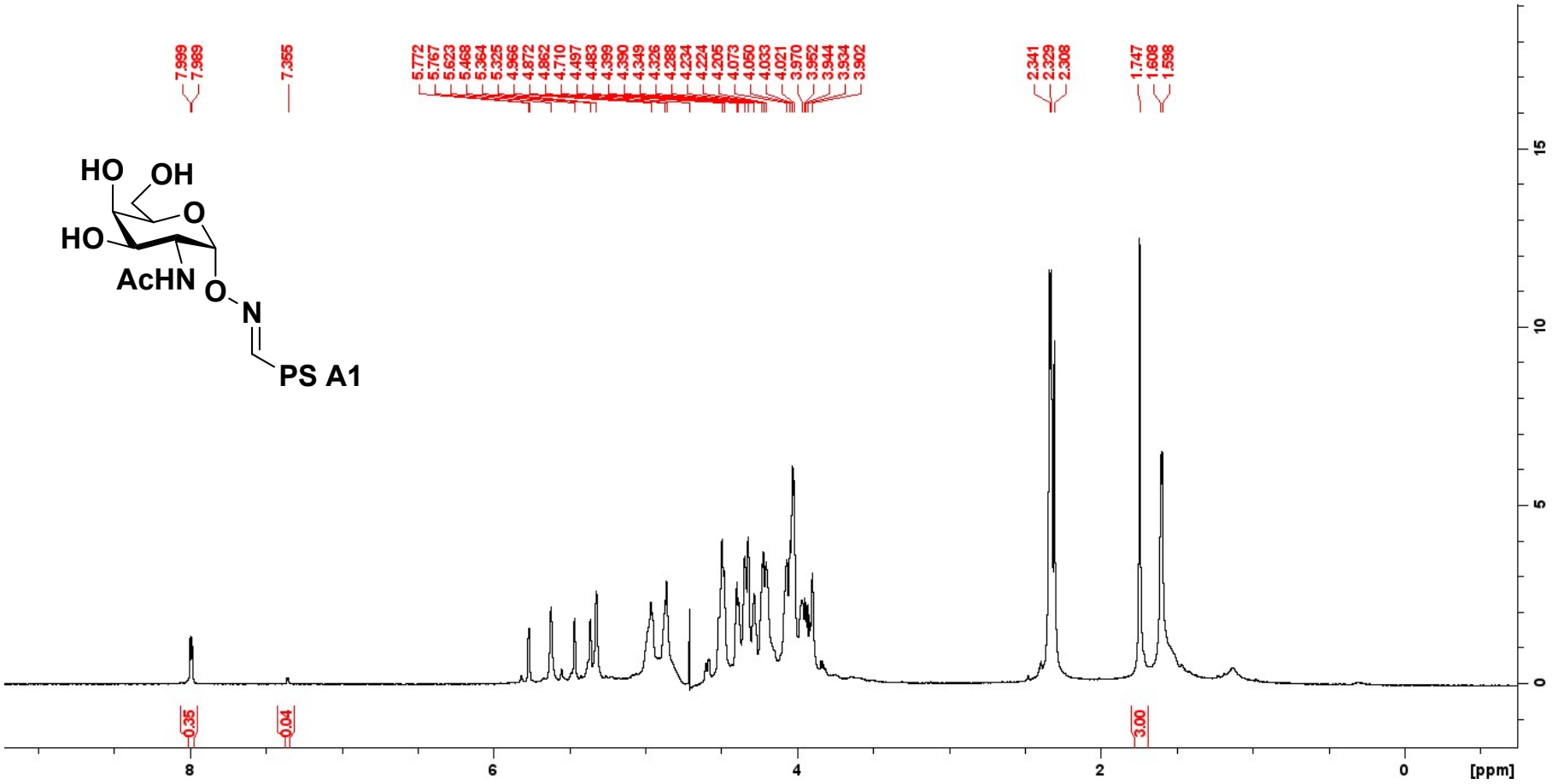
$$Antigen\ loading\ level\ (\%) = (integration\ of\ oxime\ E + integration\ of\ oxime\ Z) * 100$$

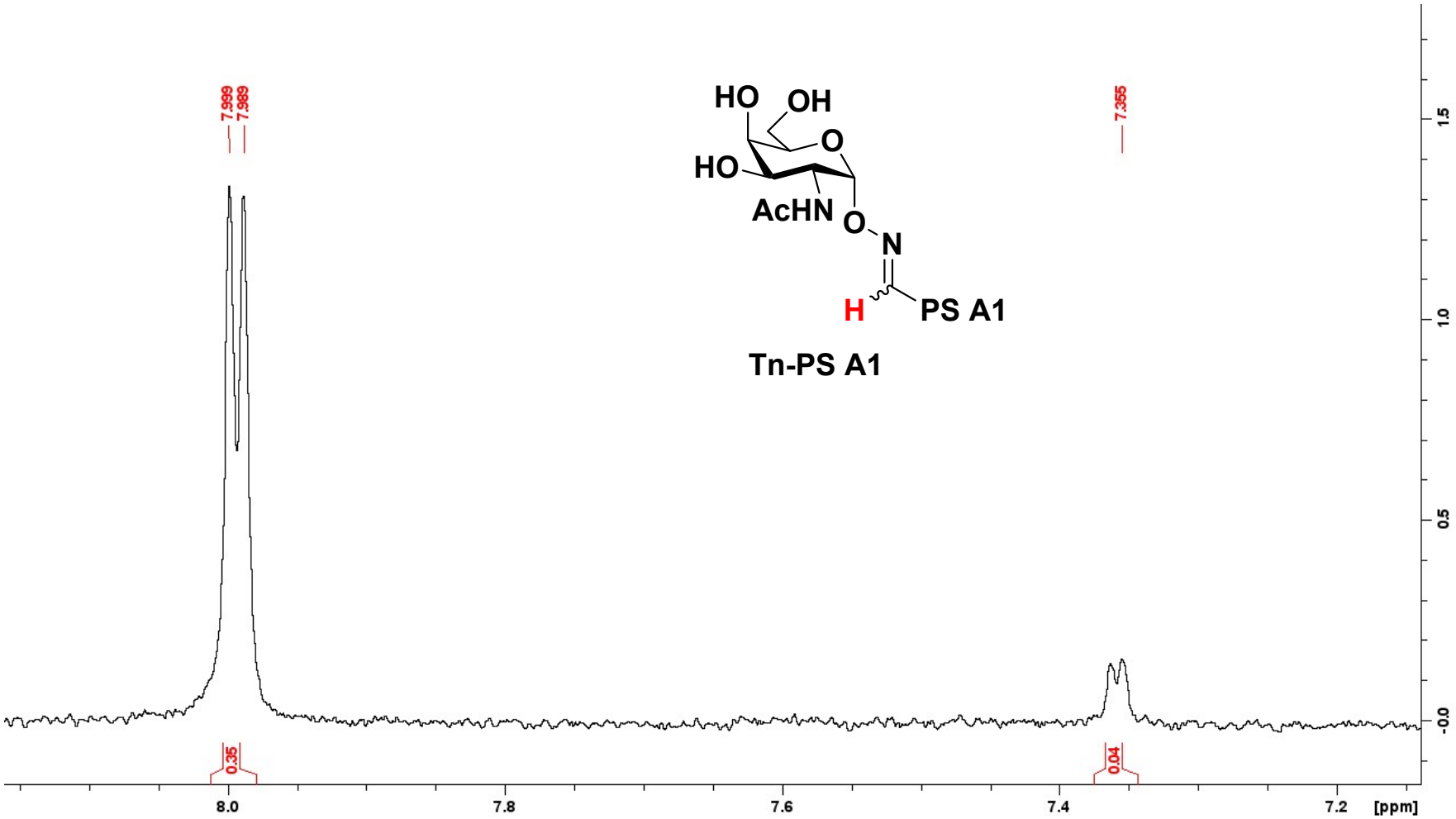
Number of antigens per molecule of PS A1 was calculated by assuming a 110 kDa/130 repeating units PS A1 molecule.

^{1}H NMR of PS A1

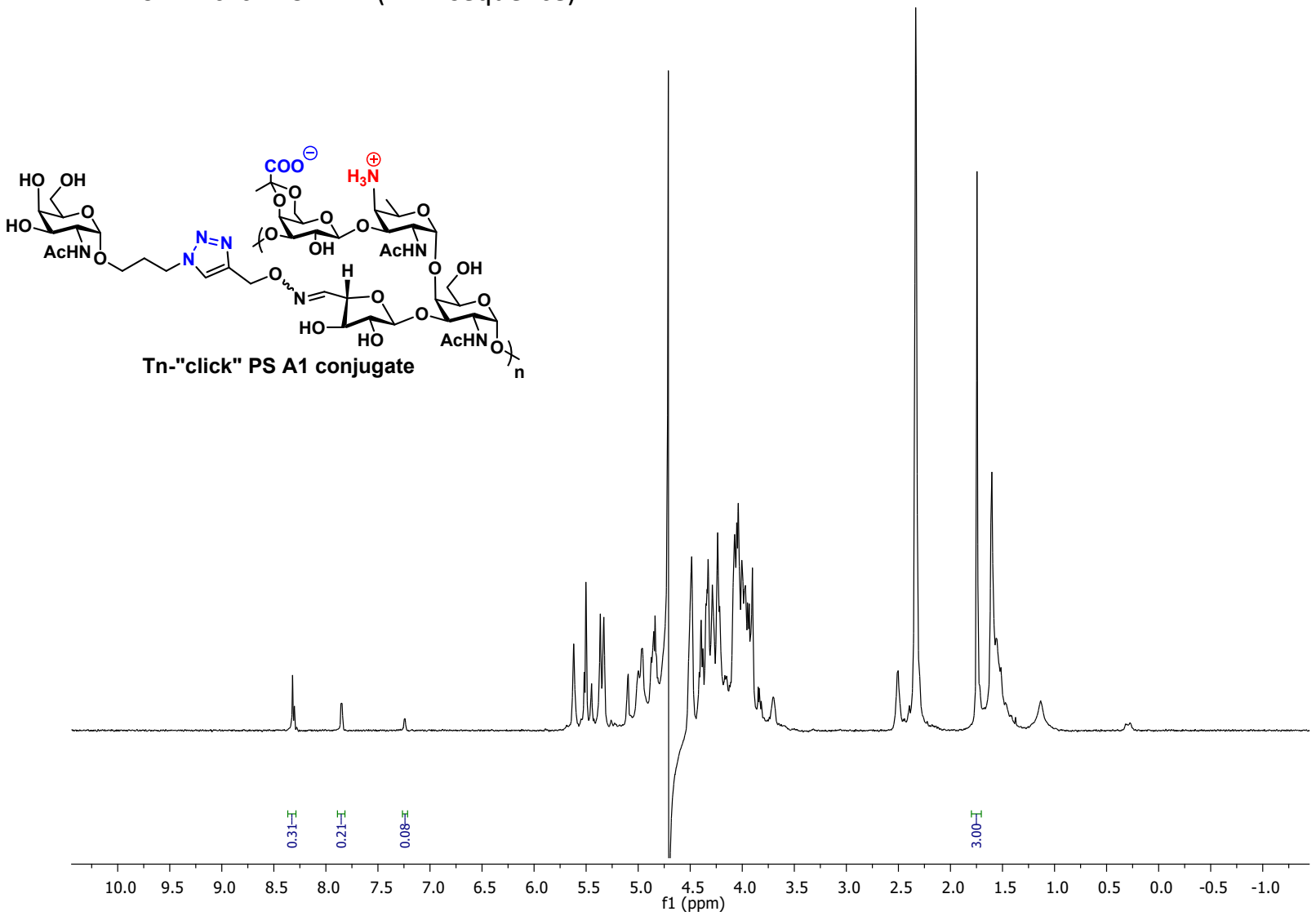


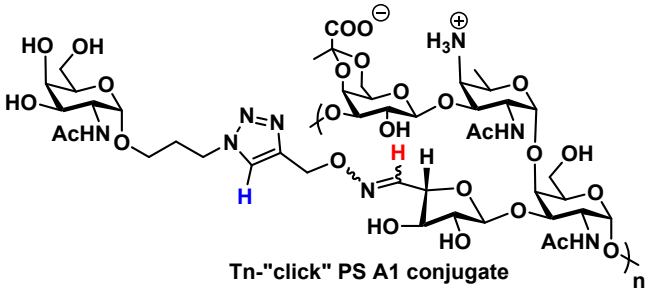
^{1}H NMR of Tn-PS A1 **A** (WET sequence)

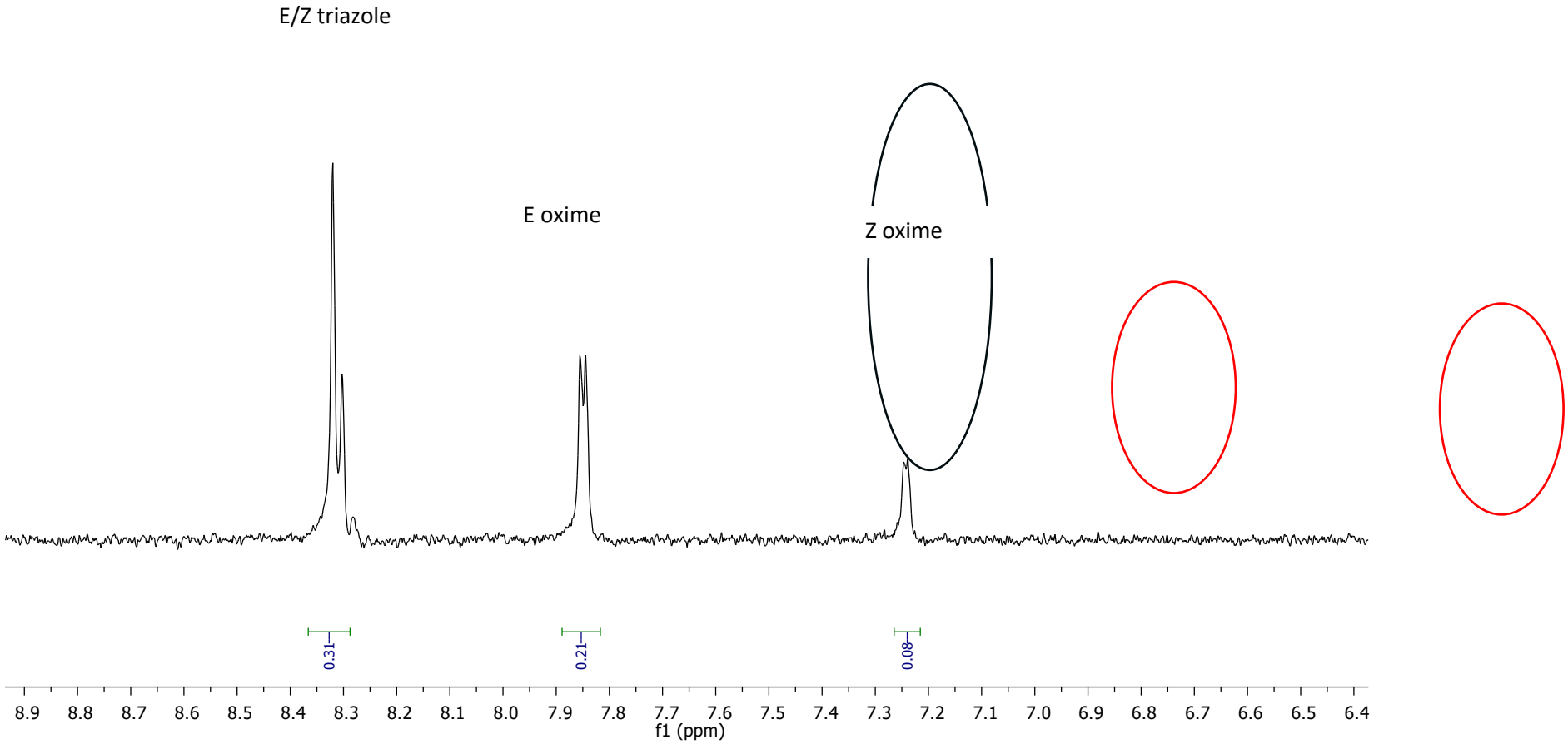



7.999
7.989
7.355
HO
OH
O
HO
AcHN
O
N
H
PS A1
Tn-PS A1
0.35
0.04
8.0
7.8
7.6
7.4
7.2
[ppm]
1.5
1.0
0.5
-0.0

^{1}H NMR of Tn-click-PS A1 **B** (WET sequence)




Tn-"click" PS A1 conjugate


E/Z triazole
E oxime
Z oxime
0.31
0.21
0.08
8.9 8.8 8.7 8.6 8.5 8.4 8.3 8.2 8.1 8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1 7.0 6.9 6.8 6.7 6.6 6.5 6.4
f1 (ppm)

Biological Evaluation

Cell Culture

All cells were cultured in an incubator at 37 °C, 5% CO_2 in Corning CLS430167 culture-treated petri dishes. MCF7 (ATCC HTB-22) and MDA-MB-231 (ATCC CRM-HTB-26) were cultivated in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco A5209501). OVCAR3 (ATCC HTB-161) and MCF10A (ATCC CRL-10317) were cultured in DMEM supplemented with 20% fetal bovine serum.

Tn-BSA General Procedure

Tn (6-40 eq.) was dissolved in phosphate buffer pH 7.0 and 2 µL of a 50% solution of diethyl squarate (DSQ) in absolute ethanol was then added. After 24 hours, the pH was then corrected to 9.0 using 2.0 M NaOH, then BSA (0.5 µmol, 33 mg) was added, and the mixture was stirred for 1-5 days at room temperature. The BSA conjugate was purified by diafiltration (4 °C, 14,000 rcf, 10-30 kDa MWCO, 8*25 min) and characterized by MALDI-TOF-MS (conjugate 1-5 mg/mL, sDHB 20 mg/mL). Results are presented in **Table 2**.

Triazole-BSA

Triazole-BSA was synthesized by first functionalizing BSA (0.15 µmol, 10 mg) with propargyl bromide (50 µL, large excess) in 1 mL of 0.5 M borate buffer pH 9.0. Alkyne-BSA was purified by diafiltration (4 °C, 14,000 rcf, 10-30 kDa MWCO, 8*25 min), lyophilized and resuspended in The alkyne-functionalized BSA was then conjugated with 3-azidopropanol (50 µL, large excess) in 0.5 mL 100 mM phosphate buffer pH 7.0, in the presence of copper sulfate (12.5 µg, 2 µmol, 0.10 mM), THPTA (100 µg, 0.50 µmol) and sodium ascorbate (0.500 mg, 5.0 mM). After 16 hours, triazole-BSA was purified by diafiltration (4 °C, 14,000 rcf, 10 kDa MWCO, 8*25 min) and characterized by MALDI-TOF-MS (conjugate 1-5 mg/mL, sDHB 20 mg/mL).

Mouse Immunizations

All animal protocols were approved by the University of Toledo institutional Animal Care and Use Committee (IACUC) and performed at the Department of Laboratory Animal Research (DLAR), an AAALAC accredited facility following all applicable USA federal regulations and AAALAC recommendations, under protocol UT-400102. Sample size was calculated based on obtainable serum volume (0.3 mL/mouse) for downstream experiments, plus two extra mice per group to account for possible exclusions. Exclusion criteria included development of multiple abscesses at vaccine injection and health complications. Mice were observed daily for signs of distress and weighed thrice weekly. No mice met exclusion criteria, and no significant side effects were observed. The study was not randomized. Vaccines were prepared by mixing 100 µL of Sigma Adjuvant System (SAS), reconstituted according to manufacturer guidelines, with 20 µg of sterile-filtered constructs Tn-PS A1 or Tn-click-PS A1 in 100 µL sterile PBS, for a total injection volume of 200 µL. Six-week old C57BL6/J male mice (Jackson Laboratories) were separated in three groups of seven. After an acclimation period of a week, mice were intraperitoneally injected with 200 µL of PBS (group 3, negative control), Tn-PS A1 (group A, positive control) or Tn-click-PS A1 (group B) on days 0, 21 and 42 and bled 10 days after the last injection. Blood was left to clot for 60 minutes then separated by centrifugation at 4 °C, 1500 rcf for 10 minutes. Serum was pooled and kept at -78 °C.

ELISA Measurement of Antibody Titers

Enzyme-linked immunosorbent assays (ELISA) were run as previously described.[6] ELISA plates (Immulon™ 4 HBX) were coated with 100 µL of 2 µg/mL antigen-BSA conjugate and incubated overnight at 4 °C, then washed twice with washing buffer (TBS, 0.05% Triton-20, pH 7.3). Extra binding sites were blocked with 150 µL of 2% BSA in TBS, incubated for 2 hours at 37 °C and washed again three times. Anti-serum was diluted in TBS at 1:300 for total Ig and 1:100 for isotyping in TBS, then serial diluted in half-log10 dilutions and incubated at 37 °C for 2 hours, then washed three times. The plate was then incubated with alkaline-phosphatase conjugated antibody targeting murine κ chain (100 µL diluted 1:2000 in TBS, southern biotech 1050-04), IgG (1:2000, Jackson ImmunoResearch 115-055-146) or IgM (1:1000, Jackson ImmunoResearch 115-055-075) for 1 hour at 37 °C, washed, and revealed with 100 µL of 1 mg/mL PNPP in diethanolamine buffer for 30 minutes at room temperature. Absorbance values were read at 405 nm using a UV-vis plate reader (Bio-Rek Powerwave™ HT). All assays were performed in triplicate. Data between the groups were compared with one-way ANOVA on R 4.5.2.

