Supporting Information for:

Affinity-Based Covalent Sialyltransferase Probes Enabled by Ligand-Directed Chemistry

Jun Yang Ong, Erianna I. Alvarado-Melendez, Joshua C. L. Maliepaard, Karli R. Reiding and Tom Wennekes

Table of contents

I. Supplementary tables

II. Equation 1 and derivation of equation 2

III. Supplementary figures

IV. Experimental

a. Materials and general methods

b. Synthetic schemes

c. Synthesis

d. Activity of recombinant sialyltransferases with CMP-sialic acid donor **22** and lactose

e. General procedure for affinity-based labelling

f. Heat inactivation and competition assay

g. Kinetic assay

h. Protein LC-MS/MS

i. Bacteria strains and culture

j. Triton X-100 extraction of Lst

k. LOS labelling

l. Lst labelling

V. References

VI. NMR spectra

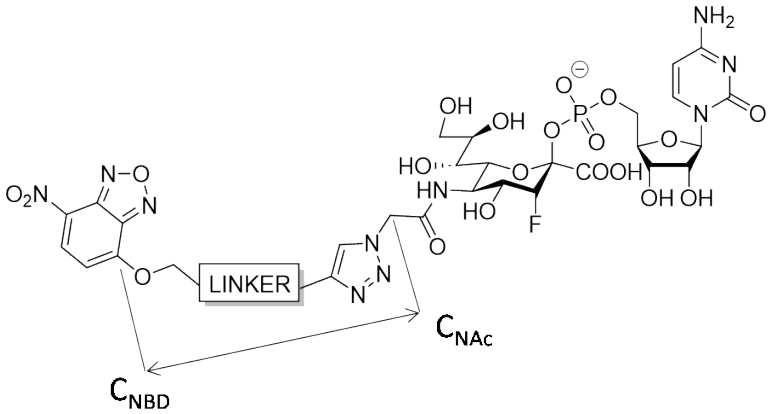
I. Supplementary table

Table S1. Sialyltransferase sequences.

Name	Full sequence as expressed (including tags)	kDa
PmST1 WT	MKNRRLNFKLFFLIIFSLFSTLSWSGTITLYLDPASLPALNQLMDFTQHNEDKTHPRIF GLSRFKIPDNIITQYQNIHFVELKDNRPTEALFTILDQYPGNIELDIHLNIAHSVQLIRPIL AYRFKHLDRVSIQRLNLYDDGSMEYVDLEKEENKDISAEIKQAEKQLSHYLLTGKIKF DNPTIARYVWQSAFPVKYHFLSTDYFEKAEFLQPLKDYLAENYQKMDWTAYQQLTP EQQAFYLTLVGFNDEVKQSLEVQQAKFIFTGTTTWEGNTDVREYYAQQQLNLLNHF THAEGDLFIGDHYKIYFKGHPRGGEINDYILNNAKNITNIPANISFEVLMMTGLLPDKV GGVASSLYFSLPKEKISHIIFTSNKQVKSKEDALNNPYVKVMRRLGIIDESQVIFWDSL KQLLEHHHHHH*	49.2
PmST1 M144D	MKNRRLNFKLFFLIIFSLFSTLSWSGTITLYLDPASLPALNQLMDFTQHNEDKTHPRIF GLSRFKIPDNIITQYQNIHFVELKDNRPTEALFTILDQYPGNIELDIHLNIAHSVQLIRPIL AYRFKHLDRVSIQRLNLYDDGSDEYVDLEKEENKDISAEIKQAEKQLSHYLLTGKIKF DNPTIARYVWQSAFPVKYHFLSTDYFEKAEFLQPLKDYLAENYQKMDWTAYQQLTP EQQAFYLTLVGFNDEVKQSLEVQQAKFIFTGTTTWEGNTDVREYYAQQQLNLLNHF THAEGDLFIGDHYKIYFKGHPRGGEINDYILNNAKNITNIPANISFEVLMMTGLLPDKV GGVASSLYFSLPKEKISHIIFTSNKQVKSKEDALNNPYVKVMRRLGIIDESQVIFWDSL KQLLEHHHHHH*	49.2
VJT-FAJ-16	MGSSHHHHHHSSGMNNDNSTTTNNNAIEIYVDRATLPTIQQMTKIVSQKTSNKKLIS WSRYPITDKSLLKKINAEFFKEQFELTESLKNIILSENIDNLIIHGNTLWSIDVVDIIKEVN LLGKNIPIELHFYDDGSAEYVRIYEFSKLPESEQKYKTSLSKNNIKFSIDGTDSFKNTIE NIYGFSQLYPTTYHMLRADIFDTTLKINPLRELLSNNIKQMKWDYFKDFNYKQKDIFY SLTNFNPKEIQEDFNKNSNKNFIFIGSNSATATAEEQINIISEAKKENSSIITNSISDYDL FFKGHPSATFNEQIINAHDMIEINNKIPFEALIMTGILPDAVGGMGSSVFFSIPKEVKNK FVFYKSGTDIENNSLIQVMLKLNLINRDNIKLISDI*	45.3
(MBP-) Pd2,6ST	MKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDG PDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALS LIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYE NGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGP WAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLT DEGLEAVNKDKPLGAVALKSYEEELVKDPRIAATMENAQKGEIMPNIPQMSAFWYA VRTAVINAASGRQTVDEALKDAQTNSSSNNNNNNNNNNLGASGSCNSDNTSLKET VSSNSADVVETETYQLTPIDAPSSFLSHSWEQTCGTPILNESDKQAISFDFVAPELKQ DEKYCFTFKGITGDHRYITNTTLTVVAPTLEVYIDHASLPSLQQLIHIIQAKDEYPSNQ RFVSWKRVTVDADNANKLNIHTYPLKGNNTSPEMVAAIDEYAQSKNRLNIEFYTNTA HVFNNLPPIIQPLYNNEKVKISHISLYDDGSSEYVSLYQWKDTPNKIETLEGEVSLLAN YLAGTSPDAPKGMGNRYNWHKLYDTDYYFLREDYLDVEANLHDLRDYLGSSAKQM PWDEFAKLSDSQQTLFLDIVGFDKEQLQQQYSQSPLPNFIFTGTTTWAGGETKEYY AQQQVNVINNAINETSPYYLGKDYDLFFKGHPAGGVINDIILGSFPDMINIPAKISFEVL MMTDMLPDTVAGIASSLYFTIPADKVNFIVFTSSDTITDREEALKSPLVQVMLTLGIVK EKDVLFWAVDELGTWSHPQFEKGGGSGGGSGGSSAWSHPQFEKGGSSAHHHHH HHHKLGTGRRFTTS*	103.1
(GFP-) ST6Gal1	MRLLTALFAYFIVALILAFSVSAKSMHHHHHHHHHMSGLNDIFEAQKIEWHEMSKGEE LFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGKLPVPWPTLVTT LTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTL VNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFKIRHNVEDGSVQ LADHYQQNTPIGDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITHGEF ASTSLYKKAGSENLYFQGRQTLGSLRGLAKAKPEASFQVWNKDSSSKNLIPRLQKI WKNYLSMNKYKVSYKGPGPGIKFSAEALRCHLRDHVNVSMVEVTDFPFNTSEWEG YLPKESIRTKAGPWGRCAVVSSAGSLKSSQLGREIDDHDAVLRFNGAPTANFQQDV GTKTTIRLMNSQLVTTEKRFLKDSLYNEGILIVWDPSVYHSDIPKWYQNPDYNFFNN YKTYRKLHPNQPFYILKPQMPWELWDILQEISPEEIQPNPPSSGMLGIIIMMTLCDQV DIYEFLPSKRKTDVCYYYQKFFDSACTMGAYHPLLYEKNLVKHLNQGTDEDIYLLGK ATLPGFRTIHC*	72.5
(MBP-)	MKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDG	76.1

Cst-II	PDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALS LIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYE NGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGP WAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLT DEGLEAVNKDKPLGAVALKSYEEELVKDPRIAATMENAQKGEIMPNIPQMSAFWYA VRTAVINAASGRQTVDEALKDAQTNSSSNNNNNNNNNNLGENLYFQSMKKVIIAGN GPSLKEIDYSRLPNDFDVFRCNQFYFEDKYYLGKKCKAVFYNPSLFFEQYYTLKHLI QNQEYETELIMCSNYNQAHLENENFVKTFYDYFPDAHLGYDFFKQLKDFNAYFKFH EIYFNQRITSGVYMCAVAIALGYKEIYLSGIDFYQNGSSYAFDTKQKNLLKLAPNFKN DNSHYIGHSKNTDIKALEFLEKTYKIKLYCLCPNSLLANFIELAPNLNSNFIIQEKNNYT KDILIPSSEAYGKFSKNINGGSGGSHHHHHHGGSGGSWSHPQFEK*	
N. gono Lst	MGSSHHHHHHSSGMGLKKVCLTVLCLIVFCFGIFYTFDRVNQGERNAVSLLKDKLF NEEGKPVNLIFCYTILQMKVAERIMAQHPGERFYVVLMSENRNEKYDYYFNQIKDKA ERAYFFYLPYGLNKSFNFIPTMAELKVKSMLLPKVKRIYLASLEKVSIAAFLSTYPDAE IKTFDDGTNNLIRESSYLGGEFAVNGAIKRNFARMMVGDWSIAKTRNASDEHYTIFK GLKNIMDDGRRKMTYLPLFDASELKAGDETGGTVRILLGSPDKEMKEISEKAAKNFN IQYVAPHPRQTYGLSGVTALNSPYVIEDYILREIKKNPHTRYEIYTFFSGAALTMKDFP NVHVYALKPASLPEDYWLKPVYALFRQADIPILTFDDKN*	44.1

Table S2. Length of the linkers.



Linker	Length (C_{NAC}-C_{NBD}) / Å
PEG0	7.33
PEG1	10.52
PEG2C	16.03
PEG3C	19.53
PEG4C	23.03

Table S3. Sequence coverage for labelled and unlabelled VJT-FAJ-16 and PmST1 using LC-MS/MS. The samples containing unlabelled VJT-FAJ-16 and PmST1 showed higher sequence coverage than the samples that were labelled with the probe.

Sample	**Sequence coverage**
VJT-FAJ-16 unlabelled	94.92%
VJT-FAJ-16 labelled	87.06%
PmST1 unlabelled	86.67%
PmST1 labelled	71.43%

II. Equation 1 and derivation of Equation 2

$$\begin{array}{l} [\mathrm{E}] + [\mathrm{L}] \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [\mathrm{EL}] \xrightarrow{k_2} [\mathrm{E}^*] \\ + \\ [\mathrm{I}] \\ k_{-3} \uparrow\downarrow k_3 \\ [\mathrm{EI}] \end{array}$$

$$\frac{\mathrm{d}[\mathrm{E}^*]}{\mathrm{dt}} = \frac{k_2}{K_M + [\mathrm{L}]}[\mathrm{E}][\mathrm{L}] \qquad (1)$$

$$\frac{\mathrm{d}[\mathrm{E}^*]}{\mathrm{dt}} = \frac{k_2}{\alpha K_M + [\mathrm{L}]}[\mathrm{E}][\mathrm{L}] \qquad (2)$$

where $K_M = \frac{k_{-1} + k_2}{k_1} = Kd + \frac{k_2}{k_1}$,

$\alpha = 1 + \frac{[\mathrm{I}]}{K_i}$ and $K_i = \frac{k_{-3}}{k_3}$

E = Enzyme, E* = labelled enzyme, I = competitive inhibitor, L = labelling probe

Equation 1 and 2. Rate equation for the kinetics of affinity-based labelling reaction without (Equation 1)[1] and with (Equation 2) competitive inhibitor. Derivation of equation 2 is presented below, and was found to be analogous to the Copeland kinetic model for reversible competitive inhibition.[2]

Steady state assumption:

$$\frac{d[EL]}{dt} = 0$$

$$k1\ [\mathrm{E}][\mathrm{L}] = k-1\ [\mathrm{EL}] + k_2\ [\mathrm{EL}]$$

$$[EL] = \frac{k1}{k-1+k2}[E][L]$$

$$\frac{d[EL]}{dt} = \frac{k1[L]}{k-1+k2}\frac{d[E]}{dt}$$

$$\frac{d[EI]}{dt} = 0$$

$$k-3\ [\mathrm{EI}] = k3\ [\mathrm{E}][\mathrm{I}]$$

$$\frac{k-3}{k3} = \frac{[E][I]}{[EI]} = Ki$$

$$[EI] = \frac{[E][I]}{Ki}$$

$$\frac{d[EI]}{dt} = \frac{[I]d[E]}{Ki\ dt}$$

Conservation of mass of enzyme, $E_T = E + EL + E^* + EI$

$$0 = \frac{d[E]}{dt} + \frac{d[EL]}{dt} + \frac{d[E^*]}{dt} + \frac{d[EI]}{dt}$$

$$0 = \frac{d[E]}{dt} + \frac{k1[L]}{k-1+k2}\frac{d[E]}{dt} + k2\ [EL] + \frac{[I]d[E]}{Ki\ dt}$$

$$0 = \frac{d[E]}{dt} + \frac{k1[L]}{k-1+k2}\frac{d[E]}{dt} + \frac{k1k2}{k-1+k2}[E][L] + \frac{[I]d[E]}{Ki\ dt}$$

$$-\frac{d[E]}{dt} - \frac{k1[L]}{k-1+k2}\frac{d[E]}{dt} - \frac{[I]d[E]}{Ki\ dt} = \frac{k1k2}{k-1+k2}[E][L]$$

$$\left(-\frac{d[E]}{dt}\right)\left(1 + \frac{k1[L]}{k-1+k2} + \frac{[I]}{Ki}\right) = \frac{k1k2}{k-1+k2}[E][L]$$

$$\left(-\frac{d[E]}{dt}\right)\left(\frac{Ki(k-1+k2) + Kik1[L] + [I](k-1+k2)}{Ki(k-1+k2)}\right) = \frac{k1k2}{k-1+k2}[E][L]$$

$$\left(-\frac{d[E]}{dt}\right) = \frac{k1k2}{k-1+k2}[E][L]\left(\frac{Ki(k-1+k2)}{Ki(k-1+k2) + Kik1[L] + [I](k-1+k2)}\right)$$

Cancel out $k-1+k2$, then divide both numerator and denominator by $Kik1$

$$\left(-\frac{d[E]}{dt}\right)=\frac{k2[E][L]}{\frac{k-1+k2}{k1}+[L]+\frac{[I]}{Ki}\left(\frac{k-1+k2}{k1}\right)}$$

$$Michaelis\ constant,\ KM=\frac{k-1+k2}{k1}$$

$$\left(-\frac{d[E]}{dt}\right)=\frac{k2[E][L]}{KM+[L]+\frac{[I]}{Ki}(KM)}$$

$$\left(-\frac{d[E]}{dt}\right)=\frac{k2[E][L]}{KM\left(1+\frac{[I]}{Ki}\right)+[L]}$$

$$Define\ degree\ of\ inhibition,\ \alpha=1+\frac{[I]}{Ki}$$

$$\left(-\frac{d[E]}{dt}\right)=\frac{d[E^*]}{dt}=\frac{k2}{\alpha KM+[L]}[E][L] \qquad (2)$$

$$where\ KM=\frac{k-1+k2}{k1}=Kd+\frac{k2}{k1}\ ,$$

$$\alpha=1+\frac{[I]}{Ki}\ and\ Ki=\frac{k-3}{k3}$$

III. Supplementary figures

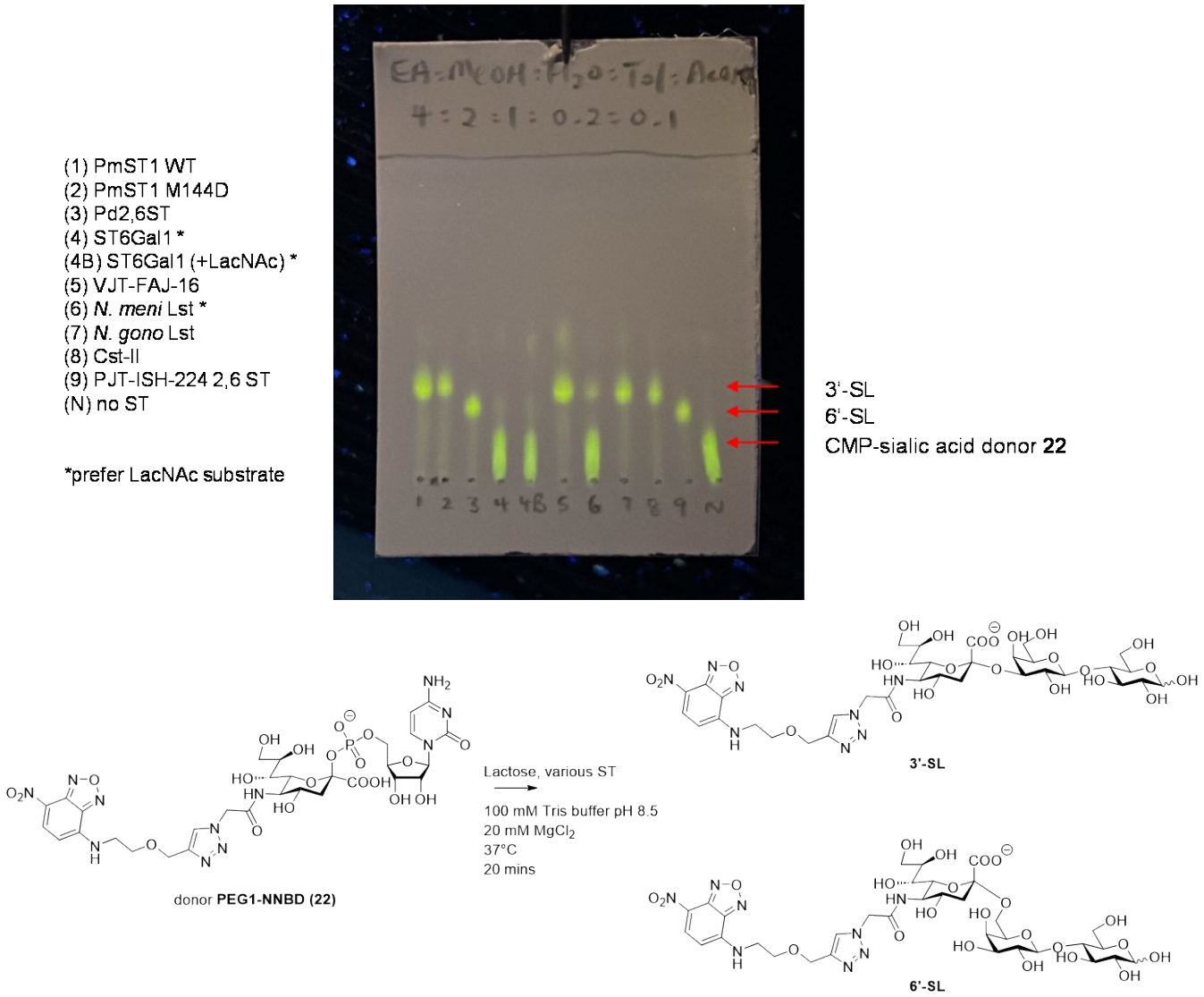


Figure S1. All enzymes were tested for their activity prior to the labelling experiment using *N*-NBD fluorescently labelled CMP-sialic acid donor **22** and lactose (except for 4B, where LacNAc-*β*-*O*-propylazide was used instead of lactose). TLC system used: ethyl acetate/methanol/water/toluene/acetic acid 4:2:1:0.2:0.1, visualised under 365 nm UV. *N. meni* Lst and PJT-ISH-224 2,6 ST were not used in affinity labelling in this paper.

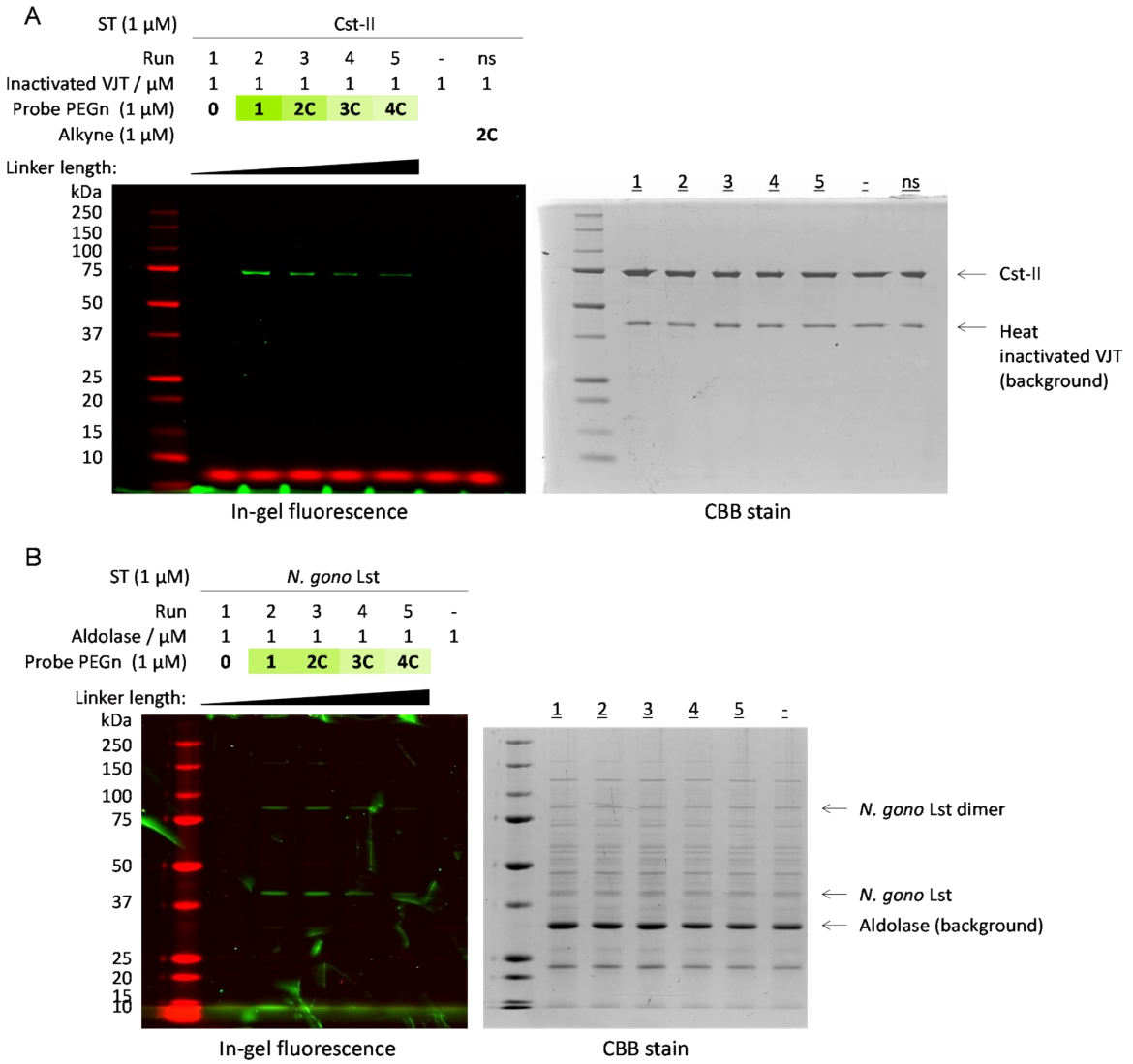


Figure S2. In-gel fluorescence was used to analyse the effect of linker length on the labelling efficiency of Cst-II and *N. gono* Lst by the affinity-based probes **17-21**. Different trend of labelling by probes with linker length ranging from PEG0 to PEG4C was observed for Cst-II and *N. gono* Lst. No off-target labelling was observed on the non-binding aldolase (the aldolase is responsible for contributing the numerous background bands in the CBB).

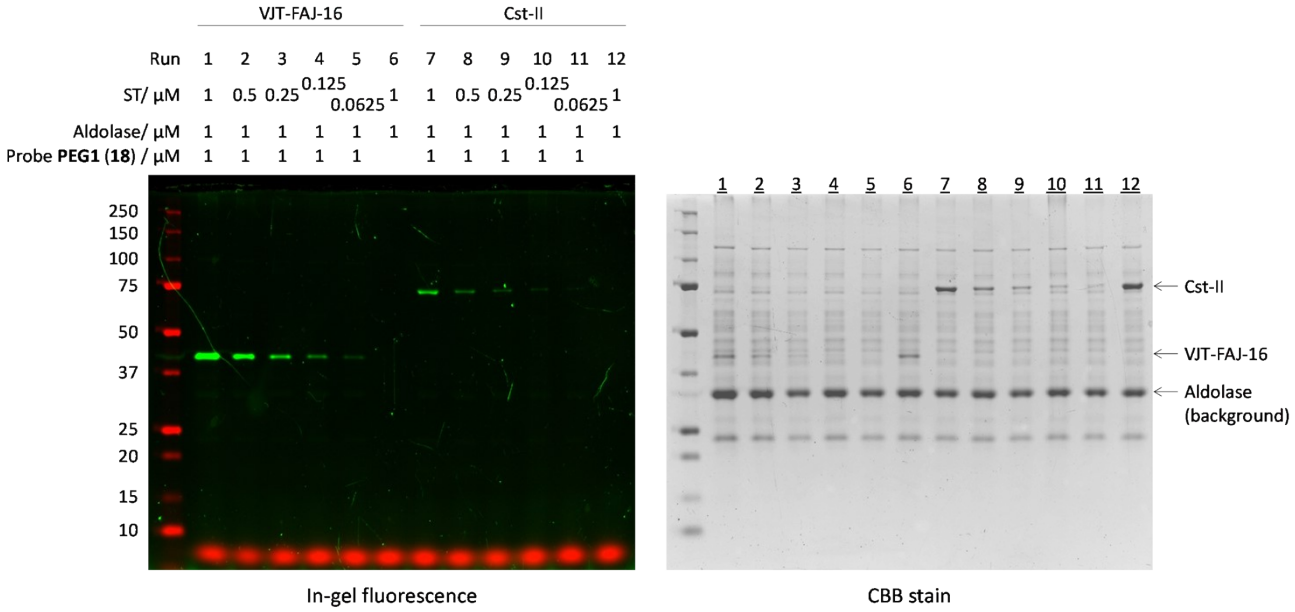


Figure S3. When 1 µM probe **PEG1** (**18**) was used, VJT-FAJ-16 could be detected down to 0.0625 µM and Cst-II down to 0.125 µM. No off-target labelling was observed on the non-binding aldolase (the aldolase is responsible for contributing the numerous background bands in the CBB).

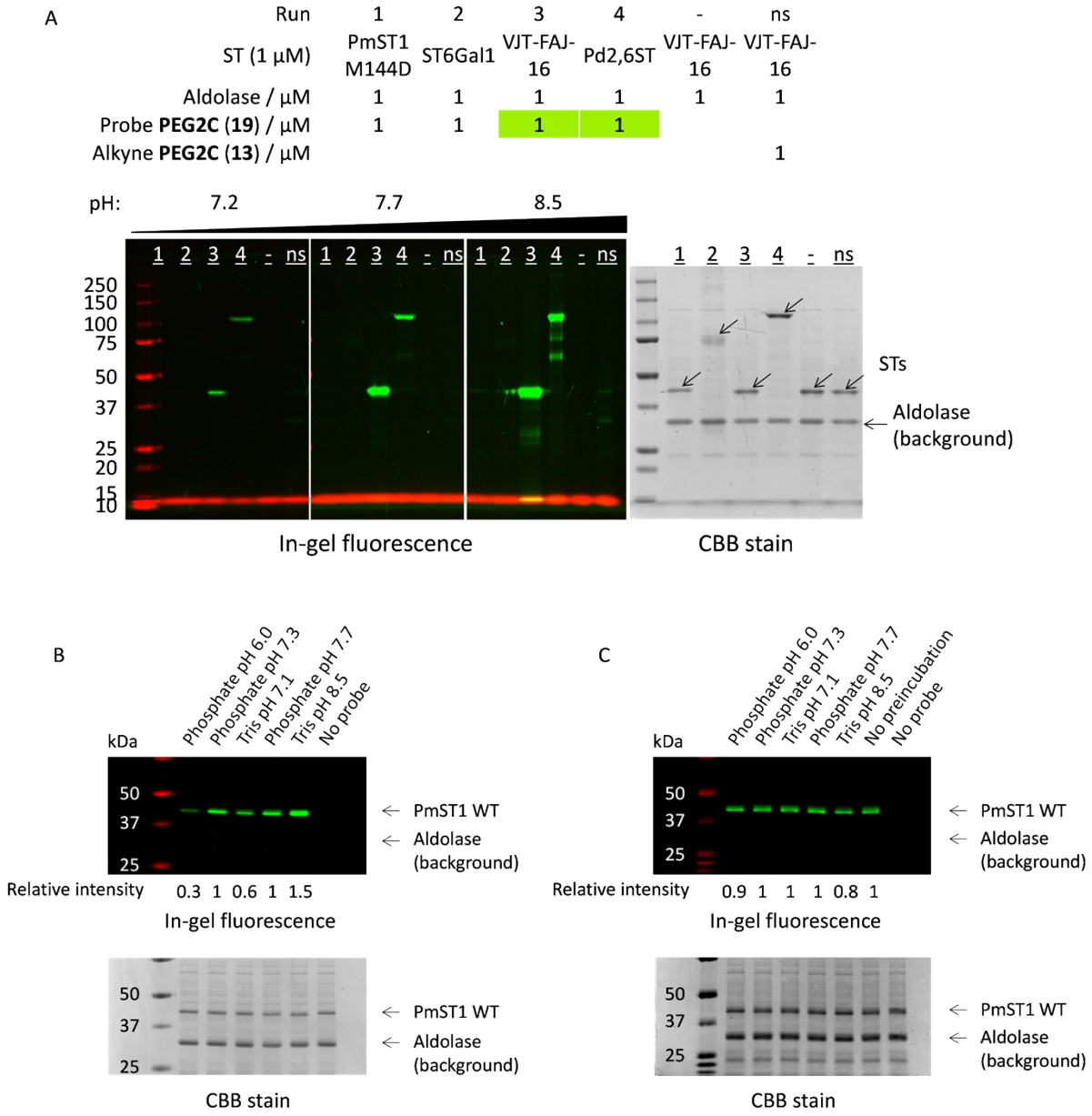


Figure S4. A) Increasing pH increased the rate of labelling, without significant loss of selectivity. B) PmST1 WT was labelled by probe **18** in 25 mM phosphate/Tris buffer at different pH. Low but noticeable level of labelling still occurs at below neutral pH, namely pH 6. C) Stability of the *O*-NBD probes at different pH was studied by preincubation of probe **18** in phosphate/Tris buffer at pH 6/7.1/7.3/7.7/8.5 at 37°C for 4 hours, then used for a labelling experiment with PmST1 WT at pH 7.2 and 37°C, with aldolase as a non-binding control. The relative labelling intensity was compared to a no-preincubation control at pH 8.5. Relative intensity was measured by ImageJ.

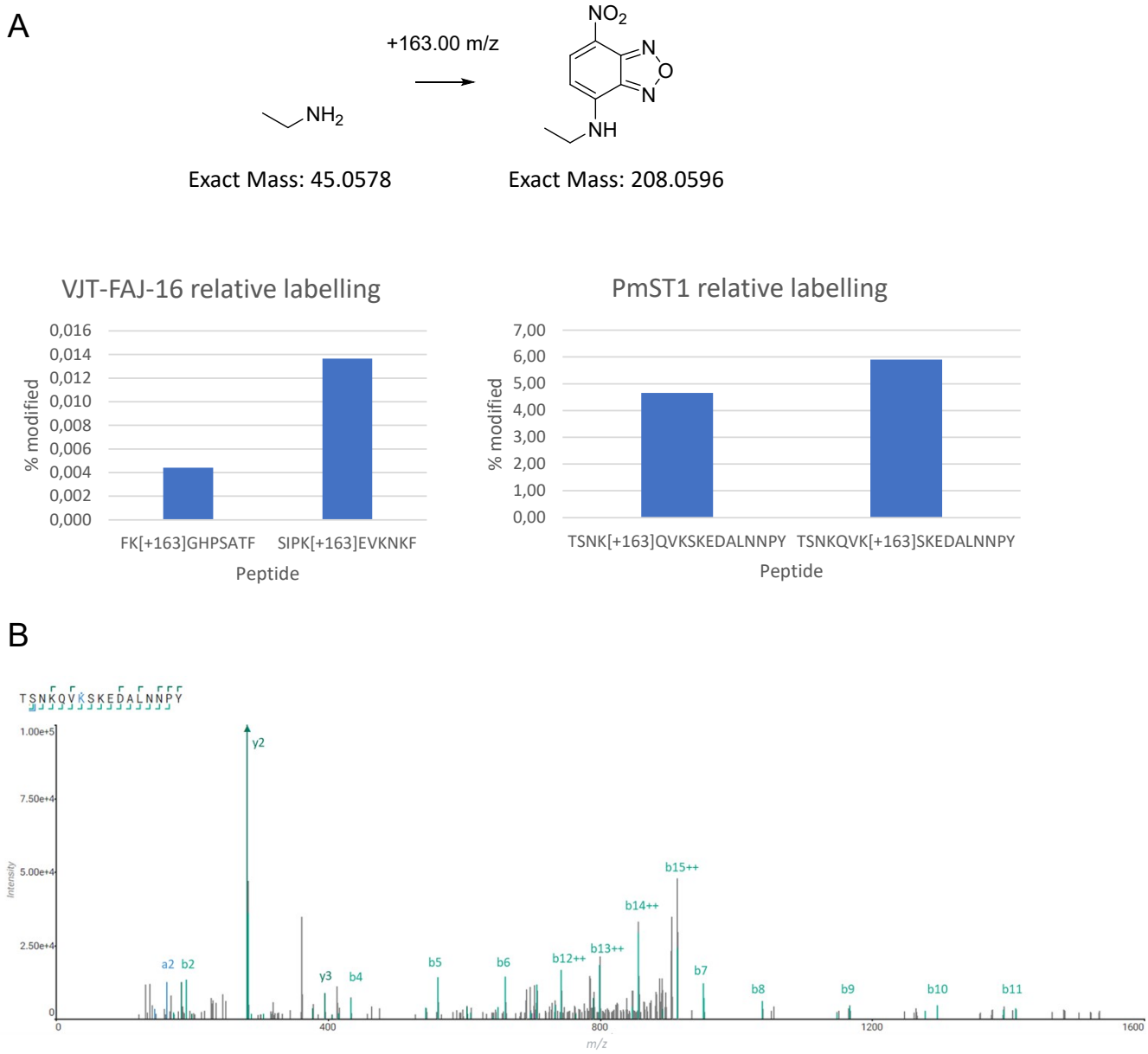


Figure S5. A) Percentage labelling of identified modification sites for VJT-FAJ-16 and PmST1. B) Annotated LC-MS/MS spectrum of identified modification site TSNKQVKSKEDALNNPY in PmST1. The series of b-ions allows for site localization of the modification.

