CHEMBIOCHEM

Supporting Information

Fluorescent Live-Cell Imaging of Metabolically Incorporated Unnatural Cyclopropene-Mannosamine Derivatives

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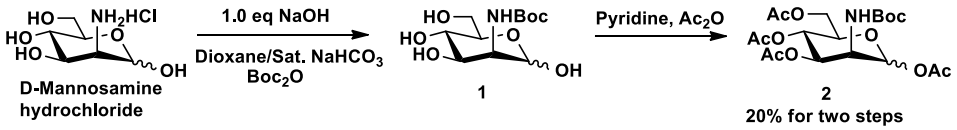
Tissue culture/cell growth conditions
SKBR3 and LS174T cells were grown in cDMEM media supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin. Cells were incubated in 5.0% carbon dioxide, 95% humidity at 37 °C. Generally, cells were grown in T-75 tissue culture flasks, seeded at densities between 500,000 and 750,000 cells per flask (cells were quantified with the Life Technologies Countess automated cell counter). The cells were trypsinized with TrypLE Express and resuspended in cDMEM. Cells were allowed to incubate for two days before supplementing with Ac_4ManNCyc (N-cyclopropeneacetylmannosamine) or Ac_4GlcNAz (N-azidoacetylglucosamine). The cells were then analyzed via confocal microscopy and flow cytometry as described below.

Live-cell microscopy
The SKBR3 and LS174T cells were incubated for two days in the presence of 100 μM of Ac_4ManNCyc and/or 50 μM Ac_4GlcNAz on a Lab-Tek chamber slide maintained in cDMEM medium . Treatment of cells with tunicamycin was done by preparing a 0.2 mg/mL stock in DMSO, which was diluted to a final working concentration of 1.2 μM tunicamycin in cDMEM (0.5% DMSO). Cells were washed 3x with phosphate-buffered saline (PBS) and incubated for 1 hour at 37 °C in 10 μM tetrazine-BODIPY TMR-X, 10 μM tetrazine-Alexa flour 488 and/or 15 μM dibenzocyclooctyne (DIBO)- Alexa Fluor 647 in cDMEM. The media was aspirated, and cells were washed twice with PBS before imaging. All photos were collected with an Olympus FV1000 confocal microscope using ImageJ 1.45j software package.

Analysis of cell surface cyclopropenes by flow cytometry
After the incubating the adherent SKBR3 cells in 100 μM of Ac_4ManNCyc (1.2 μM tunicamycin and/or 1 mM αBnGalNAc was used in experiments requiring glycosylation inhibitors) they were washed twice in PBS and then incubated in 10 μM of tetrazine-Alexa flour 488 for 1 hour at 37 °C. Control cells were not exposed to Ac_4ManNCyc and incubated in 10 μM of tetrazine-Alexa flour 488 for 1 hour at 37 °C. Cells were then resuspended in 1-2 ml of cDMEM ($5.0x10^5$ to $1.0x10^6$ per ml) using a rubber policeman. The cells were passed through a 25 gauge syringe to ameliorate excessive clumping, and subjected to analysis by flow cytometry using a 200mW 488nm blue solid state laser on the Partec Space Flow Cytometer (Partec).

Fluorescence turn-on spectroscopy
Mannosamine-cyclopropene (Ac_4ManNCyc) and tetrazine-Alexa-Fluor 488 stocks were prepared at 1 and 0.1 mM, respectively, in phosphate-buffered saline (PBS) pH 7.4. Fluorescence turn-on was measured using a Perkin Elmer LD-45 spectrometer, with the excitation wavelength set to 480/5 nm, and emission scanned over 490-620 nm (5-nm slit width) at a rate of 50 nm/min. Reaction conditions were 50 μM tetrazine-Alexa-Fluor 488 and 100 μM Ac_4ManNCyc in PBS pH 7.4 buffer at room temperature.