Complement-Dependent Cytotoxicity (CDC) Assays

CDC assays were performed as reported previously.[7] 1.0 x 10^4 cells in 100 µL DMEM medium were transferred to culture-treated 96-well plates (CytoOne® CC7682-7596) and incubated overnight at 37 °C, 5% CO_2. The plates were washed with PBS, then 100 µL of anti-serum (1:40 in DMEM) was added, and the plates were incubated for 2 hours. The wells were washed three times, and the plate was incubated for an extra hour with 100 µL of rabbit complement serum (1:10 in DMEM, Bio-Rad C12CC). Finally, 20 µL of the supernatant was transferred to a different well and mixed with 80 µL PBS and 100 µL of LDH cytotoxicity detection kit solution (Roche 11644793001) prepared following manufacturer's instructions. The plate was agitated for 30 minutes at room temperature, in the dark, and the absorbance was measured at 490 nm on a Bio-Rek Powerwave™ HT plate reader. A positive control (PC) (100% cell killing) made by replacing anti-serum and complement with 2% triton X-100 in water, and a negative control (NC) with no anti-serum or complement were added to each plate. Each assay was run in triplicate and data between the groups were compared with one-way ANOVA on R 4.5.2. The final cytotoxicity was calculated as:

$$cytotoxicity\ (\%) = \frac{Experimental\ well - NC}{PC - NC}\ X\ 100$$

Fluorescence-Activated Cell Sorting (FACS)

Medium was removed from culture plates and cells were detached using 0.25% trypsin, incubated for 5-10 minutes at 37 °C, 5% CO_2, then collected in a centrifuge tube, washed with 5 mL PBS and centrifuged (4 °C, 600 rcf, 7 min). The supernatant was discarded and 1 mL of 1:250 antiserum in PBS was added. The suspension was homogenized, incubated for 1 hour at 37 °C, then the cells were washed with PBS and centrifuged again. The cells were treated with 1 mL of 1:300 goat anti-mouse kappa FITC conjugate

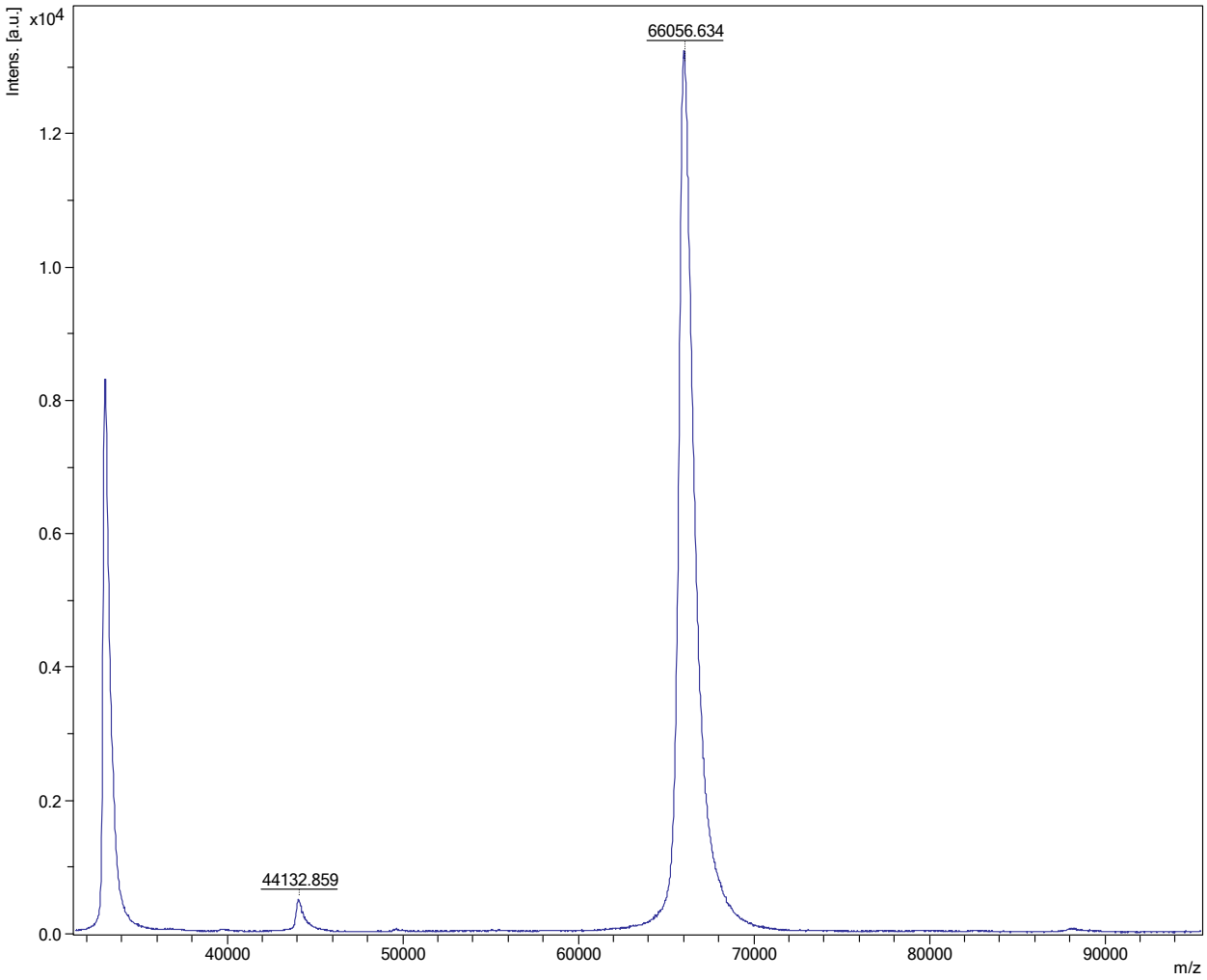
[6] K. A. Kleski, K. R. Trabbic, M. Shi, J.-P. Bourgault and P. R. Andreana, *Molecules*, 2020, **25**, 1319.

[7] M. Shi, K. A. Kleski, K. R. Trabbic, J.-P. Bourgault and P. R. Andreana, *J. Am. Chem. Soc.*, 2016, **138**, 14264–14272.

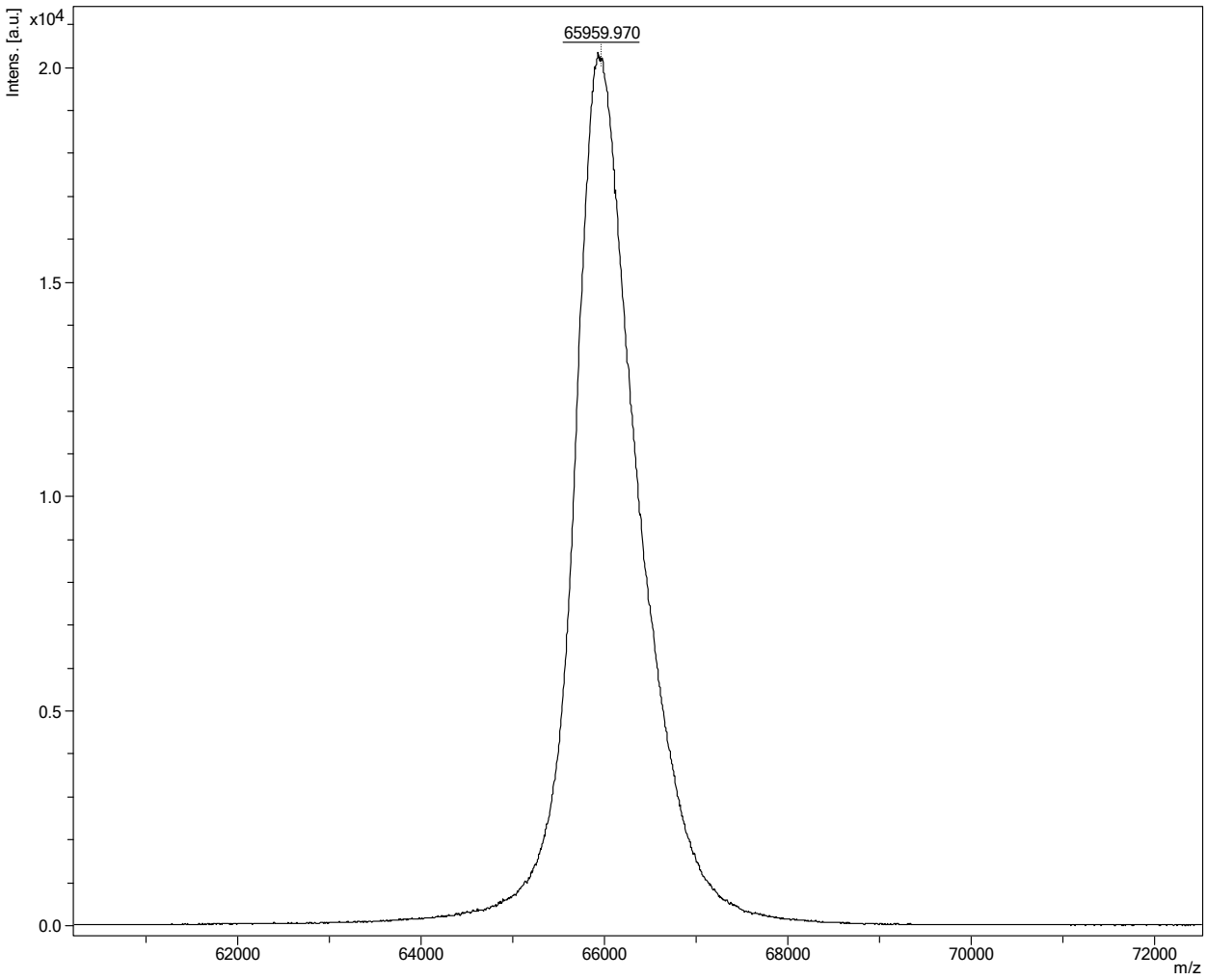
(Southern Biotech 1050-02) in PBS, incubated for 30 minutes at 37 °C, washed, centrifuged and resuspended in 2 mL PBS in a FACS tube. Samples were kept on ice for up to one hour until analysis on a BD FACSCalibur™ instrument. Data was analyzed using FlowJo™ (FlowJo LLC).

Tn-BSA MALDI Data (Table 1)

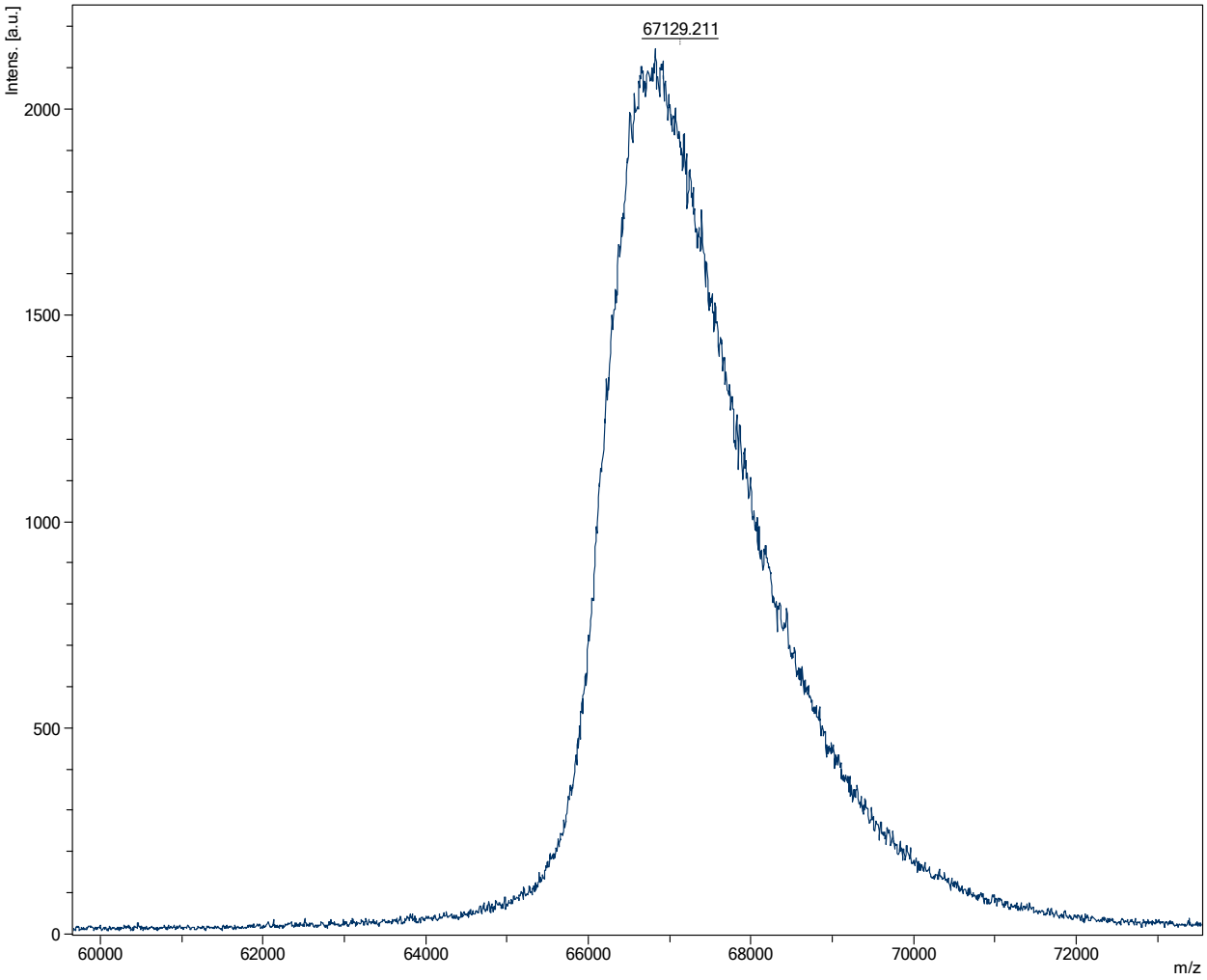
MALDI BSA Reference



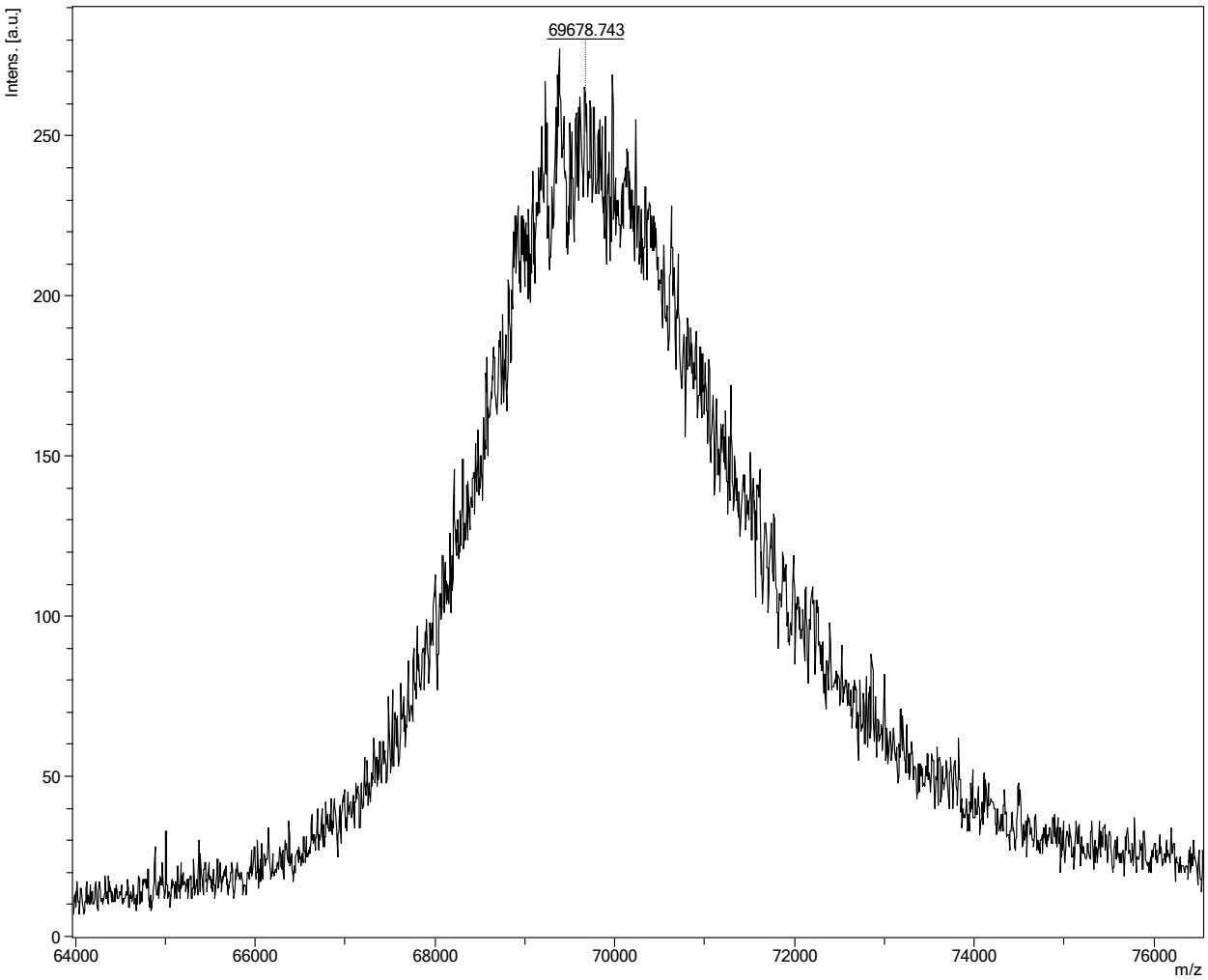
Tn-BSA One-Pot Synthesis MALDI, Table 1; Entry 1



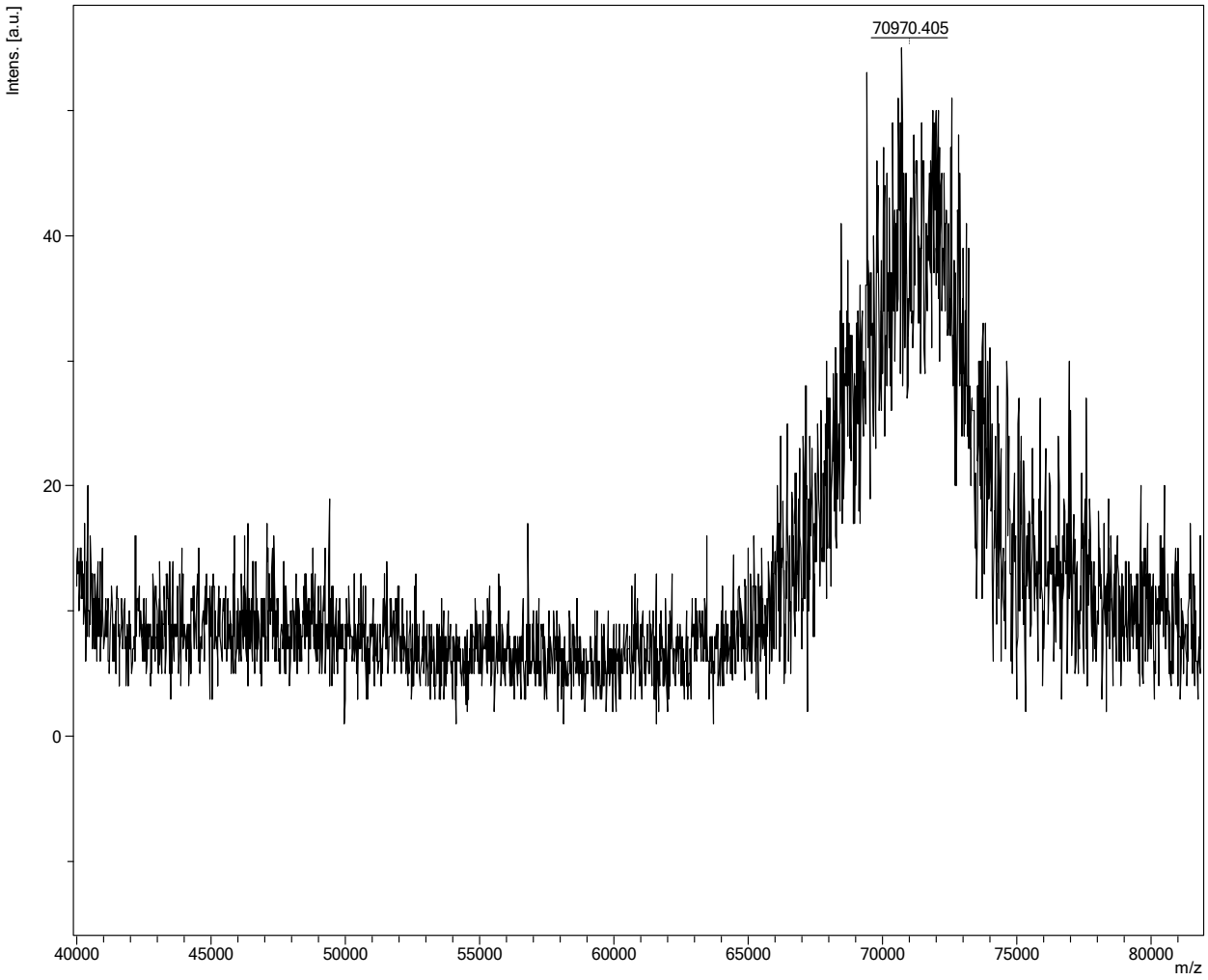
Tn-BSA One-Pot Synthesis MALDI, Table 1; Entry 2