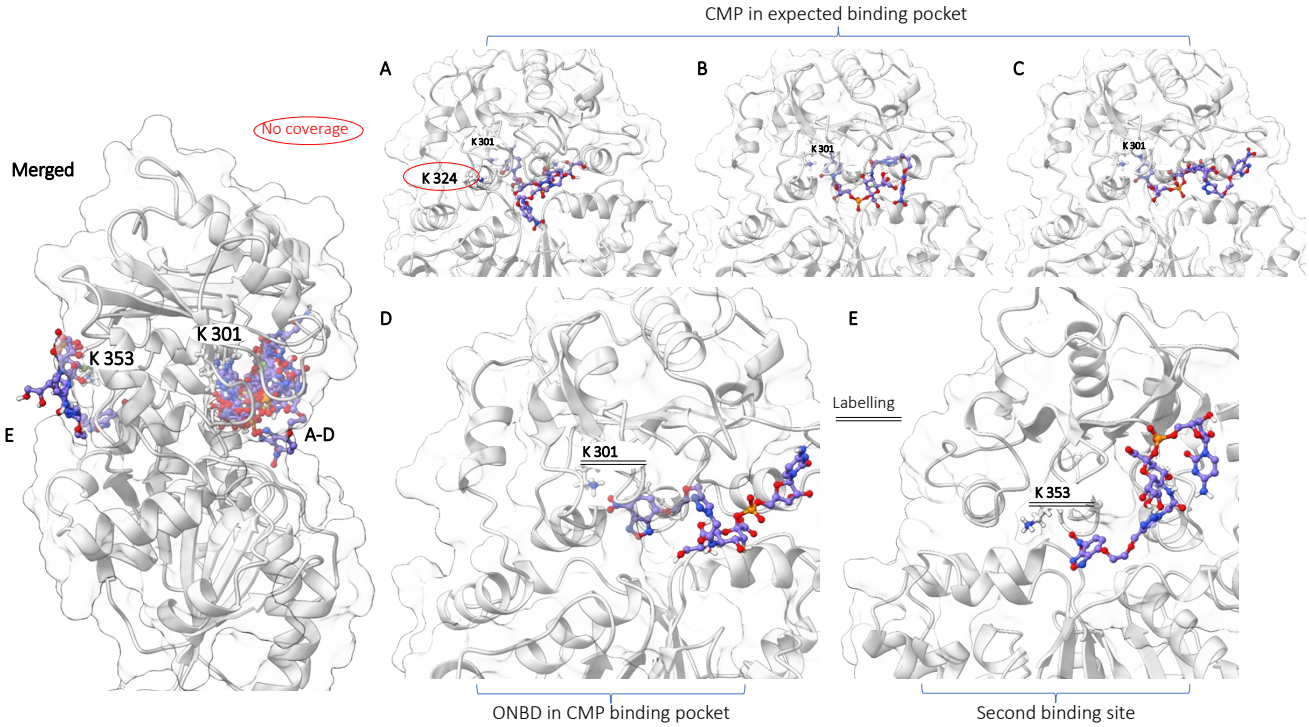


Figure S6. Molecular docking of probe **18** onto VJT-FAJ-16 revealed three major binding modes: The CMP moiety is bound in the expected binding pocket in scenario A-C, while the *O*-NBD is bound in the CMP binding pocket in scenario D. A second binding site is found on the backside of the enzyme as shown in scenario E.

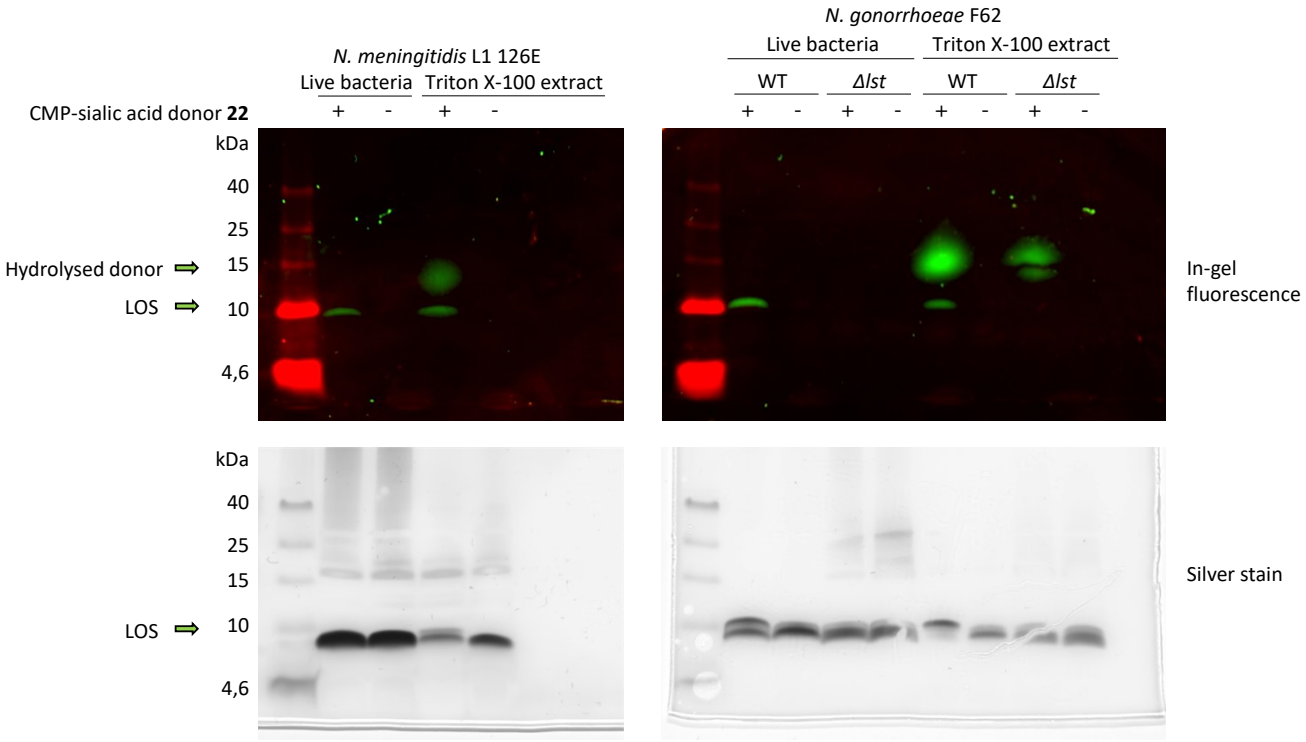


Figure S7. LOS gels from native sialyltransferase (NST) bacteria labelling of live *N. meningitidis* L1 126E*, N. gonorrhoeae* F62 and F62*Δlst*, clearly showing presence of sialyltransferase activity in both live bacteria and the Triton X-100 extract of *N. meningitidis* L1 126E*, N. gonorrhoeae* F62 but not F62*Δlst* by the fluorescent labelling and band shift of the LOS at ~10 kDa.

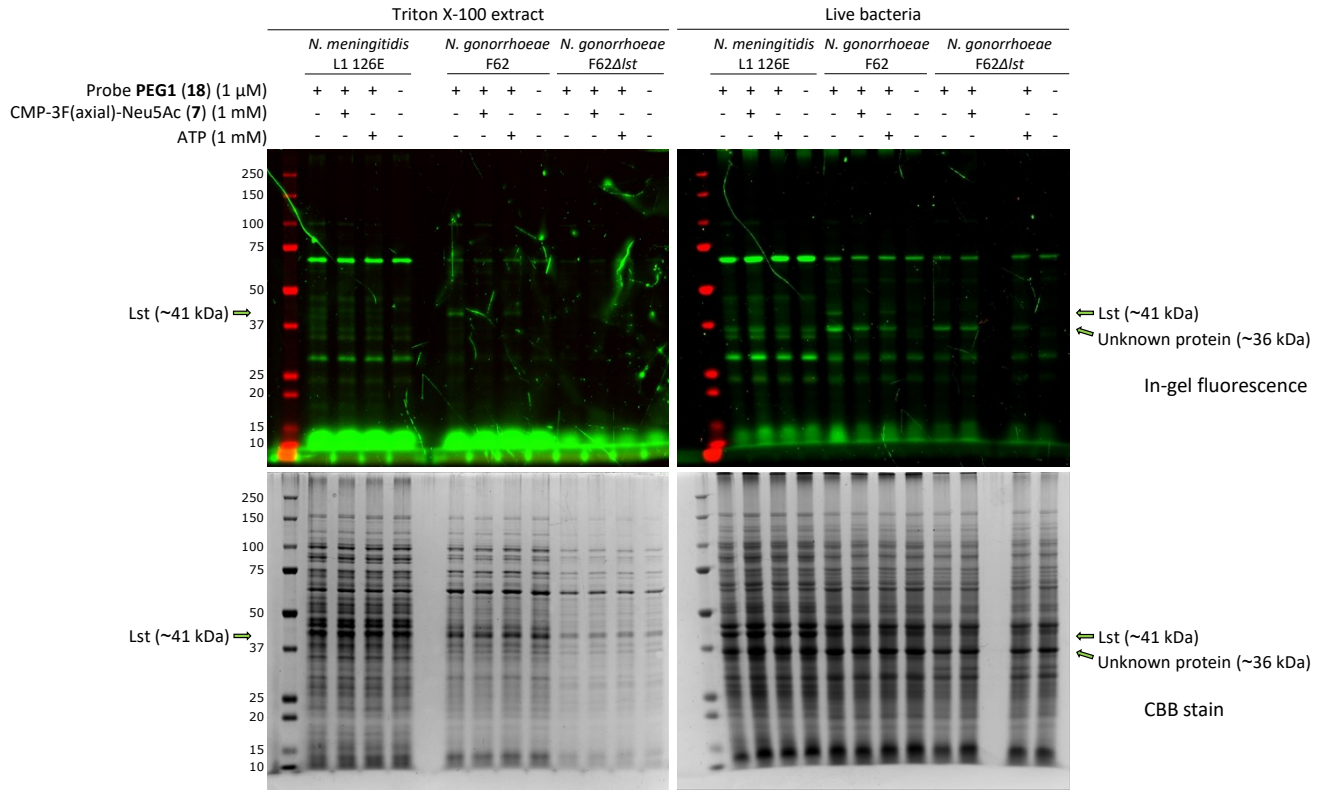


Figure S8. Full uncropped gel for the labelling of Lst in live *N. meningitidis, N. gonorrhoeae* and their Triton X-100 extracts. *N. meningitidis* L1 126E was also used for the labelling experiment but the labelling was less clear, with a very faint band at ~40 kDa observed only in the Triton X-100 extract. Since Lst knockout of *N. meningitidis* L1 126E was not available, no direct comparison could be done. Expression of Lst is known to be lower in *N. meningitidis* than *N. gonorrhoeae*.[3] *N. meningitidis* L1 126E is also encapsulated by capsular polysaccharide (CPS) of serogroup C (poly 2,9-linked sialic acids) which could explain the lower accessibility of the probe to the surface of the bacteria. Labelling of the polysialyltransferase (NmC PST) which is responsible for the CPS synthesis was not observed either, but it is known to be located on the inside of the inner membrane so it might not have been accessible in the two conditions the affinity labelling was carried out.

IV. Experimental

a. Materials and general methods

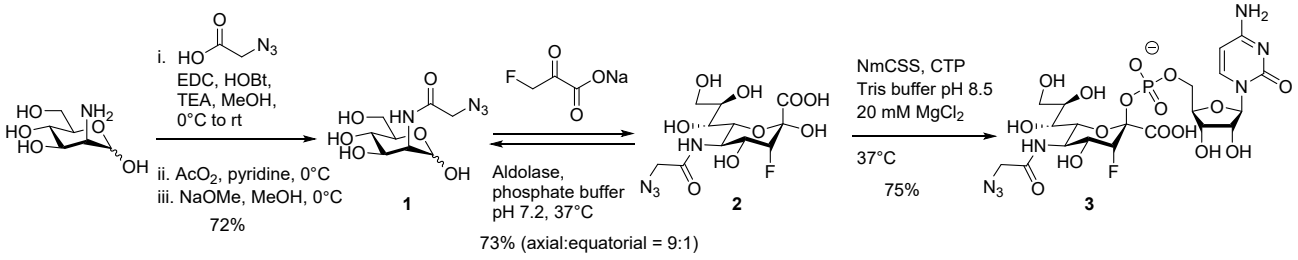
Chemicals were purchased from Merck, Thermo Fisher Scientific and Biosynth and used without further purification. Nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz Agilent spectrometer (400 and 101 MHz) or a 600 MHz Bruker Avance Neo spectrometer (600 and 125 MHz). Chemical shifts are reported in parts per million (ppm) relative to residual solvent peak. Mass spectra were recorded by ESI on a Bruker micrOTOF-QII mass spectrometer. Thin layer chromatography (TLC) was performed on aluminium-backed SiliaPlate TLC Plates F254 (Silicycle, Canada) and detected by UV (254 nm or 365 nm) where applicable and by dipping in 10% sulfuric acid in ethanol, p-anisaldehyde sugar stain or ceric ammonium molybdate stain followed by heating. Column chromatography was carried out using silica gel (40-63 µm; VWR chemicals) or C18-reversed phase silica gel (40-63 µm; Merck). Solvents were evaporated at maximum 40 °C under reduced pressure. Size exclusion chromatography was conducted using Bio-Gel P2 polyacrylamide gel from Bio-Rad, eluting with 25 mM ammonium bicarbonate buffer. Sialic acid aldolase was purchased from Biosynth (MS11080). PmST1 WT,[4] PmST1 M144D,[5] VJT-FAJ-16,[6] Pd2,6ST (as a MBP fusion),[7] ST6Gal1 (as a GFP fusion),[8] Cst-II (as a MBP fusion)[9] and N. gono Lst[10] were expressed and purified as described previously. The full protein sequences are presented in Table S1. Protein concentration was determined by absorbance at 280 nm using calculated extinction coefficient (ExPASy ProtParam tool).

Proteins were separated by SDS-PAGE using 10% Bis-Tris gels and MOPS buffer system under denaturing condition (200 V, 30 minutes). LOS was separated on a 16% Tris-Tricine gel and .[11] Precision Plus Protein™ All Blue Prestained Protein Standards (Bio-Rad, #1610373) and Spectra™ Multicolor Low Range Protein Ladder (Thermo Scientific, 26628) were used respectively as reference for protein and LOS SDS-PAGE gels. In-gel fluorescence was imaged using Bio-Rad ChemiDoc MP Imaging System on the Cy5 channel (625-650 nm excitation, 675-725 nm emission filter) for the protein ladder and the Cy2 channel (460-490 nm excitation, 518-546 nm emission filter) for the NBD labelling. Optionally, fixing of SDS-PAGE gel with 25% isopropanol and 10% acetic acid for 30 mins enhances fluorescence intensity of the NBD fluorophore and removes bromophenol blue background. Staining of protein gels was done using PageBlue™ Protein Staining Solution (Thermo Scientific, 24620) (Coomassie Brilliant Blue, CBB). Silver staining for LOS gels was performed as described previously.[11]

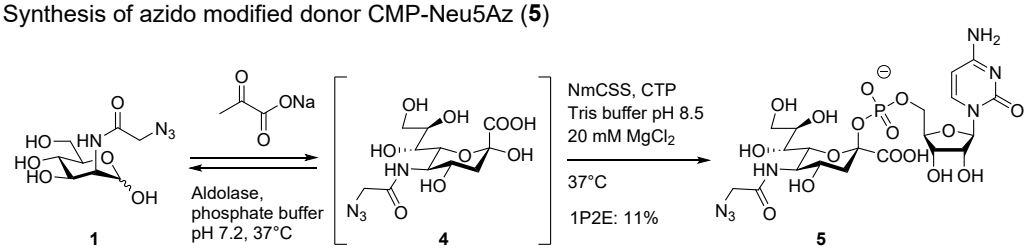
b. Synthetic schemes

Scheme S1. Synthesis of azide and alkyne precursors

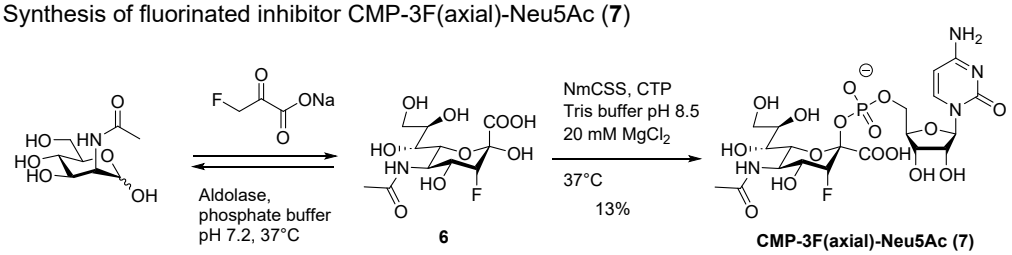
Synthesis of azido modified fluorinated inhibitor CMP-Neu3FAx5Az (**3**)



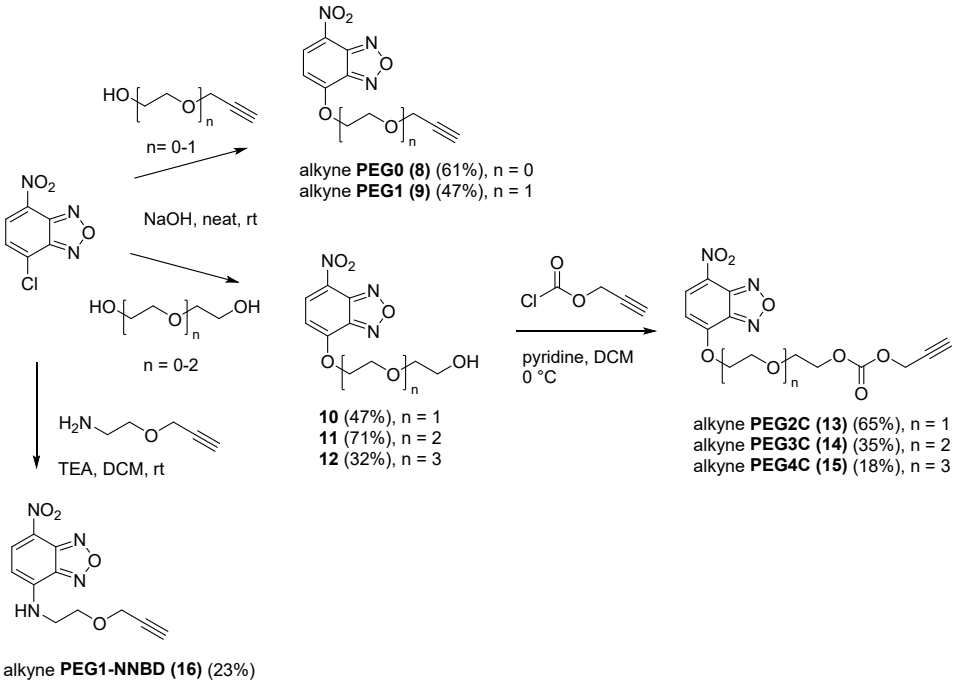
Synthesis of azido modified donor CMP-Neu5Az (**5**)



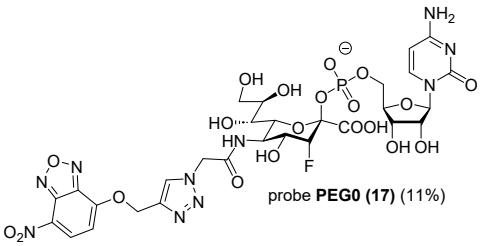
Synthesis of fluorinated inhibitor CMP-3F(axial)-Neu5Ac (**7**)

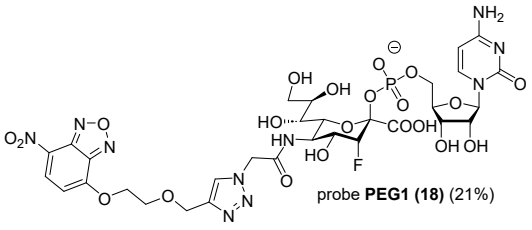


Synthesis of *O*-NBD warhead and *N*-NBD fluorophore with varying PEG linkers



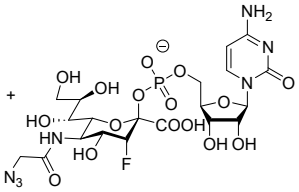
Scheme S2. Synthesis of probes and donor substrate by CuAAC





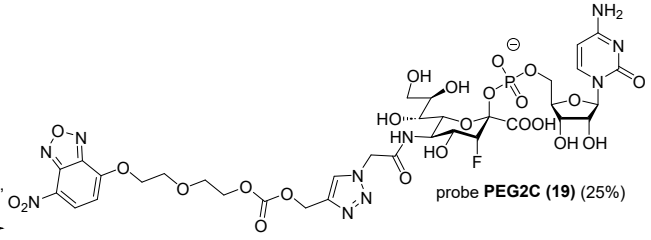
Synthesis of probes **17-21**

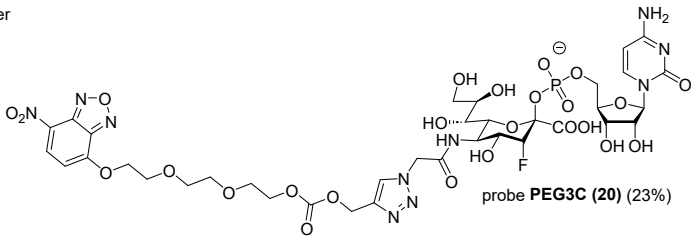
alkyne **PEG0 (8)**
alkyne **PEG1 (9)**
alkyne **PEG2C (13)**
alkyne **PEG3C (14)**
alkyne **PEG4C (15)**

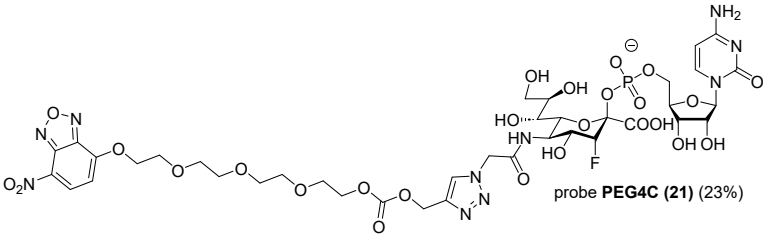


$CuSO_4$, Na ascorbate,
THPTA

phosphate buffer
pH 7.2
rt

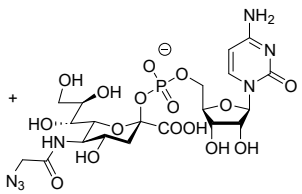






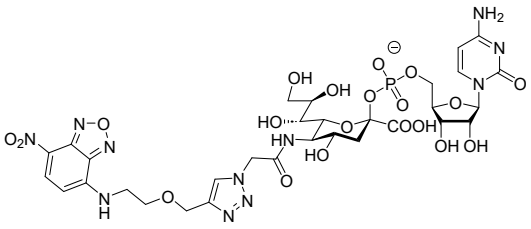
Synthesis of donor **22**

alkyne **PEG1-NNBD (16)**



$CuSO_4$, Na ascorbate,
THPTA

phosphate buffer
pH 7.2
rt



donor **PEG1-NNBD (22)** (4%)

c. Synthesis

***N*-azidoacetylmannosamine (ManNAz) (1)** Mannosamine hydrochloride (2.00 g, 9.28 mmol), azidoacetic acid (0.98 g, 9.74 mmol) and triethylaminie (2.59 mL, 18.56 mmol) were dissolved in dry MeOH (60 mL). The solution was cooled to 0°C, and HOBt (1.25 g, 9.28 mmol) and EDC hydrochloride (3.56 g, 18.56 mmol) were added. After stirring overnight at room temperature, TLC showed formation of product R_f=0.30 (CH_2Cl_2/MeOH 8:2). The product was purified roughly on the silica gel column chromatography ($CHCl_2$/MeOH 9:1 – 8:2), then acetylated by reacting with pyridine (60 mL) and acetic anhydride (60 mL) at 0°C. The reaction product was purified on the silica gel column chromatography (petroleum ether/EtOAc 70:30 – 40:60) to give peracetylated ManNAz (peracetylated **1**, Ac_4ManNAz, 1:1 mixture of anomers) (2.90 g, 6.74 mmol, 73% from mannosamine) as a white foam. R_f=0.28 (petroleum ether/EtOAc 1:1). ^{1}H and ^{13}C NMR spectra were consistent with those previously reported.[12] Zemplén deacetylation (treatment of peracetylated **1** with catalytic amount of NaOMe in dry methanol, with additional NaOMe added regularly to maintain pH 9) gave compound **1** (1.756 g, 6.70 mmol, 72% from mannosamine) as a yellowish foam and it was used without further purification. HRMS (ESI): *m/z* calcd for $C_{16}H_{22}N_4O_{10}$+Na^+: 285.0806; found: 285.0731. Note: The acetylation/column chromatography/deacetylation sequence was found to be necessary for complete purification of the azido product from HOBt impurity.

3-fluoro(axial)-*N*-azidoacetylneuraminic acid (Neu3FAx5Az) (2) ManNAz (**1**) (50 mg, 0.19 mmol) and sodium fluoropyruvate monohydrate (139 mg, 0.95 mmol) were dissolved in 1.3 mL 100 mM phosphate buffer pH 7.2. 1.5 mg of sialic acid aldolase (Carbosynth) was added and the reaction was incubated at 37°C for 72 hours. Purification over silica gel column chromatography (EtOAc/MeOH/H_2O/AcOH 5:2:0.5:0.01 – 5:2:1:0.01) afforded compound **2** (51.1 mg, 0.14 mmol, 73%) as a white foam, well separated from the minor 3-fluoro(equatorial) product. **3FEq** R_f=0.22 **3FAx** R_f=0.13 (EtOAc/MeOH/H_2O/AcOH 5:2:1:0.01). ^{1}H and ^{13}C NMR spectra were consistent with those previously reported.[13] ^{19}F NMR (376 MHz, D_2O, ppm): δ= -208.26 (dd, J = 49.3, 29.8 Hz); HRMS (ESI): *m/z* calcd for $C_{11}H_{17}FN_4O_9$+Na^+: 391.0872; found: 391.0848.

Cytidine-5'-monophospho-3-fluoro(axial)-*N*-azidoacetylneuraminic acid (CMP-Neu3FAx5Az) (3) A solution of Neu3FAx5Az (**2**) (49.0 mg, 0.13 mmol) and CTP (98.4 mg, 0.19 mmol) in 2.6 mL Tris buffer pH 8.5 with 20 mM $MgCl_2$ was added 20 µL NmCSS (2.5 mg/mL) and pyrophosphatase (1 U) and the reaction was incubated at 37°C overnight. Purification over P2 column (water) gave compound **3** (70.2 mg, 0.098 mmol, 75%) as a white powder. R_f=0.36 (EtOAc/MeOH/H_2O 4:2:1); ^{1}H NMR (600 MHz, D_2O, ppm): δ= 7.96 (d, J = 7.6 Hz, 1H, cytidine H6), 6.13 (d, J = 7.5 Hz, 1H, cytidine H5), 5.99 (d, J = 4.4 Hz, 1H, ribose H1), 4.93 (dd, J = 48.3, 2.5 Hz, 1H, Sia H3), 4.39 (t, J = 10.7 Hz, 1H, Sia H5), 4.33 (t, J = 4.9 Hz, 1H, ribose H3), 4.30 (t, J = 4.8 Hz, 1H, ribose H2), 4.28 – 4.18 (m, 5H, Sia H6 (4.27 based on HSQC) and H4 (4.23, d, J = 26.9 Hz based on HSQC), ribose H4 (4.26 based on HSQC) and H5 (4.26 and 4.23 based on HSQC)), 4.10 (s, 2H, CH2N3), 3.99 (ddd, J = 9.3, 6.5, 2.5 Hz, 1H, Sia H7), 3.90 (dd, J = 11.9, 2.5 Hz, 1H, Sia H9a), 3.65 (dd, J = 11.9, 6.6 Hz, 1H, Sia H9b), 3.47 (d, J = 9.6 Hz, 1H, Sia H8); ^{13}C NMR (151 MHz, D_2O, ppm): δ=171.28 (Sia C1), 171.02 (Sia 5-NHCO), 166.18 (cytidine C4), 157.80 (cytidine C2), 141.57 (cytidine C6), 97.88 (Sia C2), 96.57 (cytidine C5), 90.42 (dd, J = 176.5, 15.4 Hz, Sia C3), 89.05 (ribose C1), 82.81 (ribose C4), 74.24 (ribose C2), 71.42 (Sia C6), 69.68 (Sia C7), 69.32 (ribose C3), 68.66 (Sia C8), 67.70 (d, J = 17.9 Hz, Sia C4), 65.14 (ribose C5), 62.85 (Sia C9), 51.92 (CH2N3), 47.04 (Sia C5); ^{19}F NMR (376 MHz, D_2O,

ppm): δ= -208.26 (dd, J = 49.2, 28.8 Hz); HRMS (ESI): *m/z* calcd for $C_{20}H_{29}FN_7O_{16}P+H^+$: 674.1465; found: 674.1458.

Cytidine-5'-monophospho-*N*-azidoacetylneuraminic acid (CMP-Neu5Az) (5) Synthesis was done as previously reported in a one pot two enzymes system.[14] Purification over P2 column (25 mM ammonium bicarbonate) gave compound **5** (28.4 mg, 0.041 mmol, 11% over two steps) as a white foam. R_f=0.30 (EtOH/1 M ammonium bicarbonate/CH_2Cl_2 3:1:1). ^{1}H and ^{13}C NMR spectra were consistent with those previously reported.[14] HRMS (ESI): *m/z* calcd for $C_{20}H_{30}N_7O_{16}P+H^+$: 656.1559; found: 656.1548.

Cytidine-5'-monophospho-3-fluoro(axial)-*N*-cetylneuraminic acid (CMP-3F(axial)-Neu5Ac) (7) Synthesis was done as previously reported via intermediate **6**.[15] Purification over silica gel column gave compound **7** (5.0 mg, 0.0076 mmol, 13% over two steps) as a white powder. R_f=0.24 (EtOAc/MeOH/H_2O 3:2:1). ^{1}H and ^{13}C NMR spectra were consistent with those previously reported.[15] ^{19}F NMR (376 MHz, D_2O, ppm): δ= -208.16 (dd, J = 49.2, 30.1 Hz); HRMS (ESI): *m/z* calcd for $C_{20}H_{30}FN_4O_{16}P+H^+$: 633.1451; found: 633.1440.

Synthesis of 7-nitro-4-benzofurazanyl ethers (compound **8-12**) from 4-chloro-7-nitrobenzofurazan (NBD-Cl) was adapted from published procedure.[16]

Alkyne PEG0 (8) NBD-Cl (0.2 g, 1.00 mmol) was suspended in propargyl alcohol (2 mL, 34.7 mmol), then crushed NaOH (80 mg, 2.00 mmol) was added. The dark brownish green solution was stirred at room temperature for 3 hours. The reaction was acidified by adding 3 M HCl after which the solution turned red, then it was diluted with EtOAc. The organic layer was washed 3 times with water, dried over Na_2SO_4 and concentrated under reduced pressure. Purification over silica gel column chromatography (petroleum ether/EtOAc 3:1 – 2:1) afforded compound **8** (134 mg, 0.61 mmol, 61%) as a red brown solid. R_f=0.29 (petroleum ether/EtOAc 2:1); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ= 8.57 (d, J = 8.3 Hz, 1H, H5), 6.89 (d, J = 8.3 Hz, 1H, H6), 5.13 (d, J = 2.4 Hz, 2H, propargyl CH2), 2.72 (t, J = 2.5 Hz, 1H, alkyne CH); ^{13}C NMR (101 MHz, $CDCl_3$, ppm): δ= 152.90, 145.38, 144.12, 133.56, 106.09, 79.00, 77.36, 75.38, 58.32; HRMS (ESI): *m/z* calcd for $C_9H_5N_3O_4+Na^+$: 242.0172; found: 242.0224.

Alkyne PEG1 (9) Synthesised as (**8**) except propynol ethoxylate was used instead of propargyl alcohol. Purification over silica gel column chromatography (CH_2Cl_2/petroleum ether 80:20 – 100:0) afforded compound **9** (124 mg, 0.47 mmol, 47%) as an orange solid. R_f=0.25 (CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ= 8.54 (d, J = 8.3 Hz, 1H, H5), 6.75 (d, J = 8.3 Hz, 1H, H6), 4.64 – 4.57 (m, 2H, ethylene glycol CH2), 4.28 (d, J = 2.4 Hz, 2H, propargyl CH2), 4.10 – 4.04 (m, 2H, ethylene glycol CH2), 2.49 (t, J = 2.4 Hz, 1H, alkyne CH); ^{13}C NMR (101 MHz, $CDCl_3$, ppm): δ= 154.78, 145.35, 144.10, 133.96, 105.20, 79.00, 77.36, 75.48, 70.45, 67.55, 58.94; HRMS (ESI): *m/z* calcd for $C_{11}H_9N_3O_5+Na^+$: 286.0434; found: 286.0410.

Alcohol PEG2 (10) NBD-Cl (0.2 g, 1.00 mmol) was suspended in diethylene glycol (4 mL, 41.8 mmol), then crushed NaOH (80 mg, 2.00 mmol) was added. The dark brownish green solution was stirred at room temperature for 3 hours. The reaction was acidified by adding 3 M HCl after which the solution turned red, then it was diluted with water. The aqueous layer was extracted 2 times with CH_2Cl_2, and the combined organic layer was dried over

Na_2SO_4 and concentrated under reduced pressure. Purification over silica gel column chromatography (CH_2Cl_2/acetone 100:0 – 80:20) afforded compound **10** (127 mg, 0.47 mmol, 47%) as a brown liquid. R_f=0.25 (CH_2Cl_2/acetone 10:1); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ= 8.54 (d, J = 8.3 Hz, 1H, H5), 6.74 (d, J = 8.3 Hz, 1H, H6), 4.61 – 4.52 (m, 2H, ethylene glycol CH2), 4.09 – 4.02 (m, 2H, ethylene glycol CH2), 3.79 (q, J = 3.4 Hz, 2H, ethylene glycol CH2), 3.76 – 3.65 (m, 2H, ethylene glycol CH2); ^{13}C NMR (101 MHz, $CDCl_3$, ppm): δ= 154.74, 145.35, 144.13, 133.92, 105.05, 77.36, 73.03, 70.69, 68.90, 61.96; HRMS (ESI): *m/z* calcd for $C_{10}H_{11}N_3O_6$+Na^+: 292.0540; found: 292.0516.