Synthesis of 1,3,4,6-Tetra-*O*-acety-*N*-Boc-D-mannosamine 2

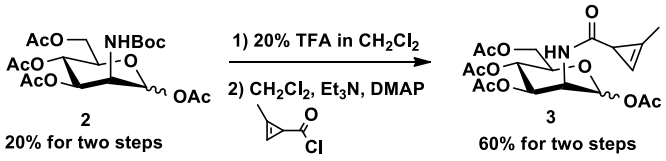


To a stirred solution of D-mannosamine hydrochloride (290 mg, 1.35 mmol) in dioxane/H_2O (4.0 mL/1.0 mL) at room temperature was added NaOH (54 mg, 1.35mmol) and 1.0 mL sat. $NaHCO_3$. Boc_2O (1.35 mL, 1.0 M in THF, 1.35 mmol) was added to the resulting solution and stirred overnight. By monitoring this reaction with LC-MS, we found the MS of one, two and three Boc products. The reaction solution was evaporated, and the residue dissolved with CH_2Cl_2 and filtered, the filtrate was concentrated, and the residue was dissolved in 2.0 mL pyridine. Ac_2O (688 mg, 6.75 mmol) was added to the solution and stirred overnight. The reaction solution was evaporated and the residue was dissolved with EtOAc, then washed by 1.0 M HCl and water. The organic layer was dried over Na_2SO_4 and concentrated to afford the crude product. The crude product was purified by flash silica column chromatography (Hexane:EtOAc = 2:1, monitored by TLC, stained by $KMnO_4$) to afford 120 mg compound **2** as white foam, in 20% yield over two steps.

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.42 (9H, s), 1.43 (9H, s), 1.97-2.13 (24H, m), 3.72-4.28 (8H, m), 4.88-5.27 (6H, m), 5.80 (1H, s); ^{13}C (100 MHz, $CDCl_3$) δ 20.88, 20.94, 21.03, 21.08, 28.40, 50.60, 50.85, 62.17, 62.30, 65.60, 65.70, 69.41, 70.29, 71.62, 73.49, 80.27, 80.59, 91.04, 92.23; HRMS $[M+Na]^+$ m/z calculated for $[C_{19}H_{29}NO_{11}Na]^+$ 470.1633, found 470.1636.

Synthesis of 1,3,4,6-Tetra-*O*-acety-*N*-cyclopropene tag-D-mannosamine 3

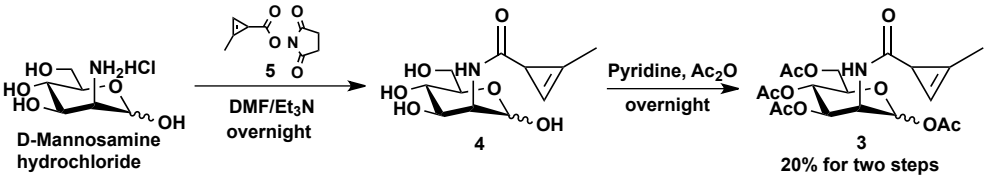
Method 1



To a stirred solution of 1,3,4,6-tetra-*O*-acety-*N*-Boc-D-mannosamine (70.0 mg, 0.16 mmol) in CH_2Cl_2 (2.0 mL) at room temperature was added CF_3COOH (0.5 mL). The reaction solution was stirred for 2.0 hours at room temperature and then evaporated to afford 1,3,4,6-tetra-*O*-acety-D-mannosamine TFA salt. This product could be stained by ninhydrin and formed a red colored spot on the TLC plate which indicated that the product possessed a free $-NH_2$ group. 1,3,4,6-tetra-*O*-acety-D-mannosamine TFA salt was dissolved in CH_2Cl_2, after adding Et_3N (32 mg, 0.32 mmol), and DMAP (3.9 mg, 0.032). Methyl cyclopropene acid chloride[1] (4.0 mg, 0.312 mmol) was added and the resulting solution was stirred for 30 minutes at room temperature. LC-MS showed the reaction was finished, and the reaction solution was evaporated to afford the crude product. The crude product was purified by preparative TLC (Hexanes:EtOAc = 1:1) to afford 40 mg compound as a white foam, in 60 % yield.