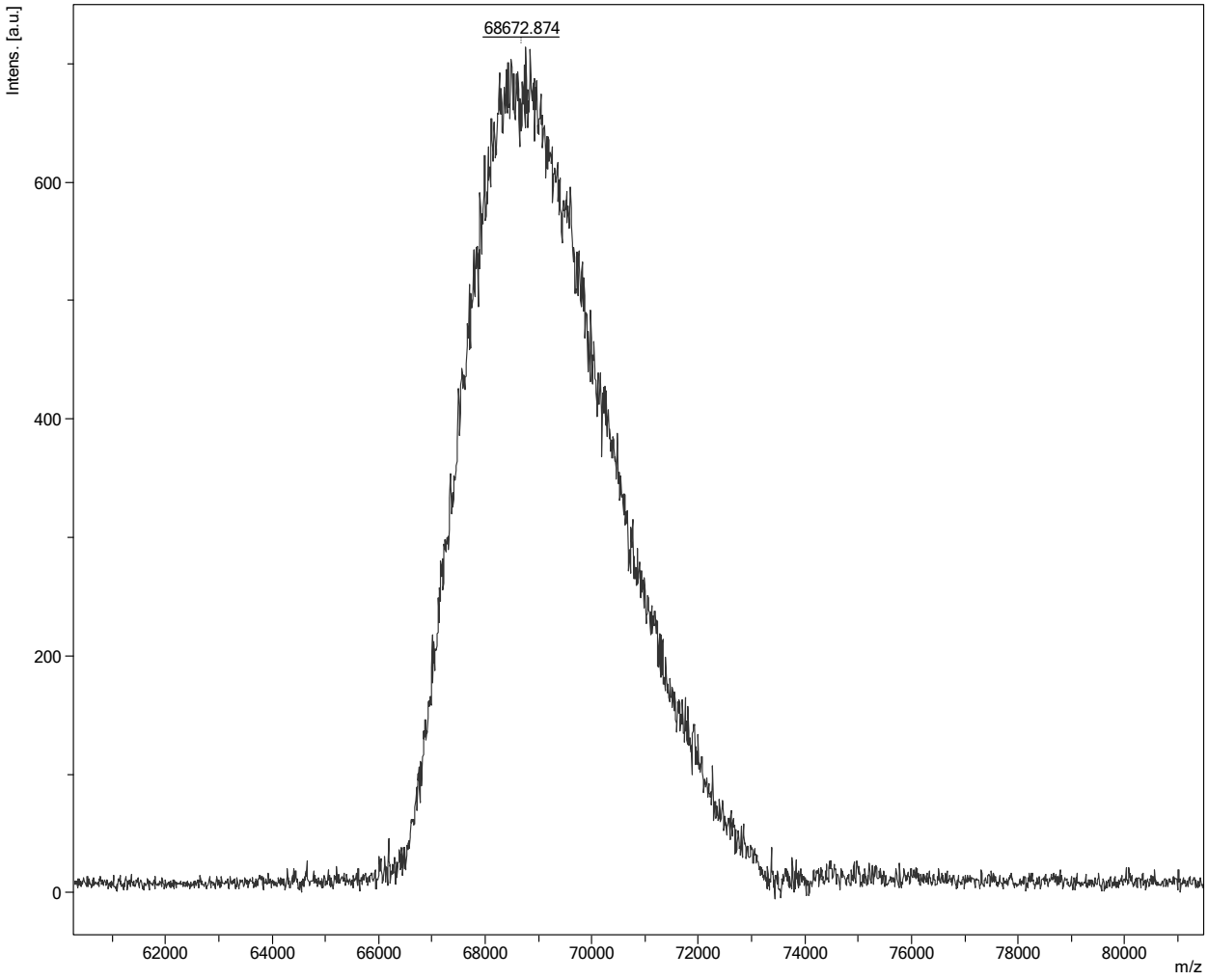
Tn-BSA One-Pot Synthesis MALDI, Table 1; Entry 3



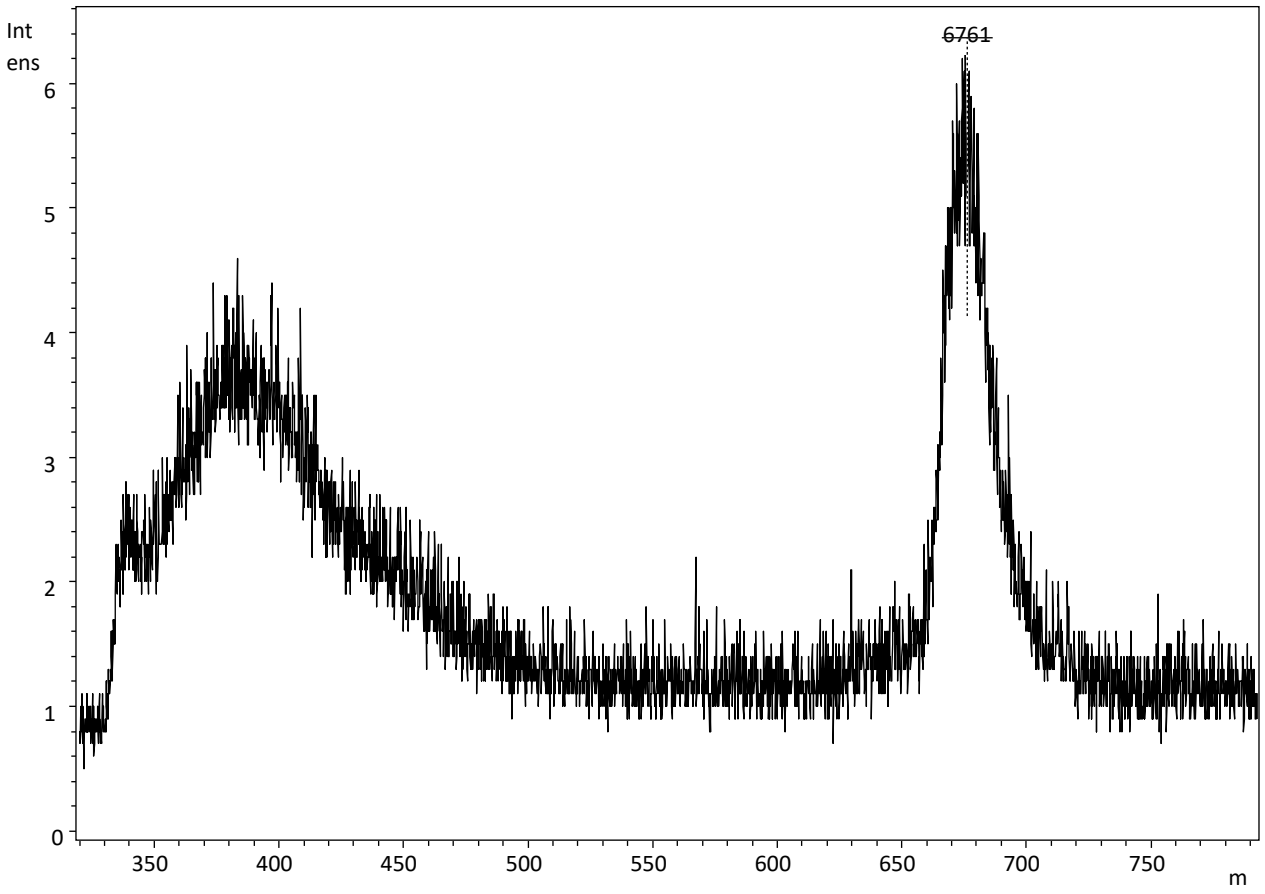
Tn-BSA One-Pot Synthesis MALDI, Table 1; Entry 4



Tn-BSA One-Pot Synthesis MALDI, Table 1; Entry 5

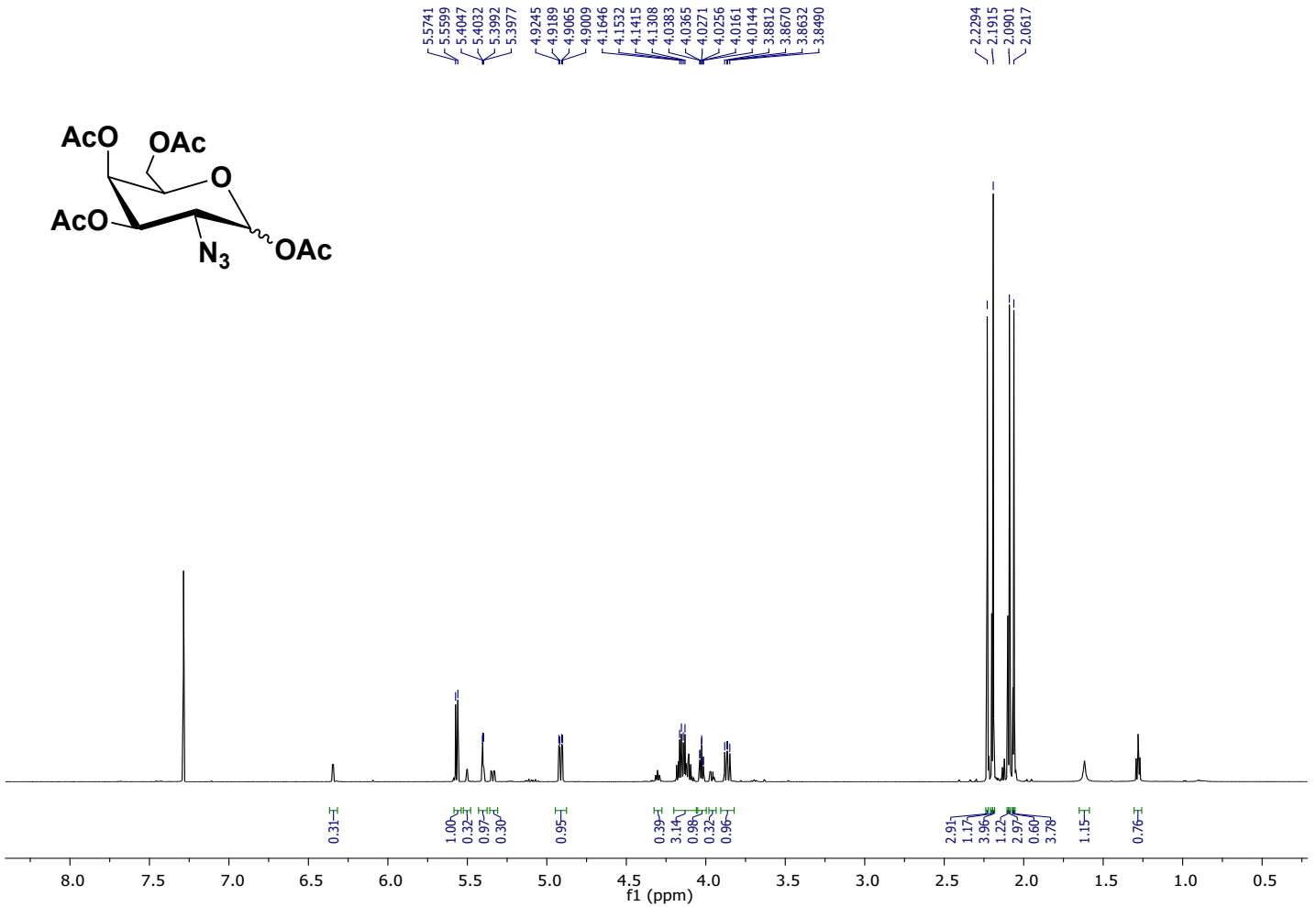


Triazole-BSA MALDI

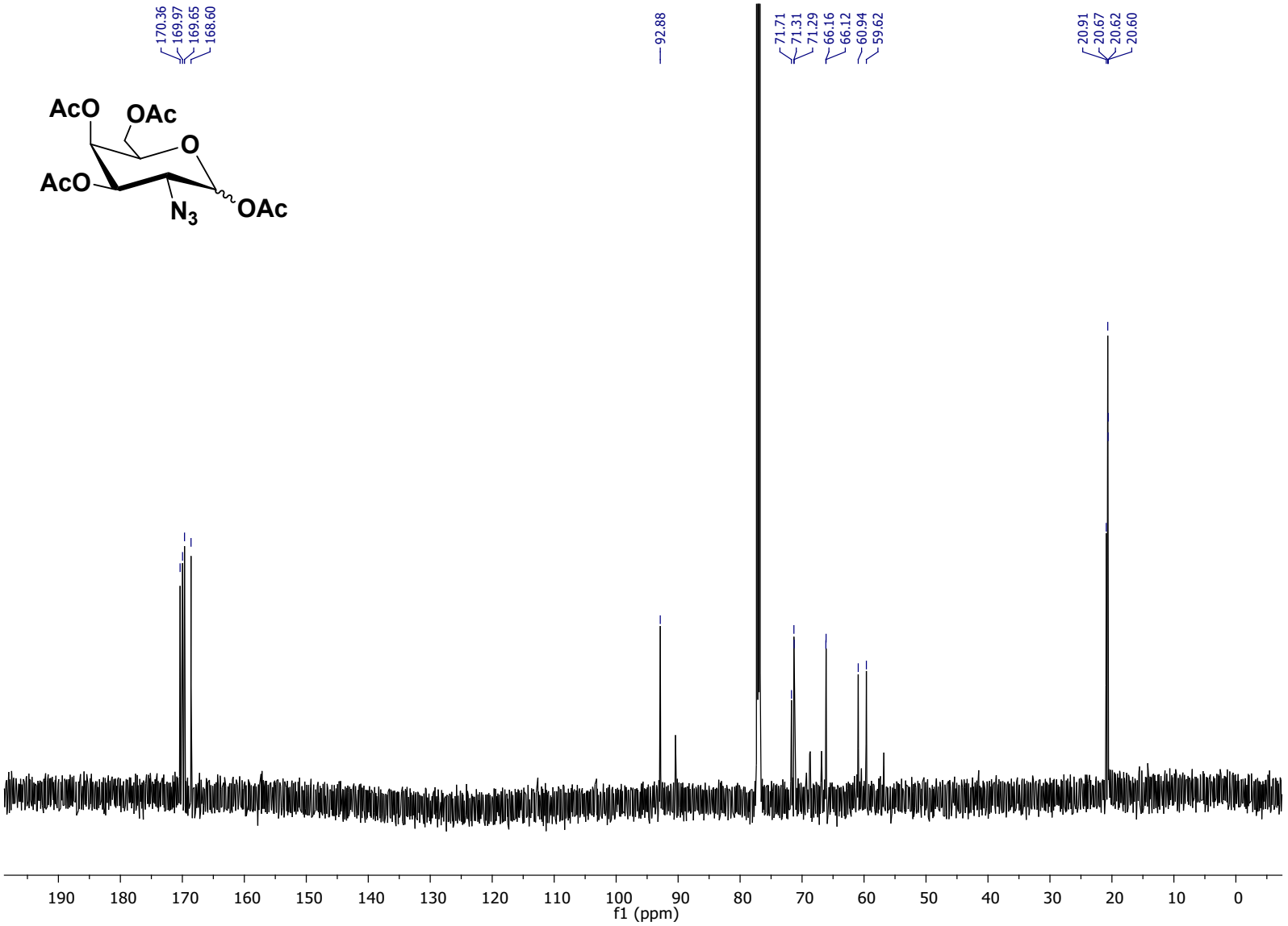


NMR Spectra

^{1}H NMR of **6**



^{13}C NMR of **6**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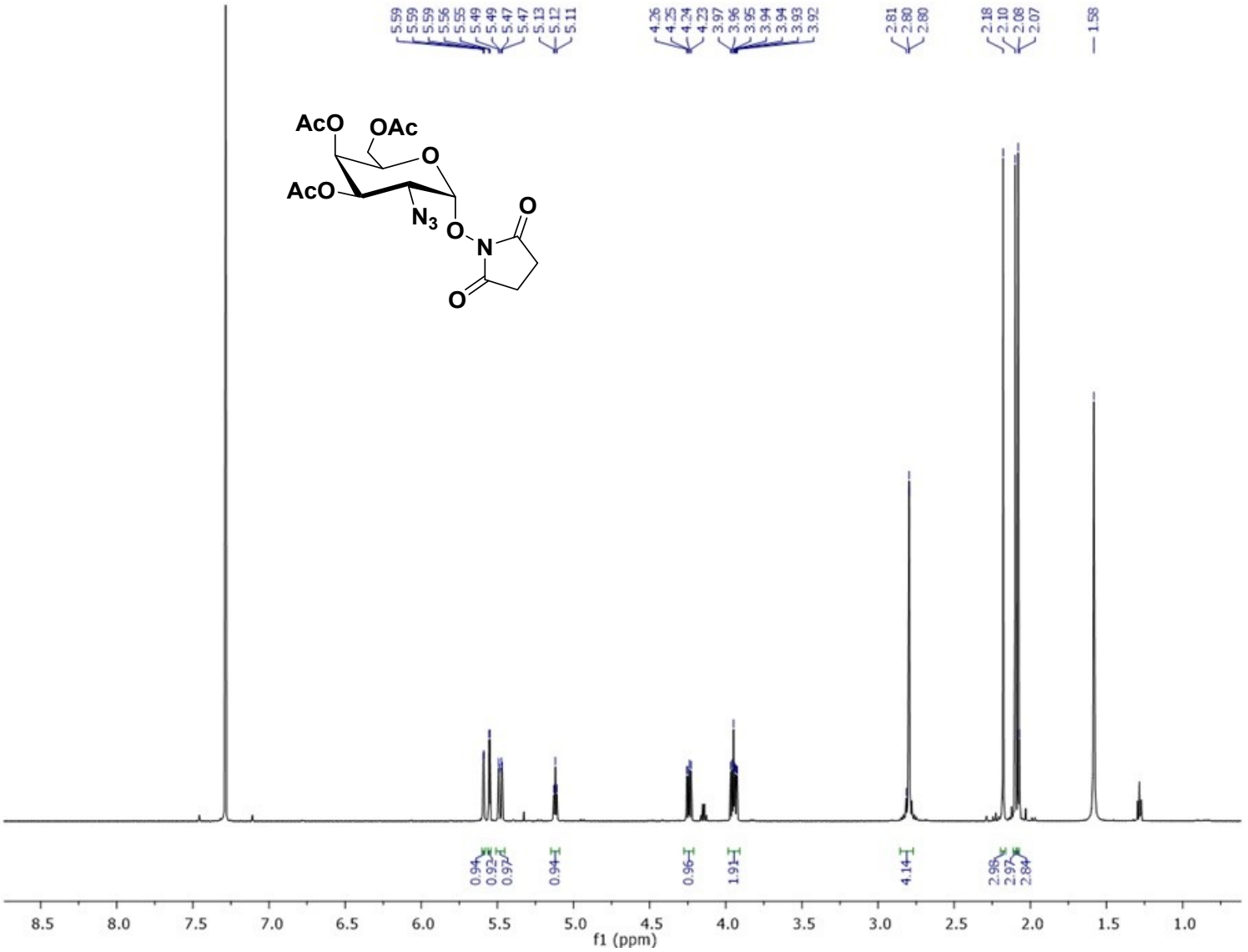
169.97
169.65
168.60
92.88
71.71
71.31
71.29
66.16
66.12
60.94
59.62
20.91
20.67
20.62
20.60
AcO
OAc
O
AcO
N3
OAc
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

^{1}H NMR of **8**


5.59
5.59
5.59
5.56
5.55
5.49
5.49
5.47
5.47
5.13
5.12
5.11
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4.25
4.24
4.23
3.97
3.96
3.95
3.94
3.94
3.93
3.92
2.81
2.80
2.80
2.18
2.10
2.08
2.07
1.58
AcO
OAc
AcO
O
N3
O
N
O
O
0.94
0.92
0.97
0.94
0.96
1.91
4.14
2.98
2.97
2.84
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
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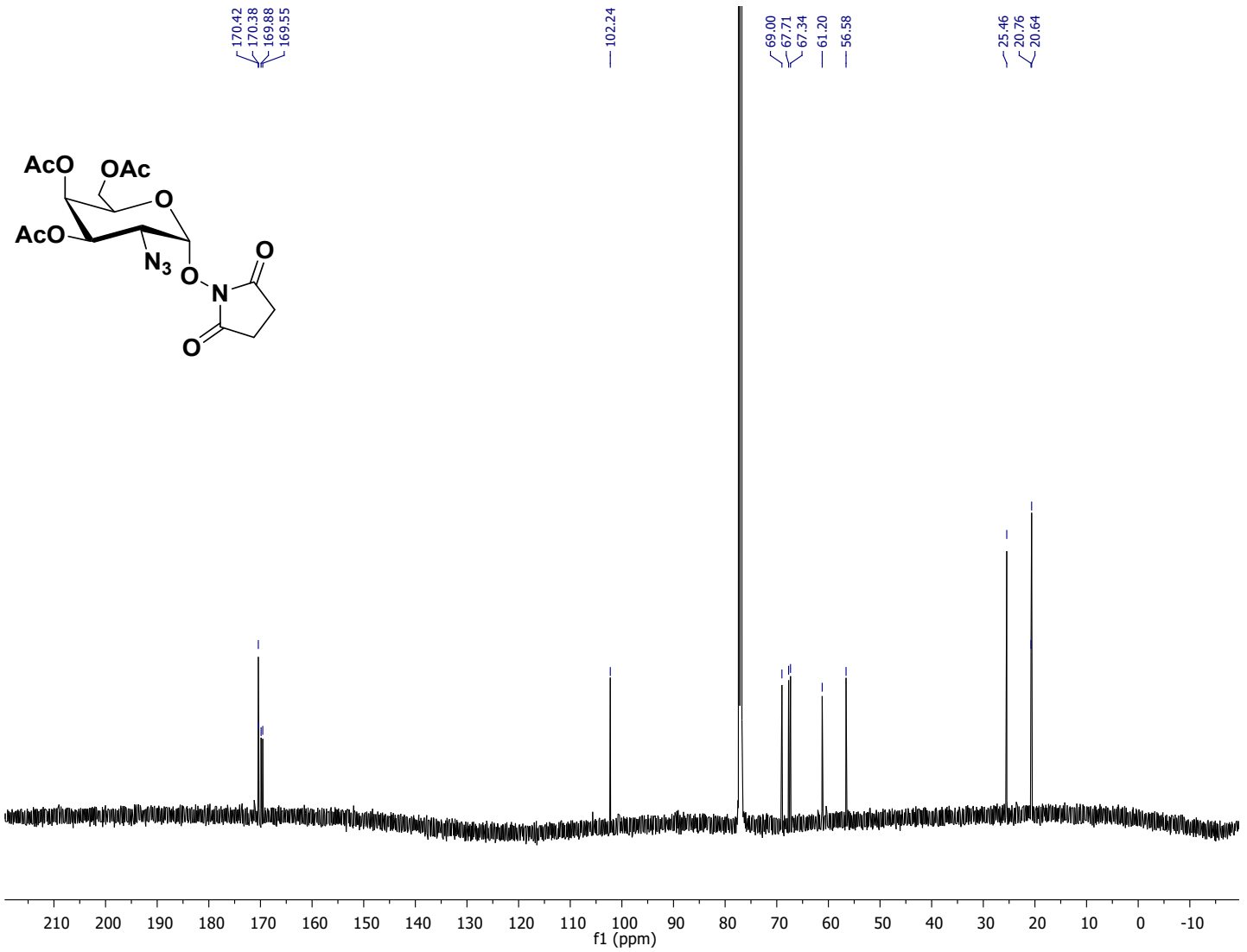
3.5
3.0
2.5
2.0
1.5
1.0
f1 (ppm)