Alcohol PEG3 (11) NBD-Cl (0.4 g, 2.00 mmol) was suspended in triethylene glycol (10.7 mL, 80.0 mmol), then crushed NaOH (160 mg, 4.00 mmol) was added. The dark brownish green solution was stirred at room temperature for 3 hours. The reaction was acidified by adding 3 M HCl after which the solution turned red, then it was diluted with water. The aqueous layer was extracted 2 times with EtOAc, and the combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. Purification over silica gel column chromatography (CH_2Cl_2/acetone 100:0 – 80:20) afforded compound **11** (441 mg, 1.41 mmol, 71%) as a brown liquid. R_f=0.15 (CH_2Cl_2/acetone 9:1);); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ= 8.52 (d, J = 8.4 Hz, 1H, H5), 6.78 (d, J = 8.4 Hz, 1H, H6), 4.60 – 4.53 (m, 2H, ethylene glycol CH2), 4.05 – 3.99 (m, 2H, ethylene glycol CH2), 3.78 – 3.72 (m, 2H, ethylene glycol CH2), 3.71 – 3.63 (m, 4H, ethylene glycol CH2), 3.60 – 3.56 (m, 2H, ethylene glycol CH2); ^{13}C NMR (101 MHz, $CDCl_3$, ppm): δ= 154.75, 145.29, 144.02, 134.30, 129.82, 105.23, 72.56, 71.12, 70.67, 70.34, 68.91, 61.73; HRMS (ESI): *m/z* calcd for $C_{12}H_{15}N_3O_7$+H^+: 314.0983; found: 314.0970.

Alcohol PEG4 (12) NBD-Cl (0.4 g, 2.00 mmol) was suspended in tetraethylene glycol (10.7 mL, 80.0 mmol), then crushed NaOH (160 mg, 4.00 mmol) was added. The dark brownish green solution was stirred at room temperature for 3 hours. The reaction was acidified by adding 3 M HCl after which the solution turned red, then it was diluted with water. The aqueous layer was extracted 2 times with EtOAc, and the combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. Purification over silica gel column chromatography (CH_2Cl_2/acetone 100:10 – 80:20) afforded compound **12** (226.1 mg, 0.63 mmol, 32%) as a brown liquid which contains 33% tetraethylene glycol impurity. This was used for the next reaction without further purification. R_f=0.25 (CH_2Cl_2/acetone 7:3); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ= 8.53 (d, J = 8.4 Hz, 1H, H5), 6.79 (d, J = 8.4 Hz, 1H, H6), 4.63 – 4.54 (m, 2H, ethylene glycol CH2), 4.06 – 3.98 (m, 2H, ethylene glycol CH2), 3.84 – 3.42 (m, 25H, ethylene glycol CH2, and tetraethylene glycol impurity); ^{13}C NMR (101 MHz, $CDCl_3$, ppm, based on HSQC): δ= 133.93, 105.05, 72.84, 70.52, 70.19, 69.02, 61.55; HRMS (ESI): *m/z* calcd for $C_{14}H_{19}N_3O_8$+H^+: 358.1245; found: 358.1222.

Alkyne PEG2C (13) Alcohol PEG2 (**10**) (20.0 mg, 0.07 mmol) was dissolved in dry CH_2Cl_2 (5 mL) and cooled to 0°C. Propargyl chloroformate (10.7 µL, 13.0 mg, 0.11 mmol) and pyridine (8.9 µL, 8.7 mg, 0.11 mmol) were diluted in CH_2Cl_2 (2 mL) and added dropwise to the reaction. The reaction was stirred at 0°C for 6 hours. The reaction was washed with 1 M HCl, and the aqueous layer was back extracted 2 times with CH_2Cl_2. The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. Purification over silica gel column chromatography (CH_2Cl_2/acetone 100:0 – 100:1) afforded compound **13** (16 mg, 0.046 mmol, 65%) as a bright orange liquid. R_f=0.75 (CH_2Cl_2/acetone 100:5); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ= 8.55 (d, J = 8.3 Hz, 1H, H5),

6.79 (d, *J* = 8.4 Hz, 1H, H6), 4.71 (d, *J* = 2.5 Hz, 2H, propargyl CH2), 4.62 – 4.53 (m, 2H, ethylene glycol CH2), 4.37 – 4.30 (m, 2H, ethylene glycol CH2), 4.06 – 3.99 (m, 2H, ethylene glycol CH2), 3.86 – 3.77 (m, 2H, ethylene glycol CH2), 2.52 (t, *J* = 2.5 Hz, 1H, alkyne CH); ^{13}C NMR (101 MHz, $CDCl_3$, ppm): *δ*= 154.78, 154.58, 145.39, 144.12, 134.12, 105.41, 77.36, 76.93, 75.94, 70.69, 69.45, 69.24, 67.38, 55.54; HRMS (ESI): *m/z* calcd for $C_{14}H_{13}N_3O_8$+H^+: 352.0775; found: 352.0737.

Alkyne PEG3C (14) Alcohol PEG3 (**11**) (55.5 mg, 0.18 mmol) was dissolved in dry CH_2Cl_2 (12 mL) and cooled to 0°C. Propargyl chloroformate (25.8 µL, 62.8 mg, 0.53 mmol) and pyridine (42.8 µL, 8.7 mg, 0.53 mmol) were diluted in CH_2Cl_2 (6 mL) and added dropwise to the reaction. The reaction was stirred at 0°C for 6 hours. The reaction was washed with 1 M HCl, and the aqueous layer was back extracted 2 times with CH_2Cl_2. The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. Purification over silica gel column chromatography (CH_2Cl_2/acetone 100:0 – 100:1) afforded compound **14** (25 mg, 0.063 mmol, 35%) as a bright orange liquid. R_f=0.50 (CH_2Cl_2/acetone 100:5); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): *δ*= 8.54 (d, *J* = 8.4 Hz, 1H, H5), 6.78 (d, *J* = 8.4 Hz, 1H, H6), 4.71 (d, *J* = 2.5 Hz, 2H, propargyl CH2), 4.60 – 4.54 (m, 2H, ethylene glycol CH2), 4.33 – 4.28 (m, 2H, ethylene glycol CH2), 4.05 – 4.01 (m, 2H, ethylene glycol CH2), 3.77 – 3.70 (m, 4H, ethylene glycol CH2), 3.68 – 3.65 (m, 2H, ethylene glycol CH2), 2.51 (t, *J* = 2.4 Hz, 1H, alkyne CH); ^{13}C NMR (101 MHz, $CDCl_3$, ppm): *δ*= 154.89, 154.62, 145.38, 144.11, 134.19, 105.22, 77.37, 77.03, 75.88, 71.15, 70.87, 70.80, 69.15, 69.00, 67.60, 55.44; HRMS (ESI): *m/z* calcd for $C_{16}H_{17}N_3O_9$+H^+: 396.1038; found: 396.1008.

Alkyne PEG4C (15) Alcohol PEG4 (**12**) (60.7 mg, 0.17 mmol) was dissolved in dry CH_2Cl_2 (12 mL) and cooled to 0°C. Propargyl chloroformate (25.8 µL, 62.8 mg, 0.53 mmol) and pyridine (42.8 µL, 8.7 mg, 0.53 mmol) were diluted in CH_2Cl_2 (6 mL) and added dropwise to the reaction. The reaction was stirred at 0°C for 6 hours. The reaction was washed with 1 M HCl, and the aqueous layer was back extracted 2 times with CH_2Cl_2. The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. Purification over silica gel column chromatography (CH_2Cl_2/acetone 100:0 – 100:5) afforded compound **15** (13 mg, 0.030 mmol, 18%) as a bright orange liquid. R_f=0.10 (CH_2Cl_2/acetone 100:5); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): *δ*= 8.54 (d, *J* = 8.4 Hz, 1H, H5), 6.79 (d, *J* = 8.4 Hz, 1H, H6), 4.71 (d, *J* = 2.5 Hz, 2H, propargyl CH2), 4.59 – 4.55 (m, 2H, ethylene glycol CH2), 4.31 (ddd, *J* = 4.8, 3.9, 2.3 Hz, 2H, ethylene glycol CH2), 4.05 – 4.01 (m, 2H, ethylene glycol CH2), 3.77 – 3.70 (m, 5H, ethylene glycol CH2), 3.68 – 3.63 (m, 5H, ethylene glycol CH2), 2.51 (t, *J* = 2.5 Hz, 1H, alkyne CH); ^{13}C NMR (101 MHz, $CDCl_3$, ppm): *δ*= 154.93, 154.64, 145.39, 144.12, 134.20, 105.25, 77.36, 77.05, 75.85, 71.18, 70.83, 70.81, 70.76, 70.72, 69.12, 68.92, 67.66, 55.42; HRMS (ESI): *m/z* calcd for $C_{18}H_{21}N_3O_{10}$+Na^+: 462.1119; found: 462.1100.

Alkyne PEG1-NNBD (16) NBD-Cl (55 mg, 0.275 mmol) and amino-PEG1-alkyne (24.8 mg, 0.25 mmol) was dissolved in dry CH_2Cl_2 (1 mL) and triethylamine (420 µL, 3 mmol) was added. The brownish green solution was stirred at room temperature for 3 hours. The reaction was acidified by adding 3 M HCl after which the solution turned red, then it was diluted with EtOAc. The organic layer was washed 3 times with water, dried over Na_2SO_4 and concentrated under reduced pressure. Purification over silica gel column chromatography (CH_2Cl_2/acetone 100:0 – 100:1) afforded compound **16** (15.3 mg, 0.06 mmol, 23%) as a brown liquid. R_f=0.2 (CH_2Cl_2, spot fluoresces yellow-green under 365 nm UV); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): *δ*= 8.47 (d, *J* = 8.6 Hz, 1H, H5), 6.21 (d, *J* = 8.6

Hz, 1H, H6), 4.26 (d, *J* = 2.4 Hz, 2H, propargyl CH2), 3.91 (dd, *J* = 5.6, 4.6 Hz, 2H, ethylene glycol CH2), 3.72 (q, *J* = 5.2 Hz, 2H, ethylene glycol CH2), 2.51 (t, *J* = 2.4 Hz, 1H, alkyne CH); ^{13}C NMR (101 MHz, $CDCl_3$, ppm): *δ*= 144.42, 143.99, 136.47, 124.42, 99.04, 78.97, 75.75, 66.99, 58.78, 43.64, 29.82; HRMS (ESI): *m/z* calcd for $C_{11}H_{10}N_4O_4$+H^+: 263.0775; found: 263.0787.

General procedure for CuAAC reaction

The CuAAC reaction was performed as published previously.[17] Briefly, the alkyne and azide (1:1 ratio, final concentration of 1.5 mM each) were mixed in 100 mM phosphate buffer pH 7.2. Premixed $CuSO_4$ with 3 equivalents of THPTA ligand (final concentration of 0.1 mM) and sodium ascorbate (final concentration of 5 mM) were added sequentially and incubated for an hour at room temperature. When the reaction was complete as indicated by ESI-MS, the reaction mixture was freeze-dried and loaded onto a C18 column with minimal amount of water. Elution was done with step gradient of 0%, 1%, 5%, 10%, 15%, 20% and 25% acetonitrile/water. Fractions containing the product were pooled together and freeze-dried. ^{1}H and HSQC NMR spectra were assigned using CMP-Neu3FAx5Az (**3**) as reference. ^{13}C NMR was assigned from HSQC NMR. Full assignment tables of all compounds are provided with the HSQC NMR spectra in section VI.

Probe **PEG0** (**17**) (Eluted at 5-10% acetonitrile/water, 0.3 mg, 11%) ^{1}H NMR (600 MHz, D_2O, ppm): *δ*= 8.77 (d, *J* = 8.4 Hz, 1H), 8.34 (s, 1H, triazole CH), 7.96 (d, *J* = 7.6 Hz, 2H), 7.18 (d, *J* = 8.5 Hz, 1H), 6.12 (d, *J* = 7.6 Hz, 1H), 5.99 (d, *J* = 4.5 Hz, 1H), 5.73 (s, 2H, NBD-O-CH2-triazole), 5.41 (d, *J* = 3.0 Hz, 2H), 4.93 (dd, *J* = 48.3, 2.3 Hz, 1H), 4.38 (t, *J* = 10.6 Hz, 1H), 4.33 (t, *J* = 4.8 Hz, 2H), 4.31 – 4.20 (m, 12H), 4.17 (dd, *J* = 10.4, 2.6 Hz, 5H), 4.00 (ddd, *J* = 9.3, 6.3, 2.5 Hz, 2H), 3.90 (dd, *J* = 11.8, 2.5 Hz, 2H), 3.74 (s, 2H), 3.66 (dd, *J* = 11.9, 6.4 Hz, 2H), 3.50 (d, *J* = 9.7 Hz, 2H); ^{13}C NMR (151 MHz, D_2O, ppm, based on HSQC): *δ*= 141.78, 136.46, 127.26, 106.61, 96.45, 90.97, 89.84, 89.03, 82.90, 74.35, 71.45, 69.67, 69.35, 68.71, 67.74, 67.74, 65.16, 63.22, 62.74, 62.74, 59.35, 52.09, 47.41; HRMS (ESI): *m/z* calcd for $C_{29}H_{34}FN_{10}O_{20}P$+$H^+$: 893.1745; found: 893.1654.

Probe **PEG1** (**18**) (Eluted at 10-15% acetonitrile/water, 0.6 mg, 21%) ^{1}H NMR (600 MHz, D_2O, ppm): *δ*= 8.72 (d, *J* = 8.6 Hz, 1H, NBD H5), 8.14 (s, 1H, triazole CH), 7.93 (d, *J* = 7.5 Hz, 1H, cytidine H6), 6.98 (d, *J* = 8.4 Hz, 1H, NBD H6), 6.09 (d, *J* = 7.6 Hz, 1H, cytidine H5), 5.97 (d, *J* = 4.4 Hz, 1H, ribose H1), 5.33 (d, *J* = 2.8 Hz, 2H, amide-CH2-triazole), 4.92 (d, *J* = 48.4 Hz, 1H, Sia H3), 4.83 (s, 2H, O-CH2-triazole), 4.64 (t, *J* = 4.2 Hz, 2H, PEG CH2), 4.38 – 4.18 (m, 8H, Sia H4, H5 and H6, ribose H2, H3, H4 and H5), 4.12 (d, *J* = 4.4 Hz, 2H, PEG CH2), 3.98 (ddd, *J* = 9.5, 6.5, 2.5 Hz, 1H, Sia H7), 3.88 (dd, *J* = 11.8, 2.5 Hz, 1H, Sia H9a), 3.73 (s, 1H, Tris buffer), 3.64 (dd, *J* = 11.9, 6.5 Hz, 1H, Sia H9b), 3.48 (d, *J* = 9.7 Hz, 1H, Sia H8); ^{13}C NMR (151 MHz, D_2O, ppm, based on HSQC): *δ*= 141.61, 136.77, 126.61, 106.29, 96.45, 90.96, 89.83, 89.03, 82.73, 74.18, 71.44, 69.99, 69.67, 69.18, 68.70, 67.89, 67.89, 67.57, 64.99, 63.22, 62.89, 62.89, 52.08, 47.41; HRMS (ESI): *m/z* calcd for $C_{31}H_{38}FN_{10}O_{21}P$+$H^+$: 937.2007; found: 937.2043.

Probe **PEG2C** (**19**) (Eluted at 5-10% acetonitrile/water, 0.8 mg, 25%) ^{1}H NMR (600 MHz, D_2O, ppm): *δ*= 8.71 (d, *J* = 8.4 Hz, 1H), 8.10 (s, 1H, triazole CH), 7.94 (d, *J* = 7.6 Hz, 1H), 6.99 (d, *J* = 8.6 Hz, 1H), 6.10 (d, *J* = 7.6 Hz, 1H), 5.97 (d, *J* = 4.5 Hz, 1H), 5.35 (d, *J* = 2.6 Hz, 2H), 5.22 (s, 2H, carbonate-O-CH2-triazole), 4.93 (dd, *J* = 48.4, 2.3

Hz, 1H), 4.64 – 4.61 (m, 2H, PEG CH2), 4.40 – 4.36 (m, 2H, PEG CH2), 4.32 (t, *J* = 5.0 Hz, 1H), 4.30 – 4.19 (m, 6H), 4.11 – 4.07 (m, 2H, PEG CH2), 4.00 (ddd, *J* = 9.3, 6.4, 2.5 Hz, 1H), 3.95 – 3.90 (m, 2H, PEG CH2), 3.90 (dd, *J* = 11.8, 2.5 Hz, 1H), 3.66 (dd, *J* = 11.9, 6.5 Hz, 1H), 3.50 (d, *J* = 9.6 Hz, 1H); ^{13}C NMR (151 MHz, D_2O, ppm, based on HSQC): *δ*= 141.64, 136.80, 126.97, 106.34, 96.51, 91.03, 89.90, 89.10, 82.81, 74.43, 71.53, 70.08, 69.76, 69.28, 68.79, 68.63, 68.47, 67.99, 67.83, 67.50, 65.09, 62.99, 62.99, 60.41, 52.19, 47.36; HRMS (ESI): *m/z* calcd for $C_{34}H_{42}FN_{10}O_{24}P+H^+$:1025.2168; found: 1025.2172.

Probe **PEG3C** (**20**) (Eluted at 5-10% acetonitrile/water, 0.8 mg, 23%) ^{1}H NMR (600 MHz, D_2O, ppm): *δ*= 8.72 (dd, *J* = 8.4, 1.4 Hz, 1H), 8.13 (s, 1H, triazole CH), 7.95 (dd, *J* = 8.1, 4.2 Hz, 1H), 7.18 (d, *J* = 8.1 Hz, 1H), 6.99 (d, *J* = 8.8 Hz, 1H), 6.11 (t, *J* = 7.5 Hz, 1H), 6.02 – 5.96 (m, 1H), 5.35 (s, 2H), 5.25 (s, 2H, carbonate-O-CH2-triazole), 4.93 (d, *J* = 48.8 Hz, 1H, Sia H3), 4.66 (d, *J* = 4.2 Hz, 1H), 4.62 (dd, *J* = 6.4, 2.6 Hz, 1H), 4.41 – 4.18 (m, 12H), 4.11 – 4.05 (m, 2H), 3.99 (t, *J* = 8.2 Hz, 1H), 3.90 (d, *J* = 11.7 Hz, 1H), 3.83 – 3.78 (m, 5H), 3.77 – 3.69 (m, 5H), 3.66 (dd, *J* = 11.7, 6.5 Hz, 2H), 3.50 (d, *J* = 8.9 Hz, 2H); ^{13}C NMR (151 MHz, D_2O, ppm, based on HSQC): *δ*= 141.64, 136.80, 126.97, 106.18, 96.51, 91.03, 89.90, 89.10, 82.81, 74.27, 71.53, 70.08, 70.08, 69.92, 69.76, 69.76, 69.44, 68.79, 68.31, 68.31, 67.99, 67.83, 67.50, 65.25, 62.99, 62.99, 60.41, 59.61, 52.19, 47.36; ^{19}F NMR (376 MHz, D_2O, ppm): *δ*= -208.28 (dd, *J* = 48.7, 29.6 Hz) HRMS (ESI): *m/z* calcd for $C_{36}H_{46}FN_{10}O_{25}P+H^+$: 1069.2430; found: 1069.2428.

Probe **PEG4C** (**21**) (Eluted at 5-10% acetonitrile/water, 0.8 mg, 23%) ^{1}H NMR (600 MHz, D_2O, ppm): *δ*= 8.72 (d, *J* = 8.4 Hz, 1H), 8.14 (s, 1H, 1H, triazole CH), 7.97 (d, *J* = 8.0 Hz, 1H), 7.19 (d, *J* = 8.8 Hz, 1H), 6.99 (d, *J* = 8.5 Hz, 1H), 6.12 (s, 1H), 5.99 (s, 1H), 5.39 – 5.31 (m, 2H), 5.29 (t, *J* = 6.4 Hz, 2H), 4.93 (d, *J* = 49.0 Hz, 1H, Sia H3), 4.70 – 4.61 (m, 10H), 4.48 – 4.18 (m, 80H), 4.08 (s, 2H), 3.99 (s, 2H), 3.90 (d, *J* = 11.9 Hz, 1H), 3.85 – 3.59 (m, 11H), 3.49 (d, *J* = 9.6 Hz, 1H); ^{13}C NMR (151 MHz, D_2O, ppm, based on HSQC): *δ*= 141.79, 136.96, 126.96, 106.18, 96.51, 91.03, 89.90, 89.10, 82.97, 74.27, 71.85, 71.53, 70.24, 70.08, 69.92, 69.76, 69.60, 69.60, 69.44, 68.79, 68.31, 68.31, 67.99, 67.66, 67.50, 65.25, 62.99, 62.83, 60.41, 59.61, 52.19, 47.36; ^{19}F NMR (376 MHz, D_2O, ppm): *δ*= -208.27 (dd, *J* = 48.9, 30.2 Hz) HRMS (ESI): *m/z* calcd for $C_{38}H_{50}FN_{10}O_{26}P+H^+$: 1113.2692; found: 1113.2685.

Donor **PEG1-NNBD** (**22)** (Eluted at 1% acetonitrile/water, 0.2 mg, 4%) R_f=0.2 (EtOAc/MeOH/H_2O 4:2:1, spot fluoresces yellow-green under 365 nm UV); ^{1}H NMR (600 MHz, D_2O, ppm): *δ*= 8.51 (d, *J* = 8.8 Hz, 1H, NBD H5), 8.05 (s, 1H, triazole CH), 7.95 (d, *J* = 7.6 Hz, 1H, cytidine H6), 6.41 (d, *J* = 9.2 Hz, 1H, NBD H6), 6.09 (d, *J* = 7.6 Hz, 1H, cytidine H5), 5.97 (d, *J* = 4.4 Hz, 1H, ribose H1), 5.23 (d, *J* = 3.7 Hz, 2H, amide-CH2-triazole), 4.73 (s, 1H, O-CH2-triazole), 4.35 (t, *J* = 5.0 Hz, 1H, ribose H3), 4.29 (t, *J* = 4.8 Hz, 1H, ribose H2), 4.26 – 4.20 (m, 3H, PEG CH2, ribose H4 and Sia H6), 4.14 (ddd, *J* = 11.3, 10.2, 4.8 Hz, 1H, Sia H4), 3.98 (t, *J* = 10.4 Hz, 1H, Sia H5), 3.96 – 3.91 (m, 3H, PEG CH2 and Sia H7), 3.87 (dd, *J* = 11.8, 2.5 Hz, 1H, Sia H9a), 3.63 (dd, *J* = 11.8, 6.5 Hz, 1H, Sia H9b), 3.45 (d, *J* = 9.6 Hz, 1H, Sia H8), 2.50 (dd, *J* = 13.3, 4.8 Hz, 1H, Sia H3 eq), 1.64 (ddd, *J* = 13.2, 11.4, 5.8 Hz, 1H, Sia H3 ax); ^{13}C NMR (151 MHz, D_2O, ppm, based on HSQC): *δ*= 141.62, 138.88, 126.47, 101.01, 96.50, 89.09, 82.96, 74.42, 71.52, 69.75, 69.43, 68.94, 67.65, 66.69, 64.92, 63.14, 62.98, 62.98, 52.35, 52.02, 41.23, 41.23; HRMS (ESI): *m/z* calcd for $C_{31}H_{40}N_{11}O_{20}P+H^+$: 918.2261; found: 918.2244.

c. Activity of recombinant sialyltransferases with CMP-sialic acid donor **22** and lactose

To lactose (10 mM) and CMP-sialic acid donor **22** (0.2 mM) in 100 mM Tris buffer (2 µL) with 20 mM $MgCl_2$, 0.1 uL of each sialyltransferase (stock solutions of 1-2 mg/mL) was added. The reactions were incubated at 37°C for 20 minutes. 0.2 µL of the reaction was spotted on the TLC plate, developed using TLC system: ethyl acetate/methanol/water/toluene/acetic acid 4:2:1:0.2:0.1 and visualised under 365 nm UV (Figure S1).

d. General procedure for affinity-based labelling

Standard labelling experiments were carried out in the presence of 1 µM sialyltransferase, 1 µM of sialic acid aldolase (as background) and 1 µM of probe (**17-21**) in 25 mM pH 7.2 phosphate buffer (or PBS) at 37°C for 2 hours. For non-specific labelling control, the alkyne (**8-9**, **13-15**) was used.

e. Heat inactivation and competition assay

E. coli lysate was prepared by lysis of *E. coli* BL21 by sonication. 1 µM sialyltransferase (VJT-FAJ-16) was spiked into the lysate before the experiment. Heat inactivation was done at 95°C for 10 mins with 1% SDS and let cooled to room temperature prior to the addition of the probe. For competition, the protein mixture was preincubated with CMP-3F(axial)-Neu5Ac or pyruvate at room temperature for 30 minutes prior to the addition of the probe. The same condition as standard labelling experiment (1 µM of probe, 25 mM pH 7.2 phosphate buffer, 37°C for 2 hours) was used. All the runs were done in parallel so that the sialyltransferase in each run was exposed to room temperature and 37°C for the same duration of time. Relative intensity of the bands was estimated using ImageJ (National Institutes of Health).

f. Kinetic assay

Assay was done in single experiment with duplicates (except for VJT-FAJ-16 with 0 µM and 100 µM inhibitor, which was done in two independent experiments with duplicates) in black 96-well half area microplates (Product Number 3694, Corning), in a final volume of 25 µL. 1 µM sialyltransferase in PBS pH 7.2 was preincubated with various concentration of competitive inhibitor at 37°C for 15 minutes, then cooled to 4°C for 20 minutes. The affinity probe was added while the plate was on ice, then the plate was incubated in the plate reader at 37°C while taking fluorescence reading (485 nm excitation, 520 nm emission, BMG POLARstar Omega) every 5 minutes for an hour, with 300 rpm shaking between reading. The initial rate was extracted from 10-35 minutes and curve fitting to Equation 2 was done using GraphPad Prism 10 to give the K_M and K_i. Values are reported as mean and SEM.

g. Protein LC-MS/MS

Proteins were separated by SDS-PAGE using 12% Bis-Tris gels and MOPS buffer system under denaturing condition (175 V). Staining of protein gels was done using Imperial Protein Staining Solution (Thermo Scientific, 24615). Protein bands were excised, reduced with DTT, alkylated with iodoacetamide and in-gel digested with chymotrypsin.[18]

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) experiments were performed on a Thermo Scientific Exploris 480 connected to a Thermo Scientific Ultimate 3000 UPLC system. Solvent A consisted of 0.1% FA, solvent B of 0.1% FA in 80% acetonitrile. A 50 cm, 75 µm ID, 1.9 µm Reprosil column was used with a 60 min gradient of 4% B at 0-1 min, 44% B at 39 min, 55% B at 44 min, 99% B at 45-50 min, 2% B at 50-60 min. After an initial MS scan at 60000 resolution, MS/MS scans were triggered for ions with an intensity of more than 2e5 and a charge state from 2+ to 6+. The MS/MS scans used higher-energy collisional dissociation (HCD) at 28% normalized collision energy (NCE) and were performed at a resolution of 15000. All scans were performed with the instrument in peptide application mode.

The LC-MS/MS data was searched using Byonic (v5.6.16, Protein Metrics) using FYWLMADE as cleavage sites, with digestion specificity set to specific and allowing for up to 10 missed cleavages. The mass error limits were set to 10 ppm for MS and 20 ppm for MS/MS. The probe was added as a variable modification with the allowance for up to 2 variable modifications per peptide. The results were filtered using a score threshold of 150 and a |logprob| threshold of 1.5. For identified modification sites, as well as their non-modified counterparts, Skyline (64-bit, v24.1.0.199) was used to integrate the MS peak areas.

h. Bacterial strains and culture

N. gonorrhoeae F62 and F62*Δlst* were a kind gift from Jos van Putten, Utrecht University[19,20] while *N. meningitidis* L1 126E was a kind gift from Nina van Sorge, Amsterdam UMC. The bacteria were grown on Chocolate Columbia agar plates and in proteose peptone medium supplemented with HEPES at 37°C with 5% CO_2.[11]

i. Triton X-100 extraction of Lst

Lst activity was extracted from *N. gonorrhoeae* and *N. meningitidis* as described previously.[3,21] Briefly, 1000 µL of OD = 0.3 (or equivalent) of liquid bacteria culture was pelleted (4000 rpm, 7 min), washed once with PBS and resuspended in 50 µL 0.5% Triton X-100 in PBS. The cell suspension was pipetted up and down at least 30 times, then the clarified cell extract was obtained after centrifugation (10000 rpm, 7 min). The Triton X-100 extract was used immediately. From the same culture, 1000 uL of OD = 0.3 (or equivalent) of liquid bacteria culture was pelleted (4000 rpm, 7 min), washed once with PBS and resuspended in 50 mM HEPES buffer pH 7.2 and used for live bacteria LOS and Lst labelling experiments.

j. LOS labelling

LOS labelling was performed similar to the native sialyltransferase (NST) bacteria labelling technique described previously.[22] Briefly, 47.5 µL of either Triton X-100 extract or live bacteria was added 2.5 µL 1 mM CMP-sialic acid donor **22** to give a final concentration of 50 µM. The labelling mixture was incubated for 2 hours at 37°C. At the end of the incubation, experiment with live bacteria was spun down (10000 rpm, 7 min), washed twice with PBS to remove the excess probes, then the pellet was resuspended in PBS, boiled for 5 minutes and treated with proteinase K (3.3 mg/mL) overnight at 55°C. On the other hand, experiment with Triton X-100 extract was directly

boiled and treated with proteinase K. Laemmli buffer (3X) was added and the samples were analysed with a 16% Tris-Tricine gel. The same gel was visualised by in-gel fluorescence and then silver staining.

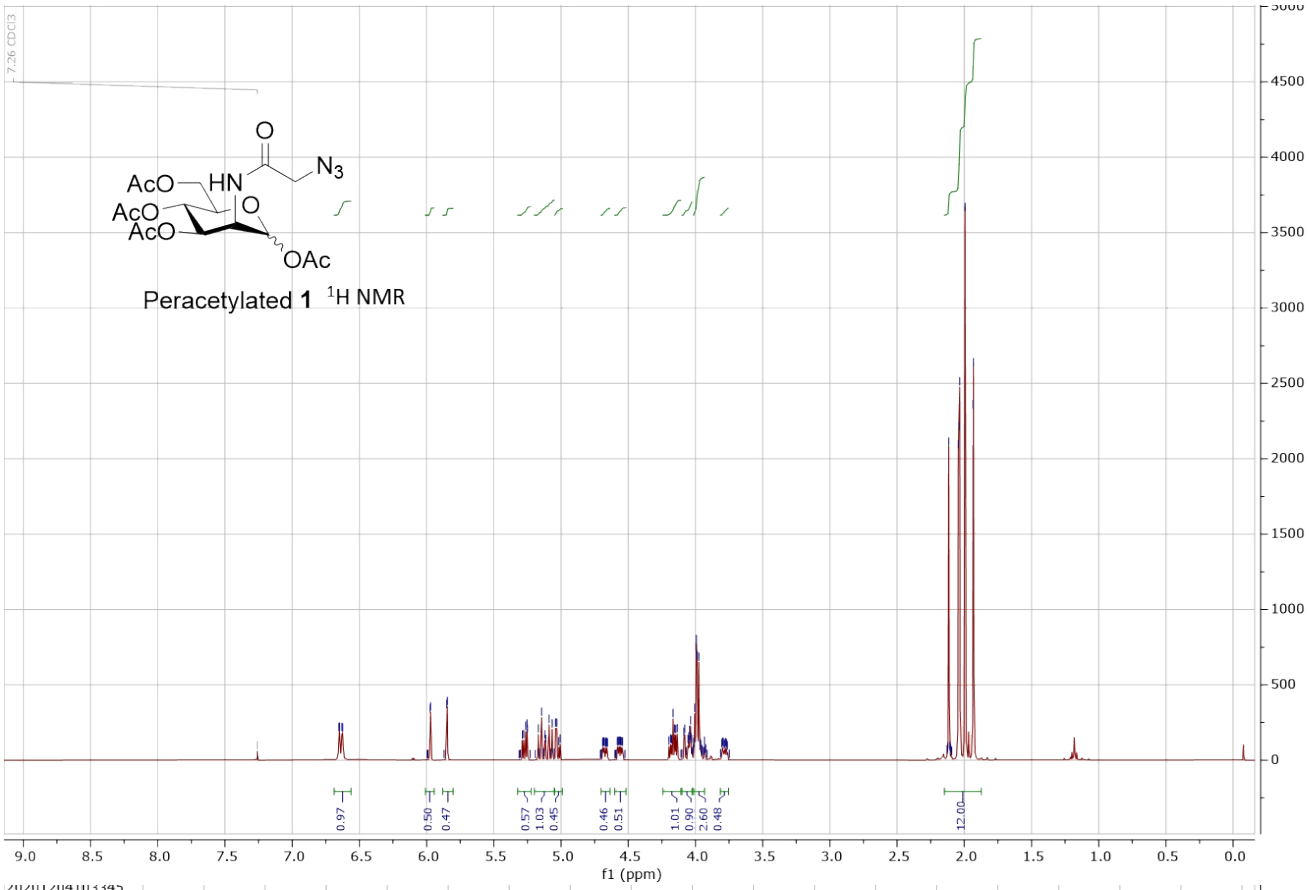
k. Lst labelling

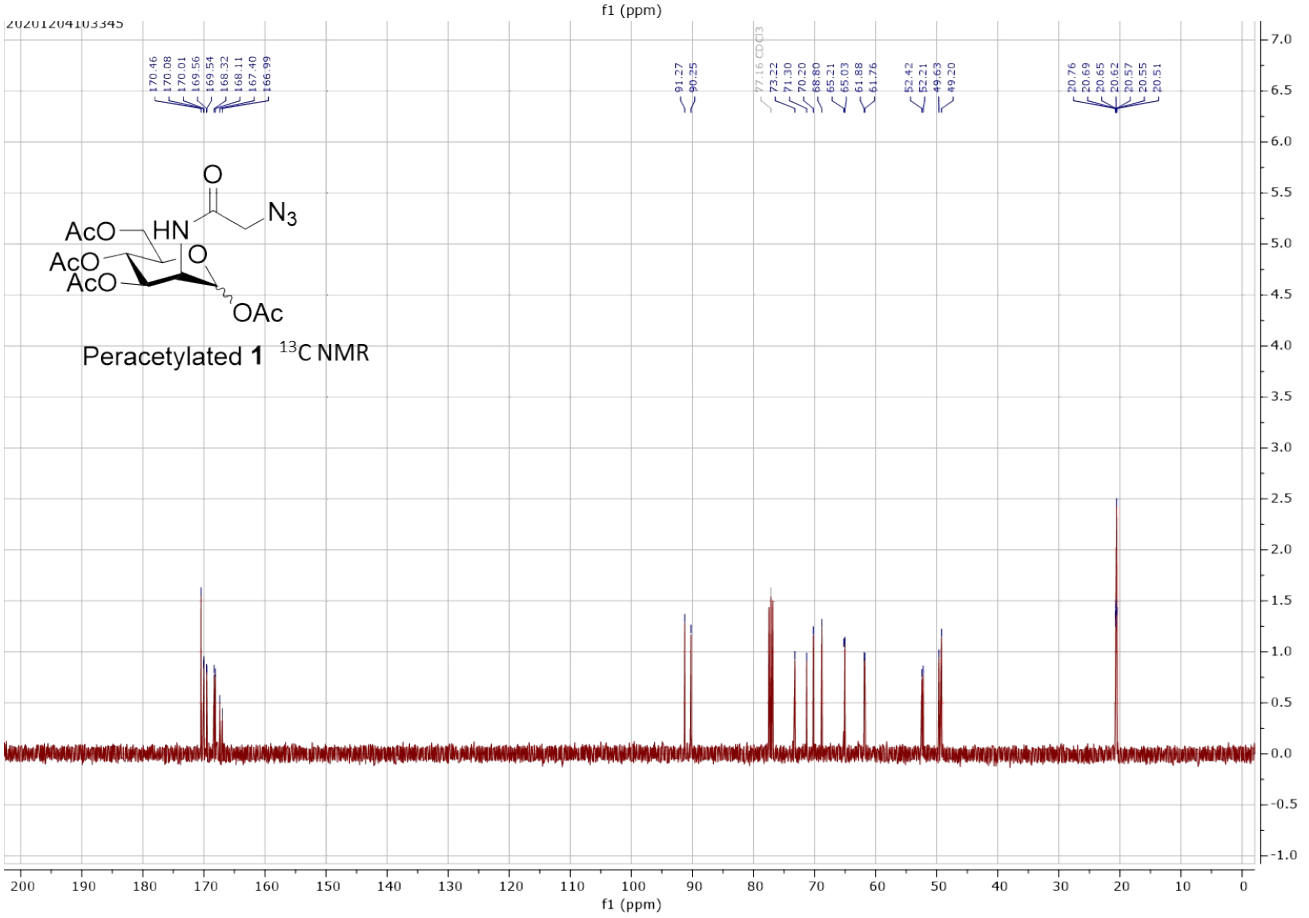
Triton X-100 extract and live bacteria were prepared as above. In each experiment, 46 uL of either Triton X-100 extract or live bacteria were first preincubated with no inhibitor, CMP-3F(axial)-Neu5Ac or ATP (final concentration 1 mM) at room temperature for 5 minutes, then probe **PEG1** (**18**) was added to a final concentration of 1 μM. The labelling mixture was incubated for 2 hours at 37°C. At the end of the incubation, experiment with live bacteria was spun down (10000 rpm, 7 min), washed twice with PBS to remove the excess probes, then the pellet was resuspended in PBS, added Laemmli buffer (3X), heated at 95°C for 10 minutes and analysed with SDS-PAGE. Experiment with Triton X-100 extract was directly added Laemmli buffer (3X), heated at 95°C for 10 minutes and analysed with SDS-PAGE. The same gel was visualised by in-gel fluorescence and then CBB staining.