Method 2

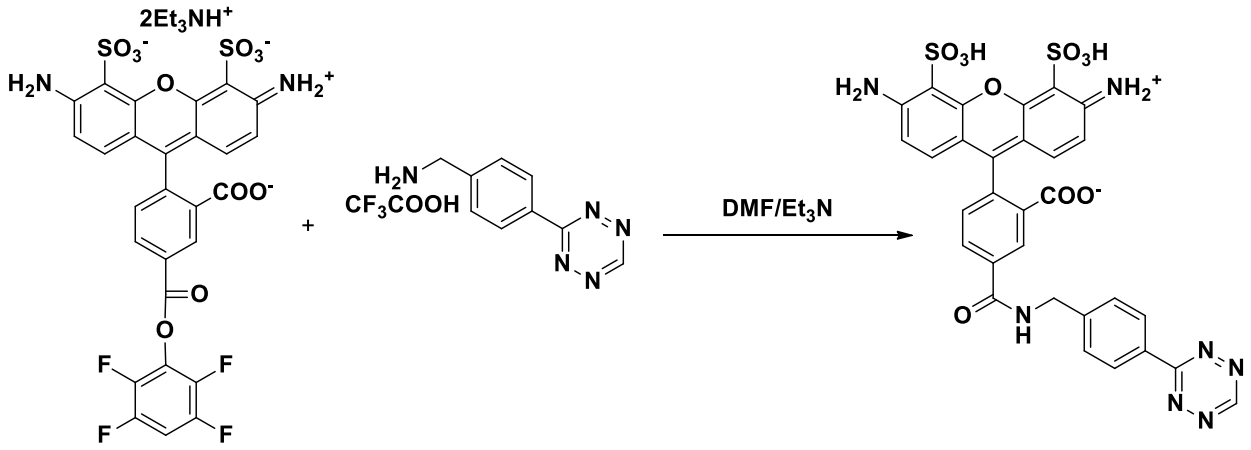
Alternative procedure



A mixture of D-mannosamine hydrochloride (5.0 mg, 0.023mmol), *N*-succinimidyl methyl cyclopropenoate **5** (5.0 mg, 0.026 mmol), and triethylamine (5.0 mg, 0.046 mmol) in DMF (0.3 ml) was stirred at room temperature overnight. The reaction mixture was concentrated *in vacuo*, and the residue was dissolved in pyridine (0.3 mL) and Ac_2O (25 mg) was added. The reaction was stirred at room temperature overnight and then was concentrated *in vacuo*, the residue was purified by prepared TLC (Hexane/EtOAc=1/1) to afford 2.0 mg of compound **3**, in 20% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.97 (32H, m), 3.99-4.07 (4H, m), 4.19-4.22 (2H, m), 4.63-4.68 (2H, m), 5.10-5.16 (2H, m), 5.25-5.31 (2H, m), 5.64-5.72 (2H, m), 5.97-5.98 (2H, m), 6.43 (1H, s), 6.45 (1H, s); ^{13}C (100 MHz, $CDCl_3$) δ 10.70, 10.85, 20.86, 20.90, 20.92, 20.96, 20.98, 21.08, 22.53, 22.56, 49.19, 49.24, 62.14, 62.30, 65.40, 65.66, 69.12, 69.30, 70.25, 92.06, 95.50, 95.99, 113.63, 114.64, 168.44, 169.87, 169.94, 170.26, 170.69, 176.22; HRMS $[M+Na]^+$ m/z calculated for $[C_{19}H_{25}NO_{10}Na]^+$ 450.1371, found 450.1373.

Synthesis of tetrazine-Alexa Flour 488



To a stirred solution of Alexa Fluor 488 5-TFP (0.5 mg) in DMF (0.5) at room temperature was added (4-(1,2,4,5-tetrazin-3-yl)phenyl)methanamine[1] (0.5 mg) and Et_3N (0.5 mg). The reaction solution was stirred at room temperature for 30 minutes. The product was purified by reverse phase TLC ($MeOH:H_2O$=1:3) directly without work-up to afford 0.4 mg product as an orange solid in quantitative yield. HRMS $[M-H]^-$ m/z calculated. for $[C_{30}H_{20}N_7O_{10}S_2]^+$ 702.0719, found 702.0718.

References

[1] J. Yang, J. Šečkutė, C. M. Cole, N. K. Devaraj, *Angew. Chem. Int. Ed.* **2012**, *51*, 7476–7479.