^{13}C NMR of **8**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67.34
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AcO
OAc
AcO
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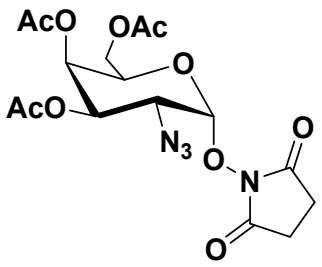
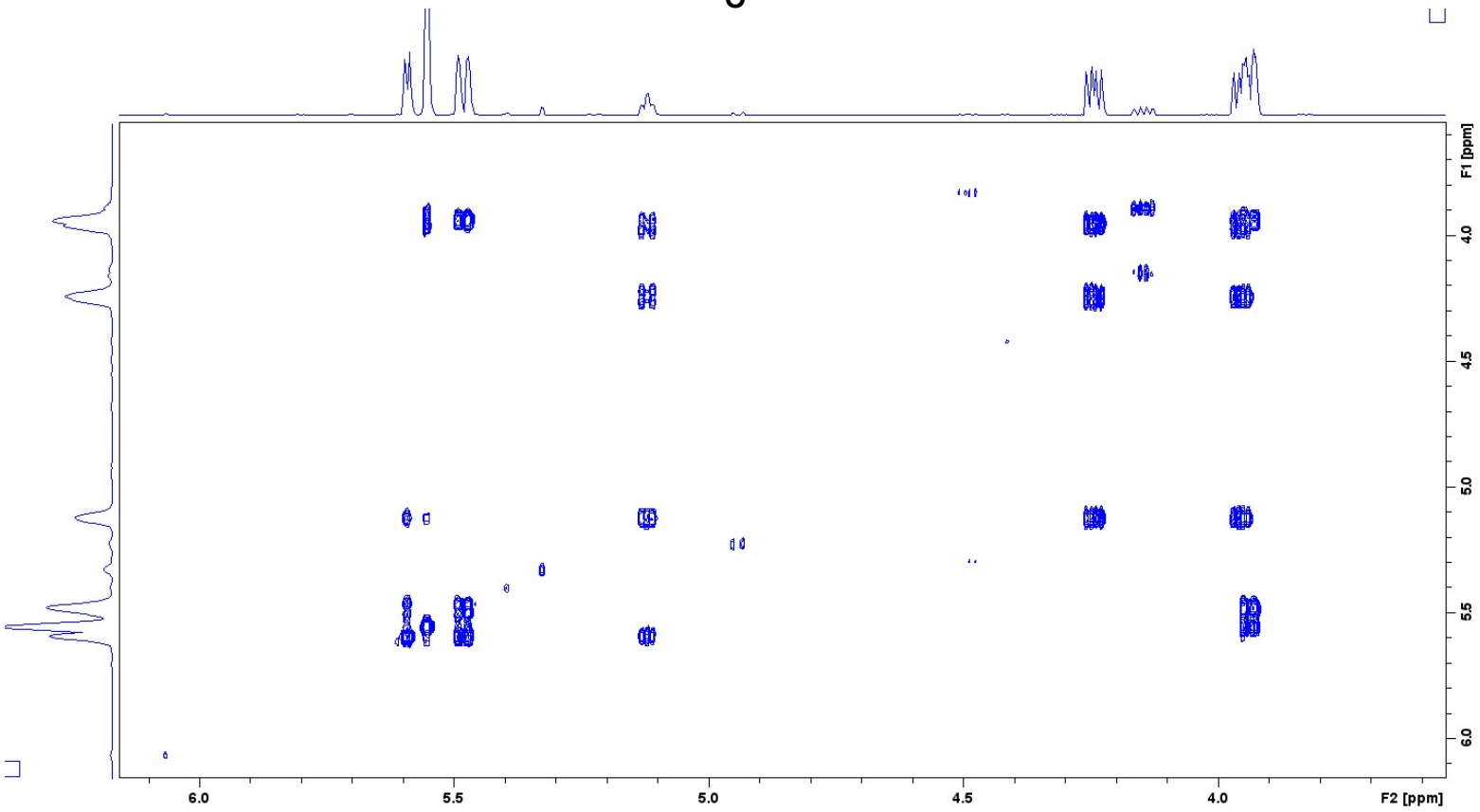
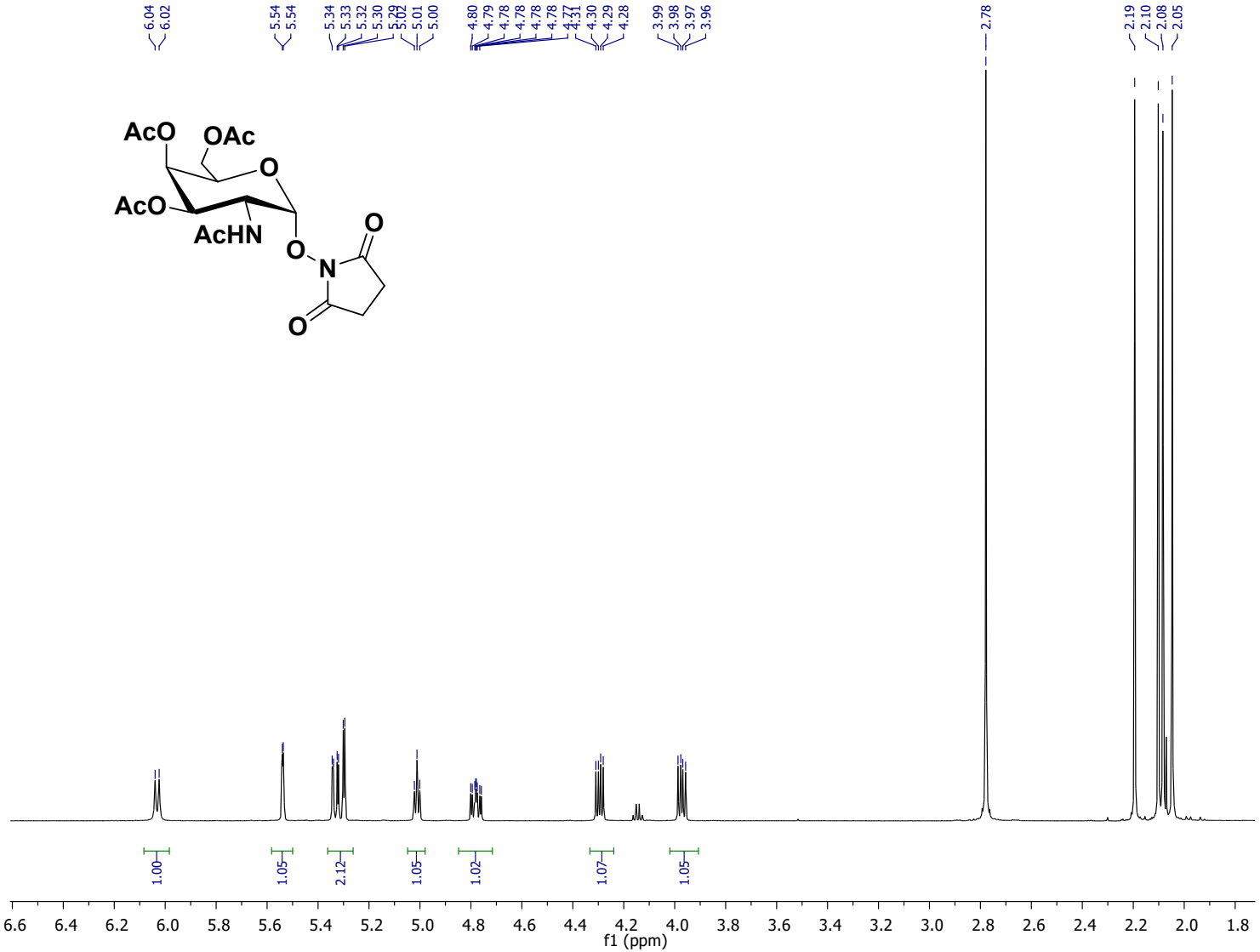
f1 (ppm)

COSY NMR of **8**





^{1}H NMR of **9**


6.04
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5.54
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5.32
5.30
5.01
5.00
4.80
4.79
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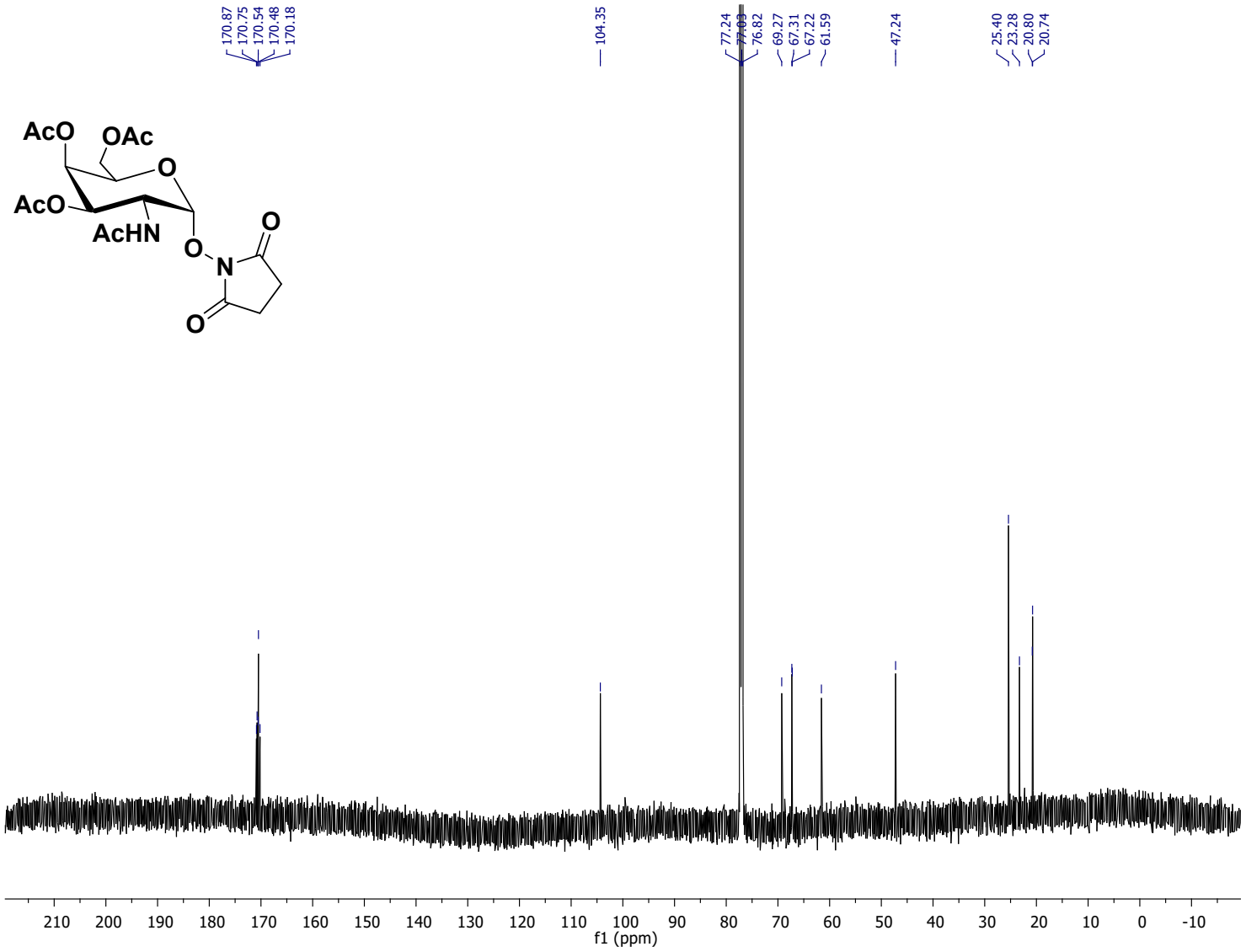
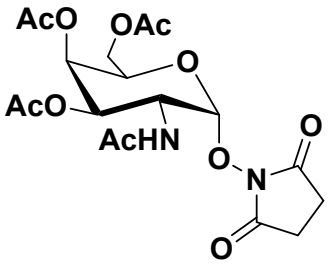
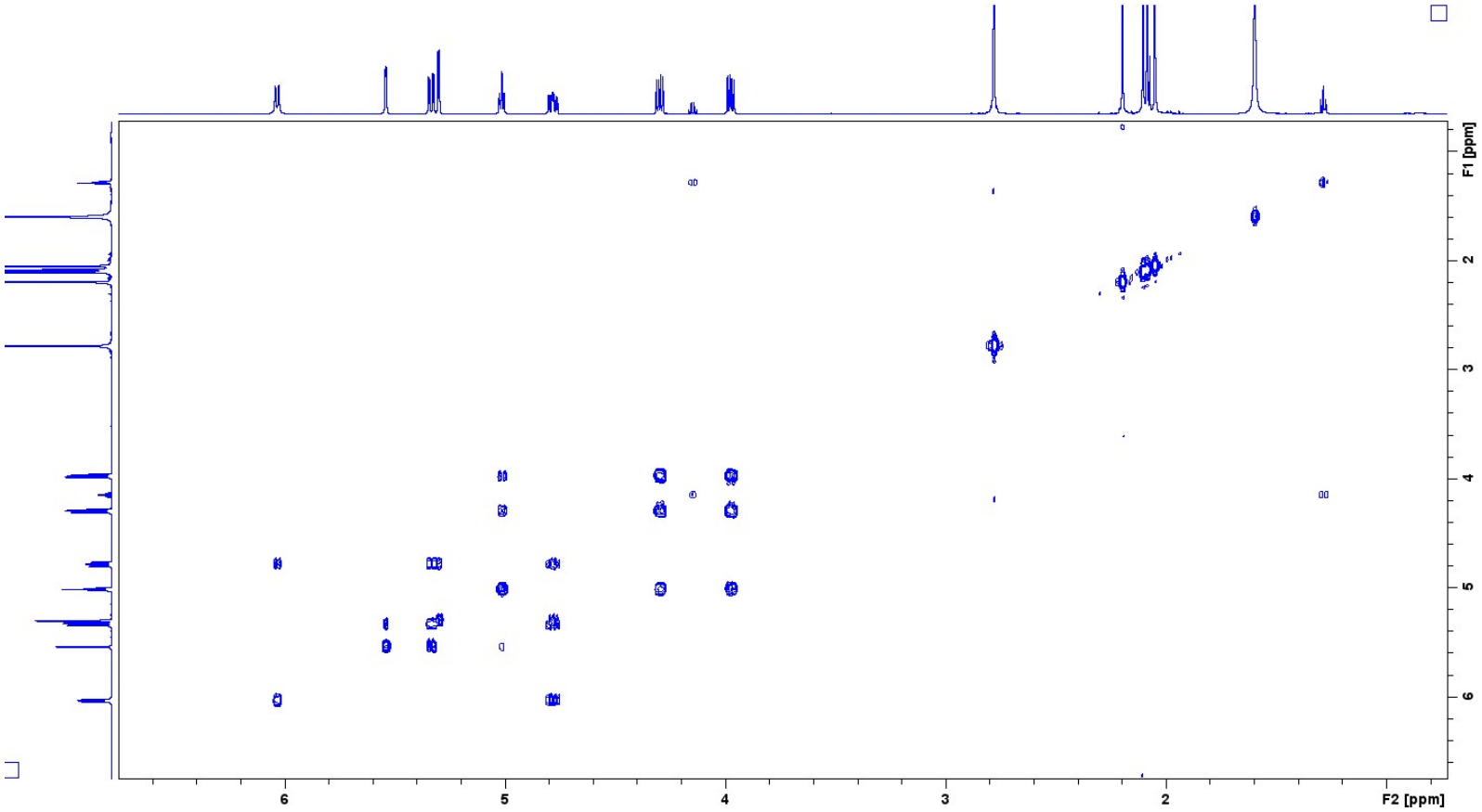
4.78
4.78
4.30
4.29
4.28
3.99
3.98
3.97
3.96
2.78
2.19
2.10
2.08
2.05
AcO
OAc
O
AcO
AcHN
O
N
O
O
1.00
1.05
2.12
1.05
1.02
1.07
1.05
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
f1 (ppm)

^{13}C NMR of **9**

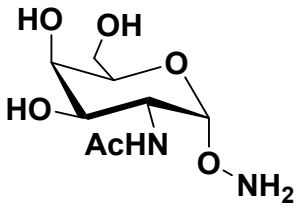

170.87
170.75
170.54
170.48
170.18
104.35
77.24
77.03
76.82
69.27
67.31
67.22
61.59
47.24
25.40
23.28
20.80
20.74
AcO
OAc
O
AcO
AcHN
O
N
O
O
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

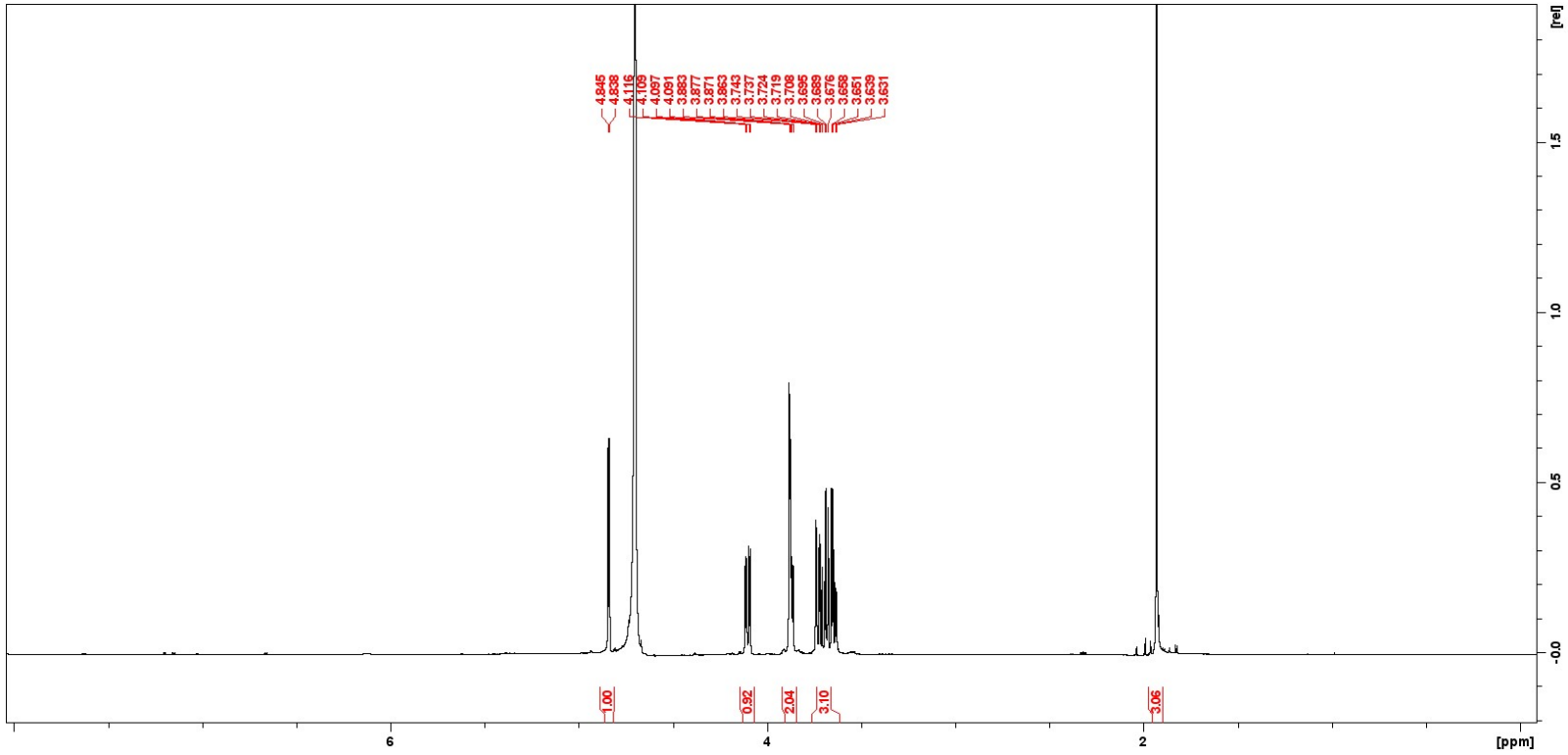
COSY NMR of **9**



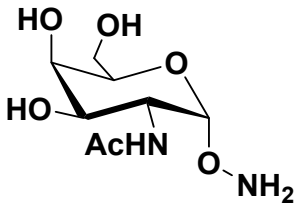


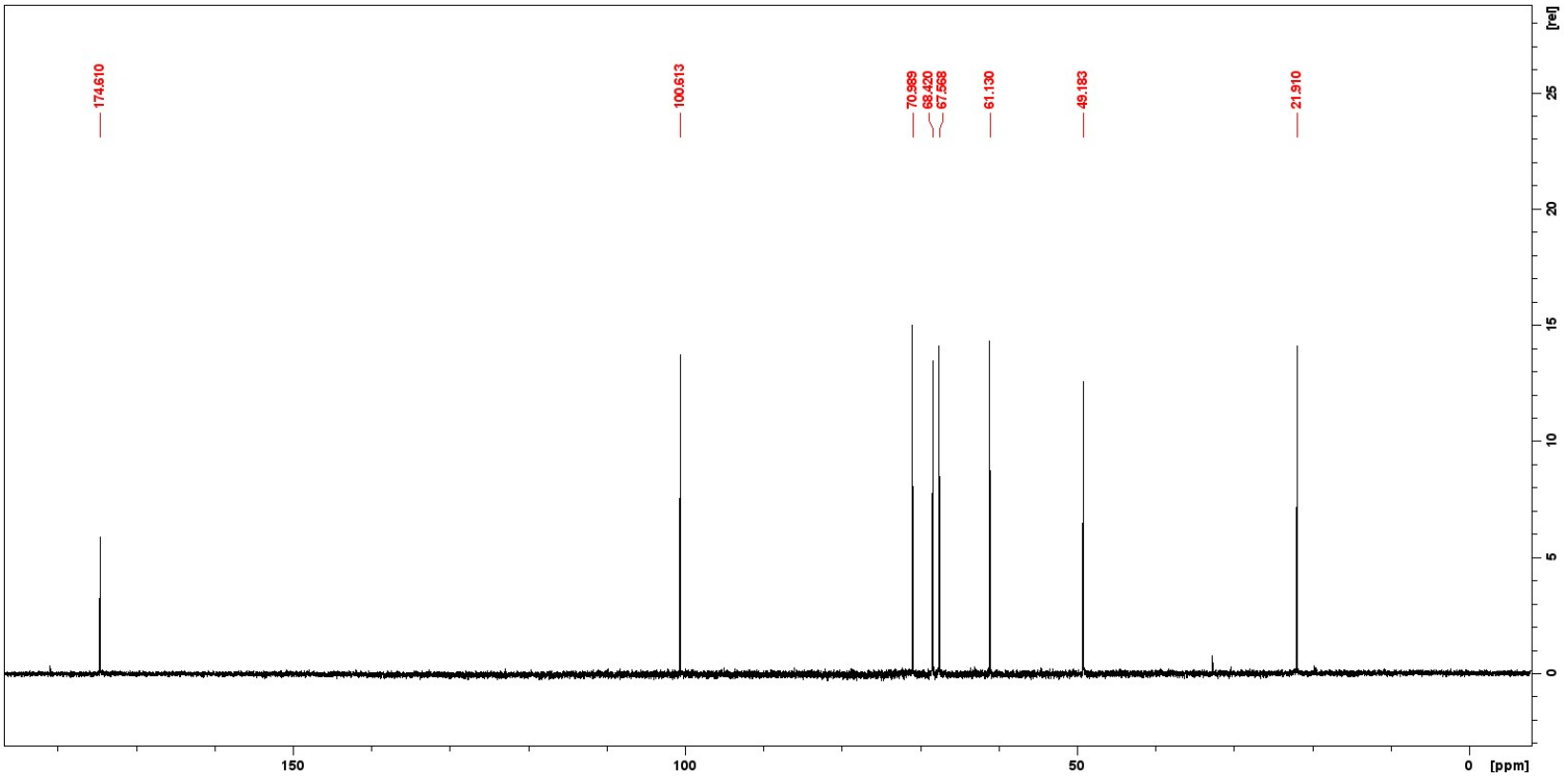
^{1}H NMR of **2**


HO
OH
O
HO
AcHN
O
NH2

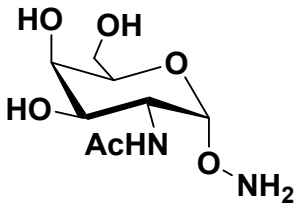

4.845
4.838
4.115
4.109
4.097
4.091
3.883
3.877
3.871
3.863
3.743
3.737
3.724
3.719
3.708
3.695
3.688
3.676
3.658
3.651
3.639
3.631
1.00
0.92
2.04
3.10
3.06
6
4
2
[ppm]
[rel]
1.5
1.0
0.5
-0.0