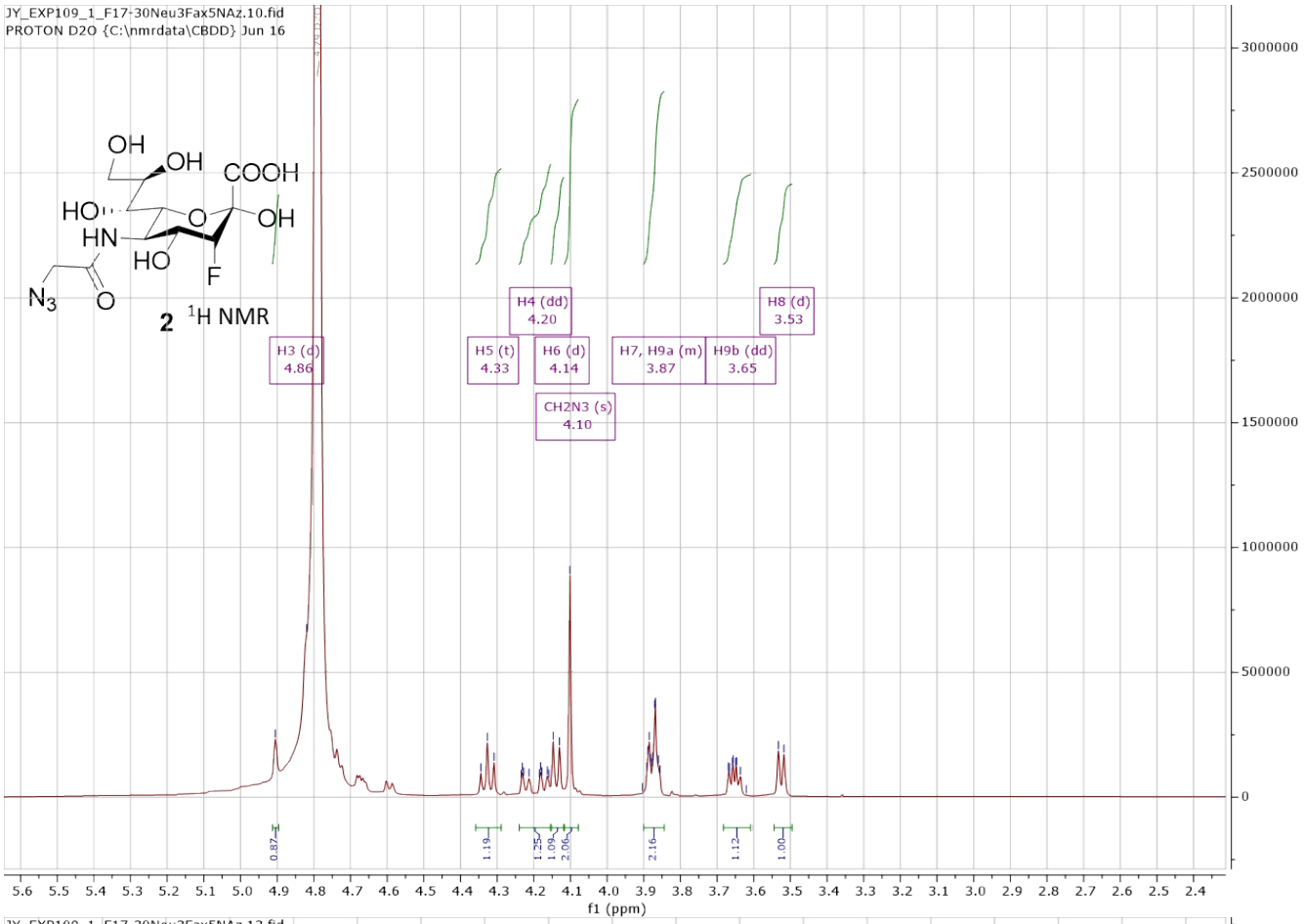
V. References

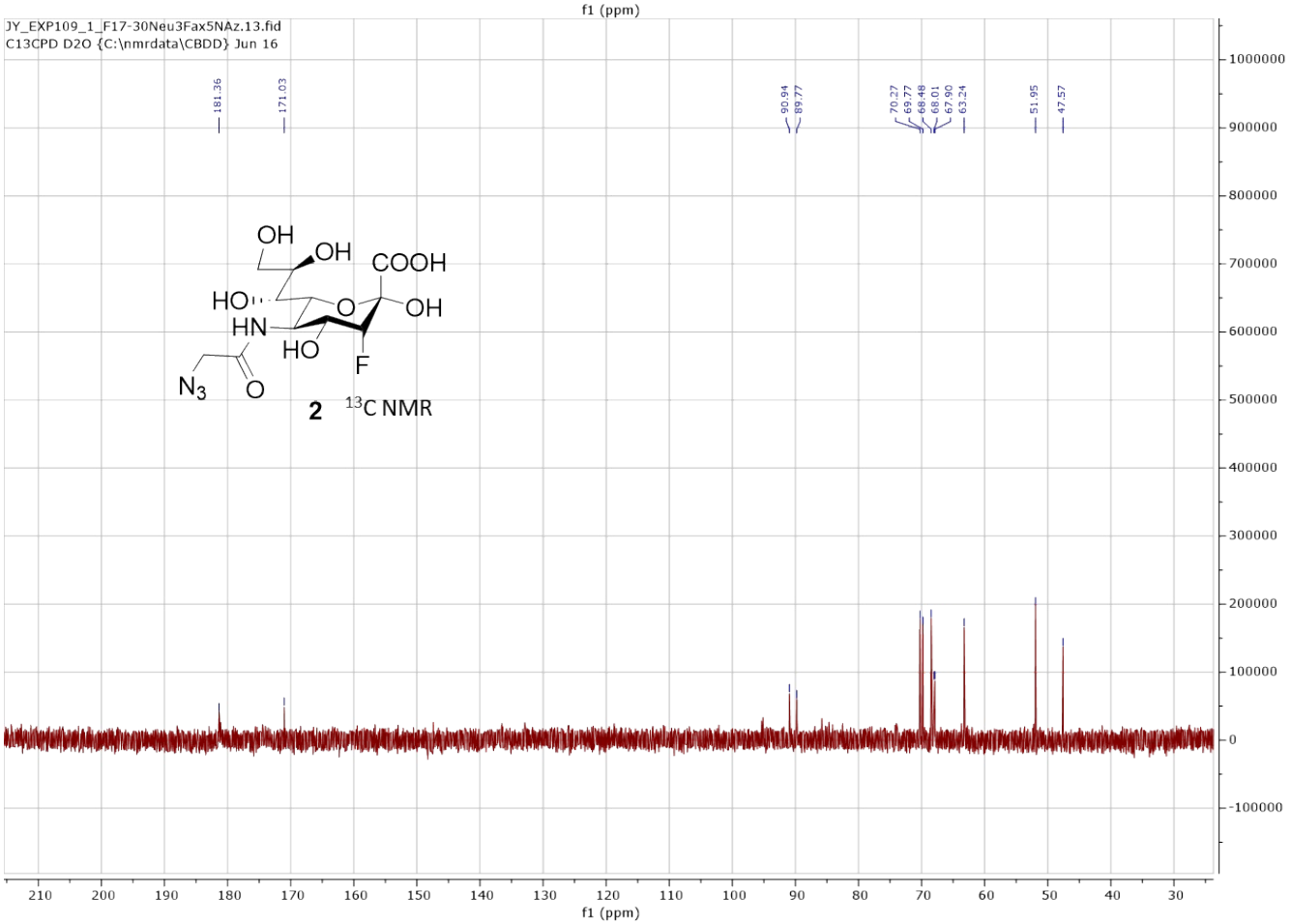
1 D. E. Fahrney and A. M. Gold, *J. Am. Chem. Soc.*, 1963, **85**, 997–1000.
2 R. A. Copeland, in *Evaluation of Enzyme Inhibitors in Drug Discovery*, Wiley, 2013, pp. 57–121.
3 M. Packiam, D. M. Shell, S. V. Liu, Y.-B. Liu, D. J. McGee, R. Srivastava, S. Seal and R. F. Rest, *Infect. Immun.*, 2006, **74**, 2637–2650.
4 H. Yu, H. Chokhawala, R. Karpel, H. Yu, B. Wu, J. Zhang, Y. Zhang, Q. Jia and X. Chen, *J. Am. Chem. Soc.*, 2005, **127**, 17618–17619.
5 G. Sugiarto, K. Lau, J. Qu, Y. Li, S. Lim, S. Mu, J. B. Ames, A. J. Fisher and X. Chen, *ACS Chem. Biol.*, 2012, **7**, 1232–1240.
6 Y. Takakura, H. Tsukamoto and T. Yamamoto, *J. Biochem.*, 2007, **142**, 403–412.
7 H. Yu, S. Huang, H. Chokhawala, M. Sun, H. Zheng and X. Chen, *Angew. Chemie - Int. Ed.*, 2006, **45**, 3938–3944.
8 K. W. Moremen, A. Ramiah, M. Stuart, J. Steel, L. Meng, F. Forouhar, H. A. Moniz, G. Gahlay, Z. Gao, D. Chapla, S. Wang, J.-Y. Yang, P. K. Prabhakar, R. Johnson, M. Dela Rosa, C. Geisler, A. V. Nairn, J. Seetharaman, S.-C. Wu, L. Tong, H. J. Gilbert, J. LaBaer and D. L. Jarvis, *Nat. Chem. Biol.*, 2018, **14**, 156–162.
9 J. Cheng, H. Yu, K. Lau, S. Huang, H. A. Chokhawala, Y. Li, V. K. Tiwari and X. Chen, *Glycobiology*, 2008, **18**, 686–697.
10 M. Gilbert, D. C. Watson, A. M. Cunningham, M. P. Jennings, N. M. Young and W. W. Wakarchuk, *J. Biol. Chem.*, 1996, **271**, 28271–28276.
11 H. de Jong, M. J. Moure, J. E. M. Hartman, G. P. Bosman, J. Y. Ong, B. W. Bardoel, G. J. Boons, M. M. S. M. Wösten and T. Wennekes, *ChemBioChem*, 2022, **23**, e202200340.
12 E. Saxon, S. J. Luchansky, H. C. Hang, C. Yu, S. C. Lee and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2002, **124**, 14893–14902.
13 S. J. Moons, E. Rossing, J. J. A. Heming, M. A. C. H. Janssen, M. Van Scherpenzeel, D. J. Lefeber, M. I. De Jonge, J. D. Langereis and T. J. Boltje, *Bioconjug. Chem.*, 2021, **32**, 1047–1051.
14 H. Yu, H. Yu, R. Karpel and X. Chen, *Bioorganic Med. Chem.*, 2004, **12**, 6427–6435.
15 H. A. Chokhawala, H. Cao, H. Yu and X. Chen, *J. Am. Chem. Soc.*, 2007, **129**, 10630–10631.
16 L. Johnson, S. Lagerkvist, P. Lindroth, M. Ahnoff and K. Martinsson, *Anal. Chem.*, 1982, **54**, 939–942.
17 V. Hong, S. I. Presolski, C. Ma and M. G. Finn, *Angew. Chem. Int. Ed. Engl.*, 2009, **48**, 9879.
18 A. Shevchenko, H. Tomas, J. Havliš, J. V. Olsen and M. Mann, *Nat. Protoc.*, 2007, **1**, 2856–2860.
19 J. Bramley, R. D. de Hormaeche, C. Constantinidou, X. Nassif, N. Parsons, P. Jones, H. Smith and J. Cole, *Microb. Pathog.*, 1995, **18**, 187–195.
20 M. J. Gill, D. P. Mcquillen, J. P. M. Van Putten, L. M. Wetzler, J. Bramley, H. Crooke, N. J. Parsons, J. A. Cole and H. Smith, *Infect. Immun.*, 1996, **64**, 3374–3378.
21 R. E. Mandrell, H. Smith, G. A. Jarvis, J. McLeod Griffiss and J. A. Cole, *Microb. Pathog.*, 1993, **14**, 307–313.
22 E. I. Alvarado-Melendez, H. Jong, J. E. M. Hartman, J. Y. Ong, M. M. S. M. Wösten and T. Wennekes, *Glycobiology*, , DOI:10.1093/glycob/cwae071.

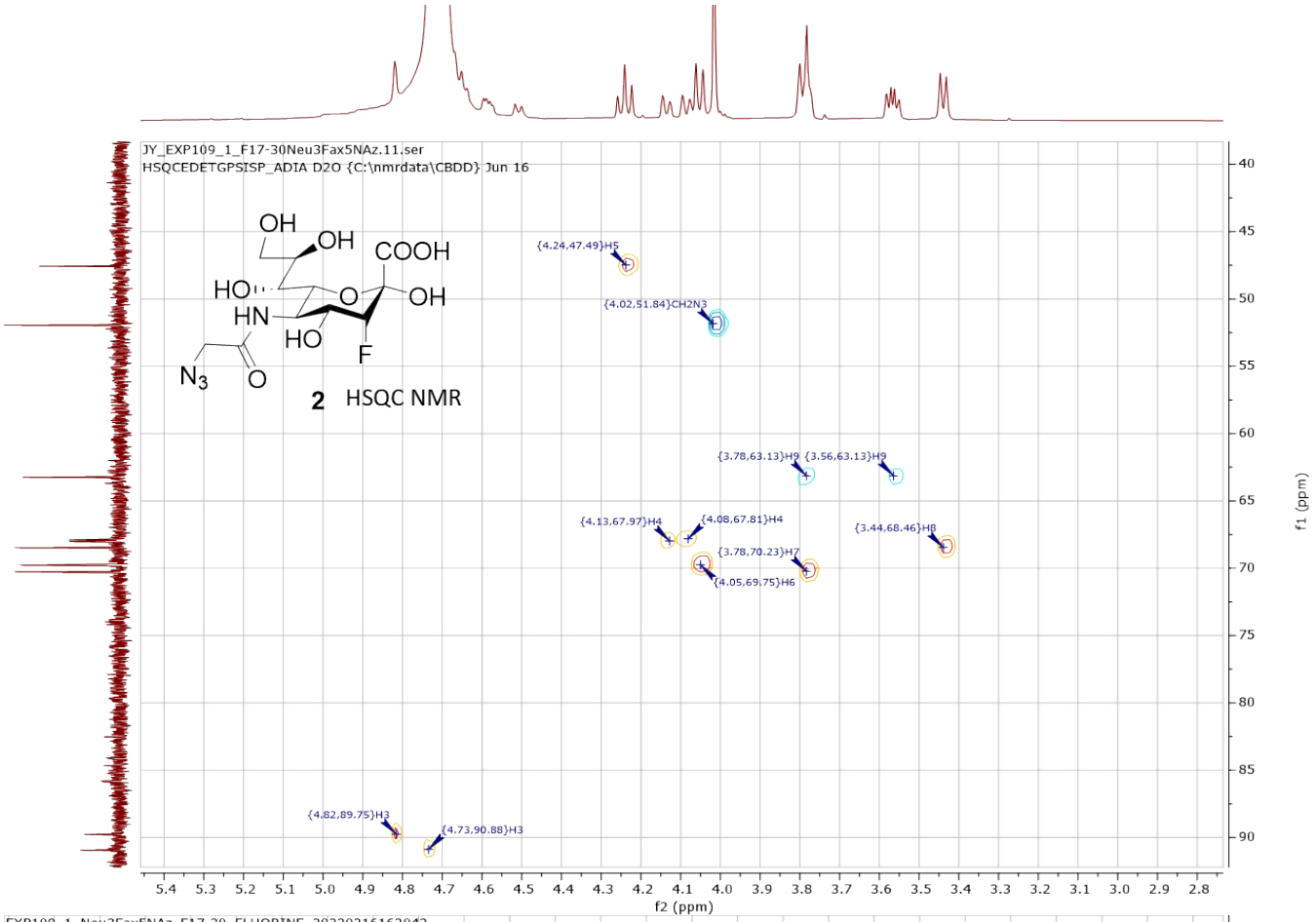
VI. NMR spectra

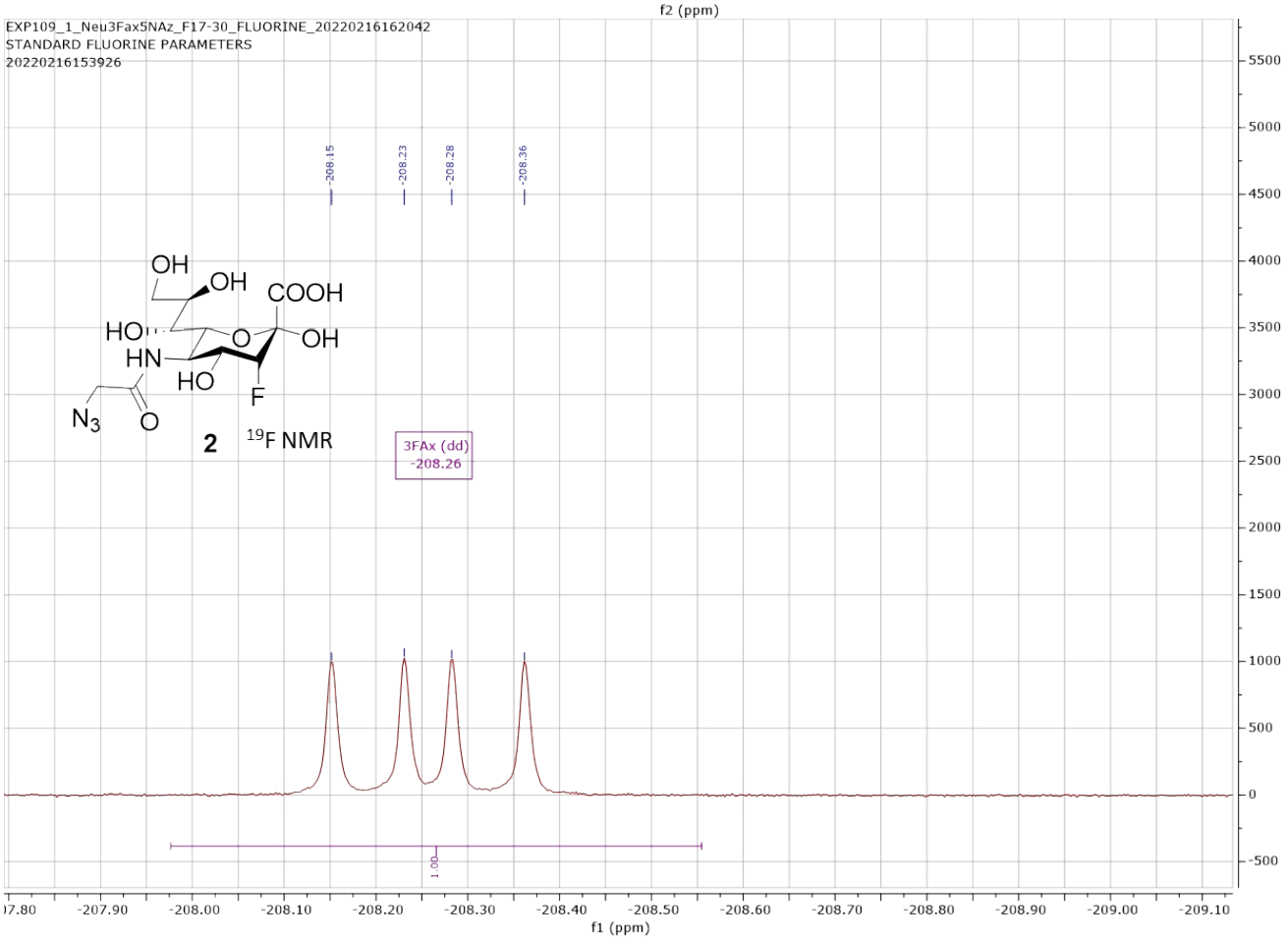


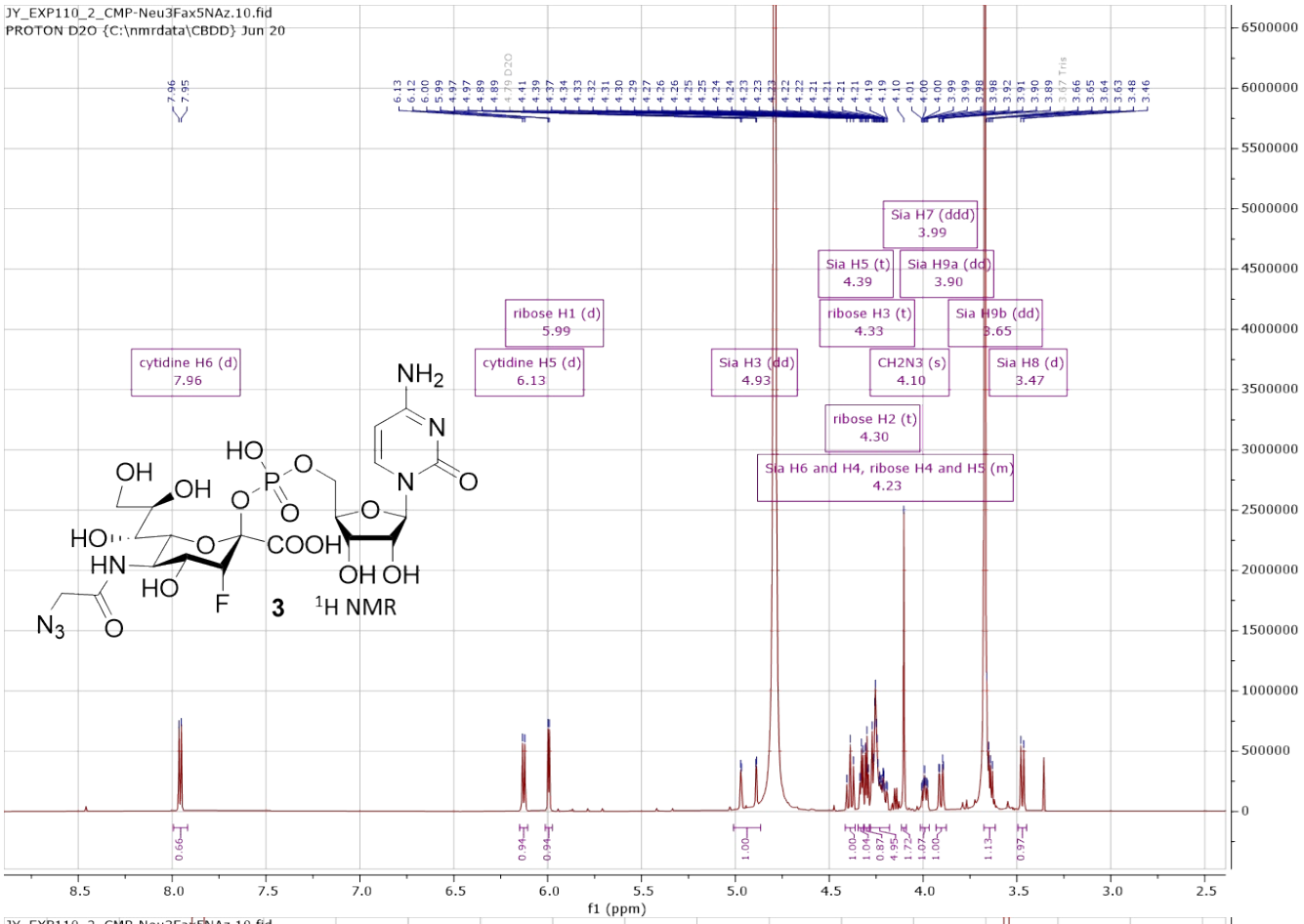


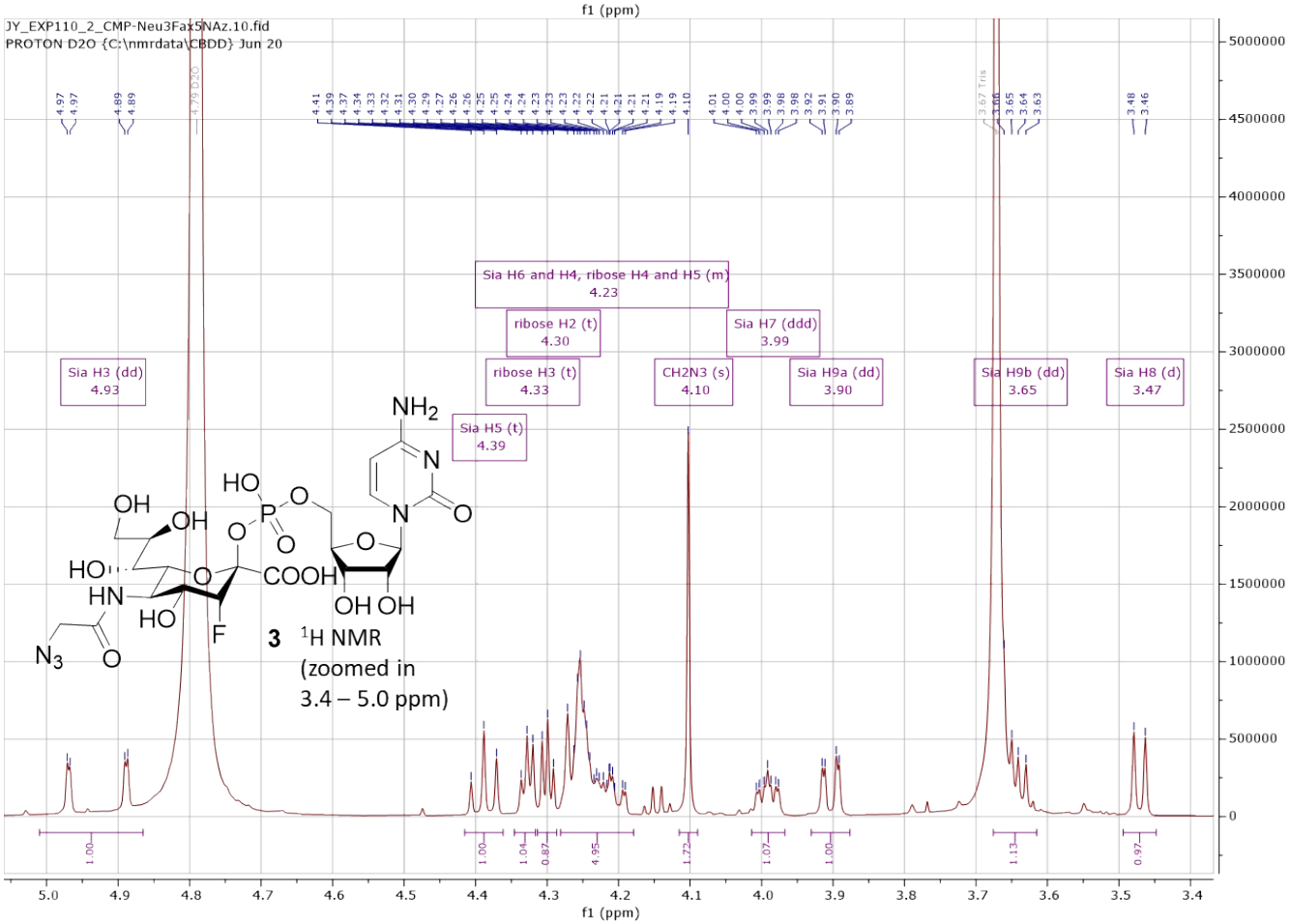

JY_EXP109_1_F17-30Neu3Fax5NAz.10.fid
PROTON D2O {C:\nmrdata\CBDD} Jun 16
OH
OH
COOH
HO
O
OH
HN
HO
F
N3
O
2 1H NMR
H3 (d) 4.86
H4 (dd) 4.20
H5 (t) 4.33
H6 (d) 4.14
CH2N3 (s) 4.10
H7, H9a (m) 3.87
H9b (dd) 3.65
H8 (d) 3.53
f1 (ppm)

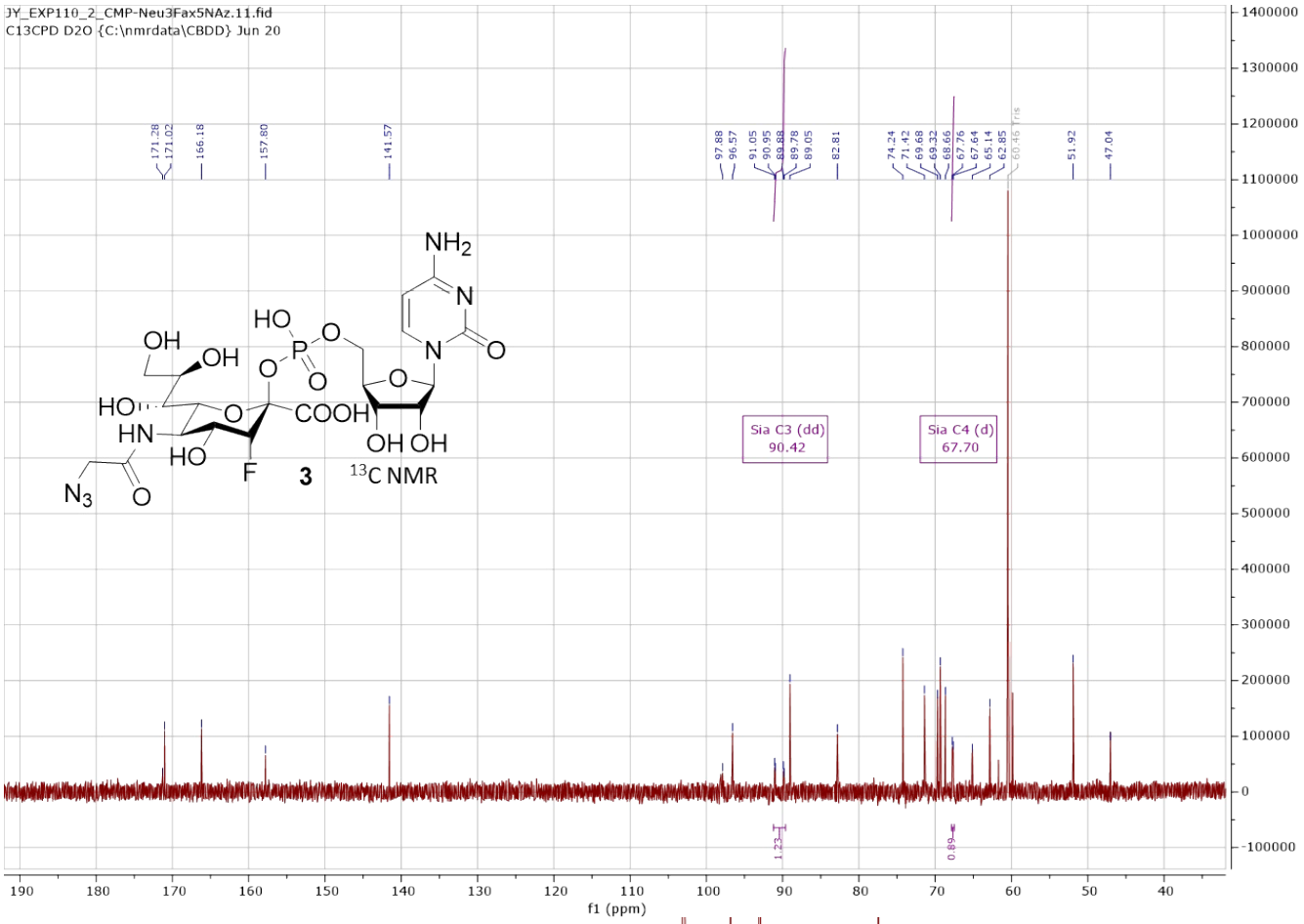

JY_EXP109_1_F17-30Neu3Fax5NAz.13.fid
C13CPD D2O {C:\nmrdata\CBDD} Jun 16
181.36
171.03
90.94
89.77
70.27
69.77
68.48
68.01
67.90
63.24
51.95
47.57
OH
OH
COOH
HO
O
OH
HN
HO
F
N3
O
2 13C NMR
f1 (ppm)