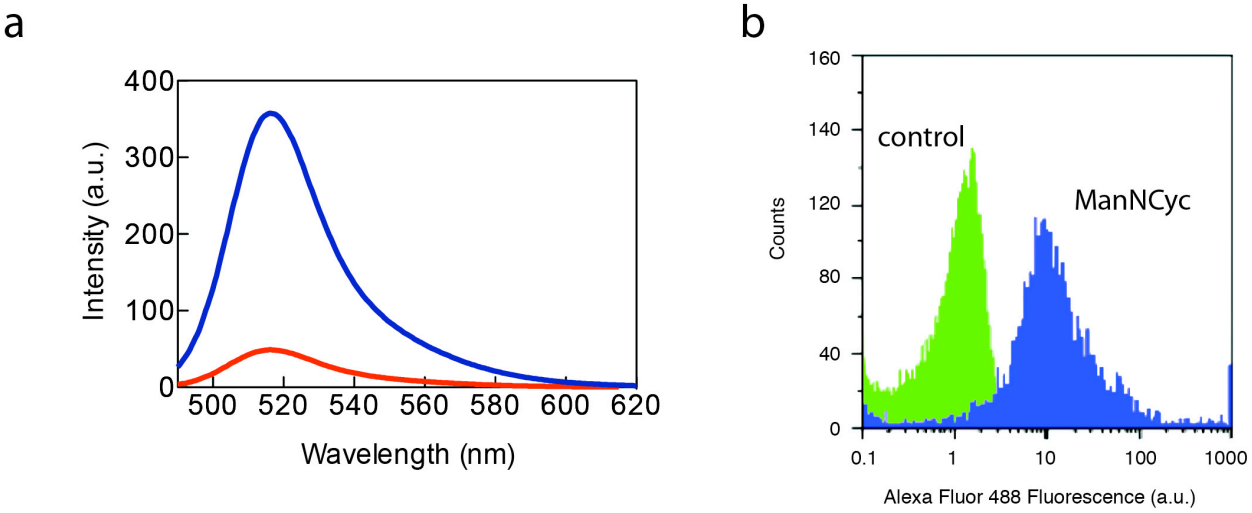


Figure S1. a) Emission spectra demonstrating the fluorogenic response of tetrazine-Alexa Fluor 488 before (red) and after incubation with Ac_4ManNCyc **3** (blue). b) Flow cytometry indicates fluorescent staining of SKBR3 cells incubated with Ac_4ManNCyc followed by tetrazine-Alexa Fluor 488. Control cells not exposed to the cyclopropene registered significantly less fluorescence intensity.

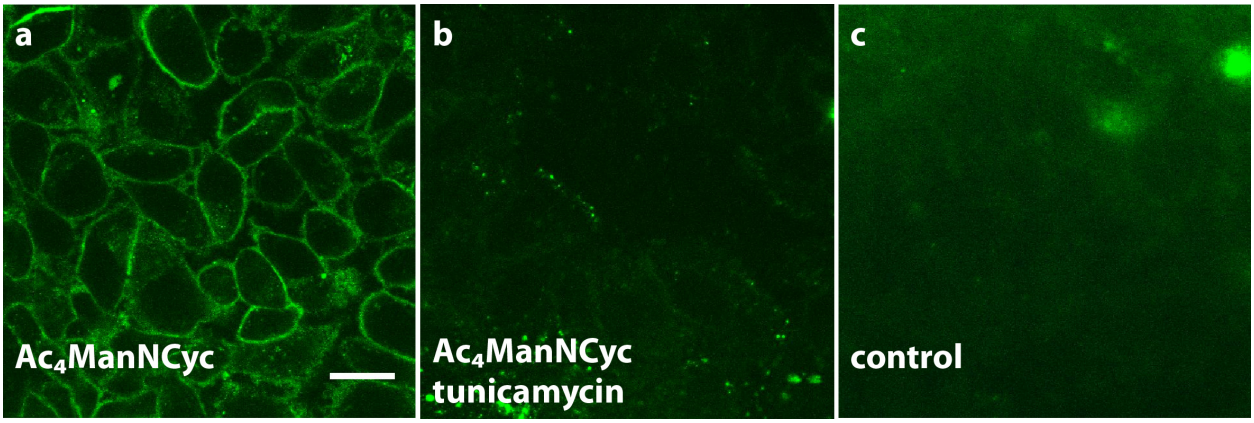


Figure S2. Imaging SKBR3 cells. Separate populations of SKBR3 cells were incubated for 48 hours with (a) Ac_4ManNCyc, (b) Ac_4ManNCyc and 1.2 μM tunicamycin, and (c) a control solution lacking a mannosamine derivative. The cells were then reacted with 10 μM tetrazine-Alexa Fluor 488 and imaged by confocal microscopy. Cells receiving only Ac_4ManNCyc showed bright surface staining while the other control populations had minimal surface staining. Scale bar denotes 20 microns.