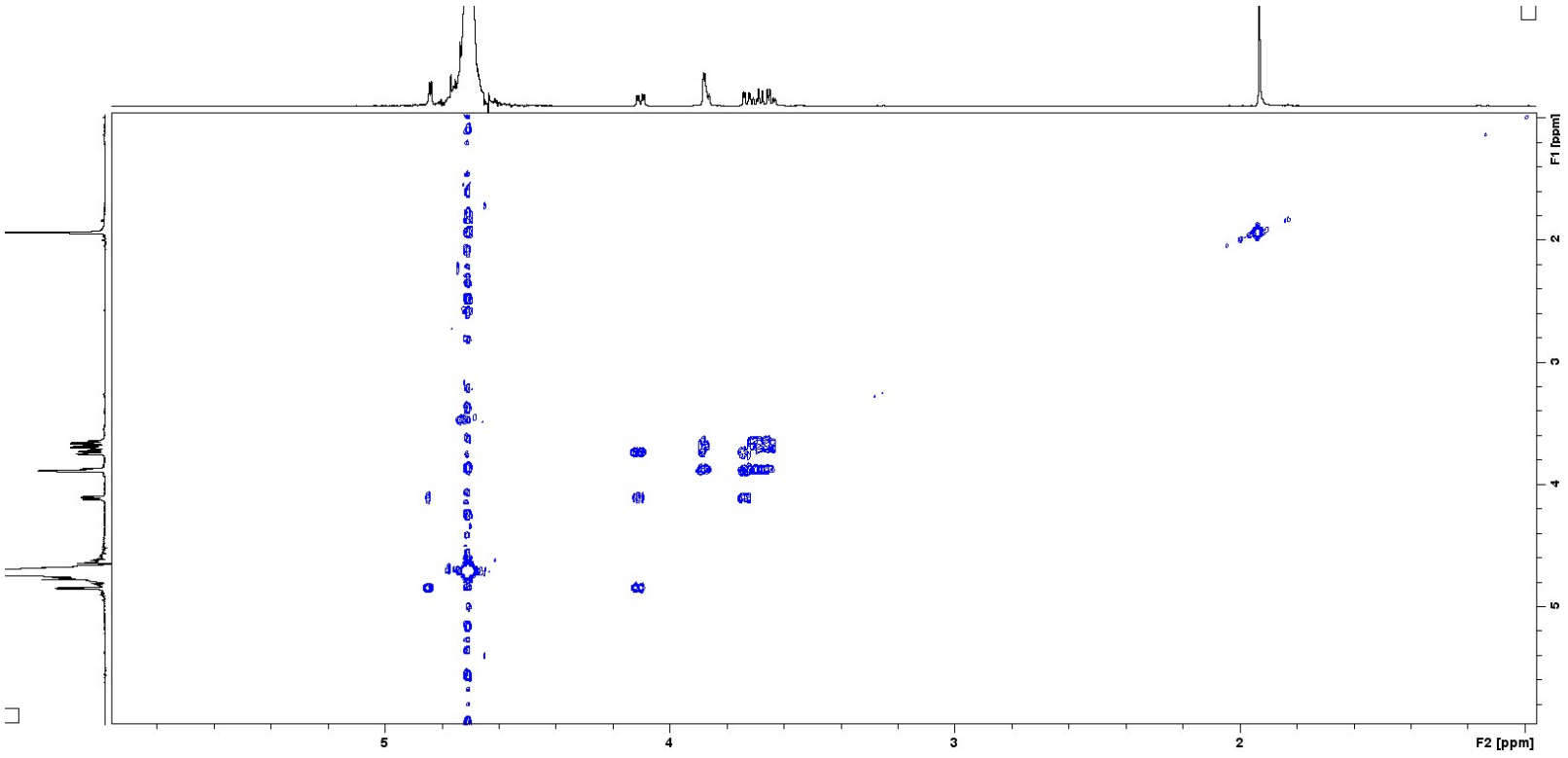
^{13}C NMR of **2**


HO
OH
O
HO
AcHN
O
NH2

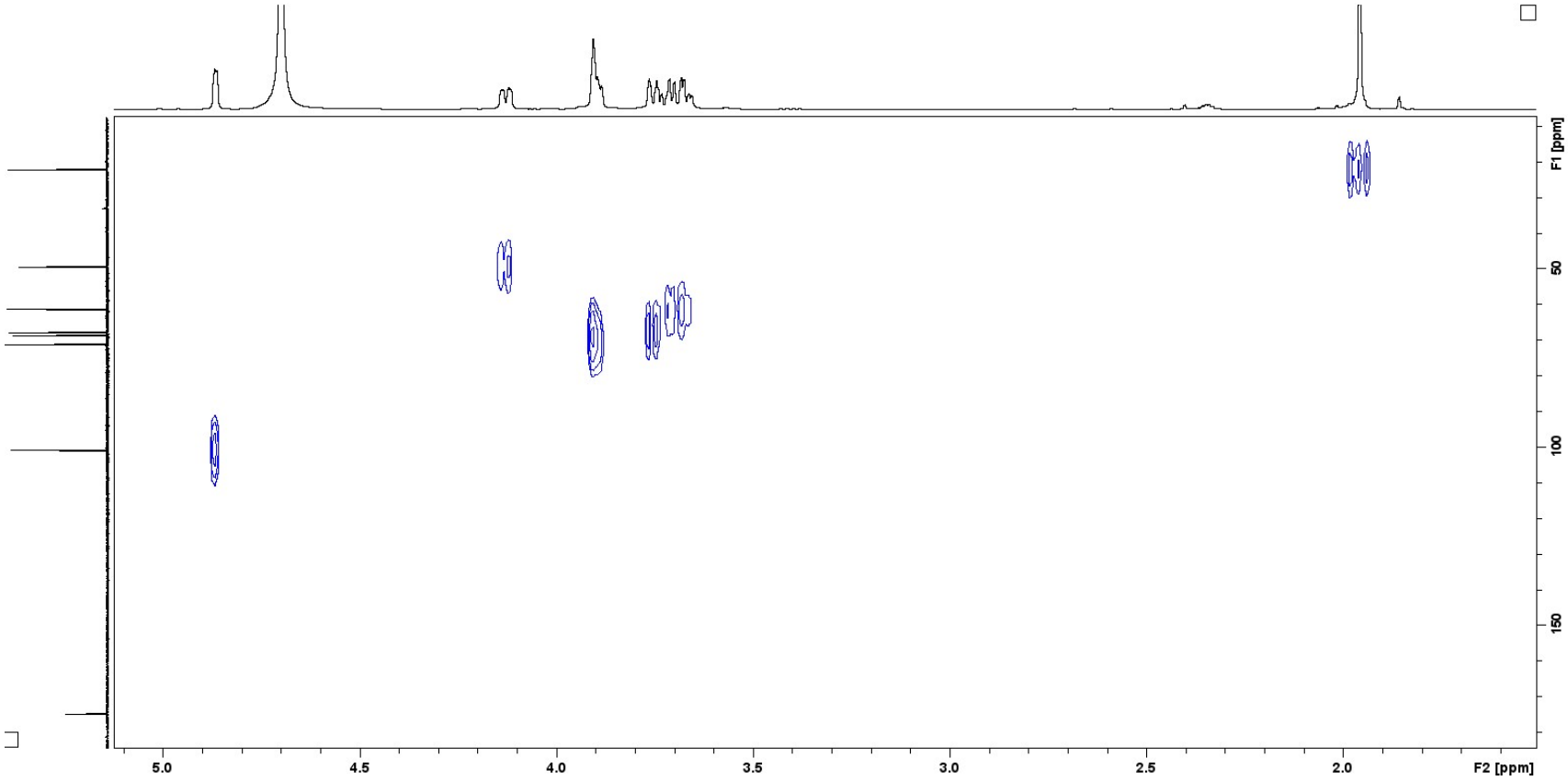

174.610
100.613
70.989
68.420
67.568
61.130
49.183
21.910
[rel]
25
20
15
10
5
0
150
100
50
0
[ppm]

COSY NMR of **2**

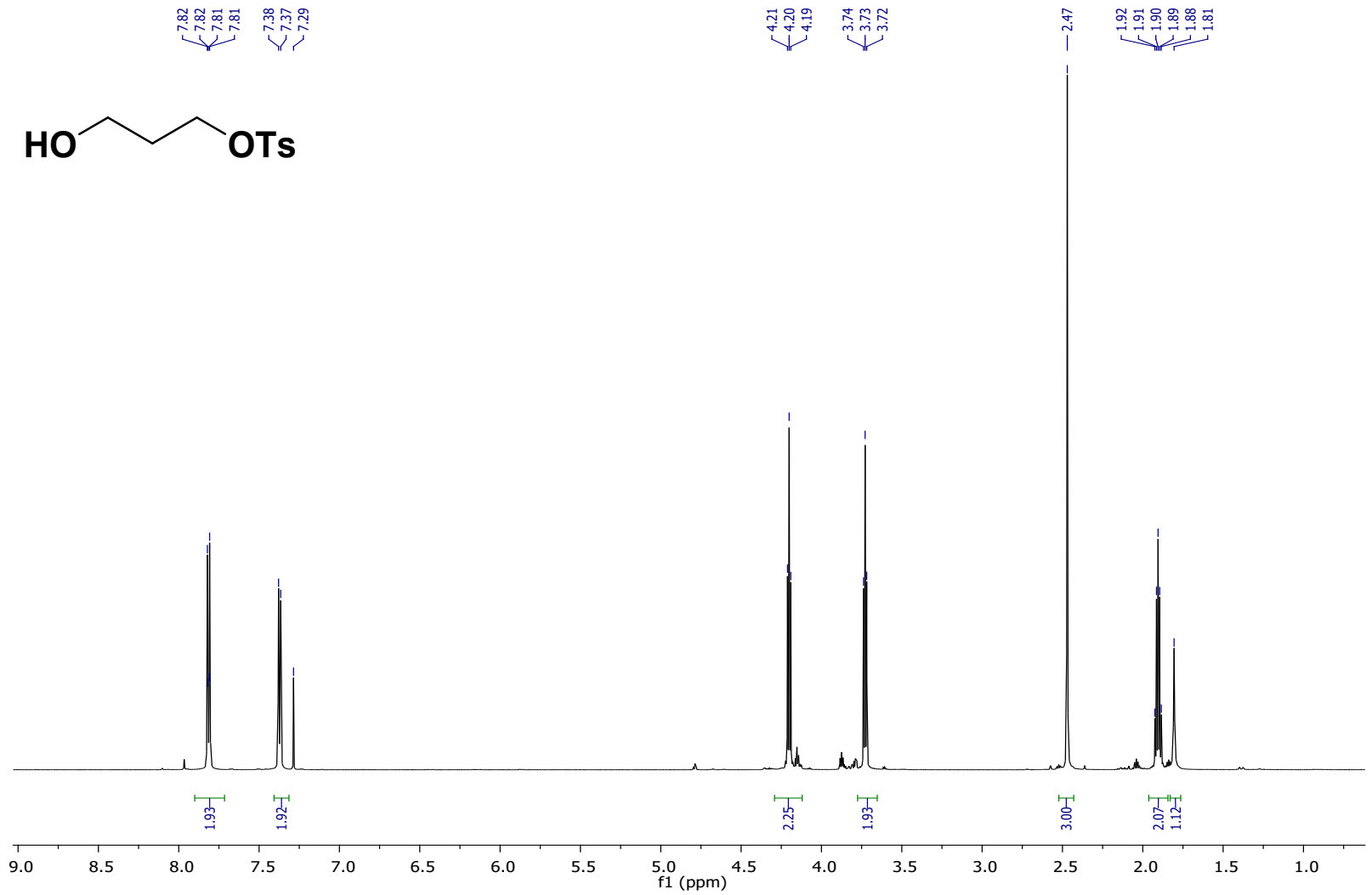




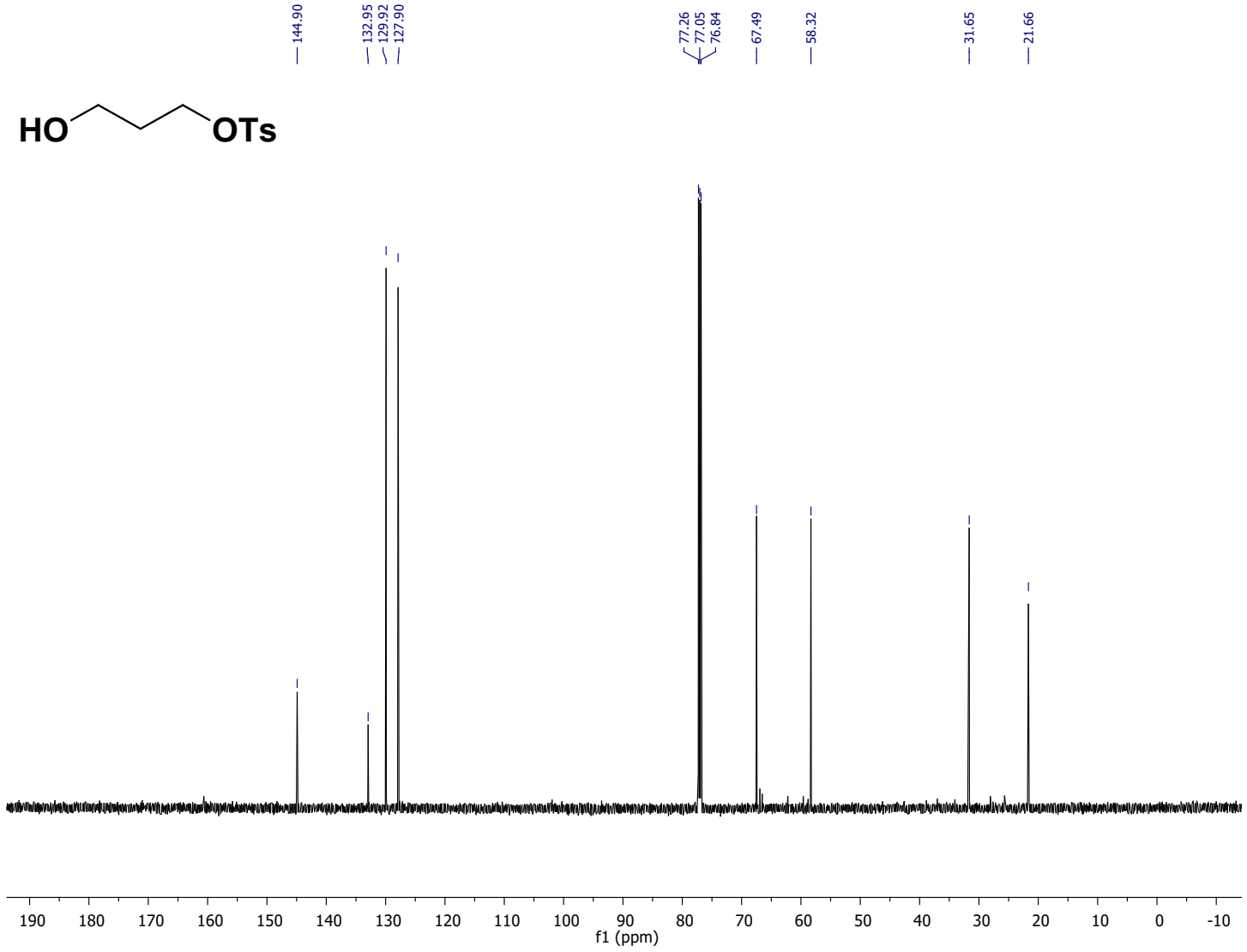
HSQC NMR of **2**



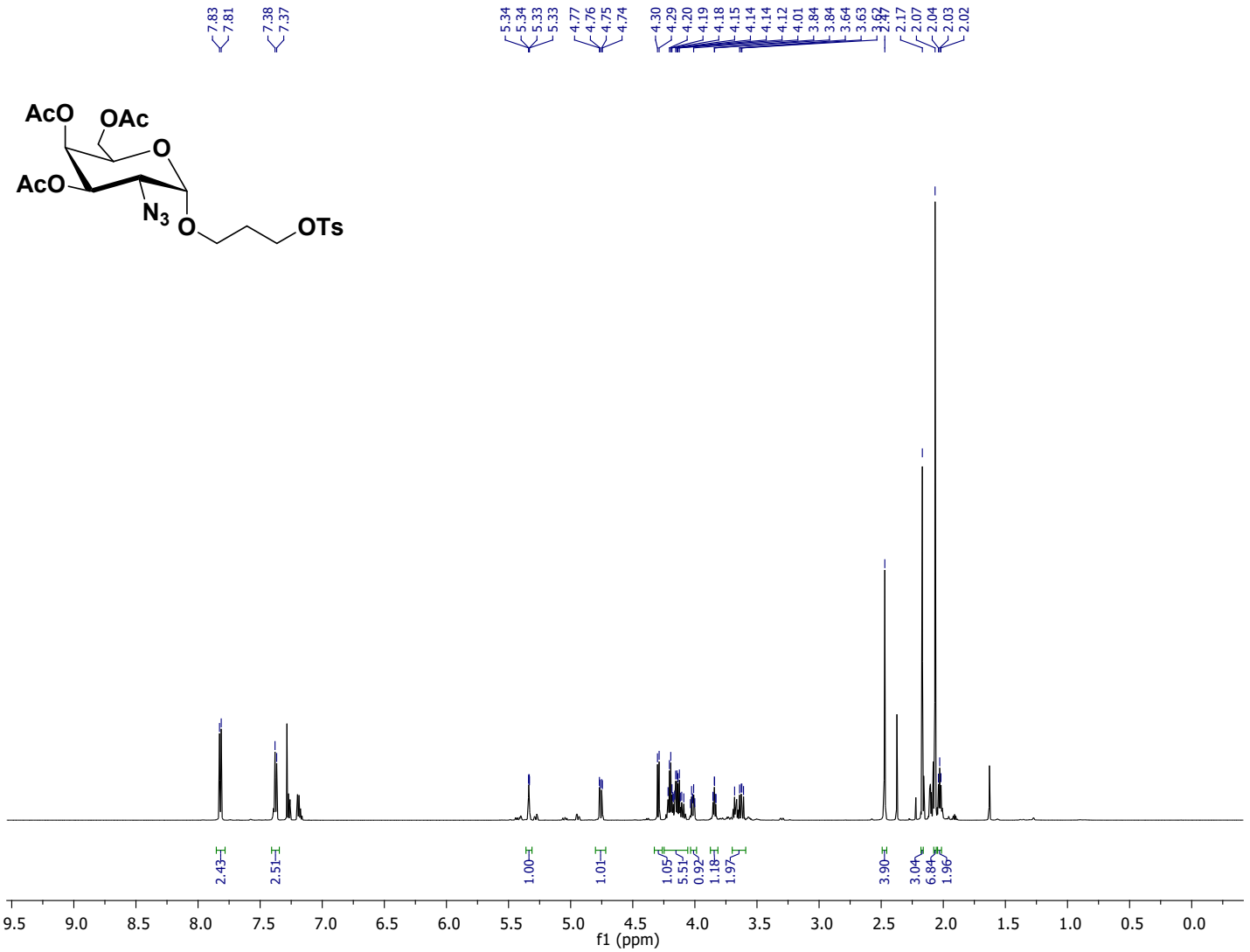
^{1}H NMR of 1-hydroxypropyl 4-methylbenzenesulfonate