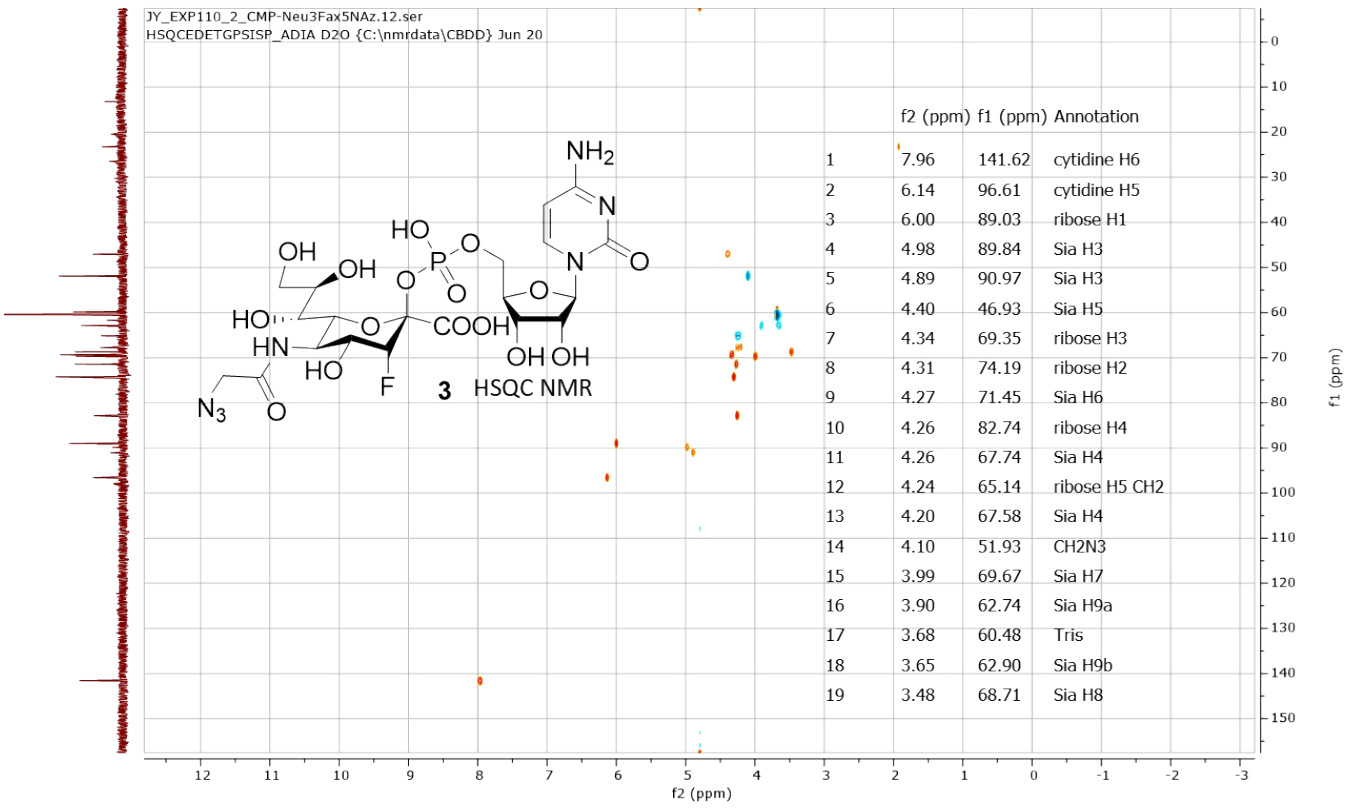

JY_EXP109_1_F17-30Neu3Fax5NAz.11.ser
HSQCEDETGPSISP_ADIA D2O {C:\nmrdata\CBDD} Jun 16
OH
OH
COOH
HO
O
OH
HN
HO
F
N3
O
2 HSQC NMR
{4.24,47.49}H5
{4.02,51.84}CH2N3
{3.78,63.13}H9
{3.56,63.13}H9
{4.13,67.97}H4
{4.08,67.81}H4
{3.44,68.46}H8
{3.78,70.23}H7
{4.05,69.75}H6
{4.82,89.75}H3
{4.73,90.88}H3
f2 (ppm)
f1 (ppm)


EXP109_1_Neu3Fax5NAz_F17-30_FLUORINE_20220216162042
STANDARD FLUORINE PARAMETERS
20220216153926
-208.15
-208.23
-208.28
-208.36
OH
OH
COOH
HO
O
OH
HN
HO
F
N3
O
2 19F NMR
3FAx (dd)
-208.26
1.00
f1 (ppm)

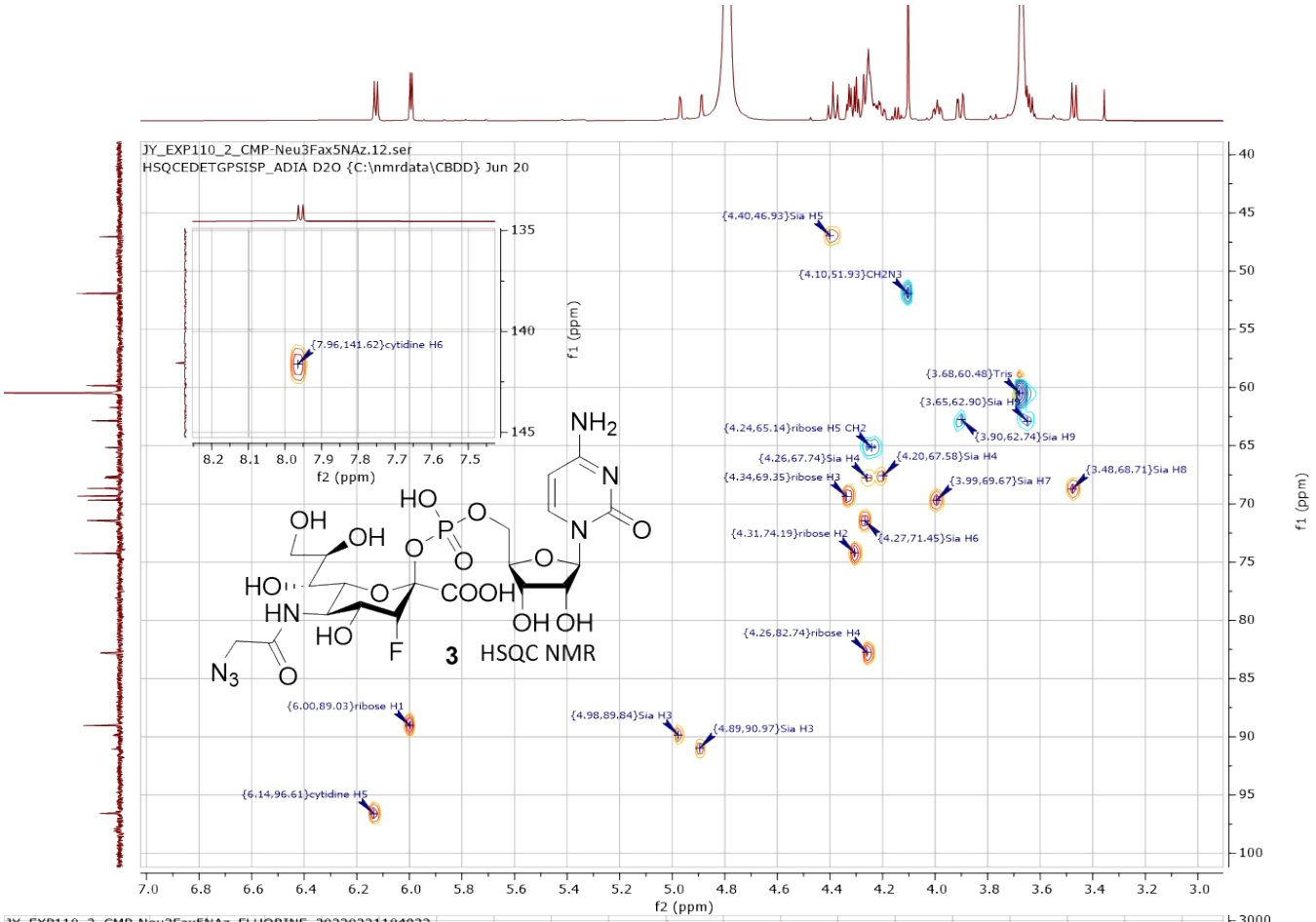

JY_EXP110_2_CMP-Neu3Fax5NAz.10.fid
PROTON D2O {C:\nmrdata\CBDD} Jun 20
cytidine H6 (d) 7.96
ribose H1 (d) 5.99
cytidine H5 (d) 6.13
Sia H3 (dd) 4.93
Sia H7 (ddd) 3.99
Sia H5 (t) 4.39
Sia H9a (dd) 3.90
ribose H3 (t) 4.33
Sia H9b (dd) 3.65
CH2N3 (s) 4.10
Sia H8 (d) 3.47
ribose H2 (t) 4.30
Sia H6 and H4, ribose H4 and H5 (m) 4.23
3 1H NMR
f1 (ppm)

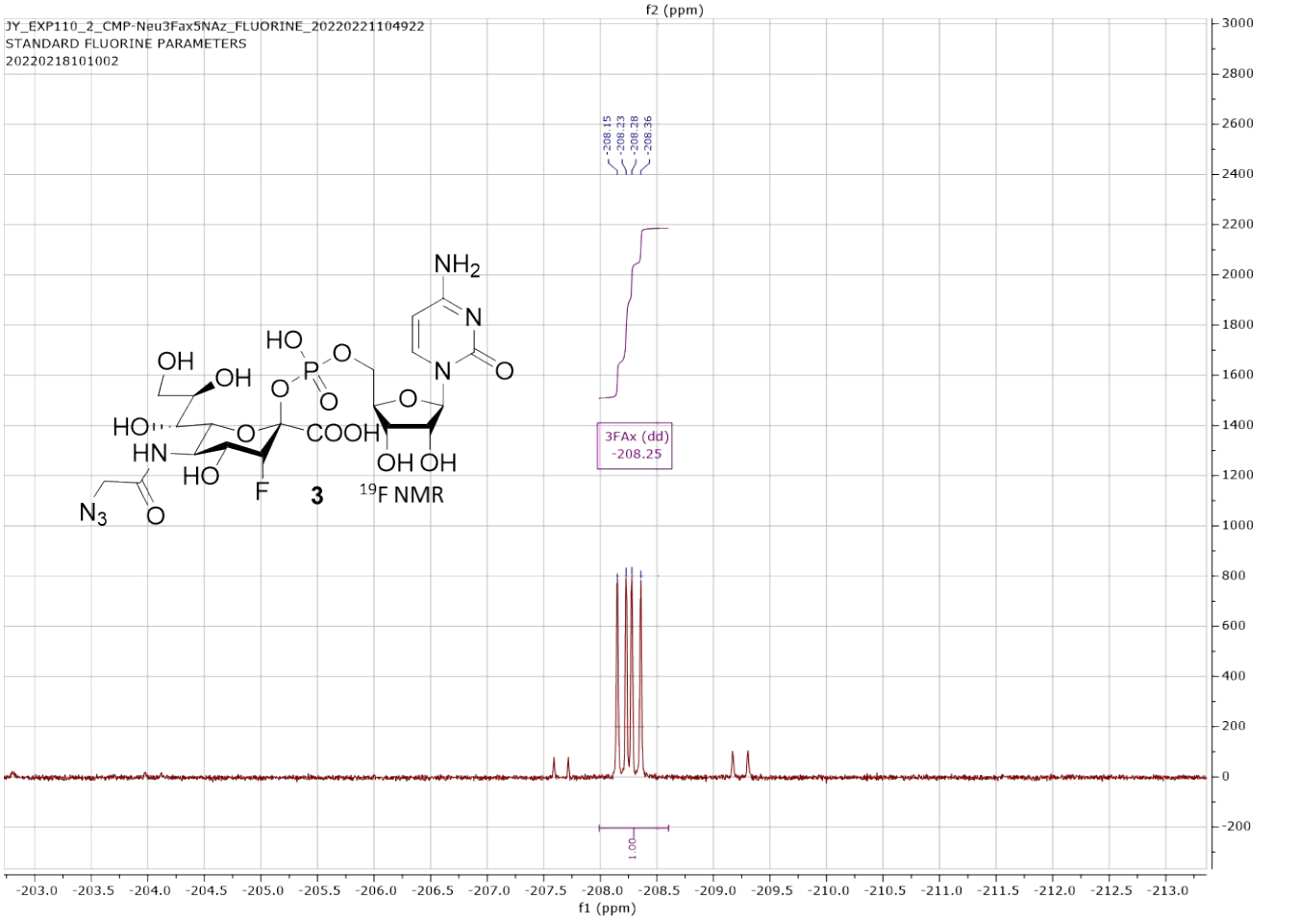

JY_EXP110_2_CMP-Neu3Fax5NAz.10.fid
PROTON D2O {C:\nmrdata\CBDD} Jun 20
Sia H6 and H4, ribose H4 and H5 (m) 4.23
ribose H2 (t) 4.30
Sia H7 (ddd) 3.99
Sia H3 (dd) 4.93
ribose H3 (t) 4.33
CH2N3 (s) 4.10
Sia H9a (dd) 3.90
Sia H9b (dd) 3.65
Sia H8 (d) 3.47
Sia H5 (t) 4.39
3 1H NMR (zoomed in 3.4 – 5.0 ppm)
f1 (ppm)

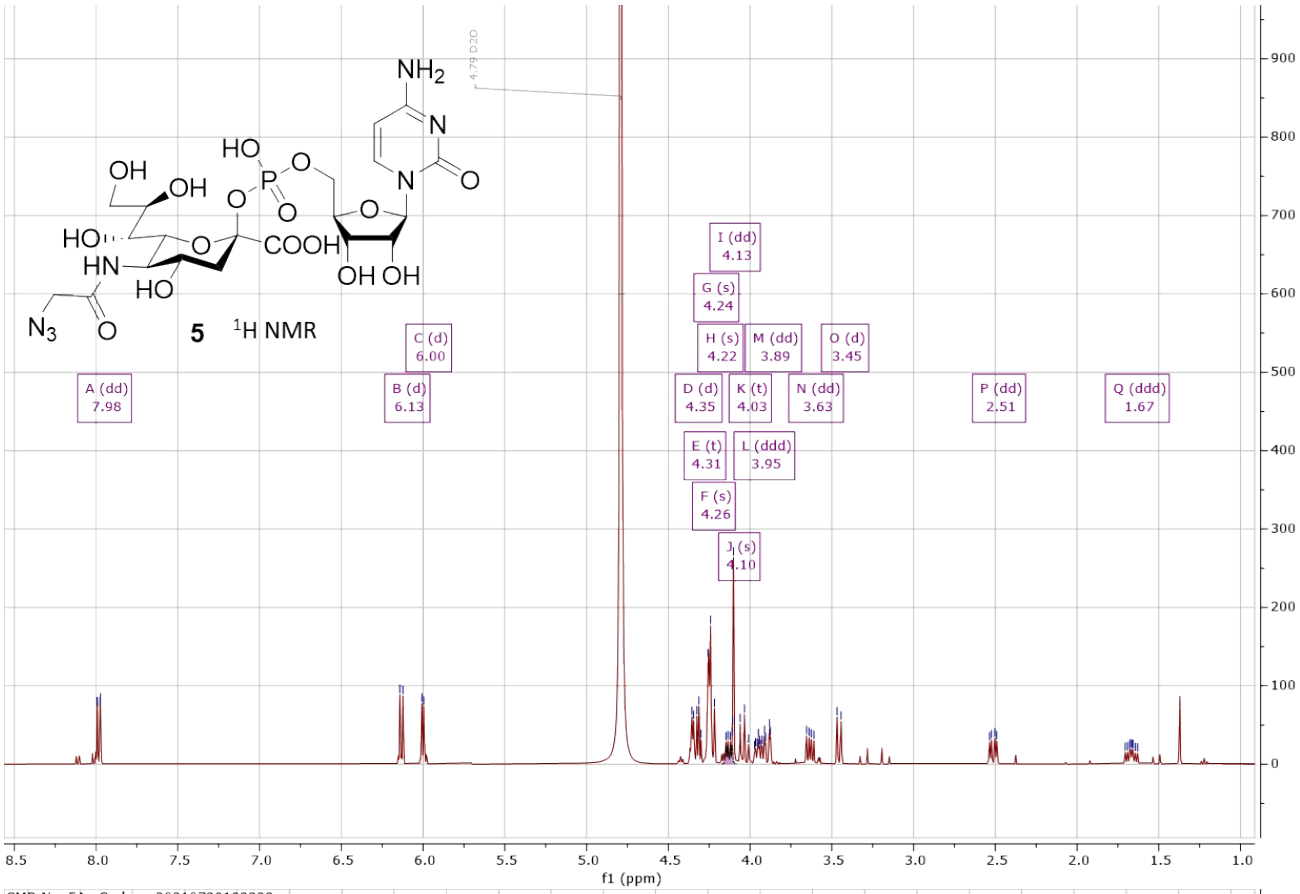


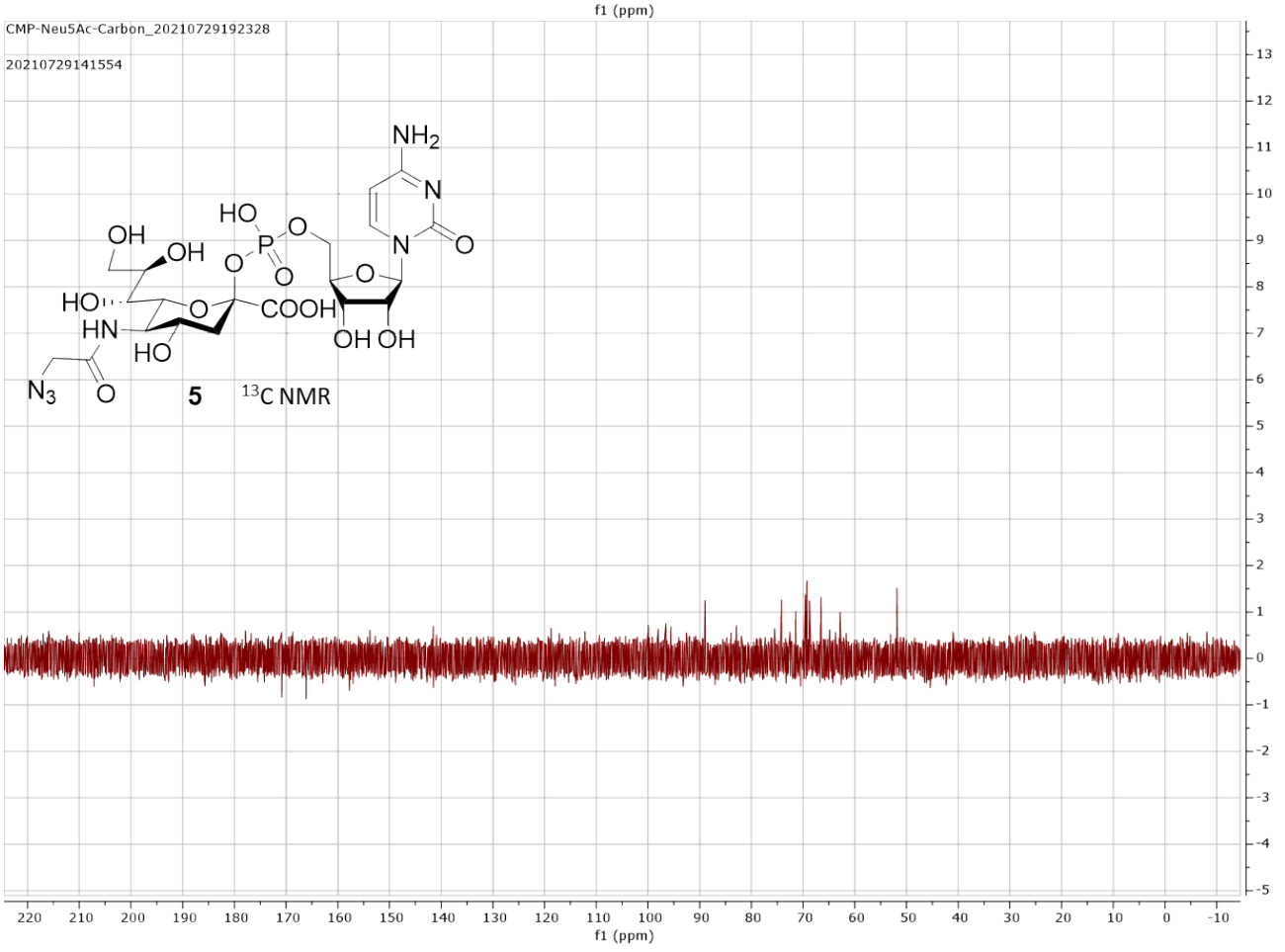


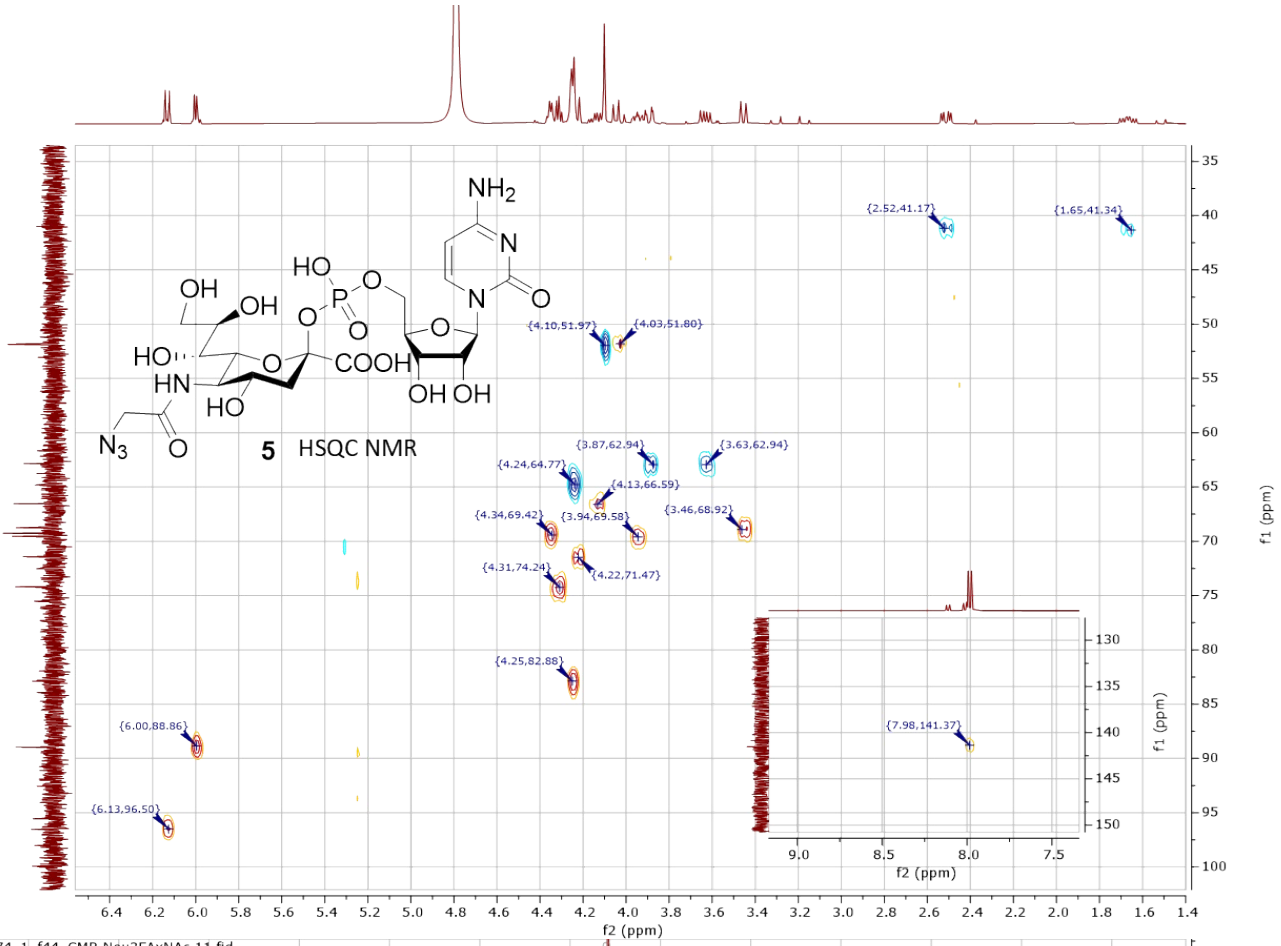
	f2 (ppm)	f1 (ppm)	Annotation
1	7.96	141.62	cytidine H6
2	6.14	96.61	cytidine H5
3	6.00	89.03	ribose H1
4	4.98	89.84	Sia H3
5	4.89	90.97	Sia H3
6	4.40	46.93	Sia H5
7	4.34	69.35	ribose H3
8	4.31	74.19	ribose H2
9	4.27	71.45	Sia H6
10	4.26	82.74	ribose H4
11	4.26	67.74	Sia H4
12	4.24	65.14	ribose H5 CH2
13	4.20	67.58	Sia H4
14	4.10	51.93	CH2N3
15	3.99	69.67	Sia H7
16	3.90	62.74	Sia H9a
17	3.68	60.48	Tris
18	3.65	62.90	Sia H9b
19	3.48	68.71	Sia H8

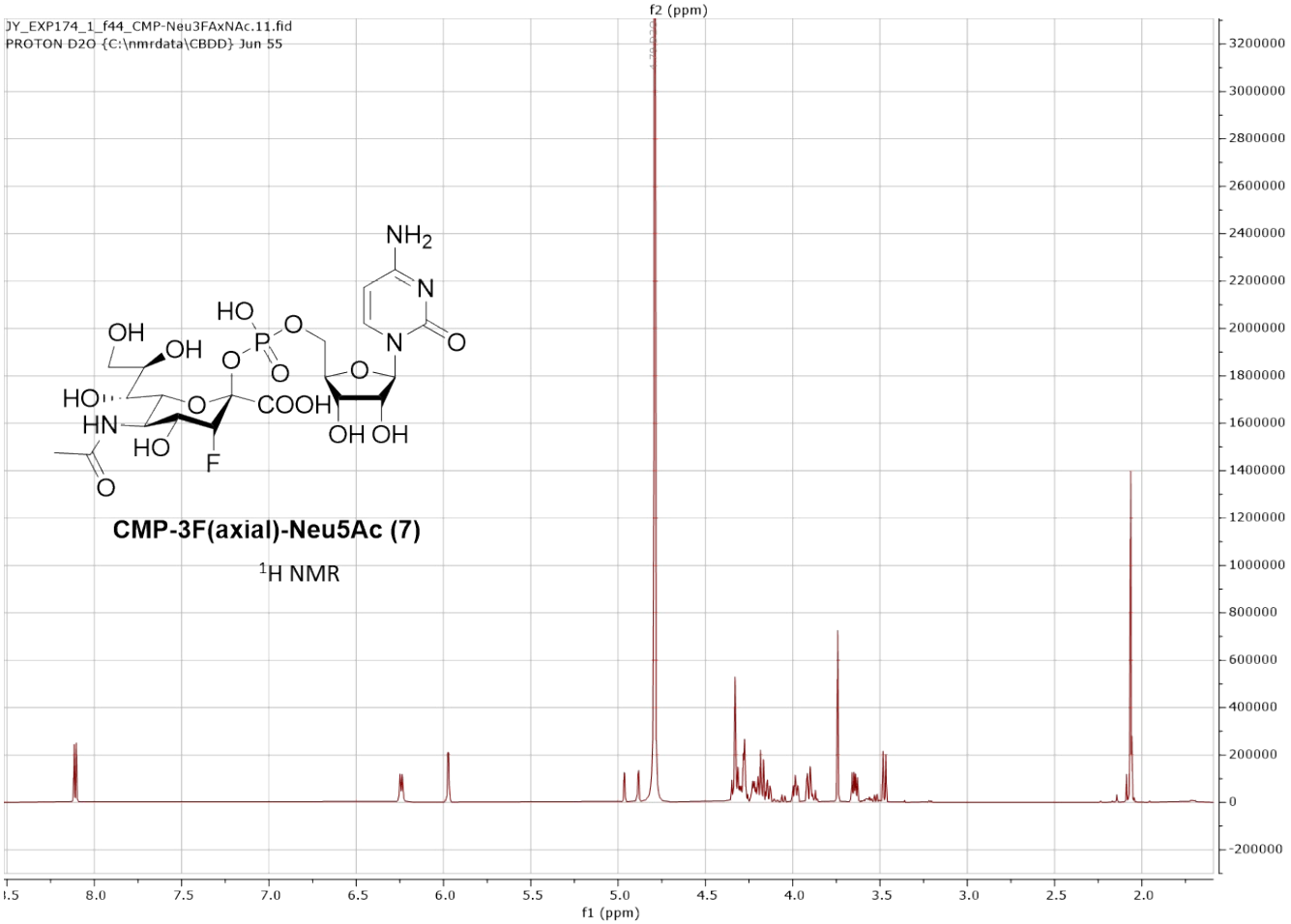

JY_EXP110_2_CMP-Neu3Fax5NAz.12.ser
HSQCEDETGPSISP_ADIA D2O {C:\nmrdata\CBDD} Jun 20
{7.96,141.62}cytidine H6
{4.40,46.93}Sia H5
{4.10,51.93}CH2N3
{3.68,60.48}Tris
{3.65,62.90}Sia H9
{3.90,62.74}Sia H9
{4.24,65.14}ribose H5 CH2
{4.26,67.74}Sia H4
{4.20,67.58}Sia H4
{4.34,69.35}ribose H3
{3.99,69.67}Sia H7
{3.48,68.71}Sia H8
{4.31,74.19}ribose H2
{4.27,71.45}Sia H6
{4.26,82.74}ribose H4
{6.00,89.03}ribose H1
{4.98,89.84}Sia H3
{4.89,90.97}Sia H3
{6.14,96.61}cytidine H5
f1 (ppm)
f2 (ppm)
3 HSQC NMR

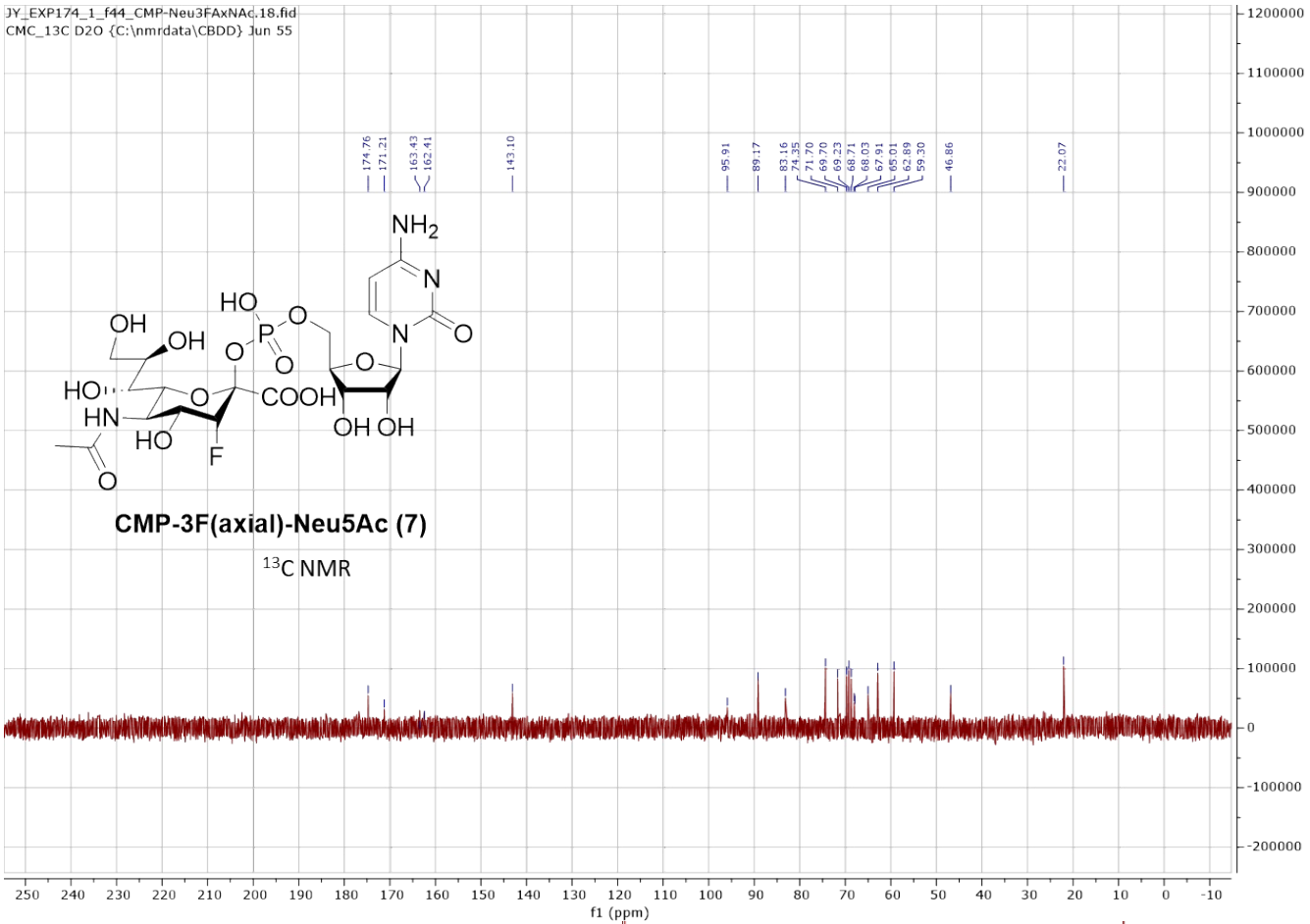

JY_EXP110_2_CMP-Neu3Fax5NAz_FLUORINE_20220221104922
STANDARD FLUORINE PARAMETERS
20220218101002
-208.15
-208.23
-208.28
-208.36
3FAx (dd)
-208.25
1.00
f1 (ppm)
3 19F NMR

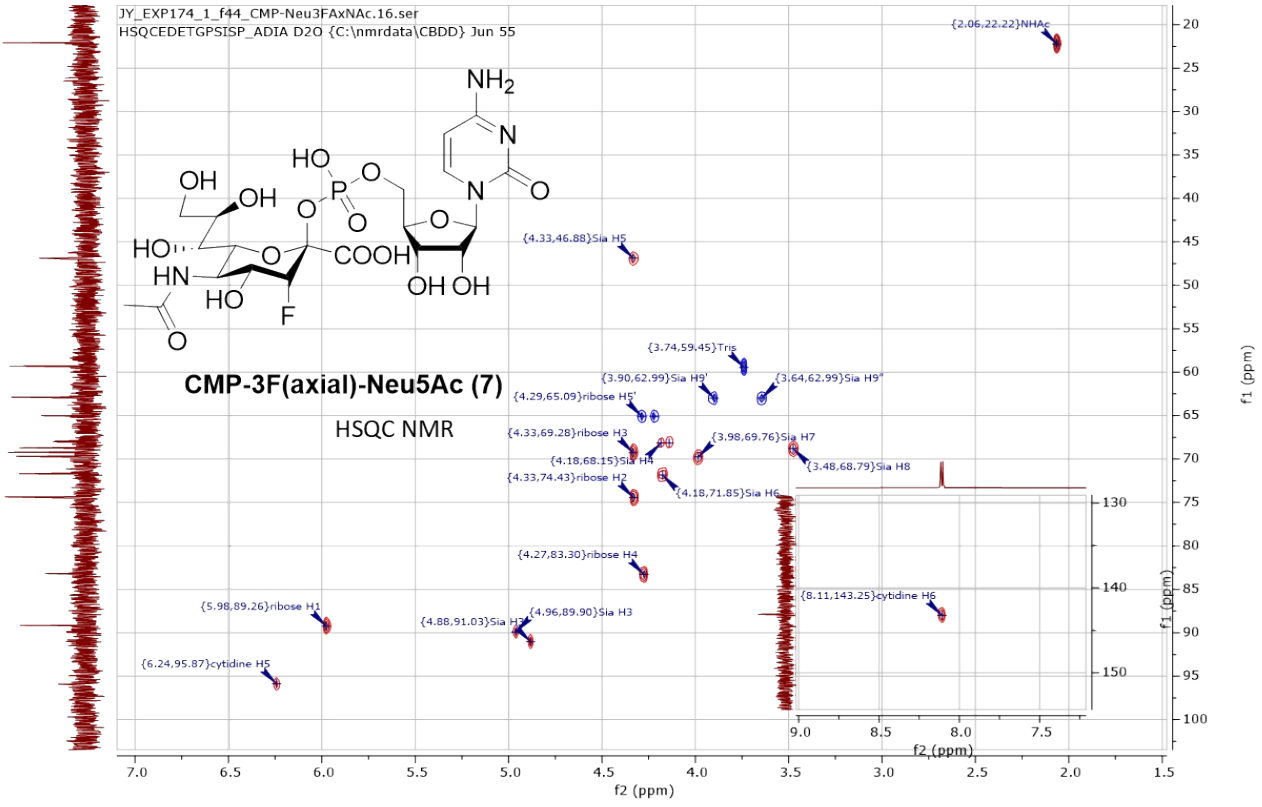

NH2
N
HO
O
P
OH
OH
HO
O
COOH
HN
HO
N3
O
OH OH
5
1H NMR
4.79 D2O
I (dd) 4.13
G (s) 4.24
H (s) 4.22
M (dd) 3.89
O (d) 3.45
C (d) 6.00
A (dd) 7.98
B (d) 6.13
D (d) 4.35
K (t) 4.03
N (dd) 3.63
P (dd) 2.51
Q (ddd) 1.67
E (t) 4.31
L (ddd) 3.95
F (s) 4.26
J (s) 4.10
f1 (ppm)