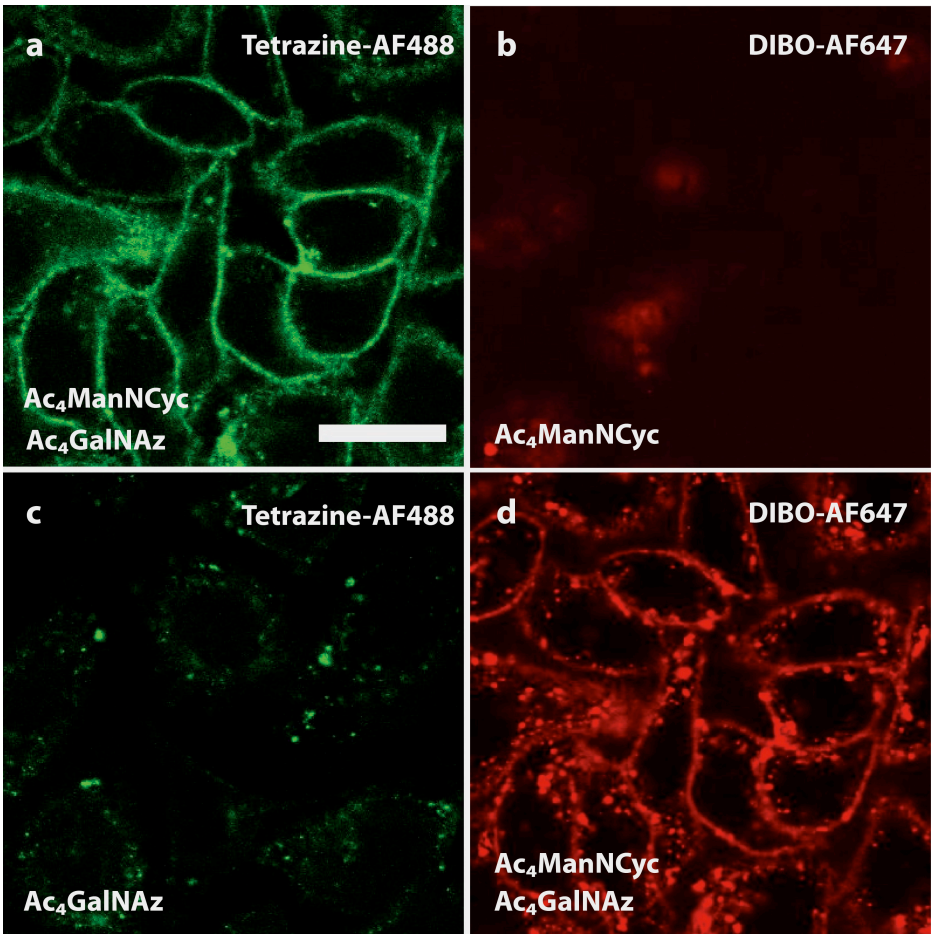
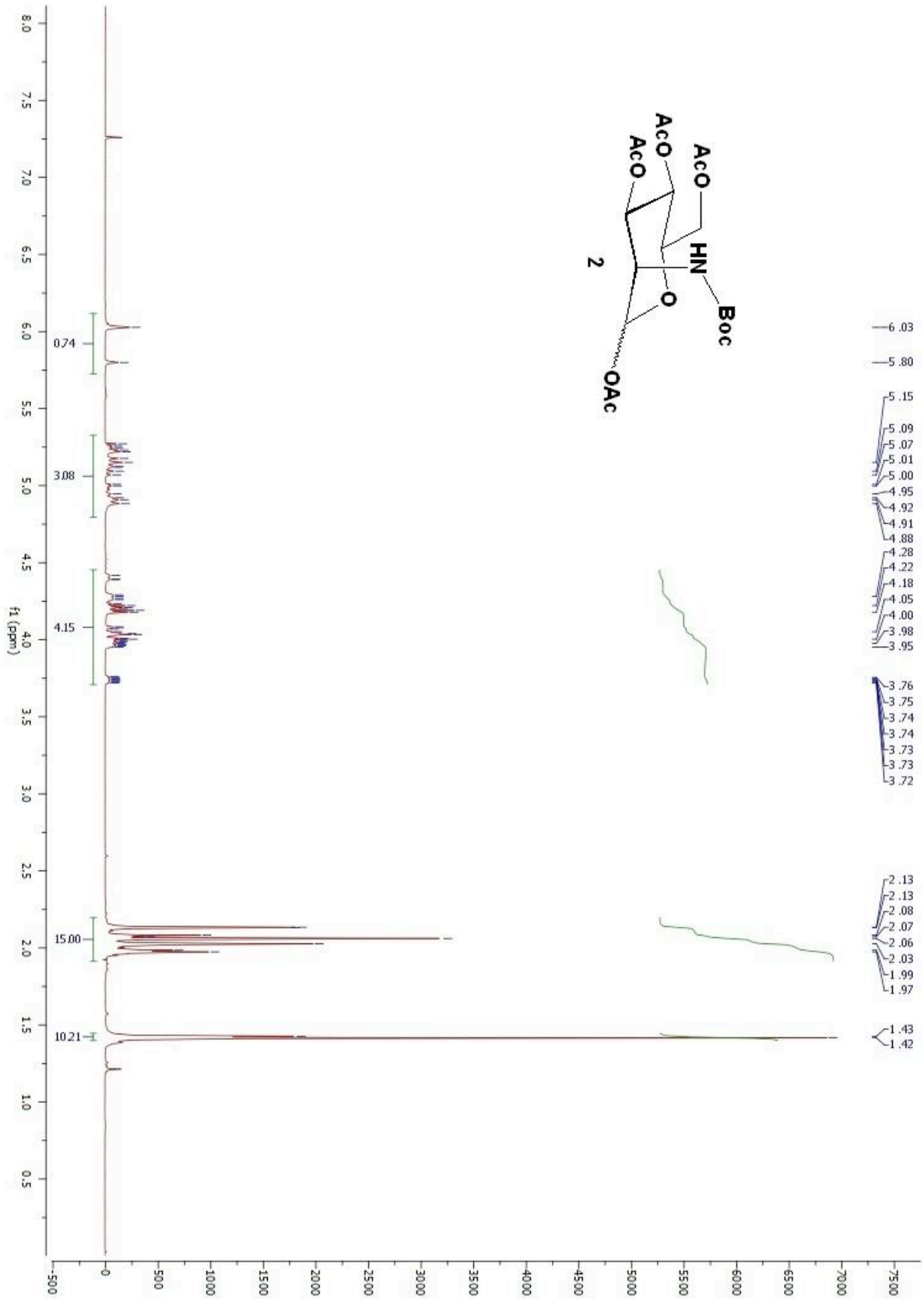