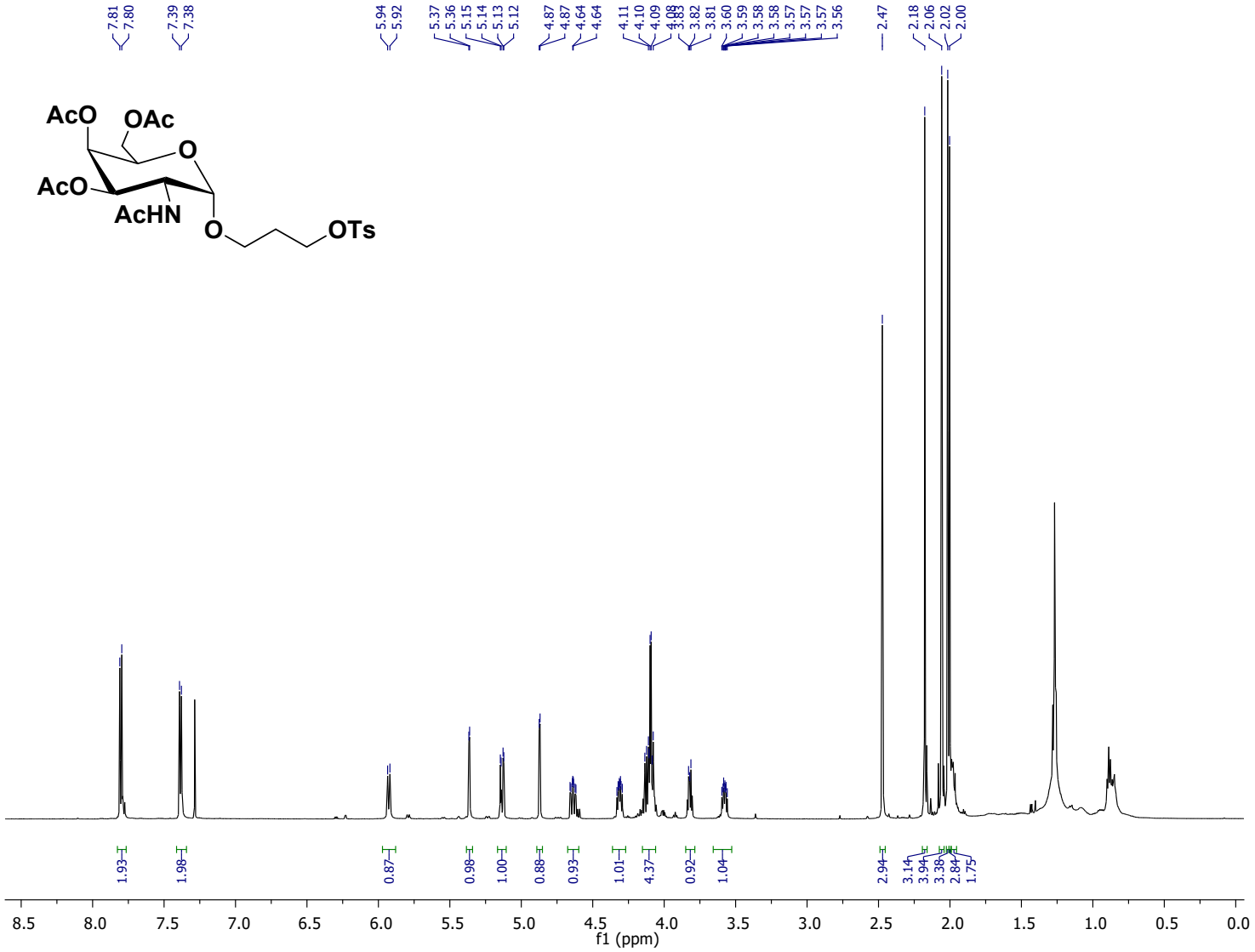
^{13}C NMR of 1-hydroxypropyl 4-methylbenzenesulfonate



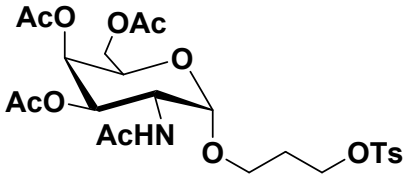
^{1}H NMR of **10**

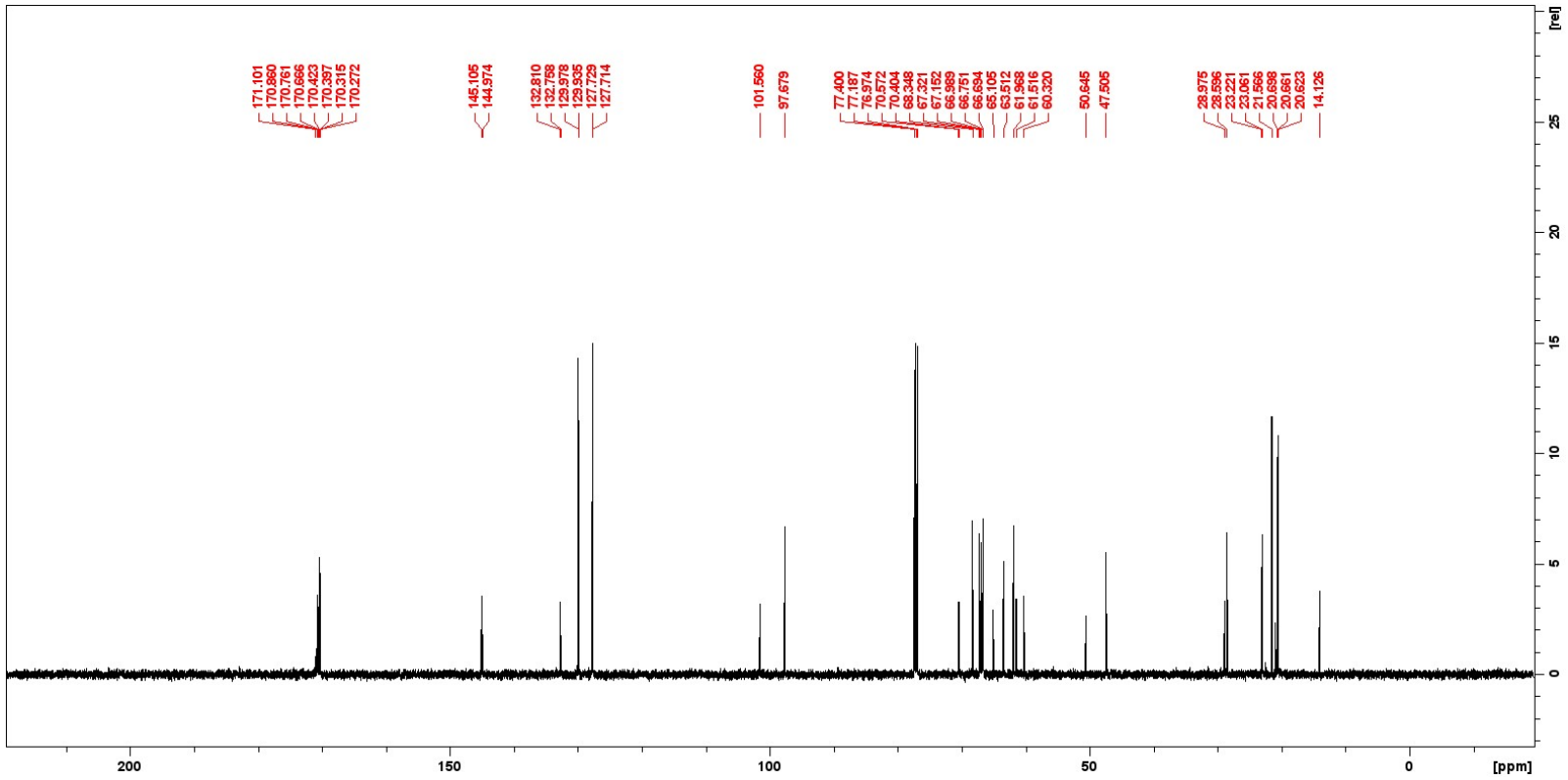

7.83
7.81
7.38
7.37
5.34
5.34
5.33
5.33
4.77
4.76
4.75
4.74
4.30
4.29
4.20
4.19
4.18
4.15
4.14
4.14
4.12
4.01
3.84
3.84
3.64
3.63
2.17
2.07
2.04
2.03
2.02
AcO
OAc
O
AcO
N3
O
OTs
2.43
2.51
1.00
1.01
1.05
5.51
0.92
1.18
1.97
3.90
3.04
6.84
1.96
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

^{1}H NMR of **13**

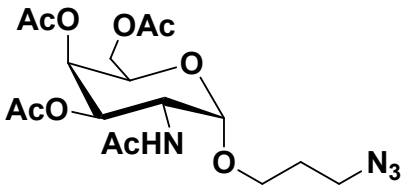

7.81
7.80
7.39
7.38
5.94
5.92
5.37
5.36
5.15
5.14
5.13
5.12
4.87
4.87
4.64
4.64
4.11
4.10
4.09
3.83
3.82
3.81
3.60
3.59
3.58
3.58
3.57
3.57
3.57
3.56
2.47
2.18
2.06
2.02
2.00
AcO
OAc
O
AcO
AcHN
O
OTs
1.93
1.98
0.87
0.98
1.00
0.88
0.93
1.01
4.37
0.92
1.04
2.94
3.14
3.94
3.38
2.84
1.75
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

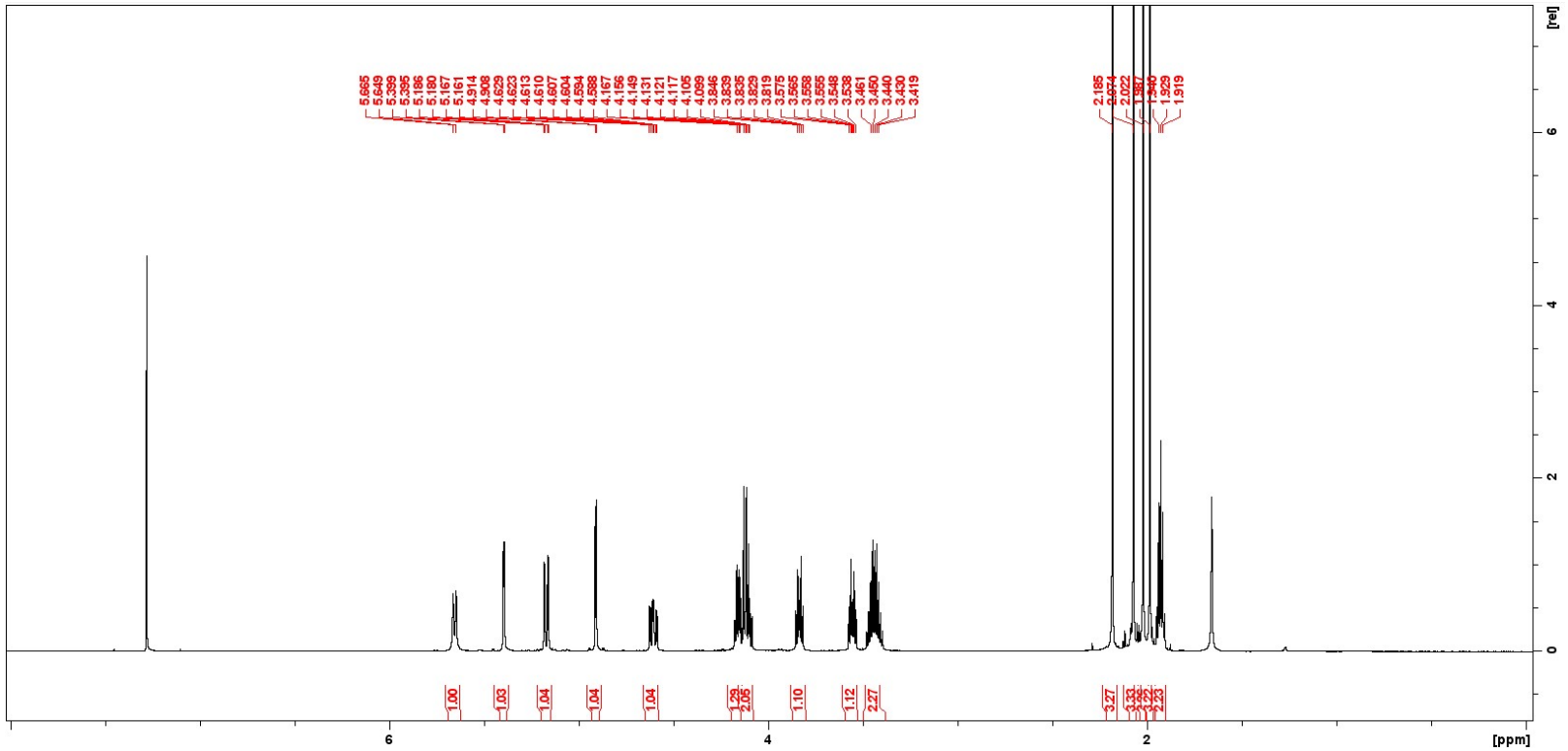
^{13}C NMR of **13**


AcO
OAc
O
AcO
AcHN
O
OTs

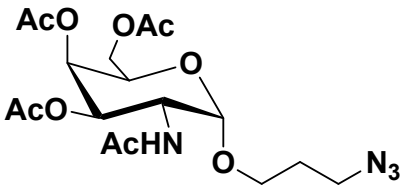

171.101
170.860
170.761
170.666
170.423
170.397
170.315
170.272
145.105
144.974
132.810
132.768
129.978
129.935
127.729
127.714
101.560
97.679
77.400
77.187
76.974
70.572
70.404
68.348
67.321
67.152
66.989
66.751
66.694
65.105
63.512
61.968
61.516
60.320
50.645
47.505
28.975
28.596
23.221
23.061
21.566
20.698
20.661
20.623
14.126
[rel]
25
20
15
10
5
0
200
150
100
50
0
[ppm]

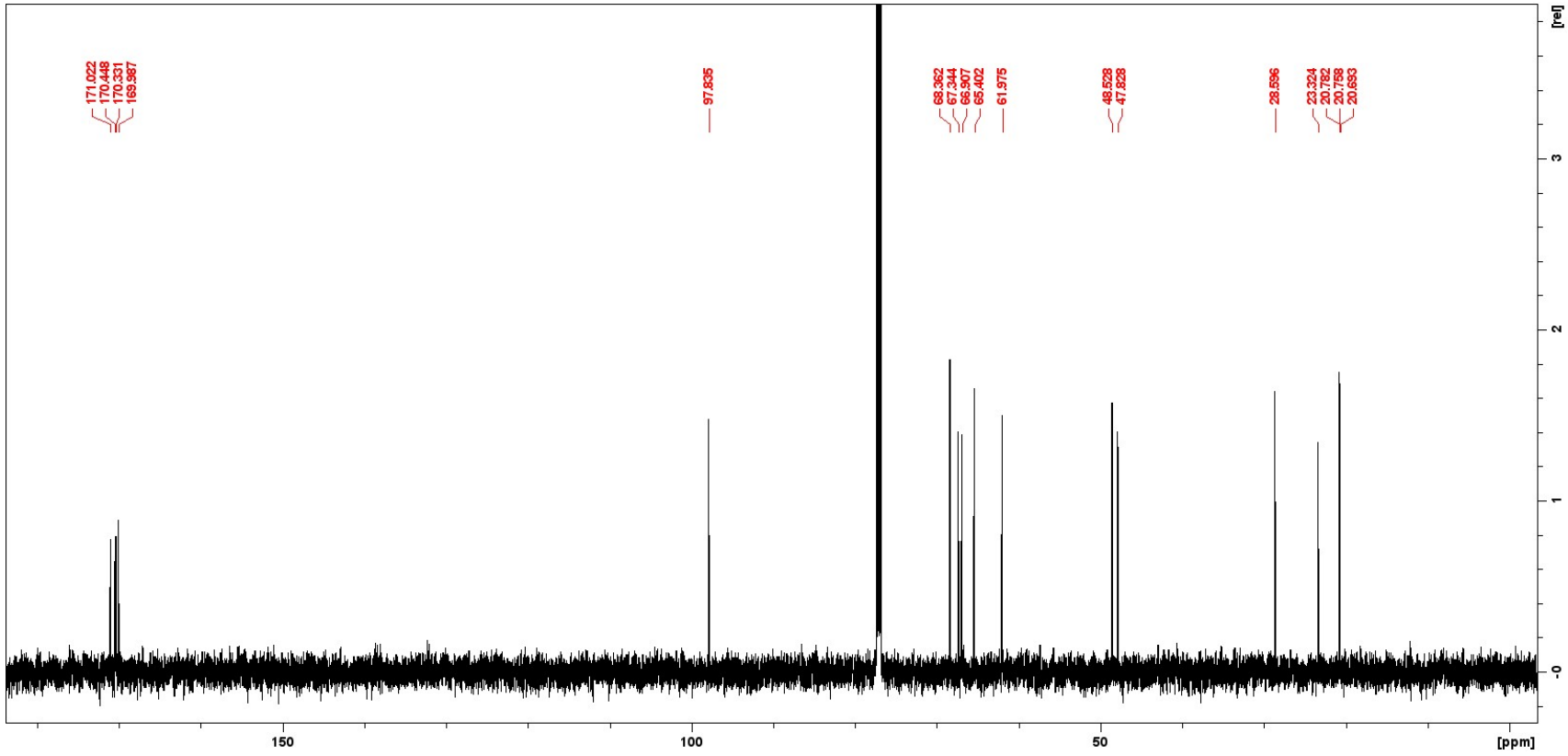
^{1}H NMR of **11**


AcO
OAc
O
AcO
AcHN
O
N3

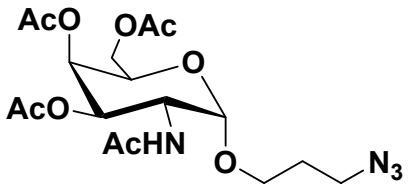

5.665
5.649
5.399
5.395
5.186
5.180
5.167
5.161
4.314
4.308
4.629
4.623
4.613
4.610
4.607
4.604
4.594
4.588
4.167
4.155
4.149
4.131
4.121
4.117
4.105
4.099
3.846
3.839
3.835
3.829
3.819
3.575
3.565
3.558
3.555
3.548
3.538
3.461
3.450
3.440
3.430
3.419
2.185
2.014
2.022
1.987
1.940
1.929
1.919
1.00
1.03
1.04
1.04
1.04
1.28
2.05
1.10
1.12
2.27
3.27
3.33
2.22
2.23
6
4
2
[ppm]
[rel]
0
2
4
6

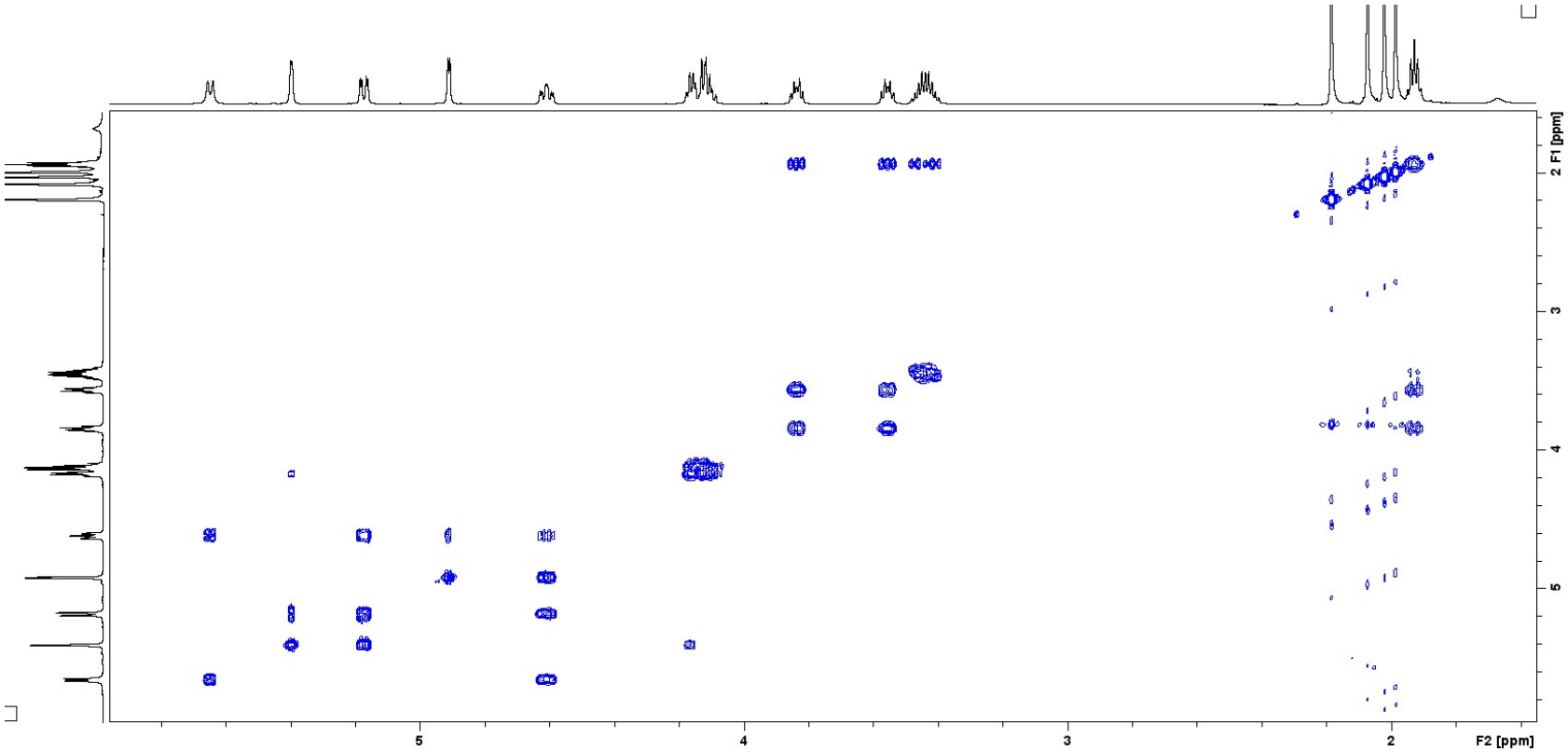
^{13}C NMR of **11**


AcO
OAc
O
AcO
AcHN
O
N3

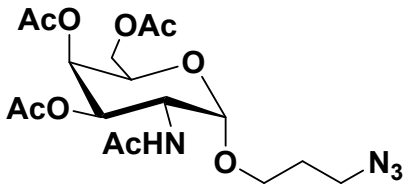

171.022
170.448
170.331
169.987
97.835
68.362
67.344
66.907
65.402
61.975
48.528
47.828
28.596
23.324
20.782
20.758
20.693
[rel]
3
2
1
-0
150
100
50
[ppm]