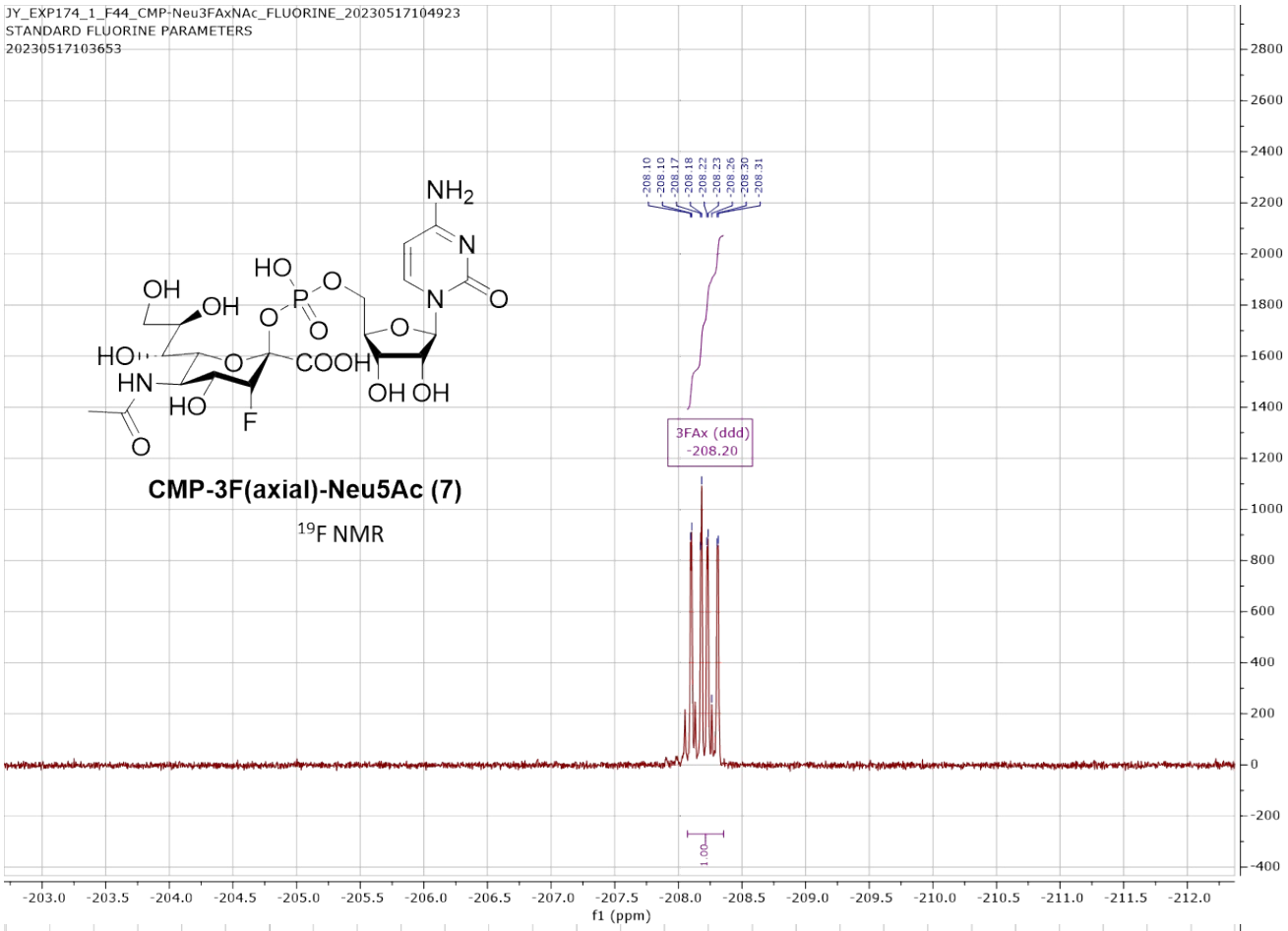

CMP-Neu5Ac-Carbon_20210729192328
20210729141554
NH2
N
HO
O
P
OH
OH
HO
O
COOH
HN
HO
N3
O
OH OH
5
13C NMR
f1 (ppm)

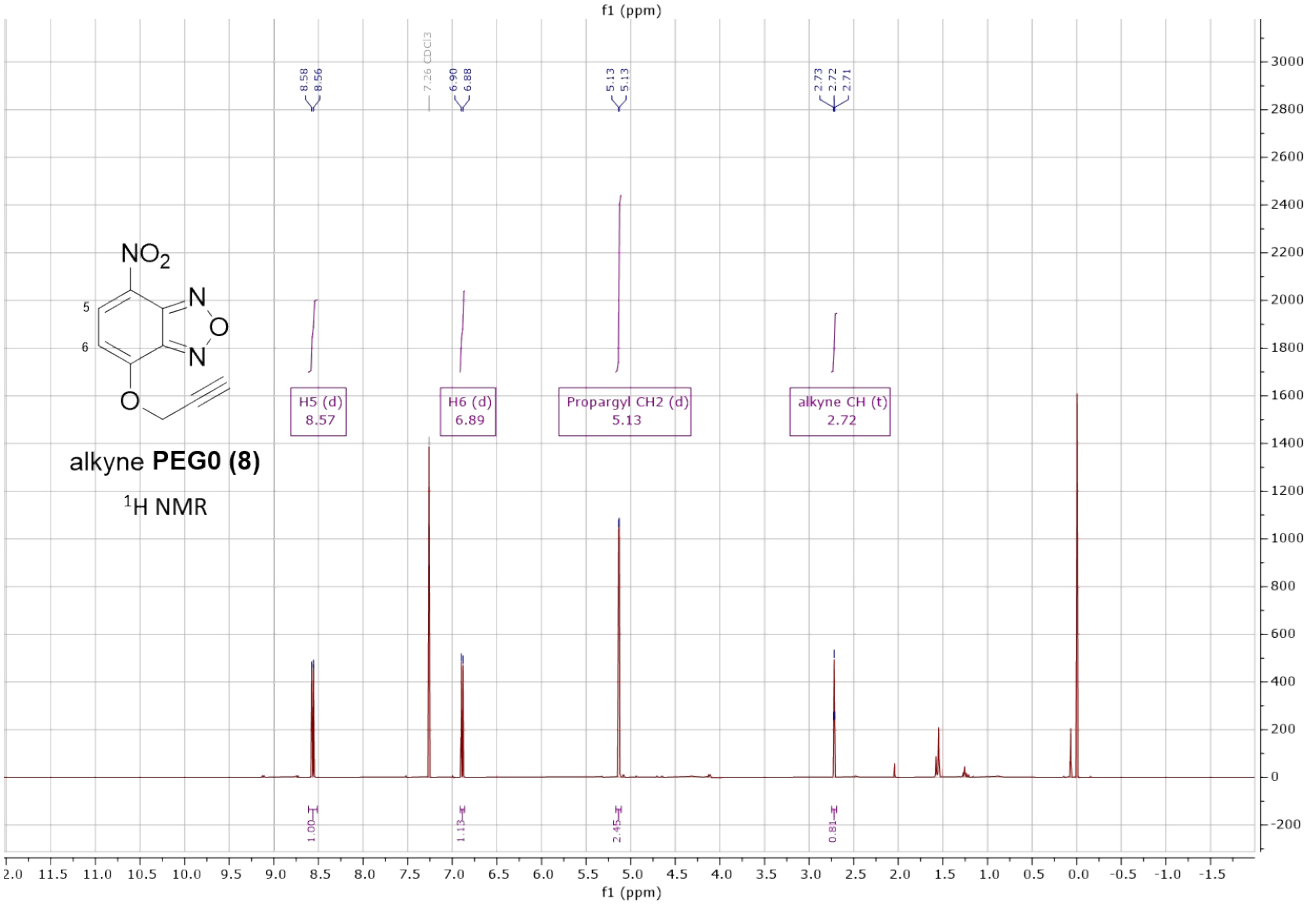

5 HSQC NMR
f2 (ppm)
f1 (ppm)

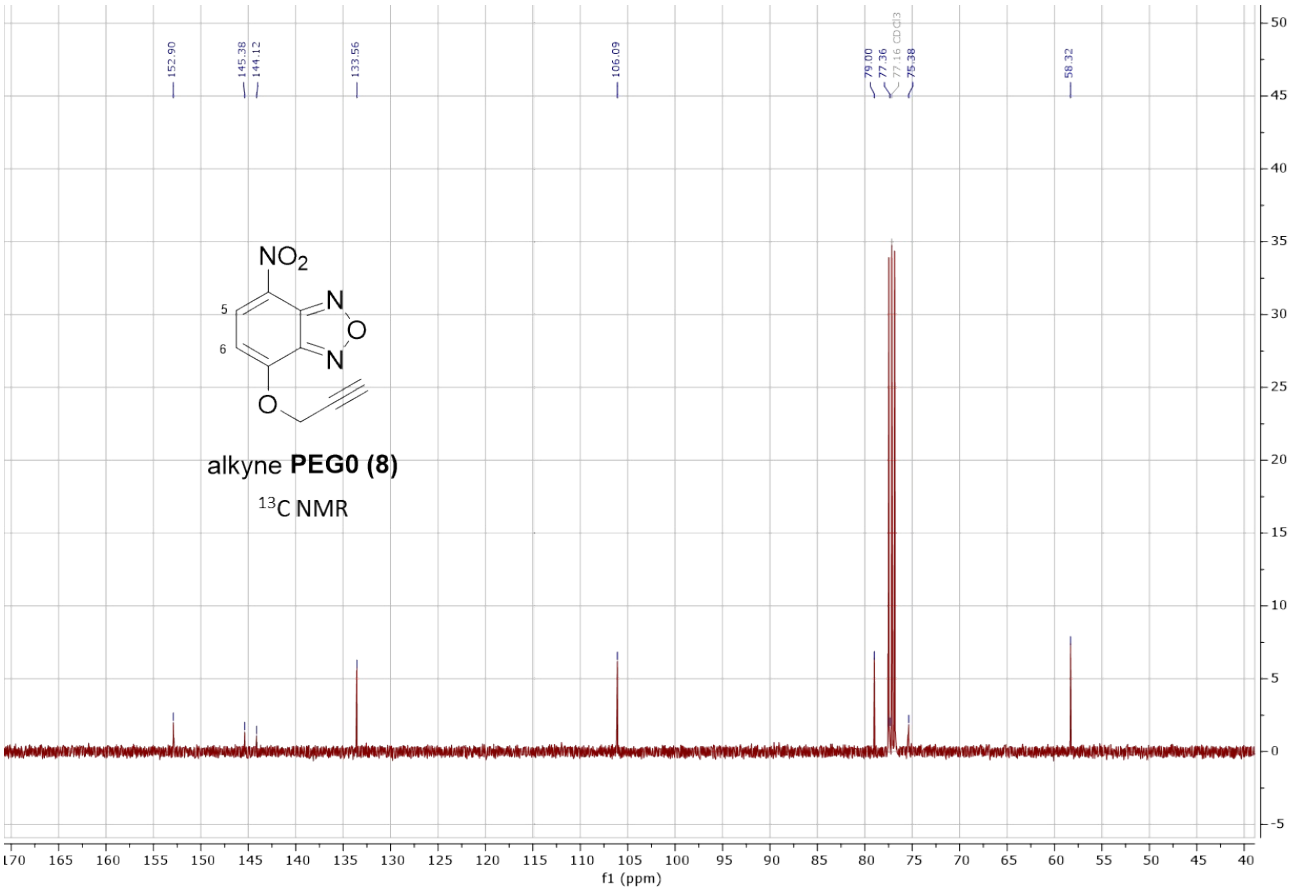

JY_EXP174_1_f44_CMP-Neu3FAxNAc.11.fid
PROTON D2O {C:\nmrdata\CBDD} Jun 55
CMP-3F(axial)-Neu5Ac (7)
1H NMR
f1 (ppm)

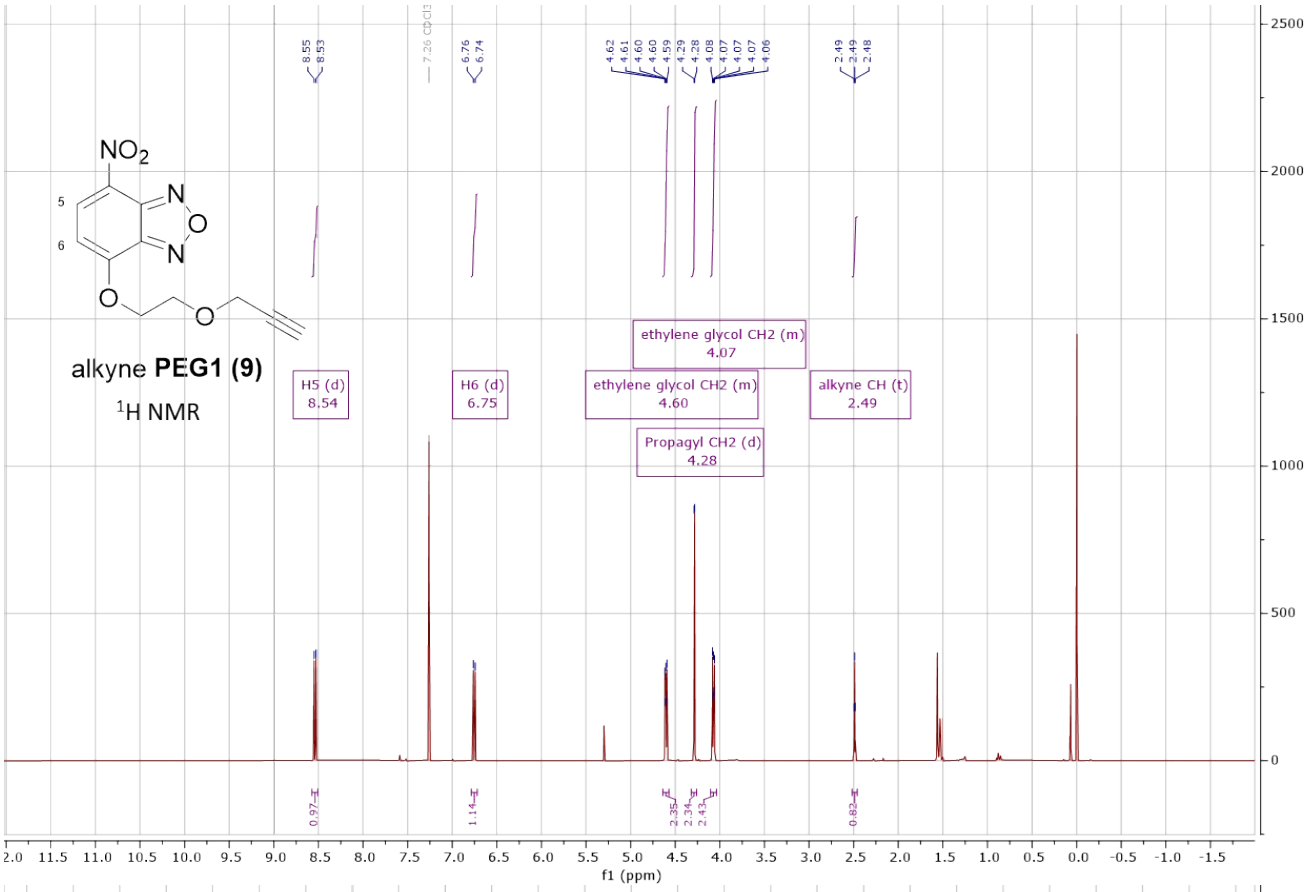

JY_EXP174_1_f44_CMP-Neu3FAxNAc.18.fid
CMC_13C D2O {C:\nmrdata\CBDD} Jun 55
174.76
171.21
163.43
162.41
143.10
95.91
89.17
83.16
74.35
71.70
69.70
69.23
68.71
68.03
67.91
65.01
62.89
59.30
46.86
22.07
CMP-3F(axial)-Neu5Ac (7)
13C NMR
f1 (ppm)

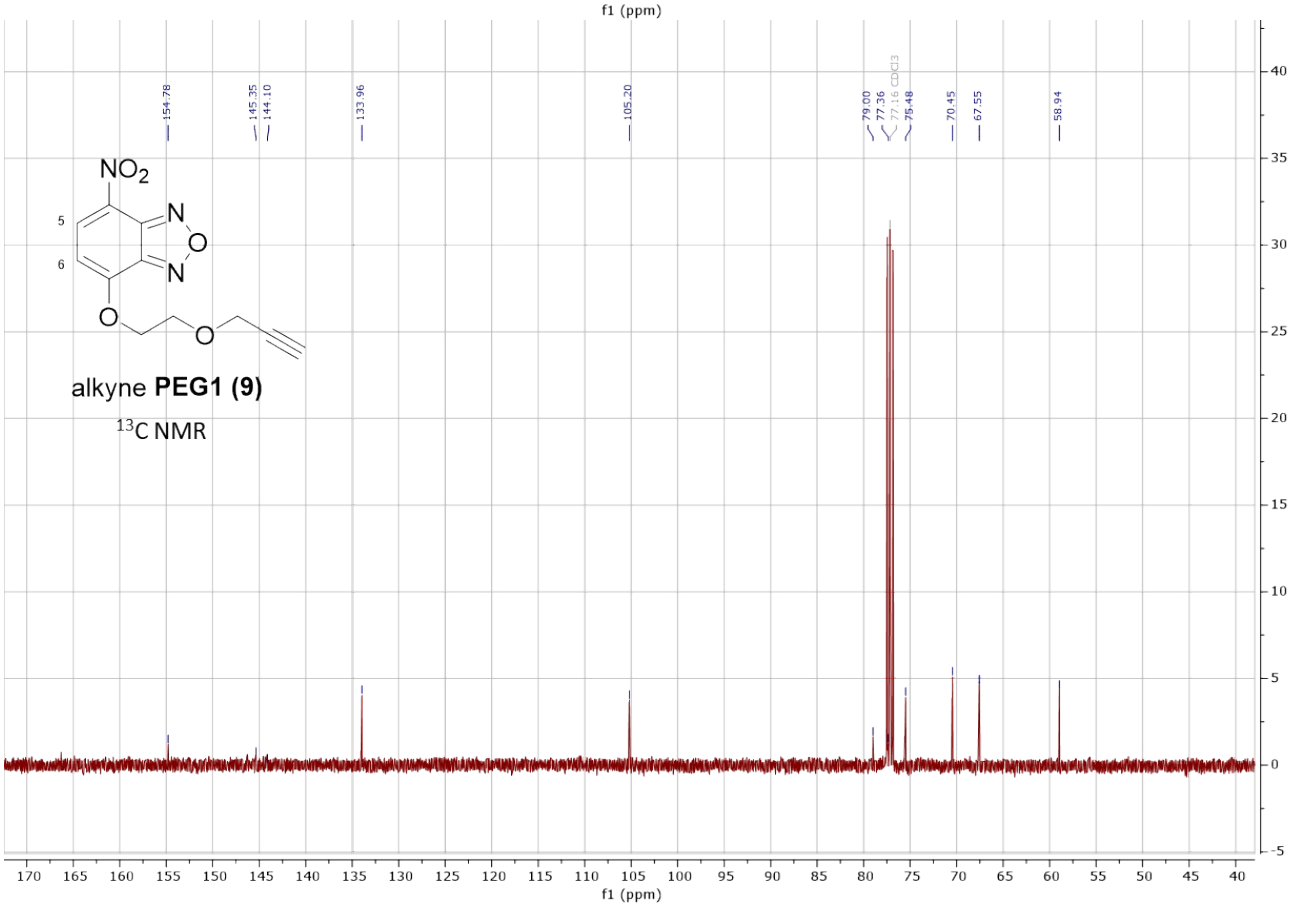

JY_EXP174_1_f44_CMP-Neu3FAxNAc.16.ser
HSQCEDETGPSISP_ADIA D2O {C:\nmrdata\CBDD} Jun 55
{2.06,22.22}NHAc
{4.33,46.88}Sia H5
{3.74,59.45}Tris
{3.90,62.99}Sia H9'
{3.64,62.99}Sia H9''
{4.29,65.09}ribose H5'
{4.33,69.28}ribose H3
{3.98,69.76}Sia H7
{4.18,68.15}Sia H4
{3.48,68.79}Sia H8
{4.33,74.43}ribose H2
{4.18,71.85}Sia H6
{4.27,83.30}ribose H4
{8.11,143.25}cytidine H6
{5.98,89.26}ribose H1
{4.88,91.03}Sia H2
{4.96,89.90}Sia H3
{6.24,95.87}cytidine H5
CMP-3F(axial)-Neu5Ac (7)
HSQC NMR
f2 (ppm)
f1 (ppm)

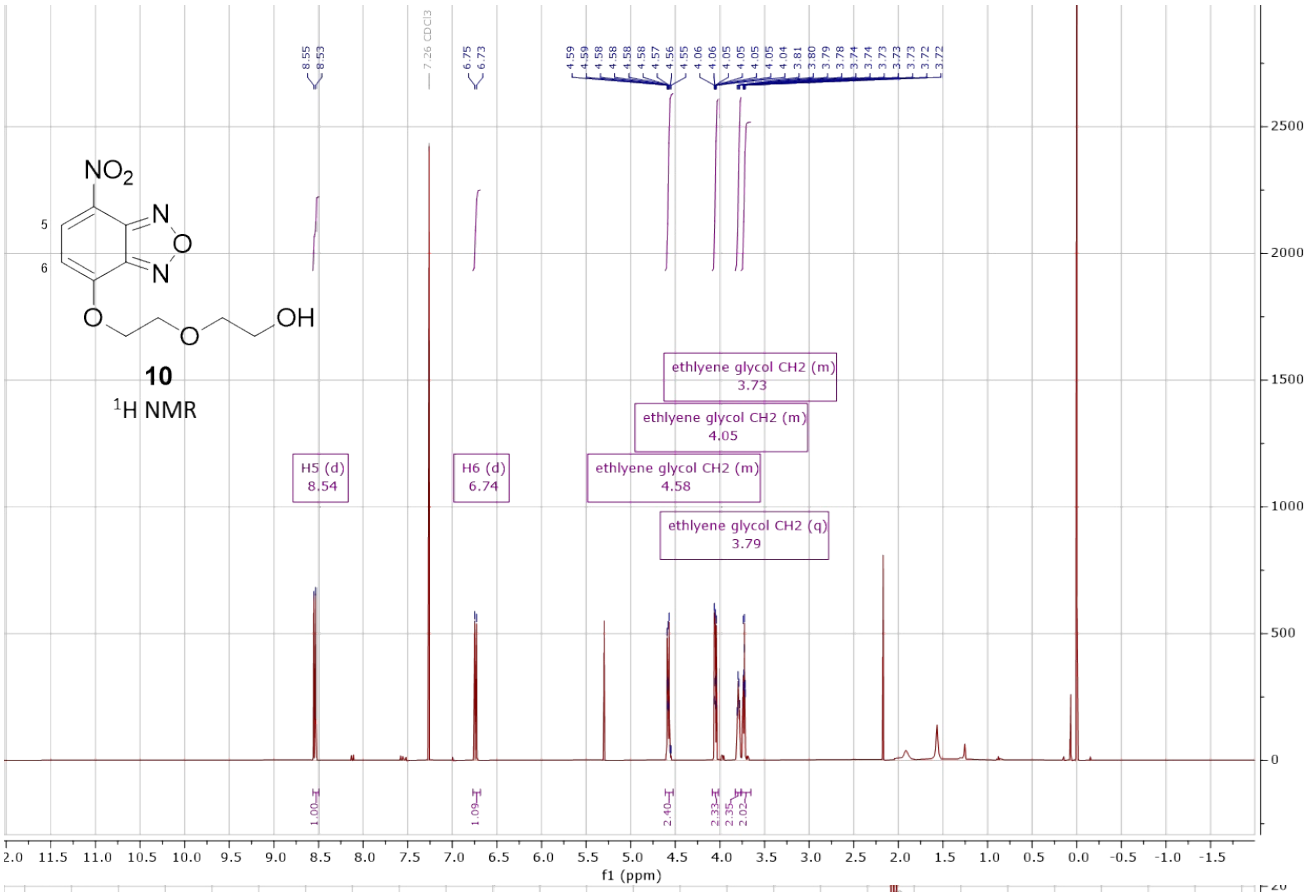

JY_EXP174_1_F44_CMP-Neu3FAxNAc_FLUORINE_20230517104923
STANDARD FLUORINE PARAMETERS
20230517103653
3FAx (ddd)
-208.20
CMP-3F(axial)-Neu5Ac (7)
19F NMR
f1 (ppm)

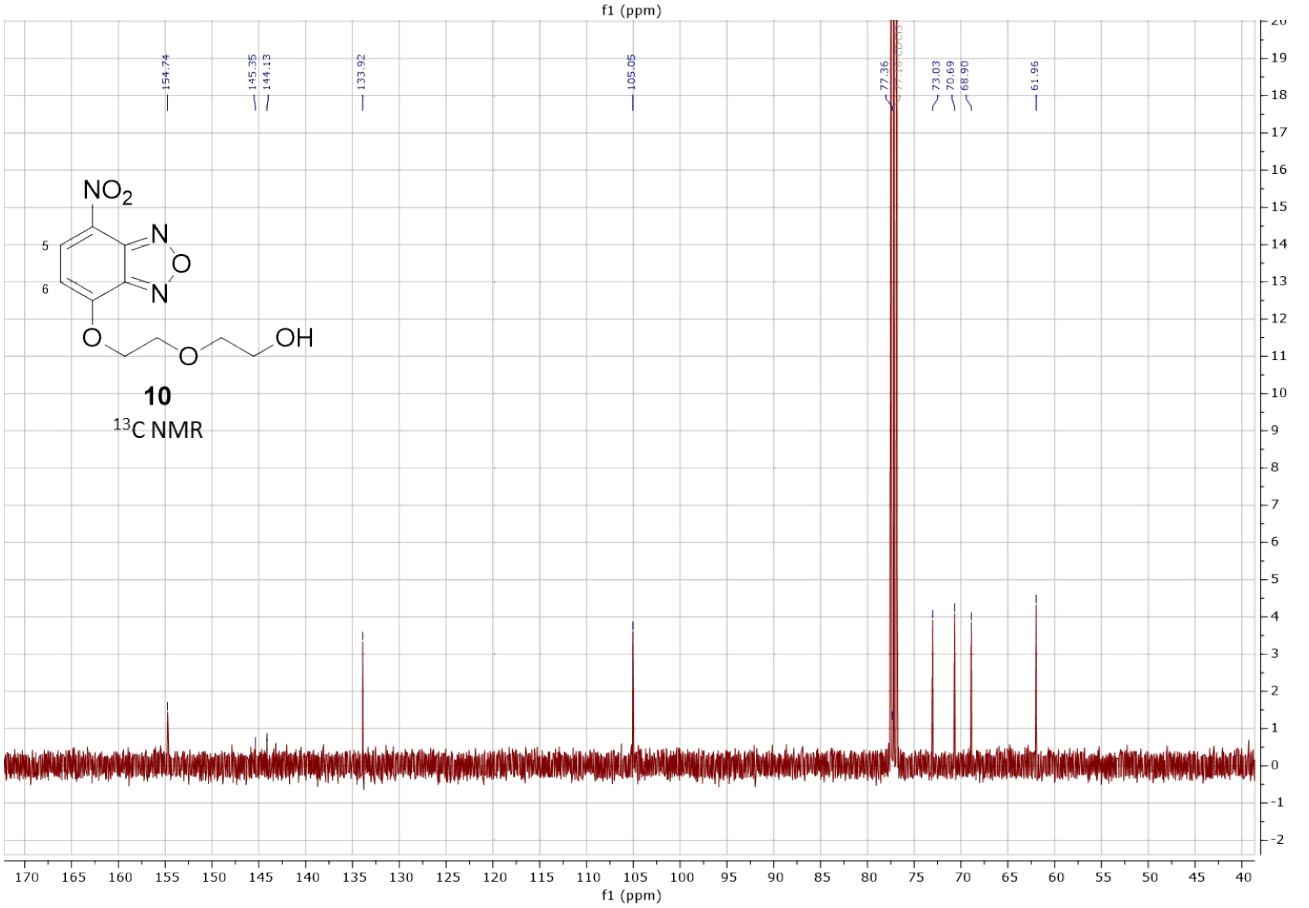

H5 (d)
8.57
H6 (d)
6.89
Propargyl CH2 (d)
5.13
alkyne CH (t)
2.72
alkyne PEG0 (8)
1H NMR
f1 (ppm)

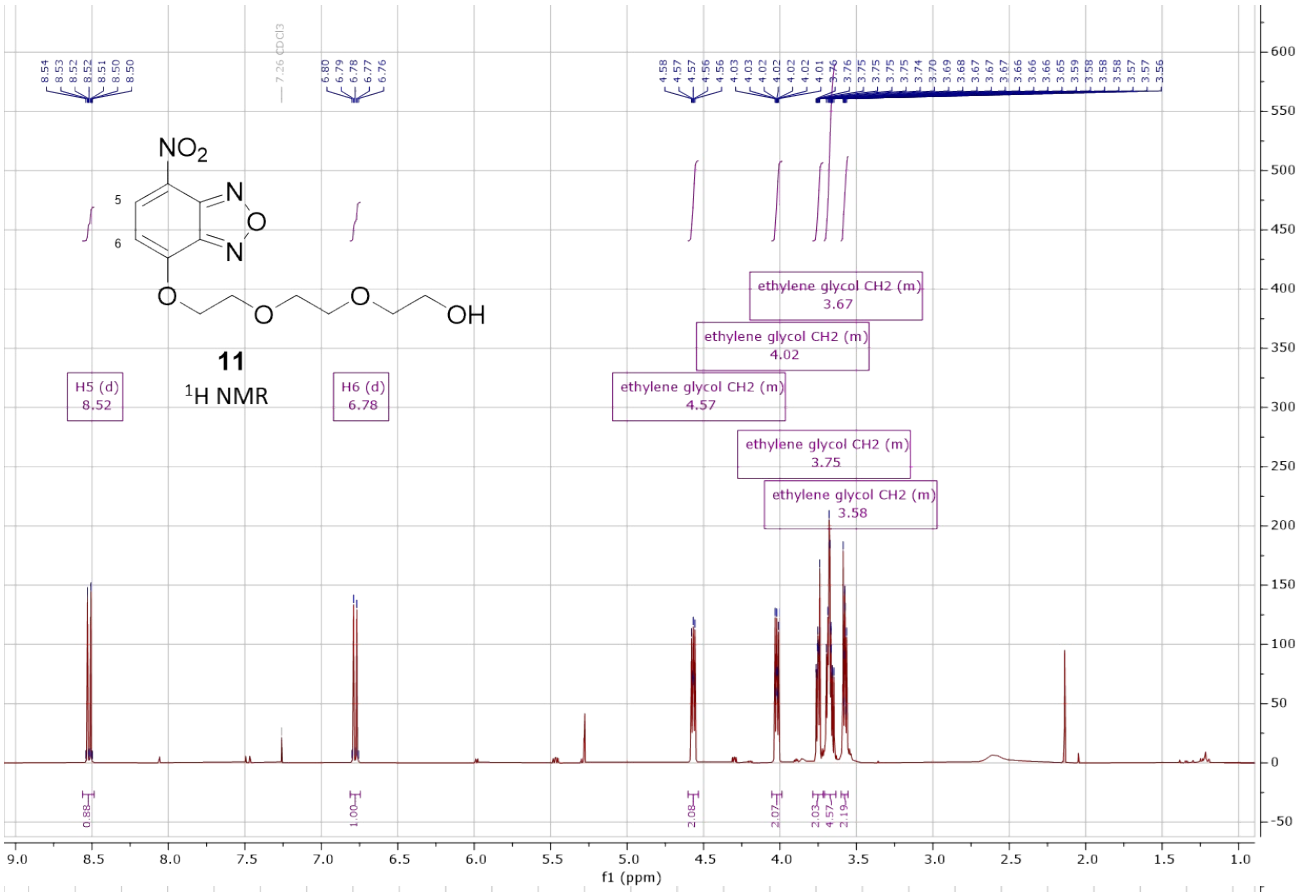

152.90
145.38
144.12
133.56
106.09
79.00
77.36
77.16 CDCl3
75.38
58.32
NO2
N
O
N
O
5
6
alkyne PEG0 (8)
13C NMR
f1 (ppm)