Figure S3: Cross reactivity studies. SKBR3 cell surfaces were incubated for 48 hours with Ac_4ManNCyc and Ac_4GalNAz reacted with both tetrazine-Alexa Fluor 488 and DIBO Alexa Fluor 647 and imaged by confocal microscopy in (a) the 488 channel and (d) the 647 channel. Cells that were incubated for 48 hours with Ac_4ManNCyc and reacted with DIBO Alexa Fluor 647 (b) or incubated with Ac_4ManNAz and reacted with tetrazine-Alexa Fluor 488 (c) showed minimal surface staining, similar to the background signal due to nonspecific binding of dye. Scale bar denotes 20 microns.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AcO
HN
Boc
O
OAc
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f1 (ppm)
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4.15
15.00
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6.03
5.80
5.15
5.09
5.07
5.01
5.00
4.95
4.92
4.91
4.88
4.28
4.22
4.18
4.05
4.00
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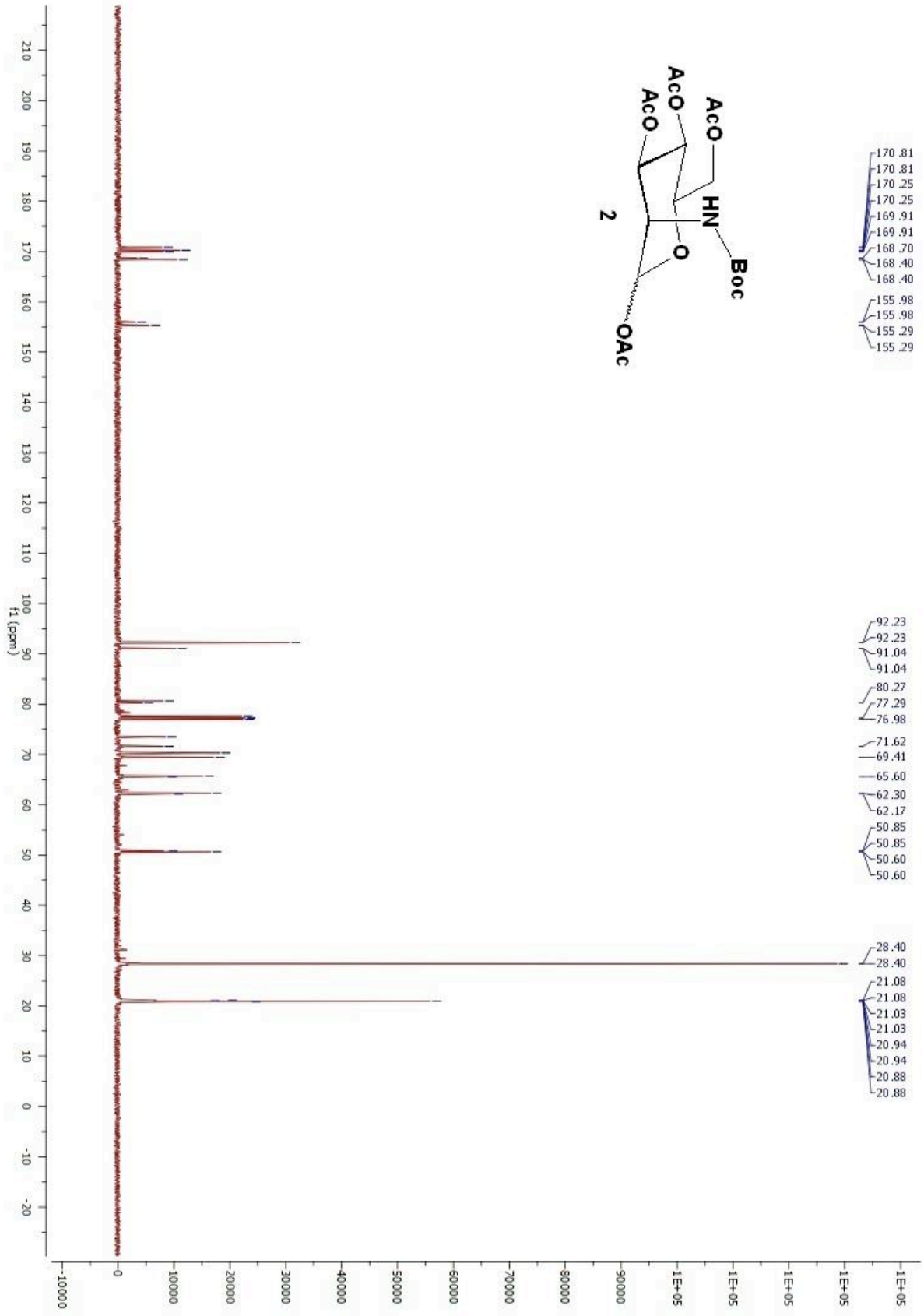
1.97
1.43
1.42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AcO
AcO
HN
Boc
O
OAc
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170.81
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168.40
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155.98
155.98
155.29
155.29
92.23
92.23
91.04
91.04
80.27
77.29
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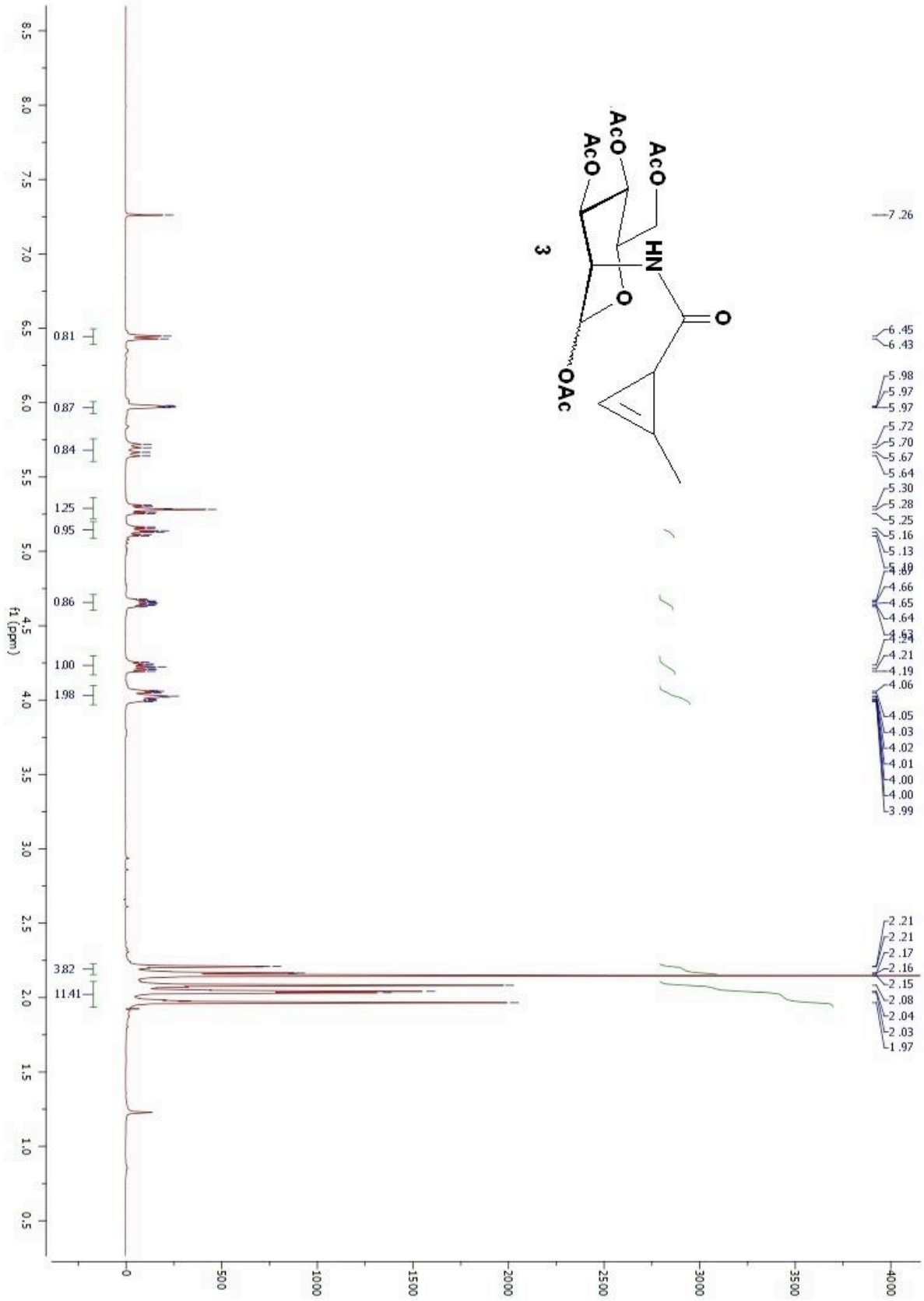
20.88
f1 (ppm)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AcO