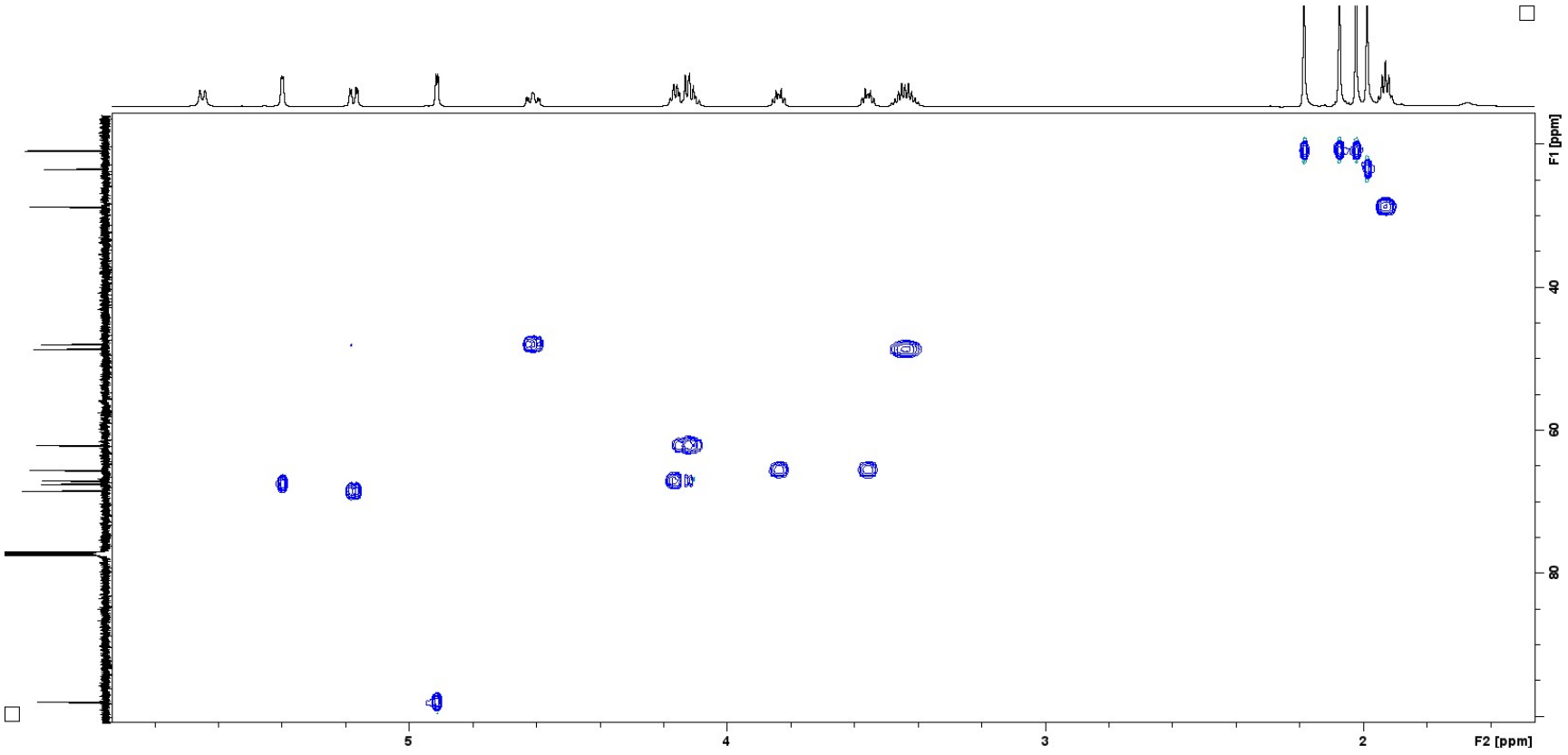
COSY NMR of **11**



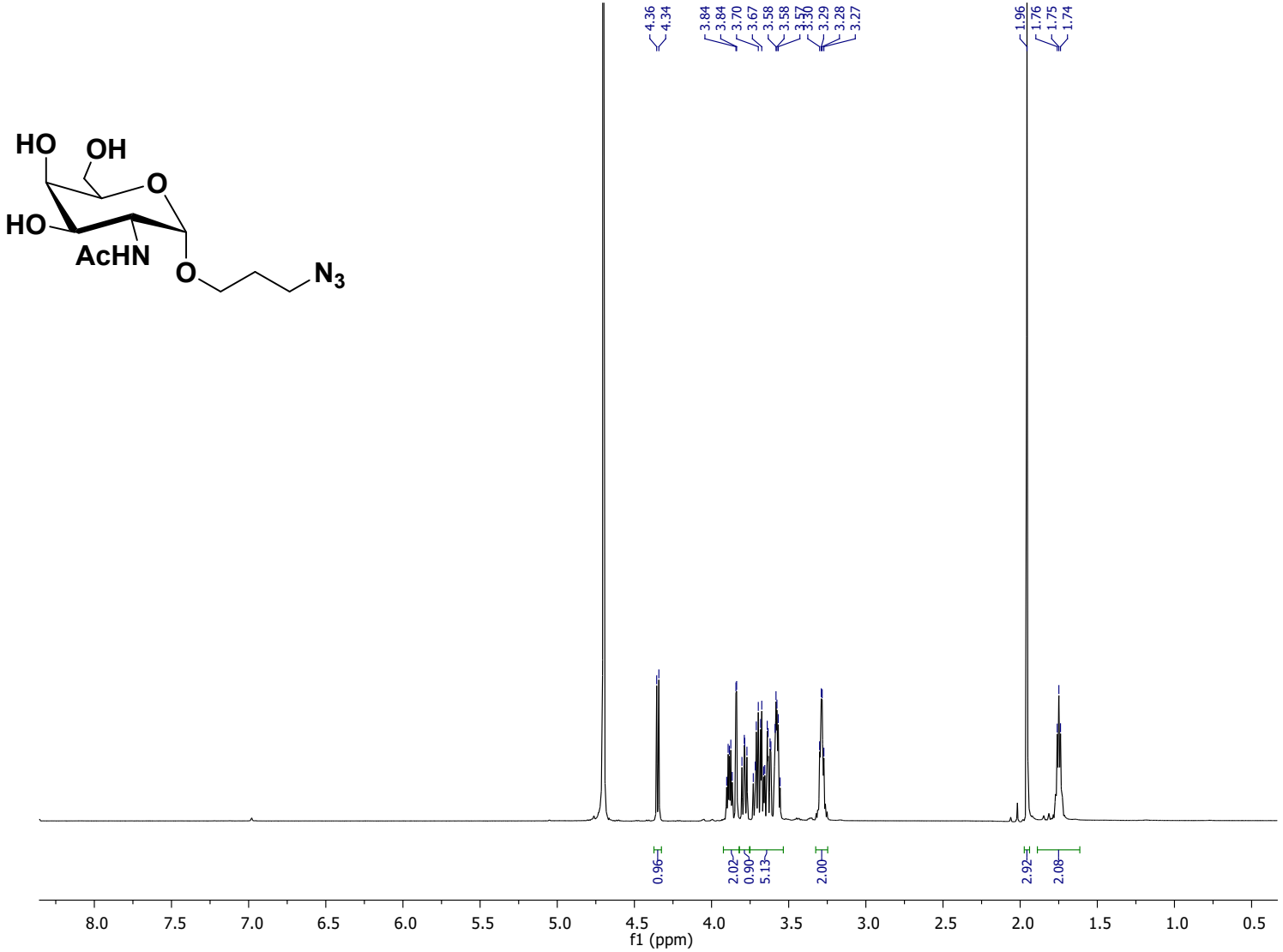


HSQC NMR of **11**

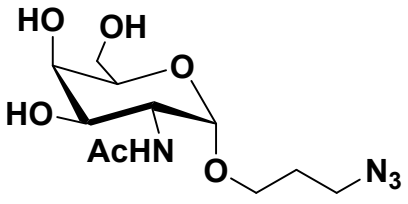


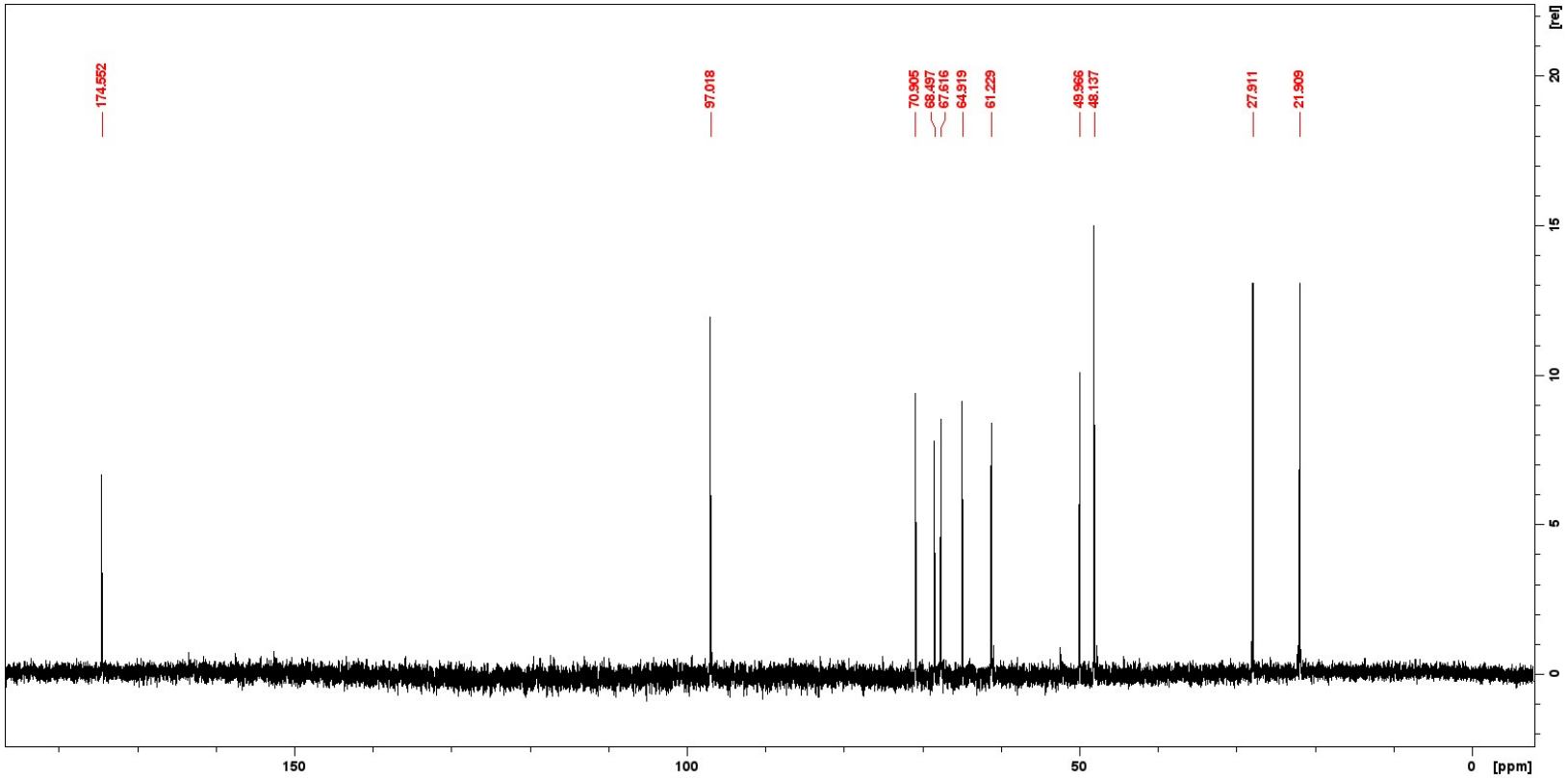


^{1}H NMR of **5**

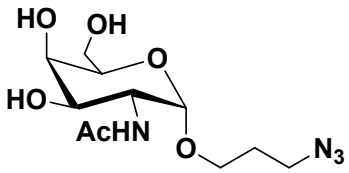

HO
OH
O
HO
AcHN
O
N3
4.36
4.34
3.84
3.84
3.70
3.67
3.58
3.58
3.57
3.30
3.29
3.28
3.27
1.96
1.76
1.75
1.74
0.96
2.02
0.90
5.13
2.00
2.92
2.08
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)

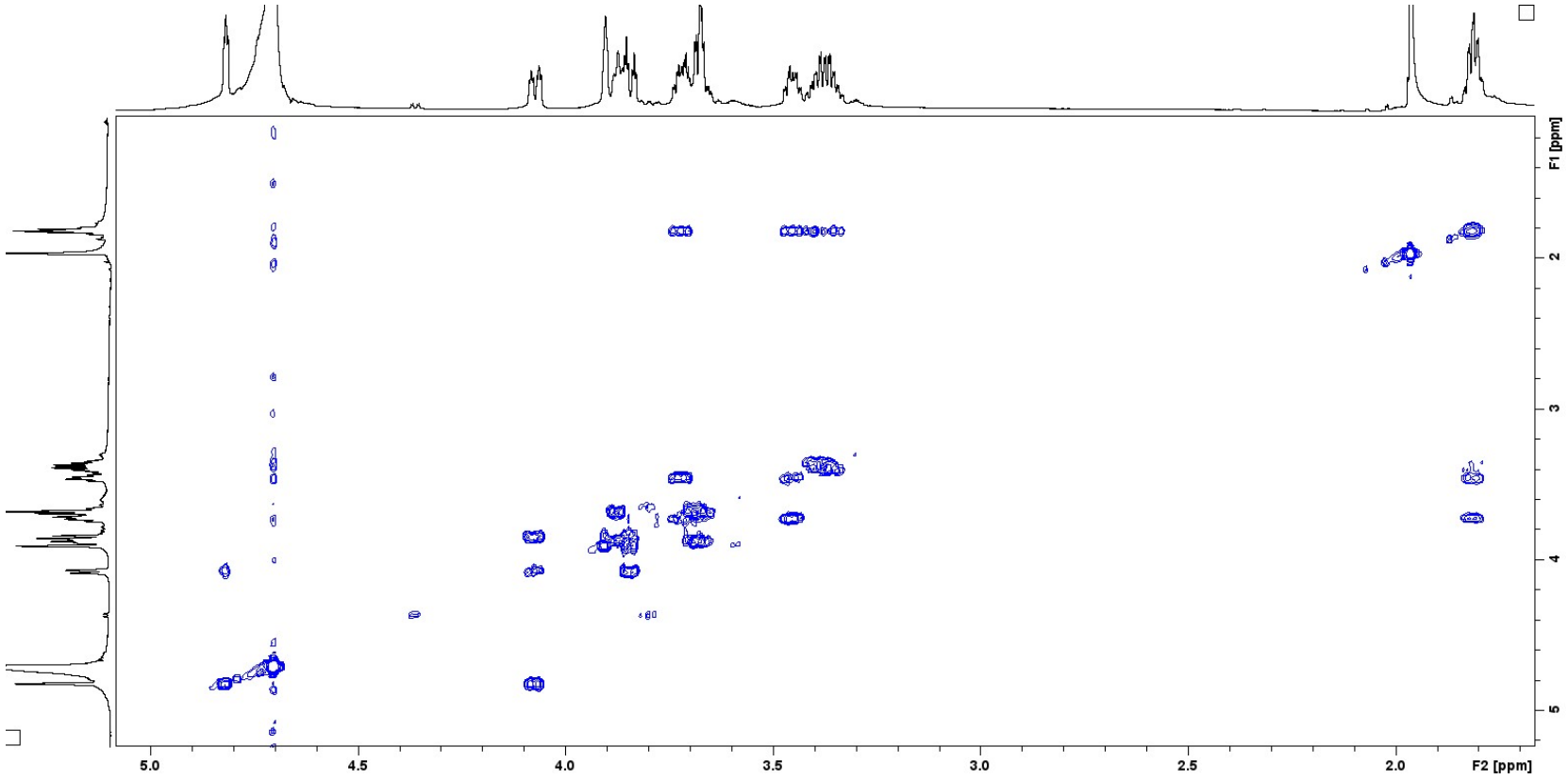
^{13}C NMR of **5**


HO
OH
O
HO
AcHN
O
N3

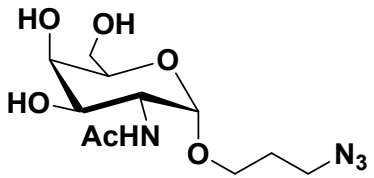

174.552
97.018
70.905
68.497
67.616
64.919
61.229
49.966
48.137
27.911
21.909
[rel]
20
15
10
5
0
150
100
50
0
[ppm]

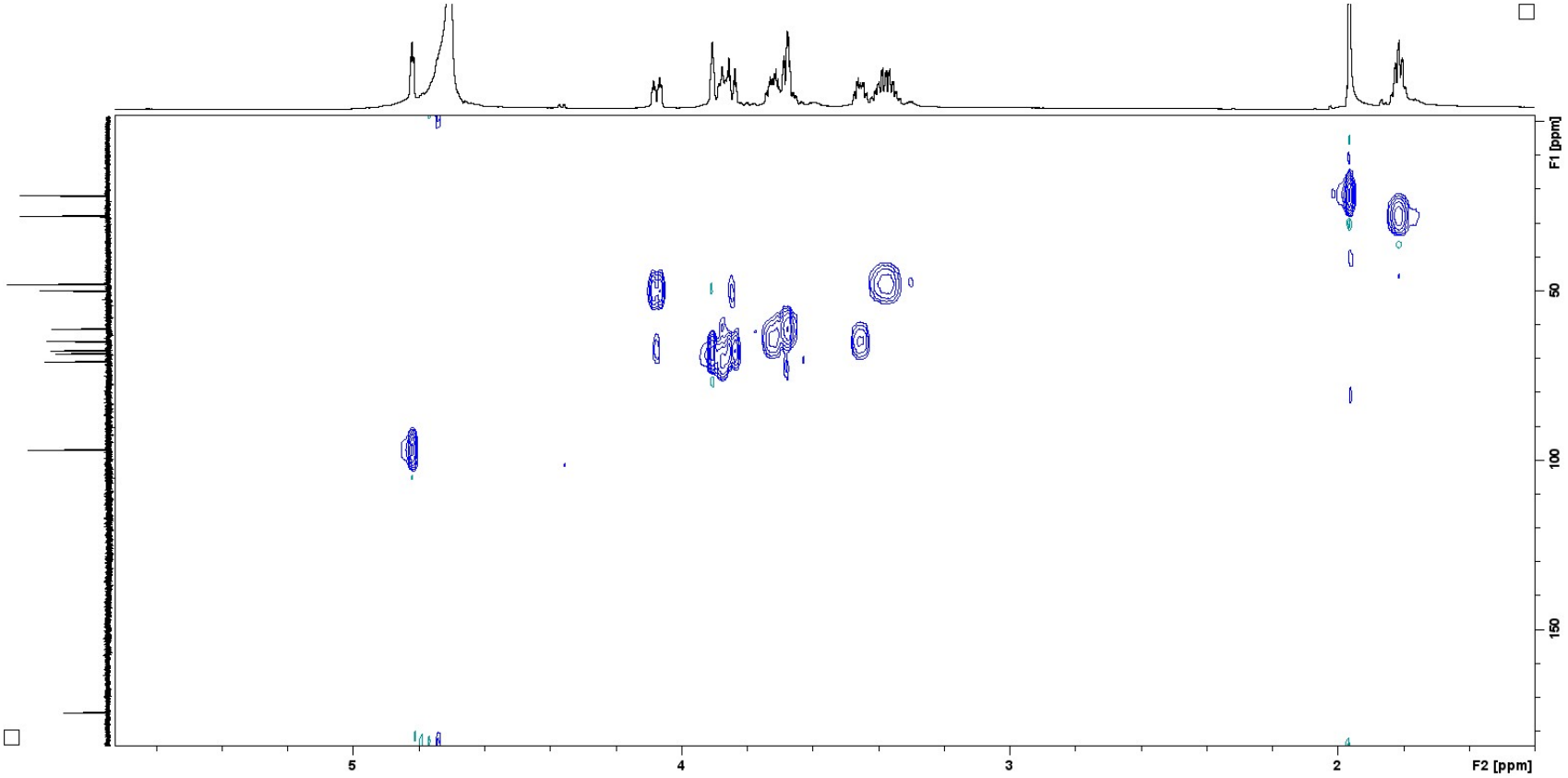
COSY NMR of **5**



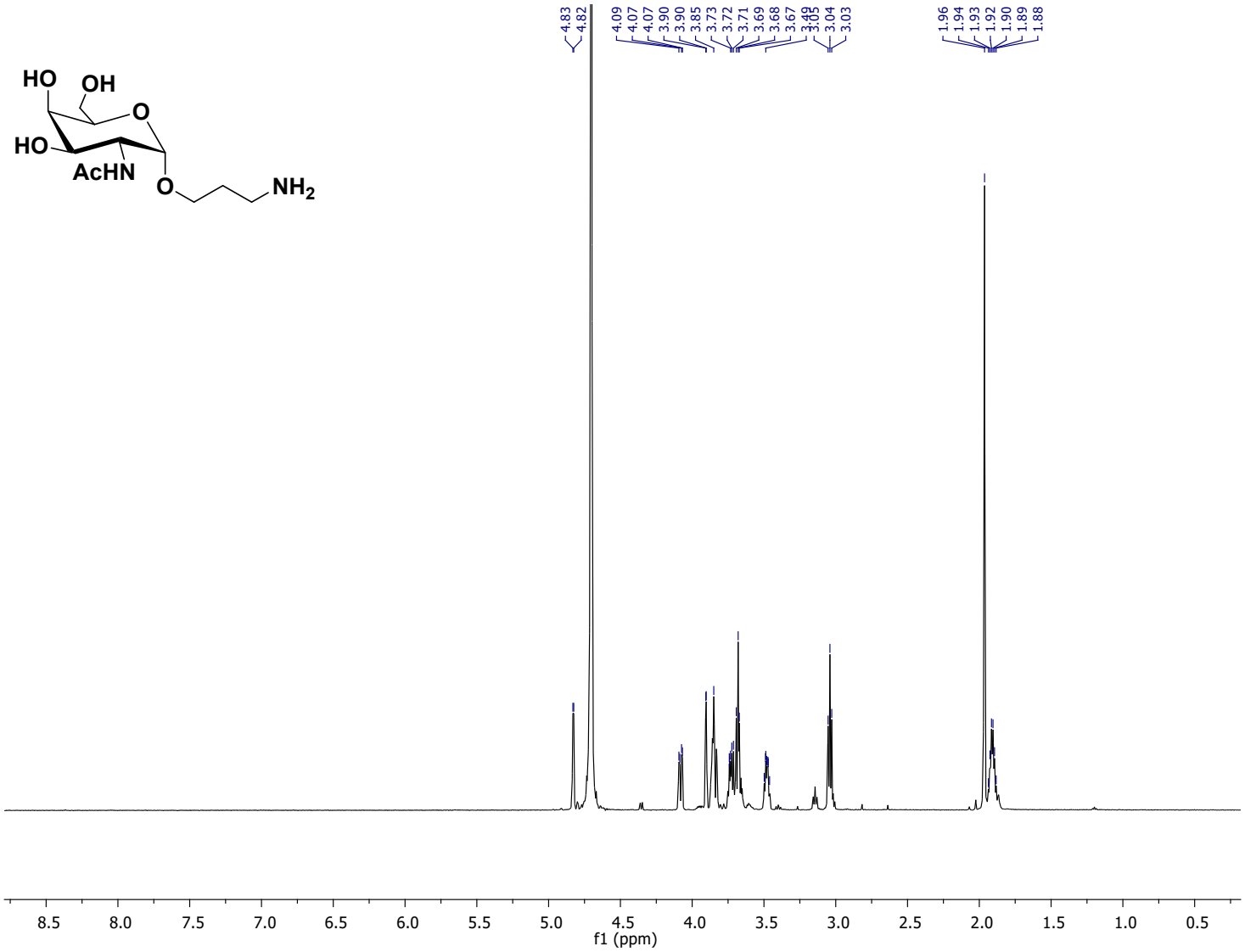


HSQC NMR of **5**

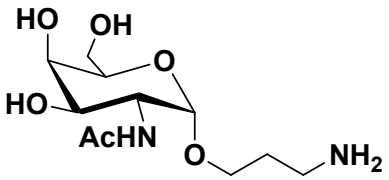




^{1}H NMR of **12**

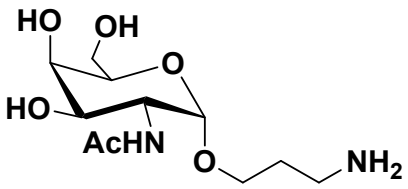

HO
OH
O
HO
AcHN
O
NH2
4.83
4.82
4.09
4.07
4.07
3.90
3.90
3.85
3.73
3.72
3.71
3.69
3.68
3.67
3.04
3.03
1.96
1.94
1.93
1.92
1.90
1.89
1.88
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)