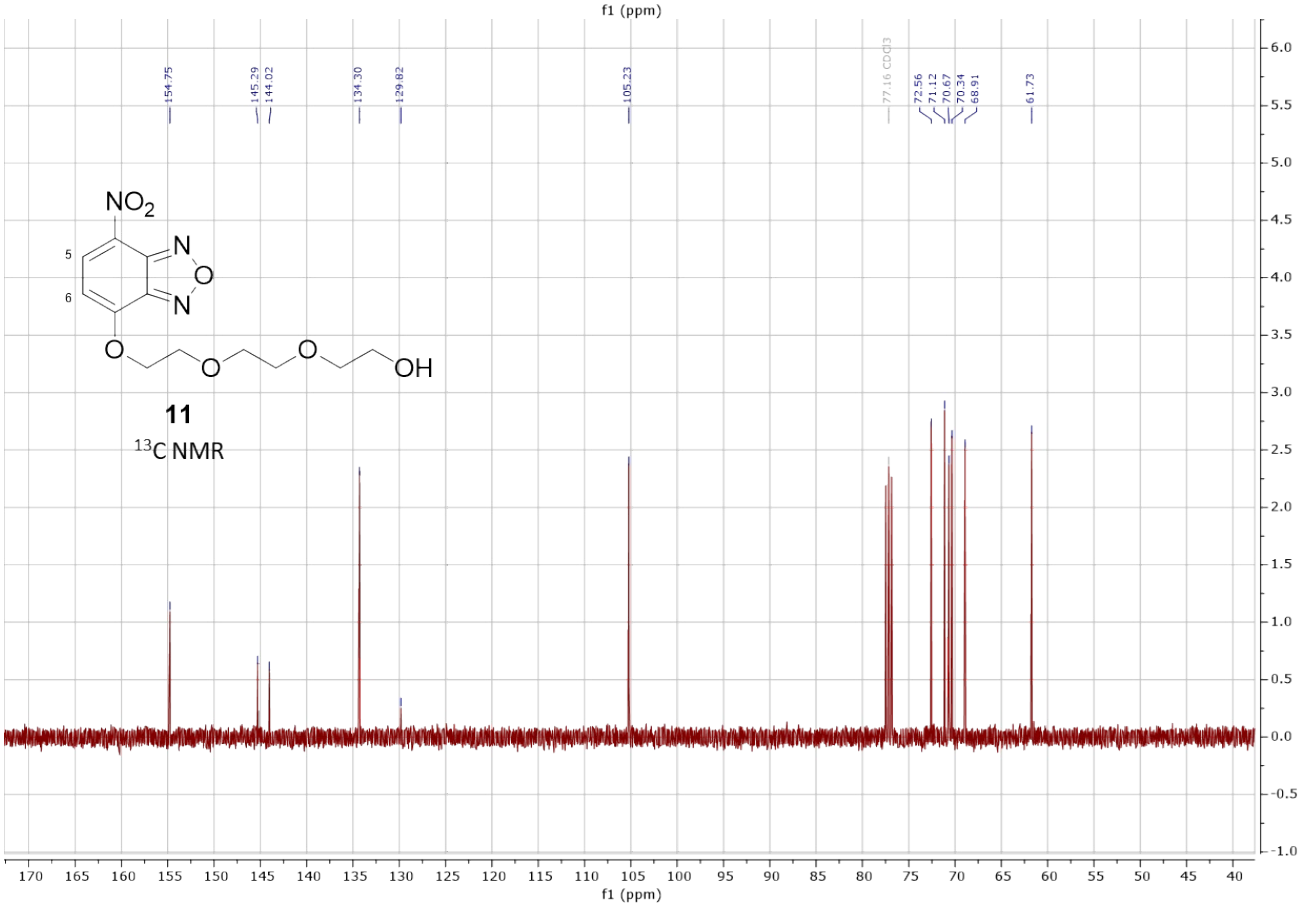

alkyne PEG1 (9)
1H NMR
H5 (d)
8.54
H6 (d)
6.75
ethylene glycol CH2 (m)
4.60
ethylene glycol CH2 (m)
4.07
Propagyl CH2 (d)
4.28
alkyne CH (t)
2.49
f1 (ppm)

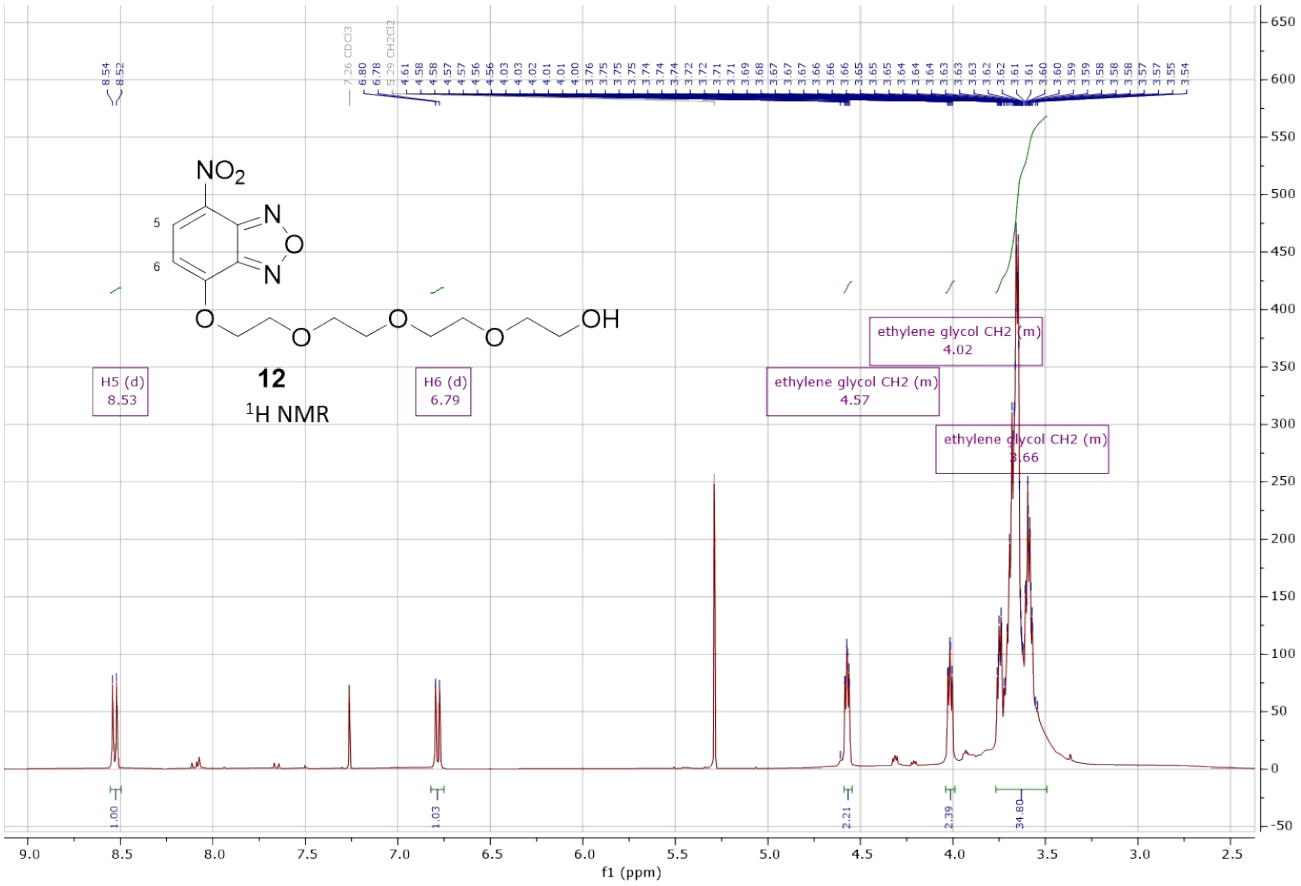

alkyne PEG1 (9)
13C NMR
f1 (ppm)

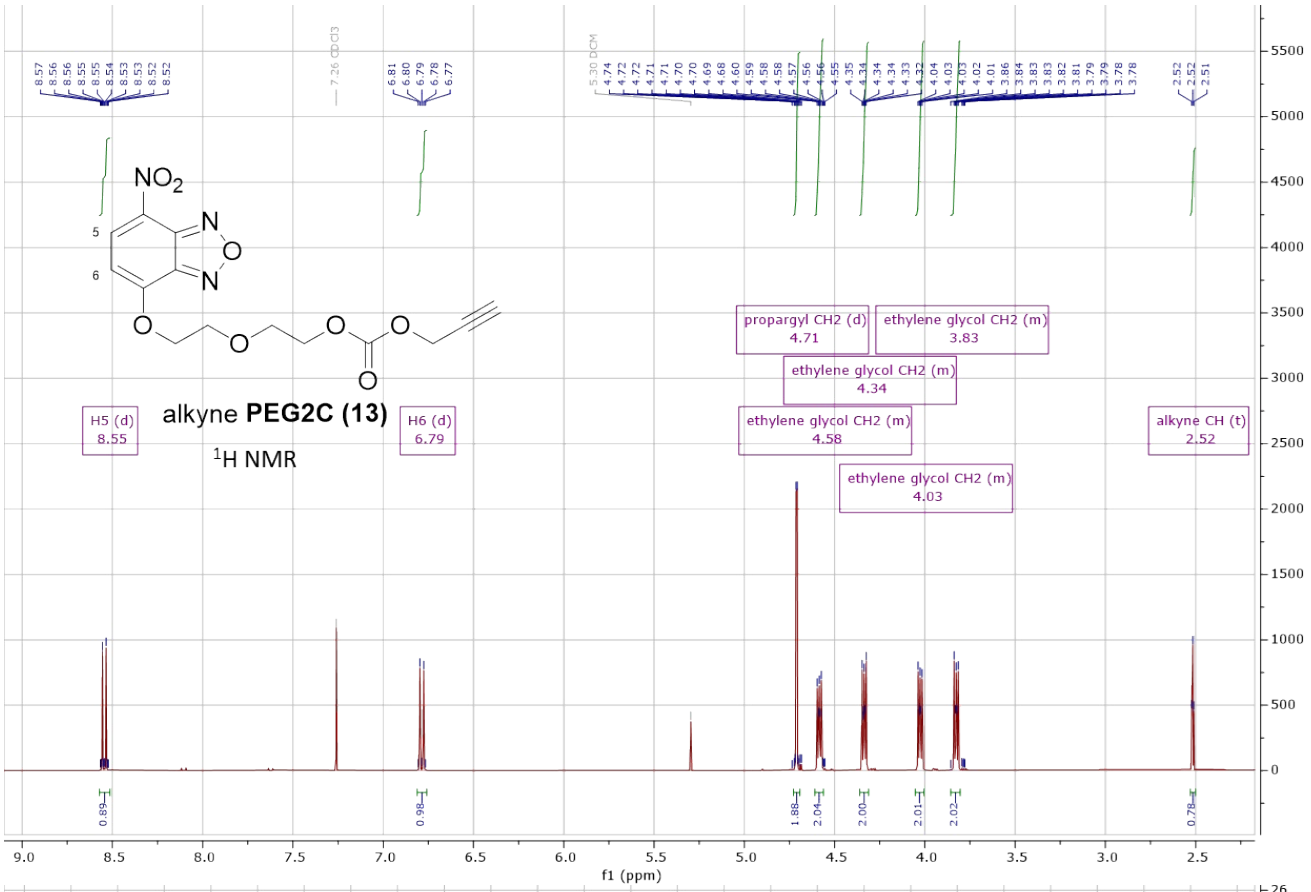

NO2
N
O
N
5
6
O
O
OH
10
1H NMR
ethlyene glycol CH2 (m)
3.73
ethlyene glycol CH2 (m)
4.05
H5 (d)
8.54
H6 (d)
6.74
ethlyene glycol CH2 (m)
4.58
ethlyene glycol CH2 (q)
3.79
f1 (ppm)

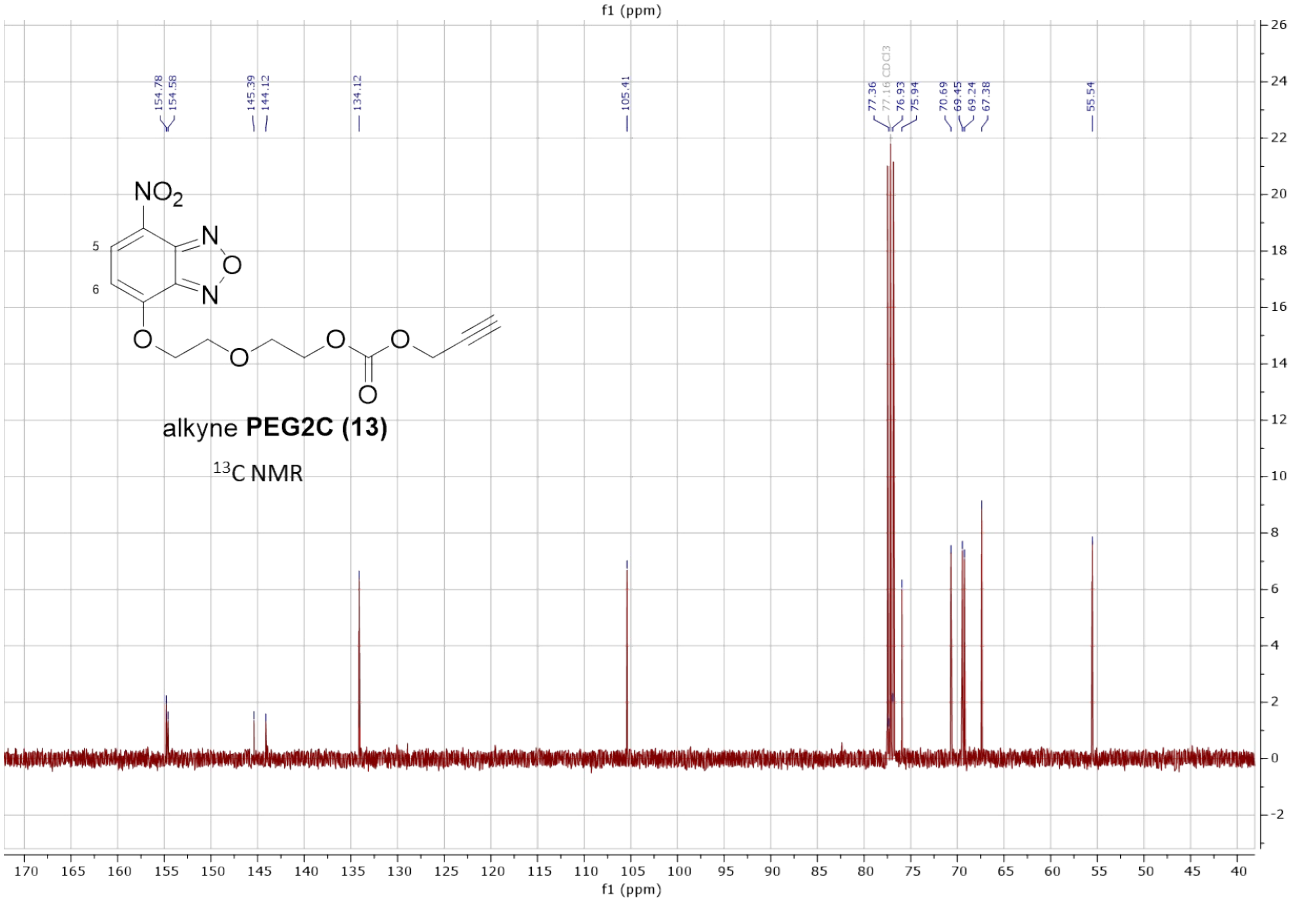

NO2
N
O
N
5
6
O
O
OH
10
13C NMR
f1 (ppm)

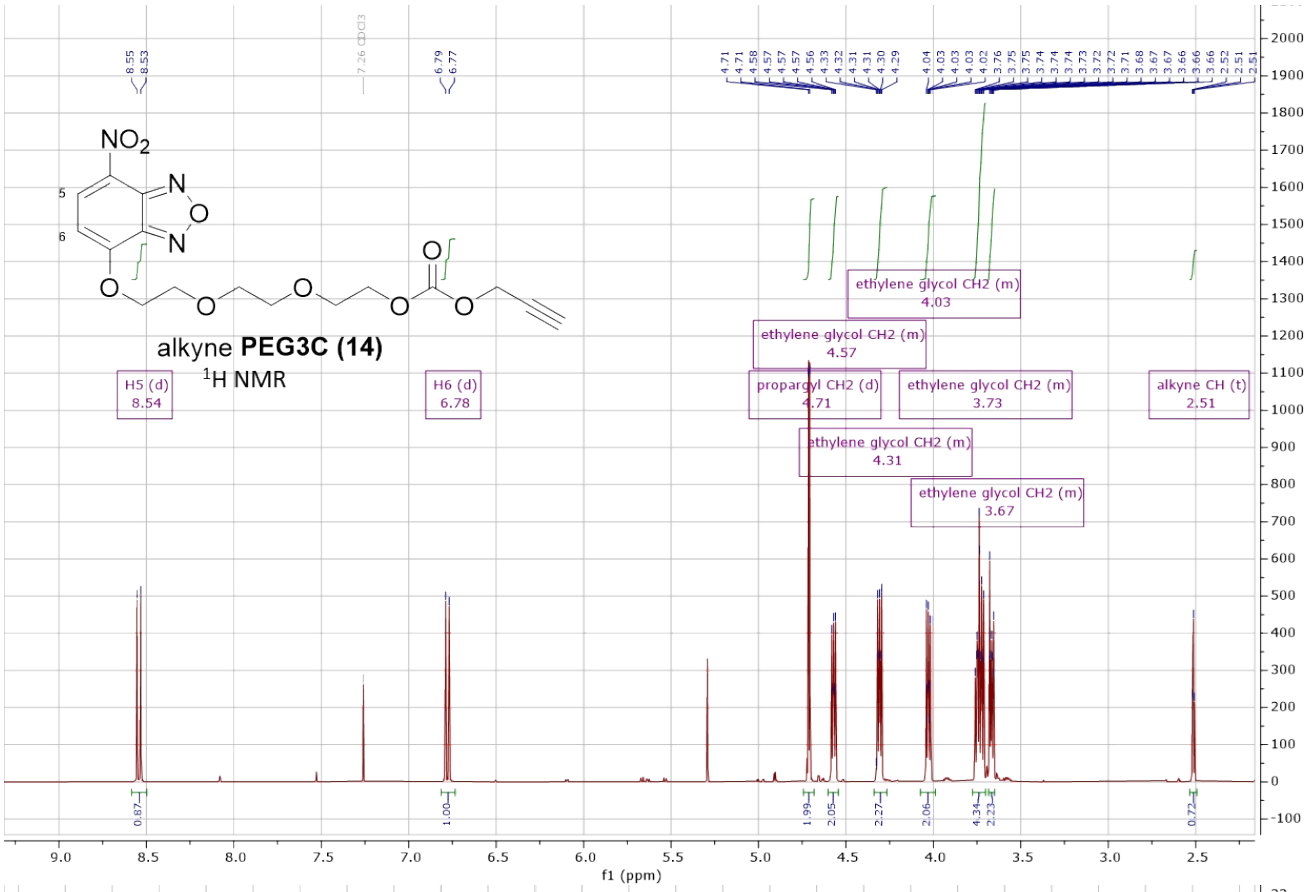

NO2
N
O
N
5
6
O
O
O
OH
11
1H NMR
H5 (d)
8,52
H6 (d)
6.78
ethylene glycol CH2 (m)
3.67
ethylene glycol CH2 (m)
4.02
ethylene glycol CH2 (m)
4.57
ethylene glycol CH2 (m)
3.75
ethylene glycol CH2 (m)
3.58
f1 (ppm)

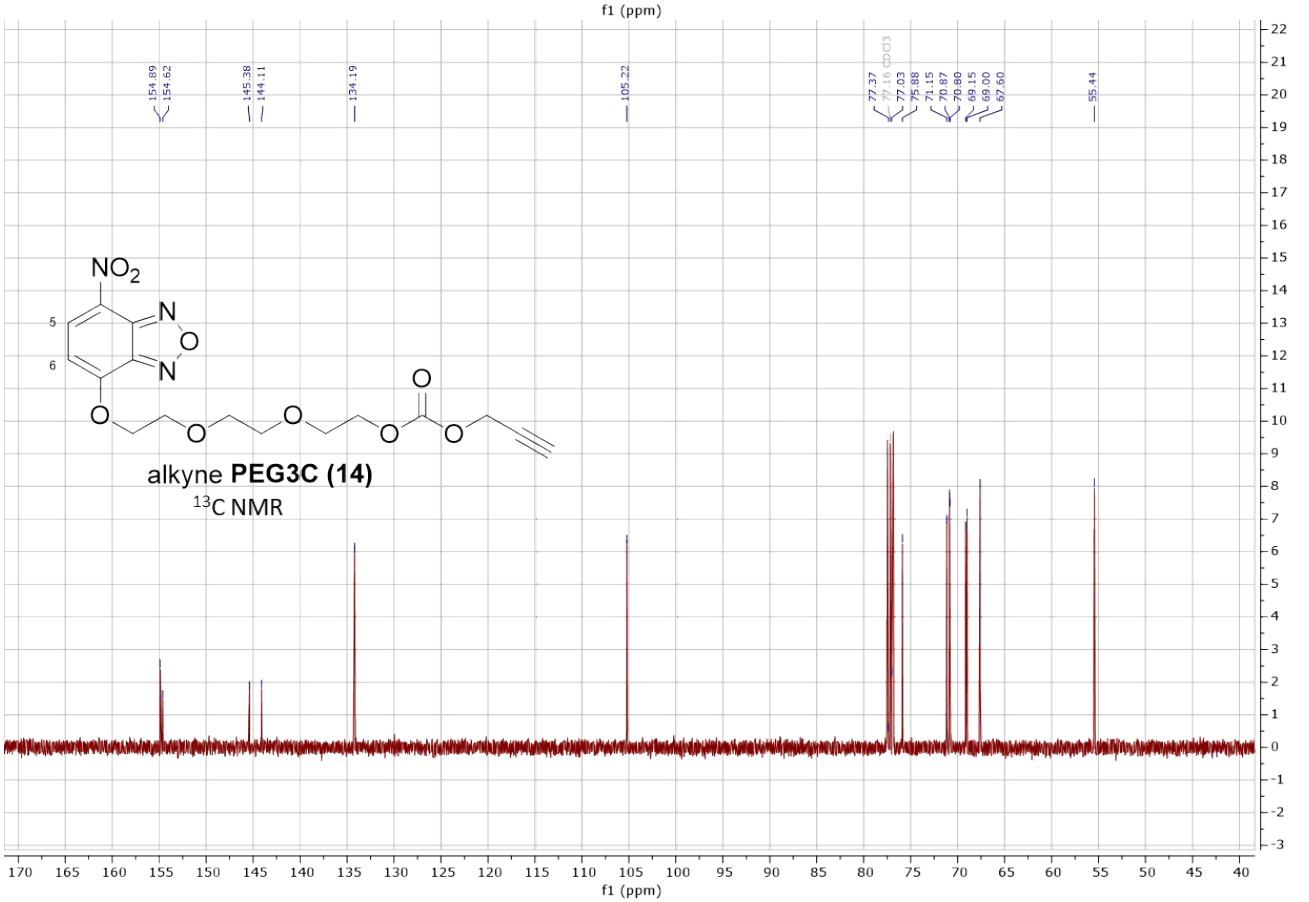

NO2
N
O
N
5
6
O
O
O
OH
11
13C NMR
f1 (ppm)

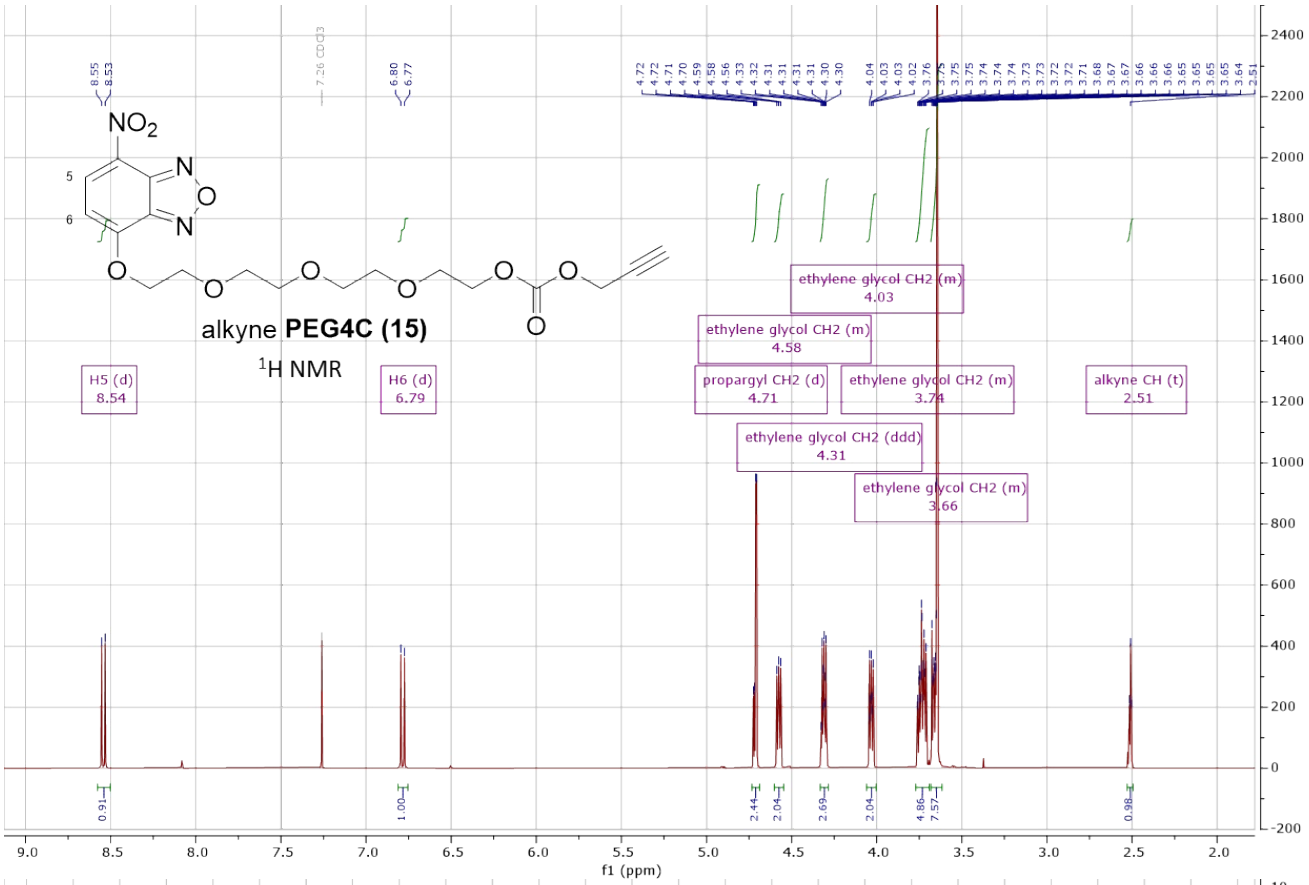

NO2
N
O
N
5
6
O
O
O
O
OH
12
1H NMR
H5 (d)
8.53
H6 (d)
6.79
ethylene glycol CH2 (m)
4.57
ethylene glycol CH2 (m)
4.02
ethylene glycol CH2 (m)
3.66
1.00
1.03
2.21
2.39
34.80
f1 (ppm)

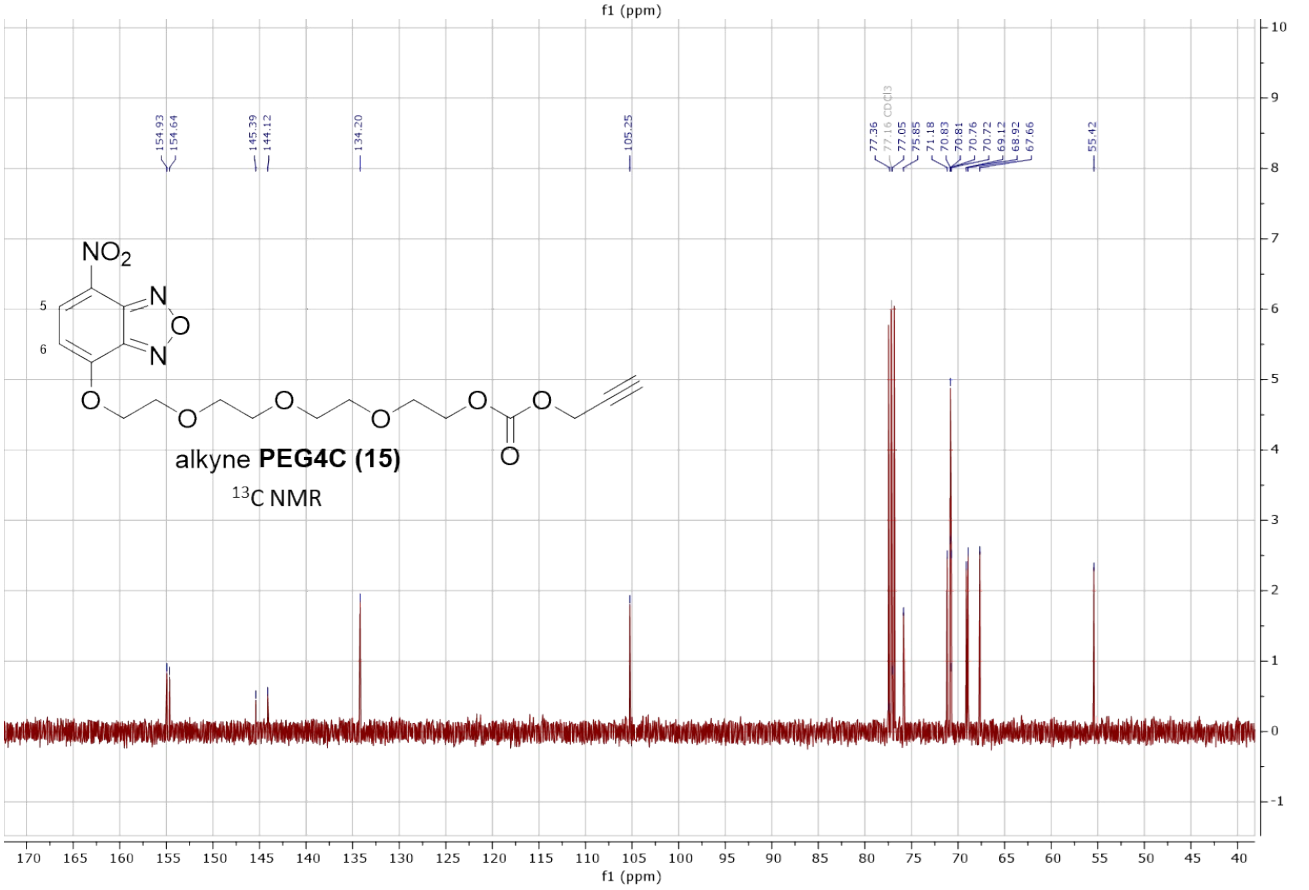

alkyne PEG2C (13)
1H NMR
H5 (d) 8.55
H6 (d) 6.79
propargyl CH2 (d) 4.71
ethylene glycol CH2 (m) 3.83
ethylene glycol CH2 (m) 4.34
ethylene glycol CH2 (m) 4.58
ethylene glycol CH2 (m) 4.03
alkyne CH (t) 2.52
f1 (ppm)

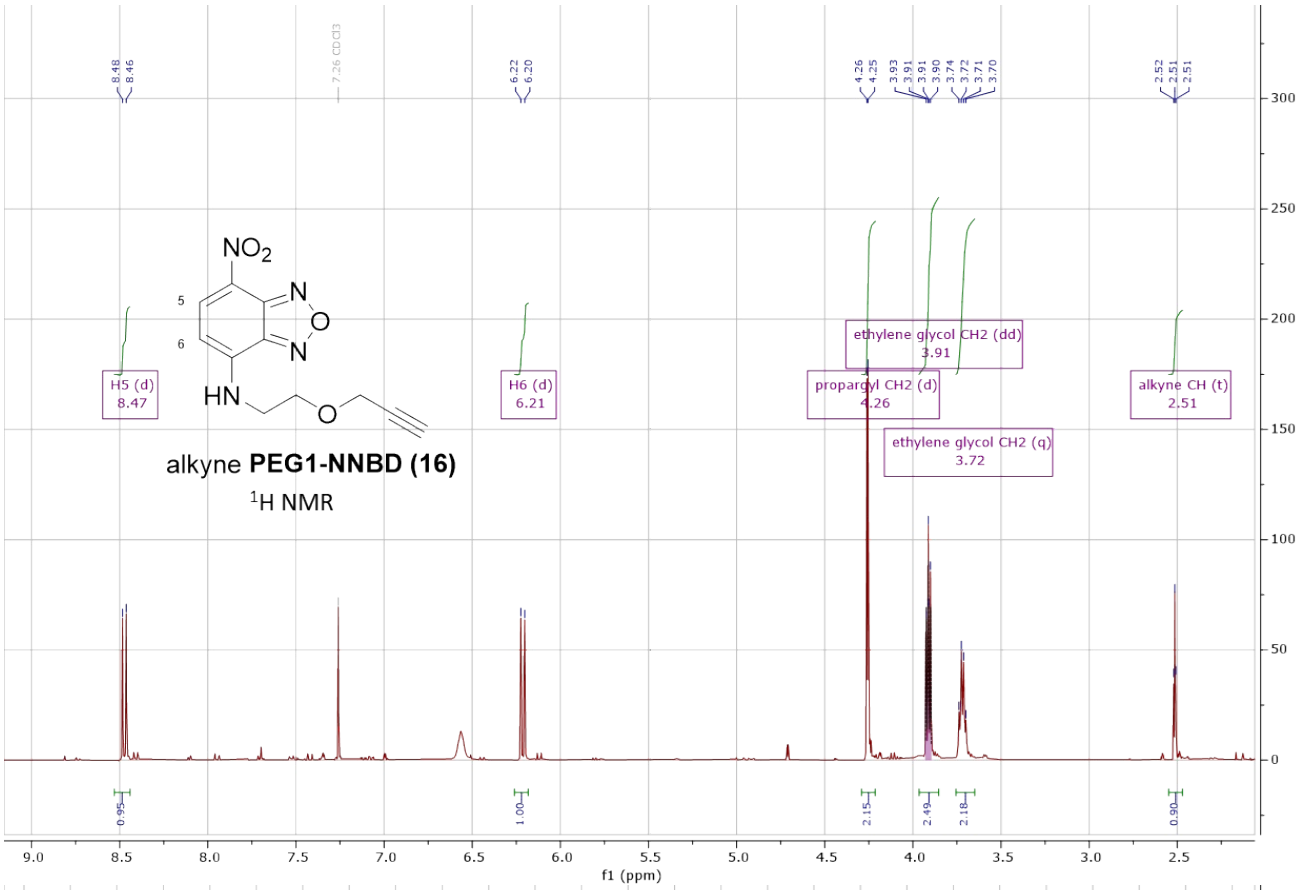

alkyne PEG2C (13)
13C NMR
f1 (ppm)