AcO
HN
O
O
OAc
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f1 (ppm)
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0.95
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1.00
1.98
3.82
11.41
7.26
6.45
6.43
5.98
5.97
5.97
5.72
5.70
5.67
5.64
5.30
5.28
5.25
5.16
5.13
4.66
4.65
4.64
4.24
4.21
4.19
4.06
4.05
4.03
4.02
4.01
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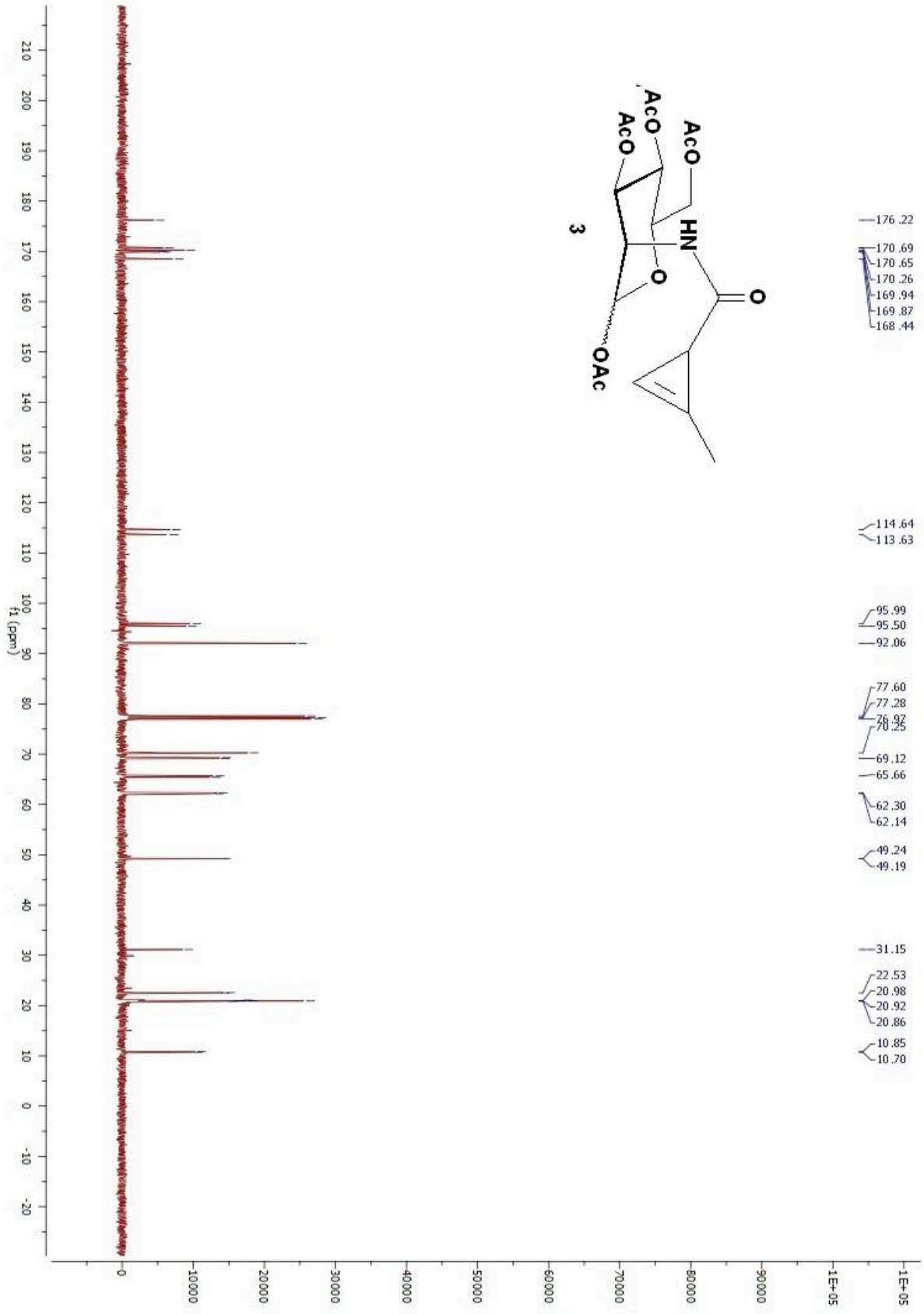
2.04
2.03
1.97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AcO
HN
O
O
OAc
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170.26
169.94
169.87
168.44
114.64
113.63
95.99
95.50
92.06
77.60
77.28
76.97
70.25
69.12
65.66
62.30
62.14
49.24
49.19
31.15
22.53
20.98
20.92
20.86
10.85
10.70
f1 (ppm)