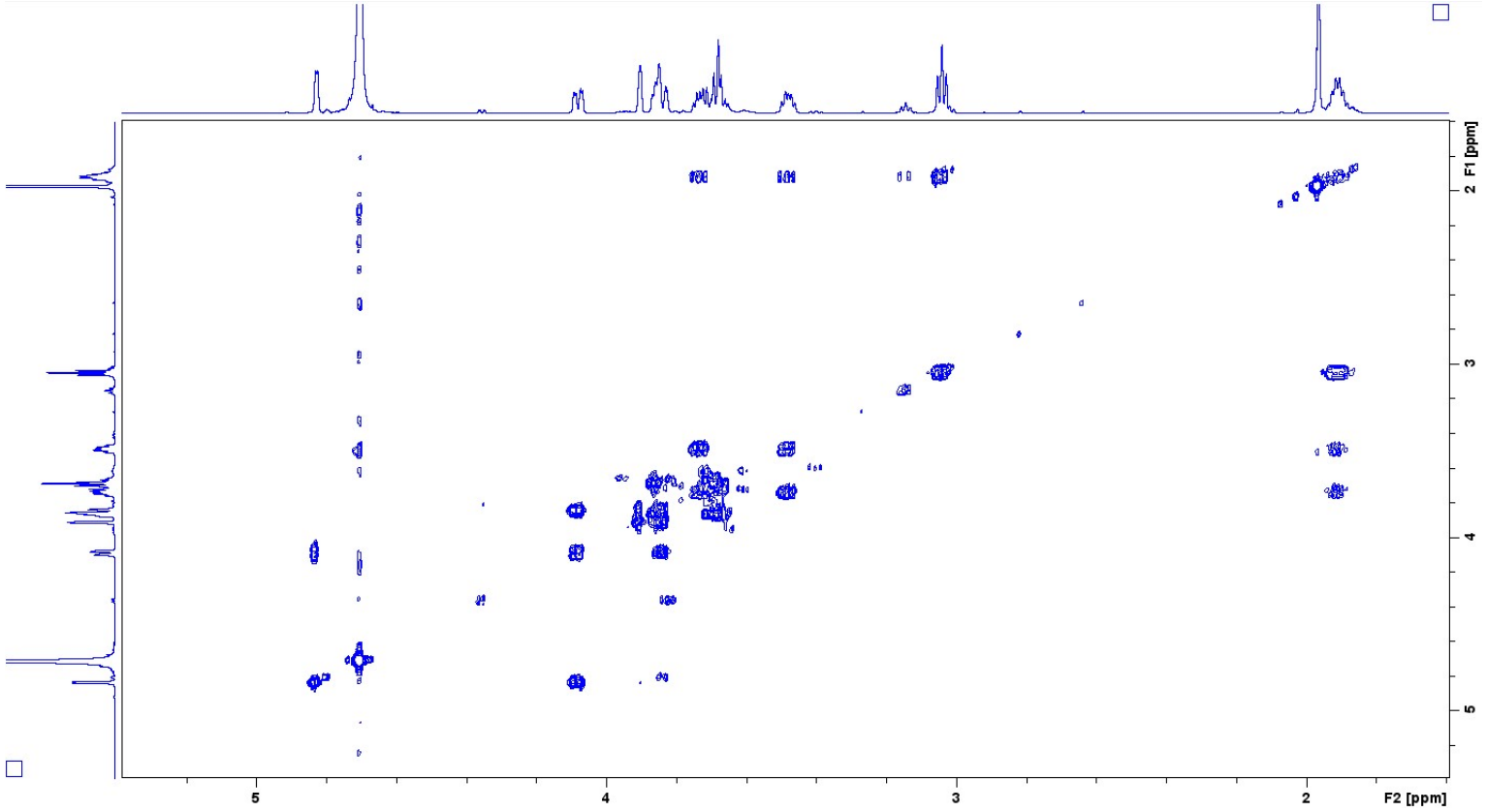
^{13}C NMR of **12**


HO
OH
O
HO
AcHN
O
NH2

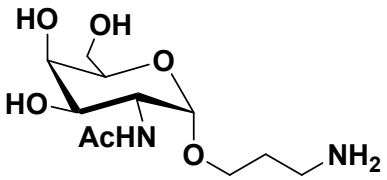
97.05
71.05
68.49
67.54
64.97
61.32
49.86
37.16
26.72
21.89
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

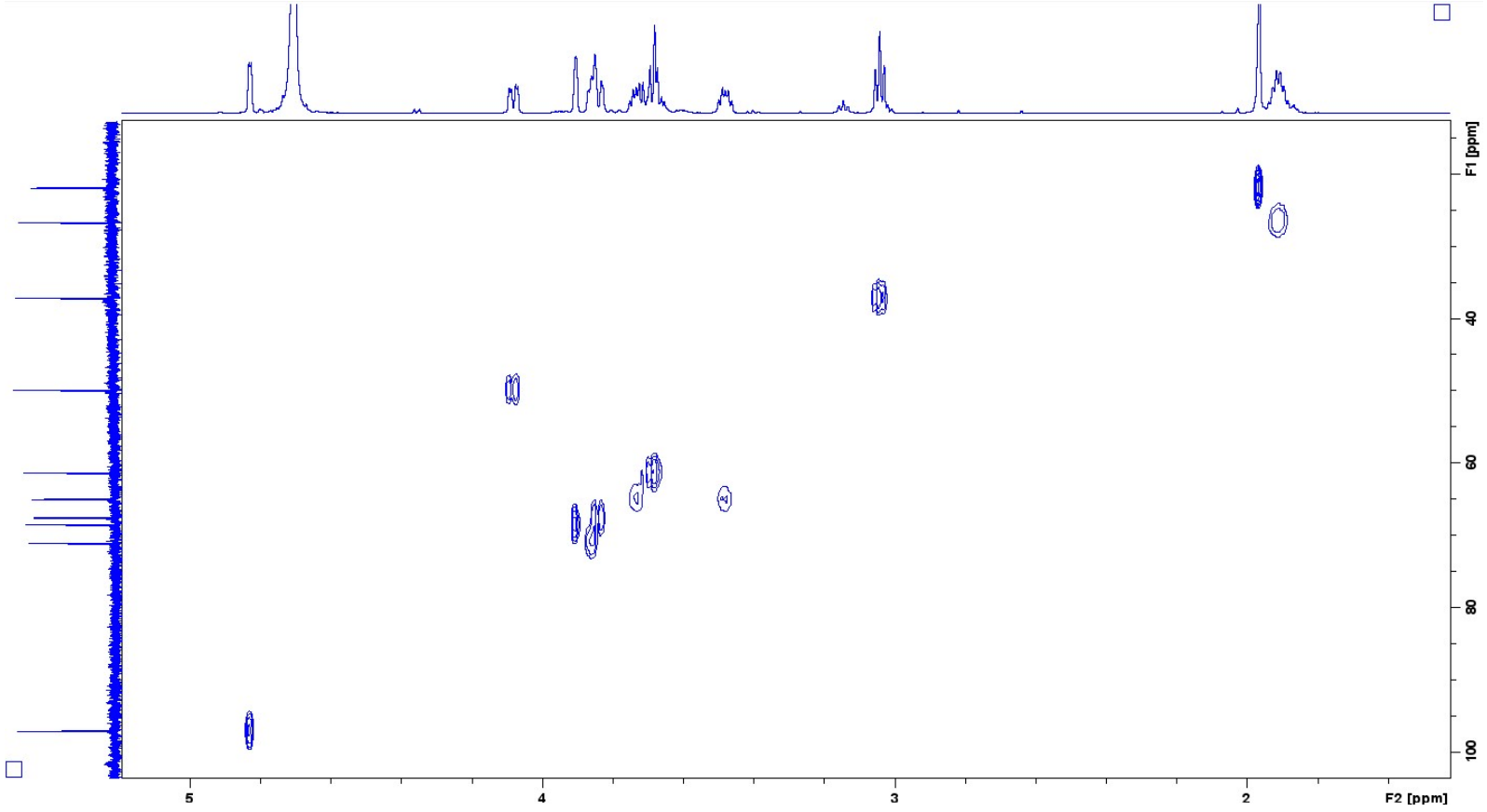
COSY NMR of **12**



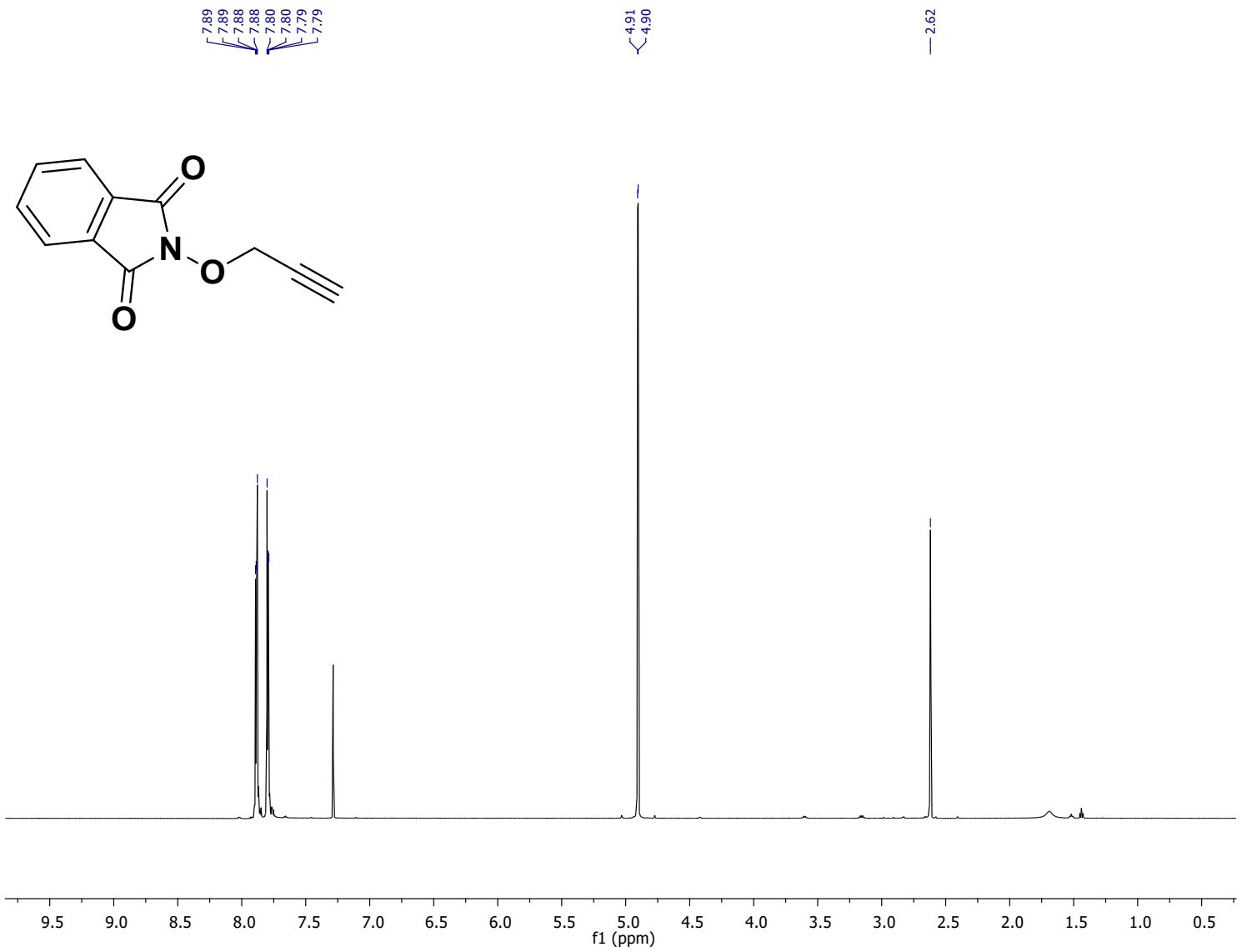


HSQC NMR of **12**

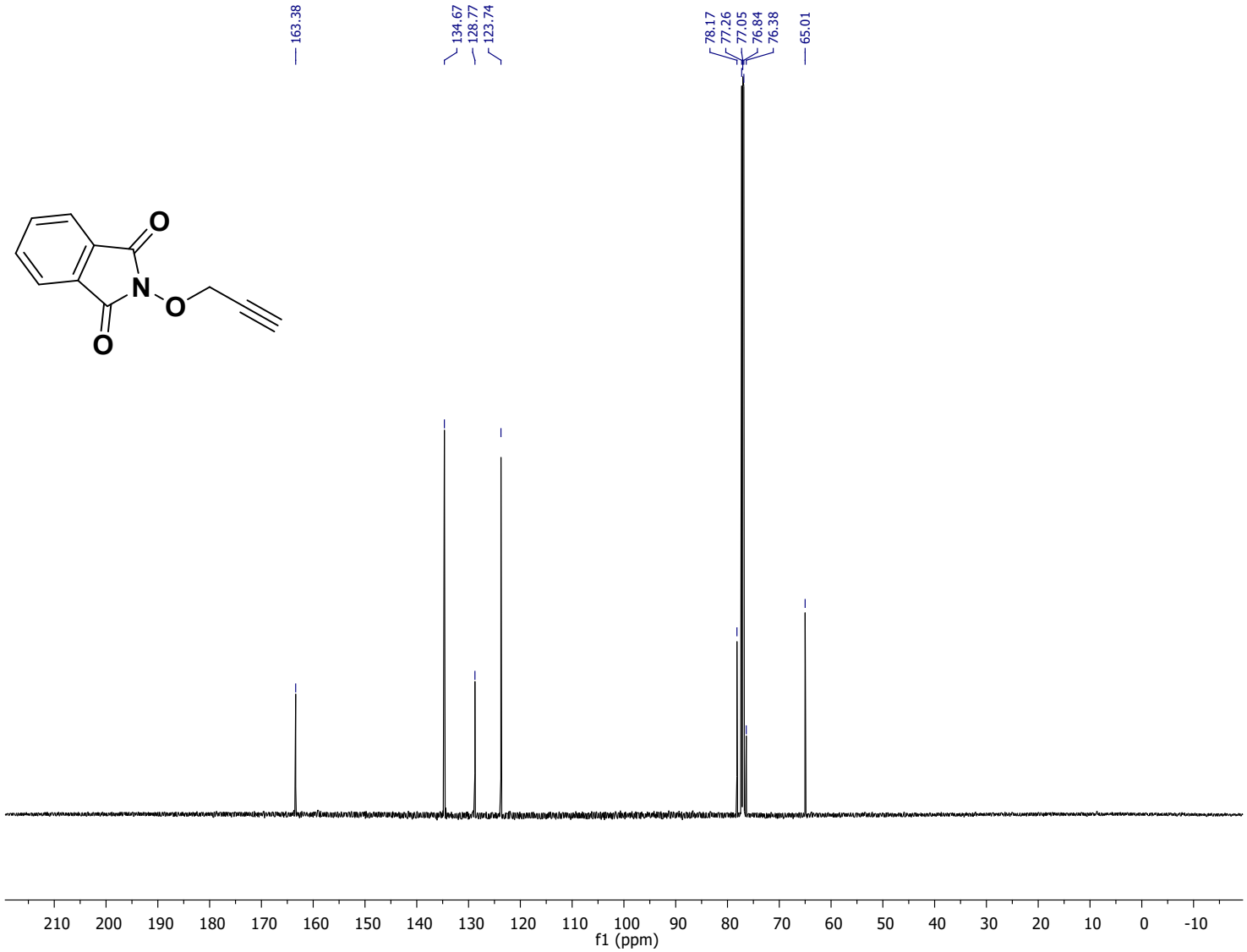




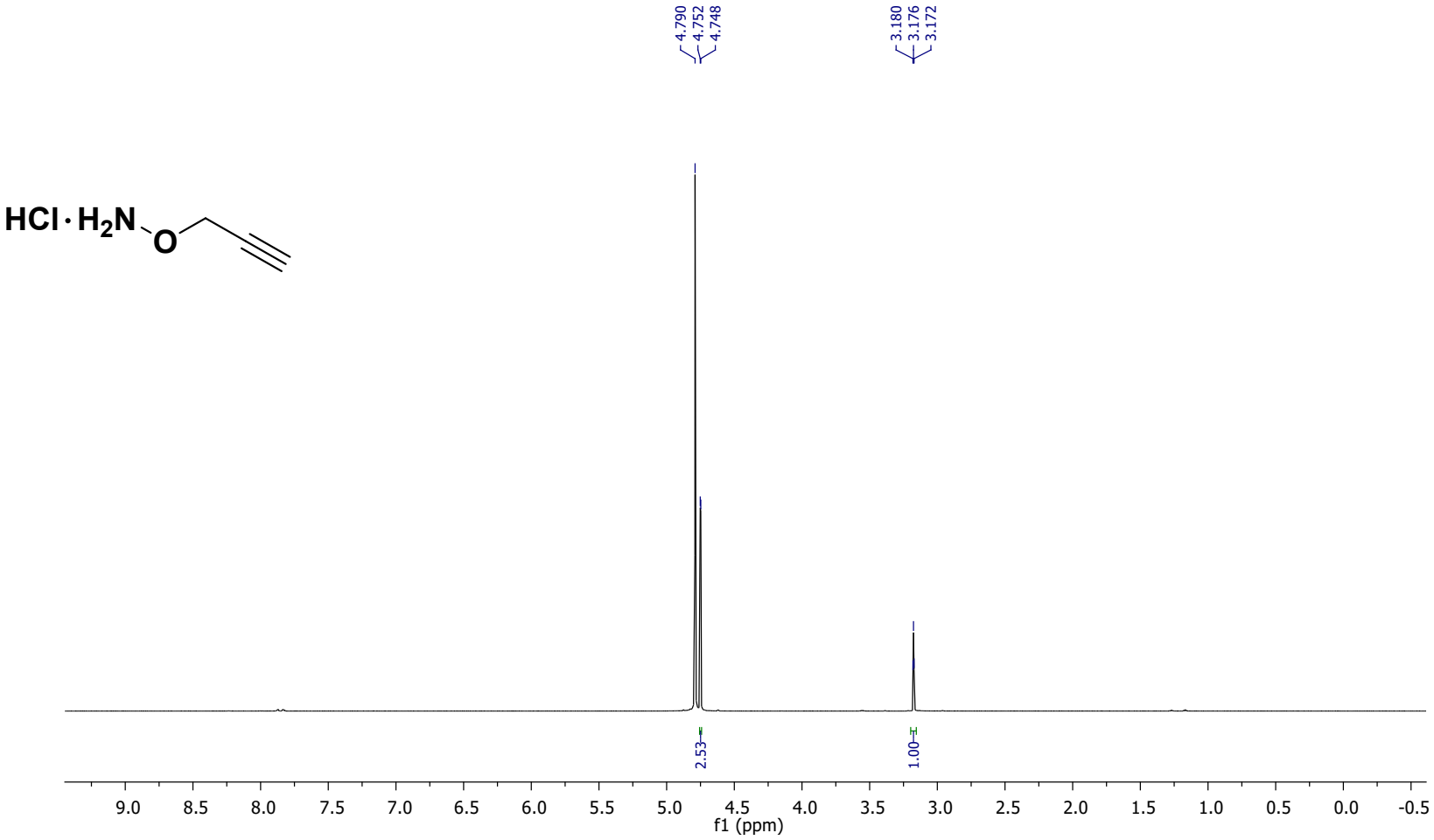
^{1}H NMR of *N*-(propargyloxy)phthalimide



^{13}C NMR of *N*-(propargyloxy)phthalimide



^{1}H NMR of *O*-2-propynylhydroxylamine hydrochloride **3**



^{13}C NMR of *O*-2-propynylhydroxylamine hydrochloride **3**