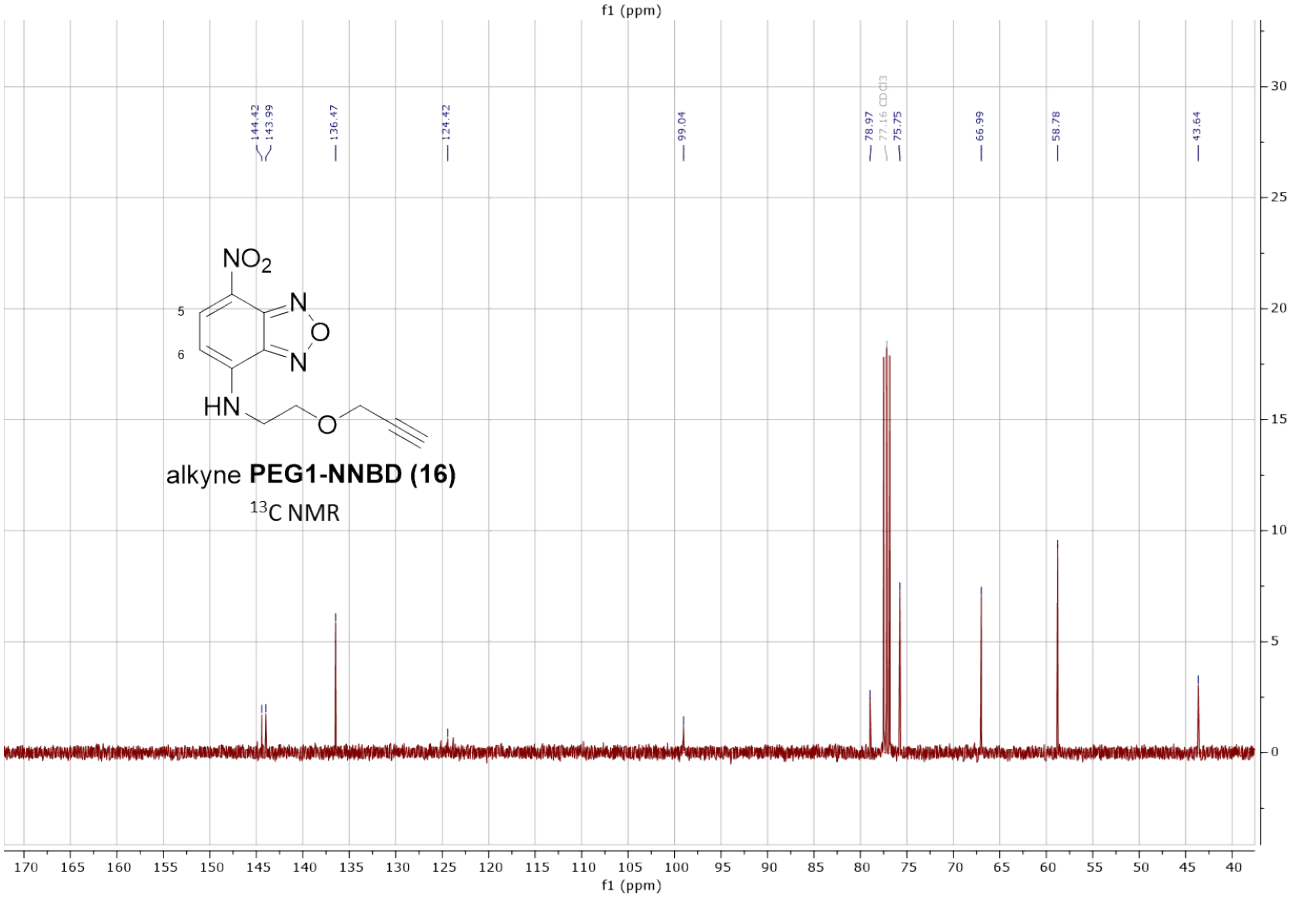

alkyne PEG3C (14)
1H NMR
H5 (d) 8.54
H6 (d) 6.78
propargyl CH2 (d) 4.71
ethylene glycol CH2 (m) 4.57
ethylene glycol CH2 (m) 4.03
ethylene glycol CH2 (m) 4.31
ethylene glycol CH2 (m) 3.73
ethylene glycol CH2 (m) 3.67
alkyne CH (t) 2.51
f1 (ppm)

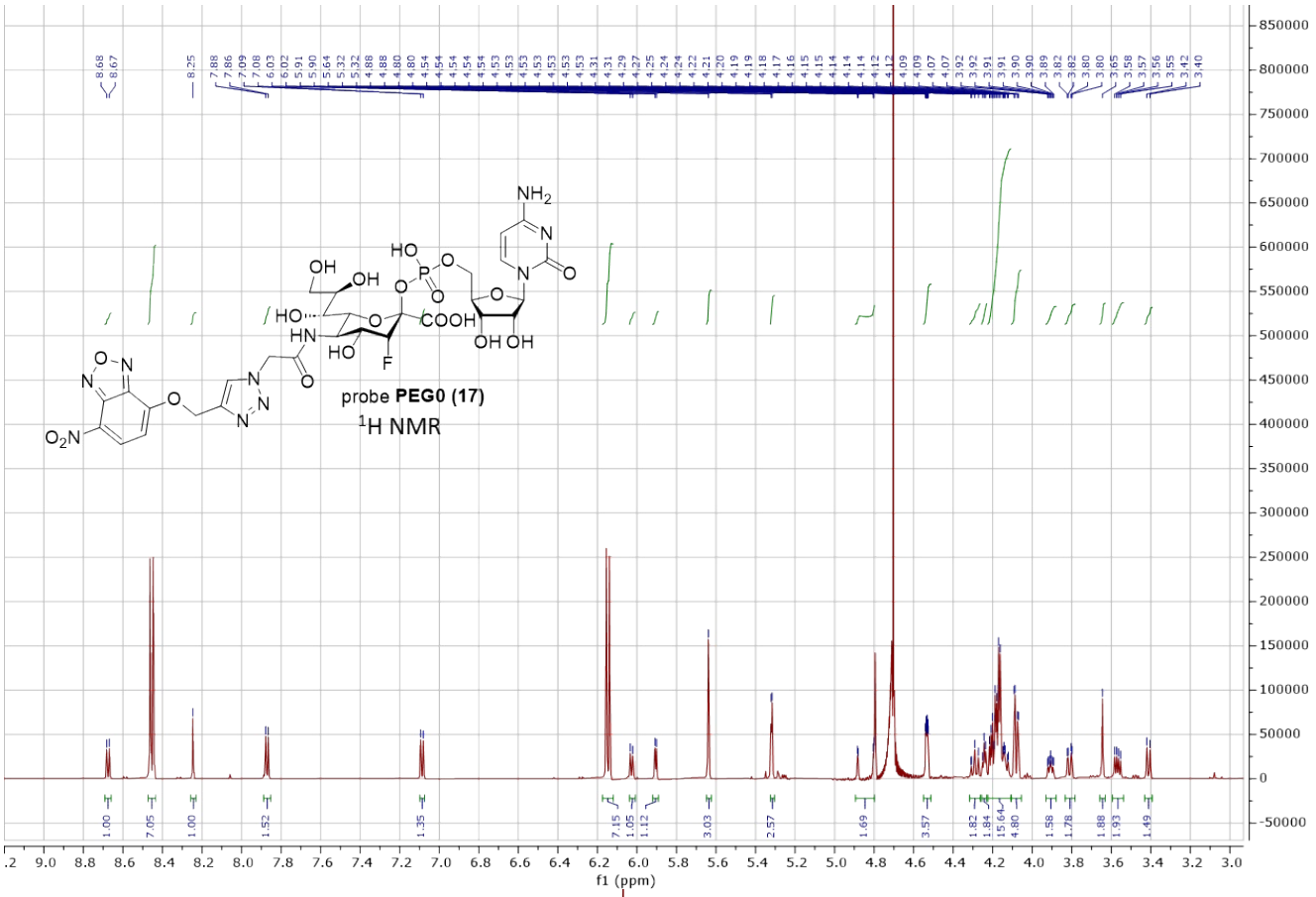

alkyne PEG3C (14)
13C NMR
f1 (ppm)

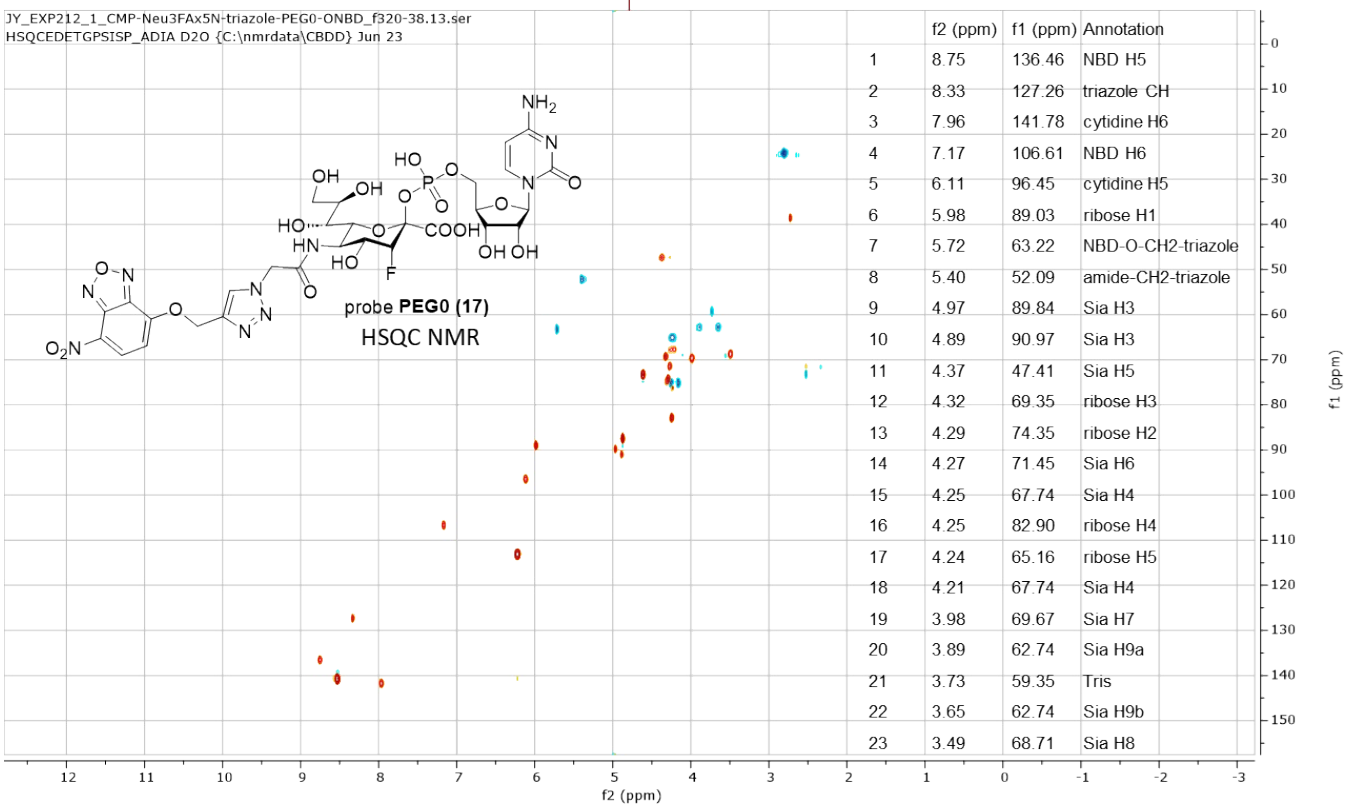

alkyne PEG4C (15)
1H NMR
H5 (d) 8.54
H6 (d) 6.79
ethylene glycol CH2 (m) 4.03
ethylene glycol CH2 (m) 4.58
propargyl CH2 (d) 4.71
ethylene glycol CH2 (m) 3.74
alkyne CH (t) 2.51
ethylene glycol CH2 (ddd) 4.31
ethylene glycol CH2 (m) 3.66
f1 (ppm)


alkyne PEG4C (15)
13C NMR
f1 (ppm)

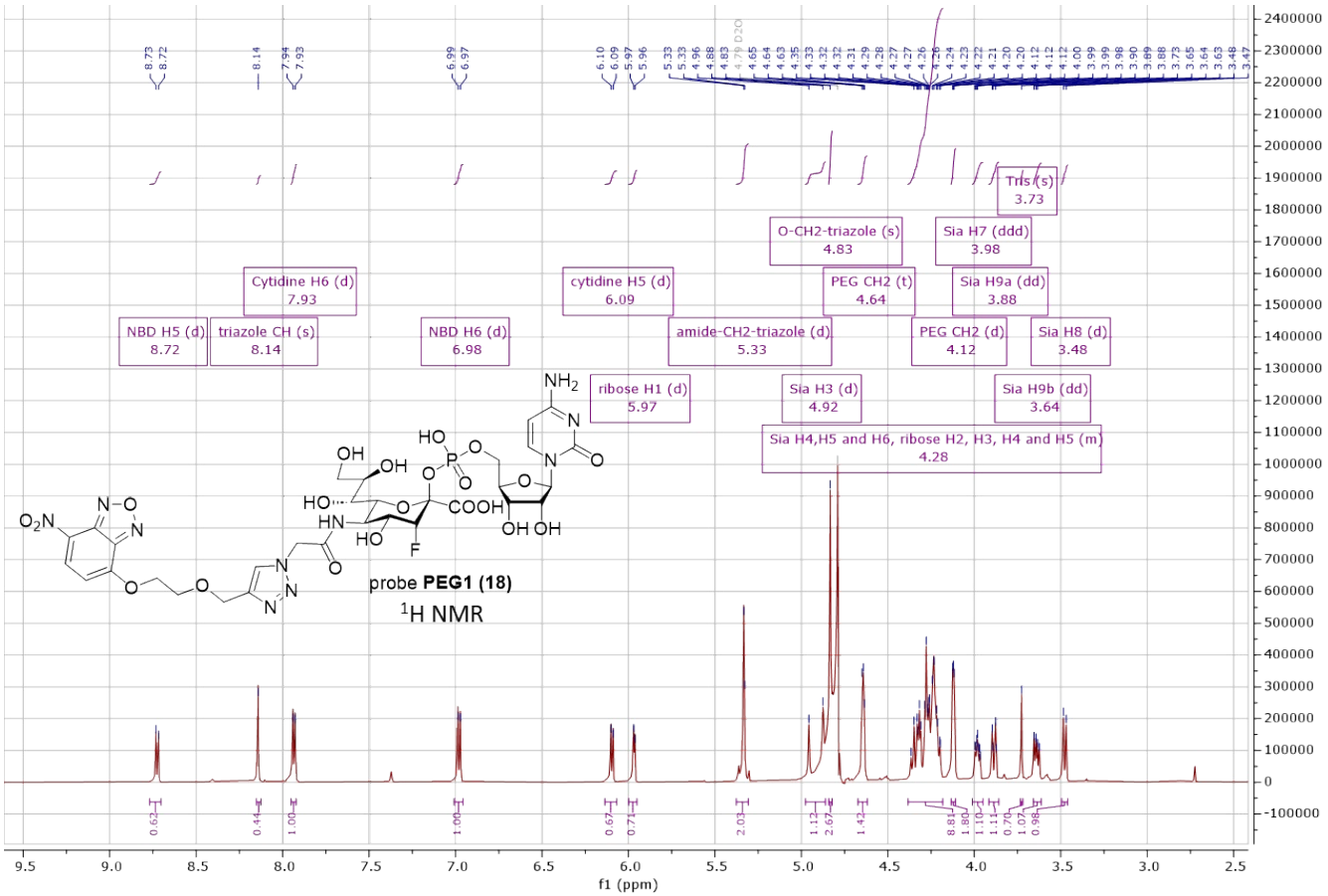

8.48
8.46
7.26 CDCl3
6.22
6.20
4.26
4.25
3.93
3.91
3.91
3.90
3.74
3.72
3.71
3.70
2.52
2.51
2.51
NO2
N
O
N
5
6
HN
O
H5 (d)
8.47
H6 (d)
6.21
ethylene glycol CH2 (dd)
3.91
propargyl CH2 (d)
4.26
ethylene glycol CH2 (q)
3.72
alkyne CH (t)
2.51
alkyne PEG1-NNBD (16)
1H NMR
0.99
1.00
2.15
2.49
2.18
0.90
f1 (ppm)

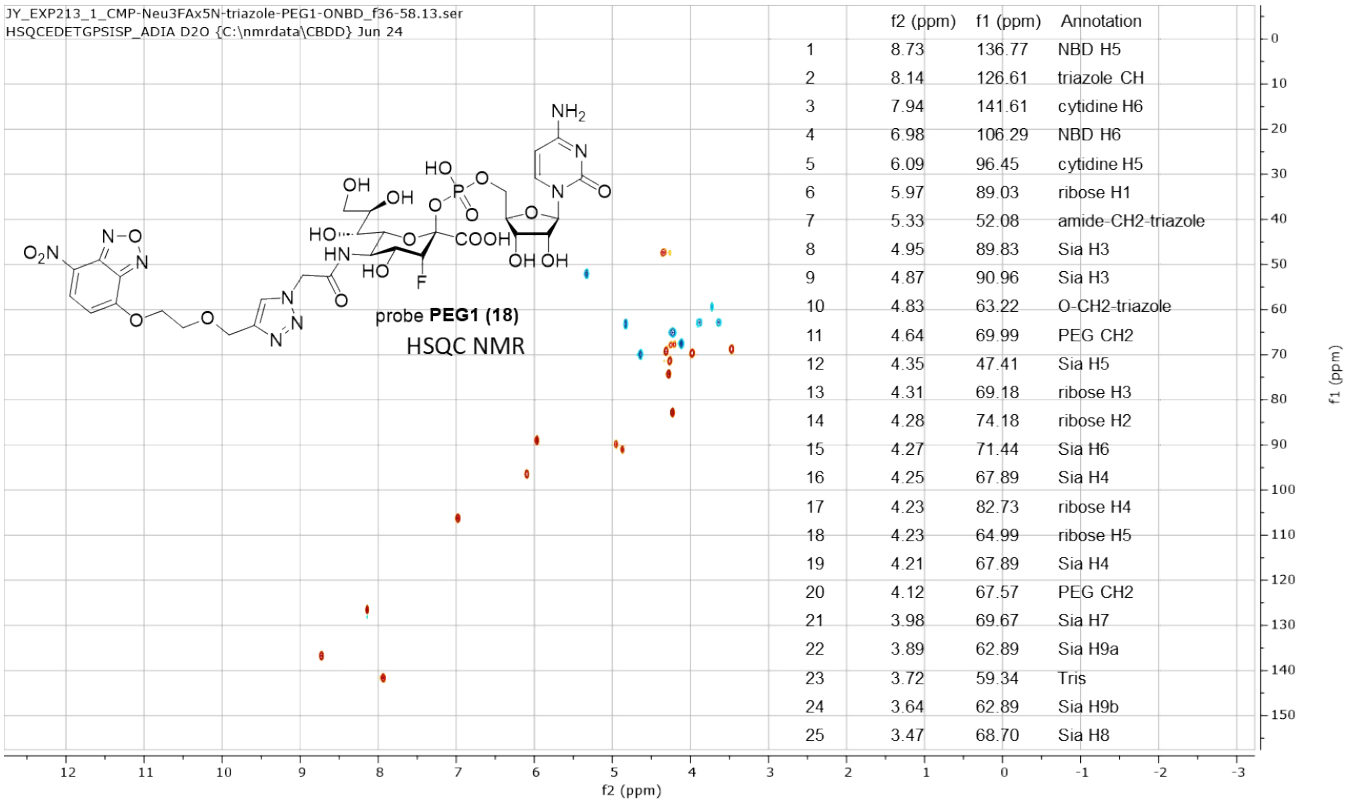

144.42
143.99
136.47
124.42
99.04
78.97
77.16 CDCl3
75.75
66.99
58.78
43.64
NO2
N
O
N
5
6
HN
O
alkyne PEG1-NNBD (16)
13C NMR
f1 (ppm)



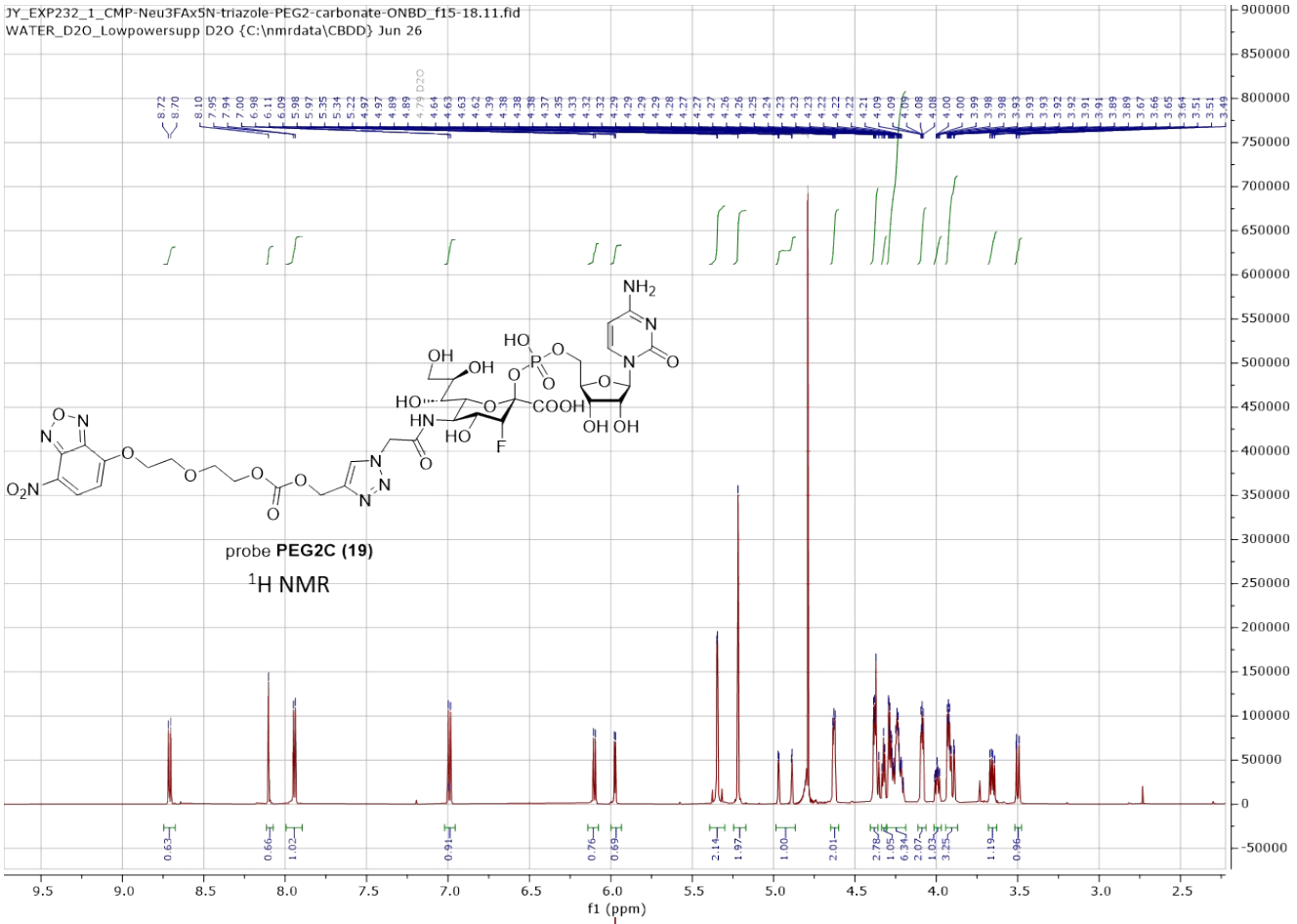


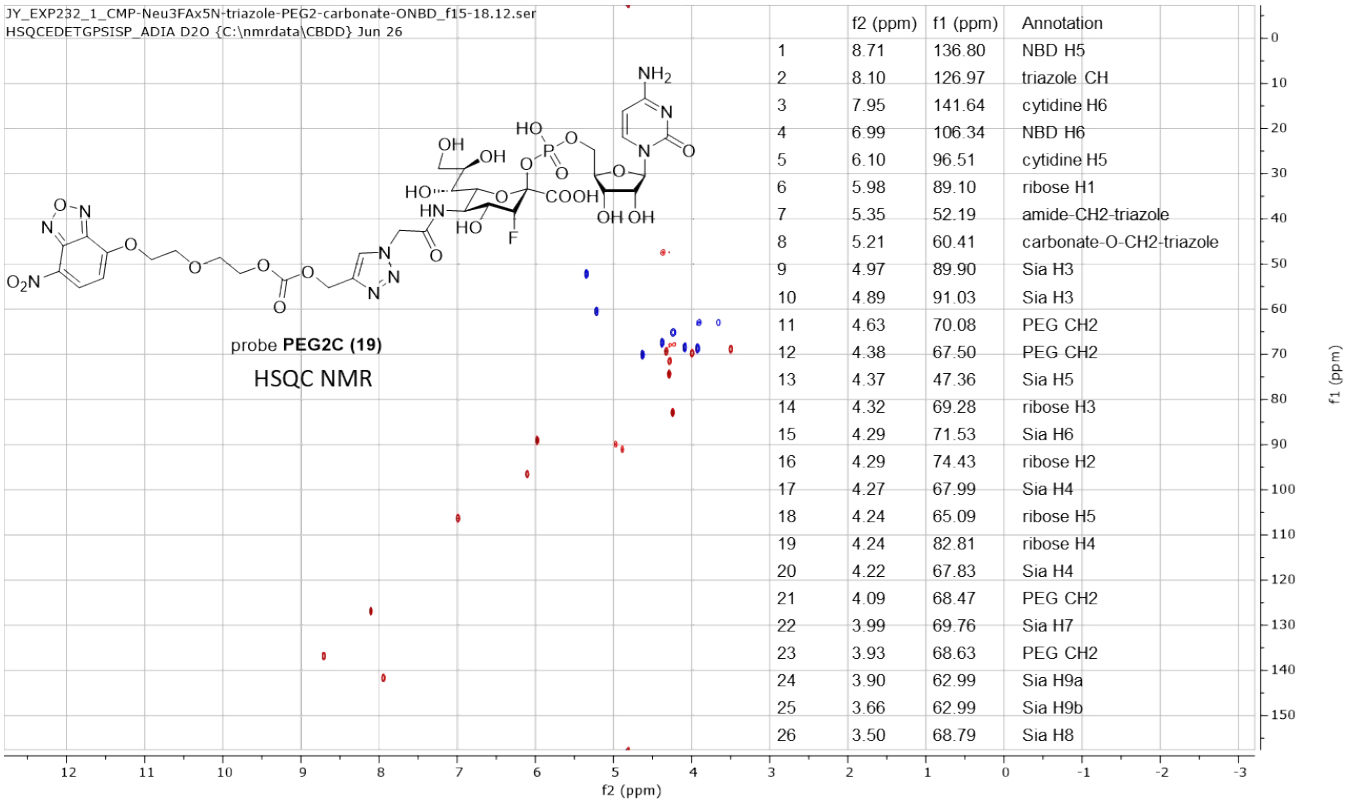
	f2 (ppm)	f1 (ppm)	Annotation
1	8.75	136.46	NBD H5
2	8.33	127.26	triazole CH
3	7.96	141.78	cytidine H6
4	7.17	106.61	NBD H6
5	6.11	96.45	cytidine H5
6	5.98	89.03	ribose H1
7	5.72	63.22	NBD-O-CH2-triazole
8	5.40	52.09	amide-CH2-triazole
9	4.97	89.84	Sia H3
10	4.89	90.97	Sia H3
11	4.37	47.41	Sia H5
12	4.32	69.35	ribose H3
13	4.29	74.35	ribose H2
14	4.27	71.45	Sia H6
15	4.25	67.74	Sia H4
16	4.25	82.90	ribose H4
17	4.24	65.16	ribose H5
18	4.21	67.74	Sia H4
19	3.98	69.67	Sia H7
20	3.89	62.74	Sia H9a
21	3.73	59.35	Tris
22	3.65	62.74	Sia H9b
23	3.49	68.71	Sia H8



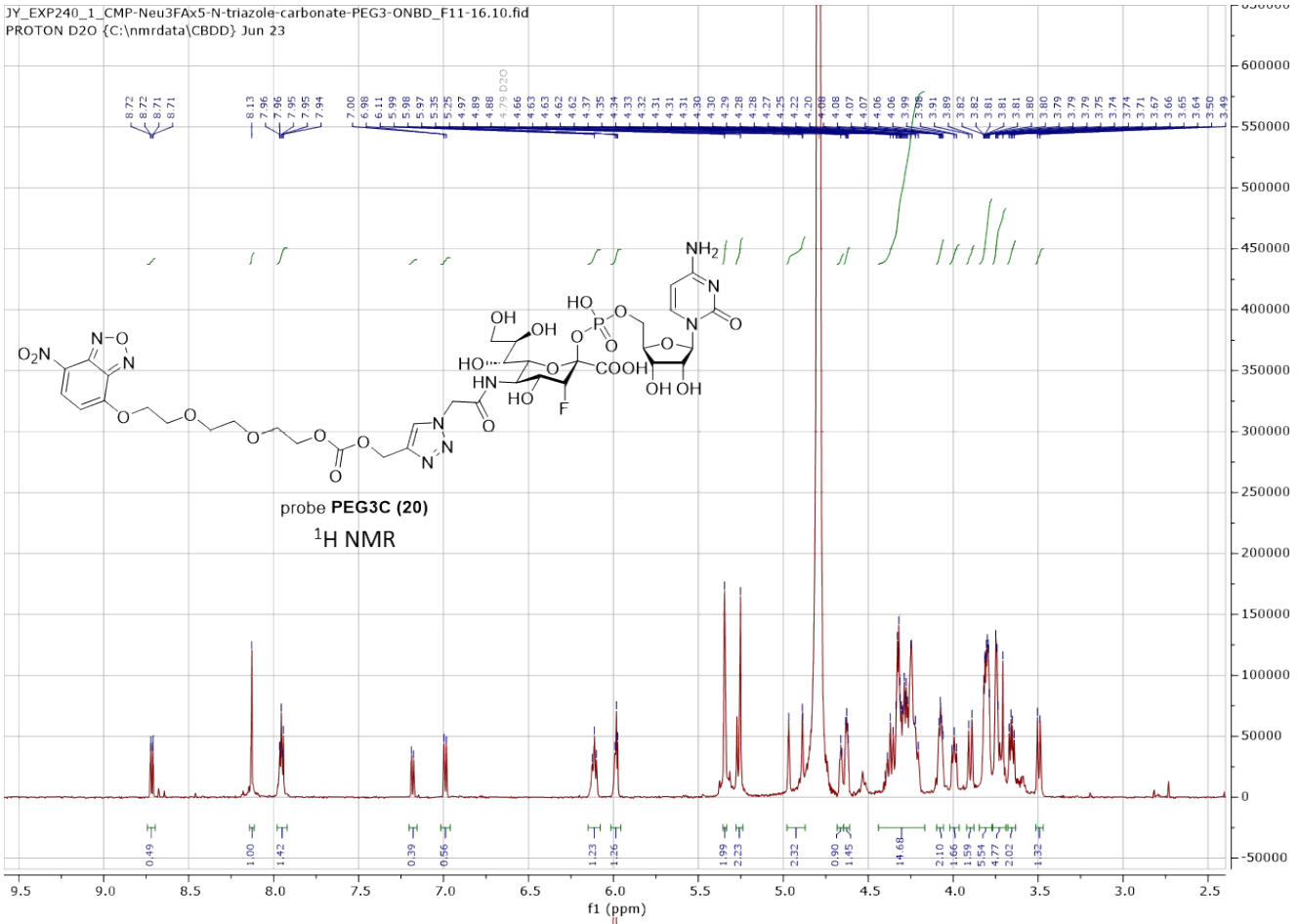


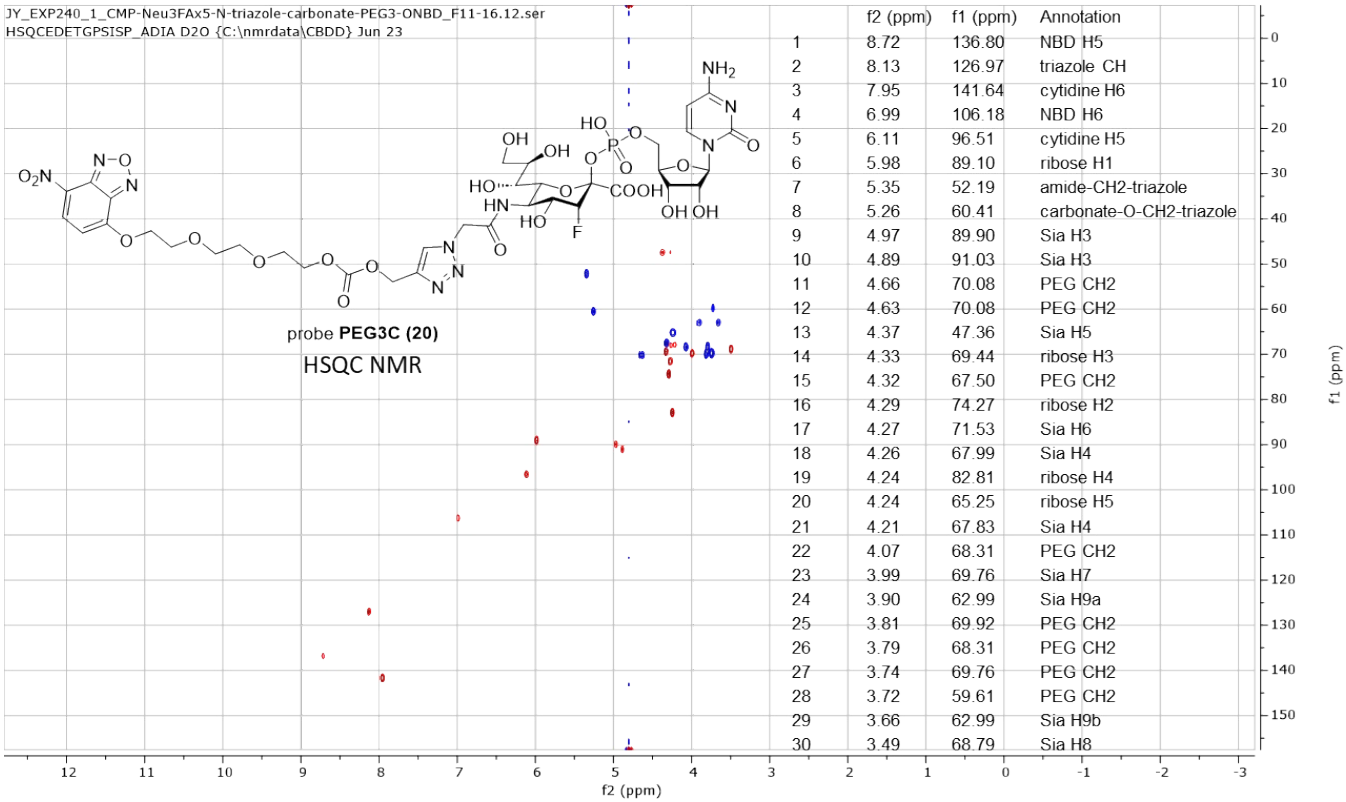
	f2 (ppm)	f1 (ppm)	Annotation
1	8.73	136.77	NBD H5
2	8.14	126.61	triazole CH
3	7.94	141.61	cytidine H6
4	6.98	106.29	NBD H6
5	6.09	96.45	cytidine H5
6	5.97	89.03	ribose H1
7	5.33	52.08	amide-CH2-triazole
8	4.95	89.83	Sia H3
9	4.87	90.96	Sia H3
10	4.83	63.22	O-CH2-triazole
11	4.64	69.99	PEG CH2
12	4.35	47.41	Sia H5
13	4.31	69.18	ribose H3
14	4.28	74.18	ribose H2
15	4.27	71.44	Sia H6
16	4.25	67.89	Sia H4
17	4.23	82.73	ribose H4
18	4.23	64.99	ribose H5
19	4.21	67.89	Sia H4
20	4.12	67.57	PEG CH2
21	3.98	69.67	Sia H7
22	3.89	62.89	Sia H9a
23	3.72	59.34	Tris
24	3.64	62.89	Sia H9b
25	3.47	68.70	Sia H8



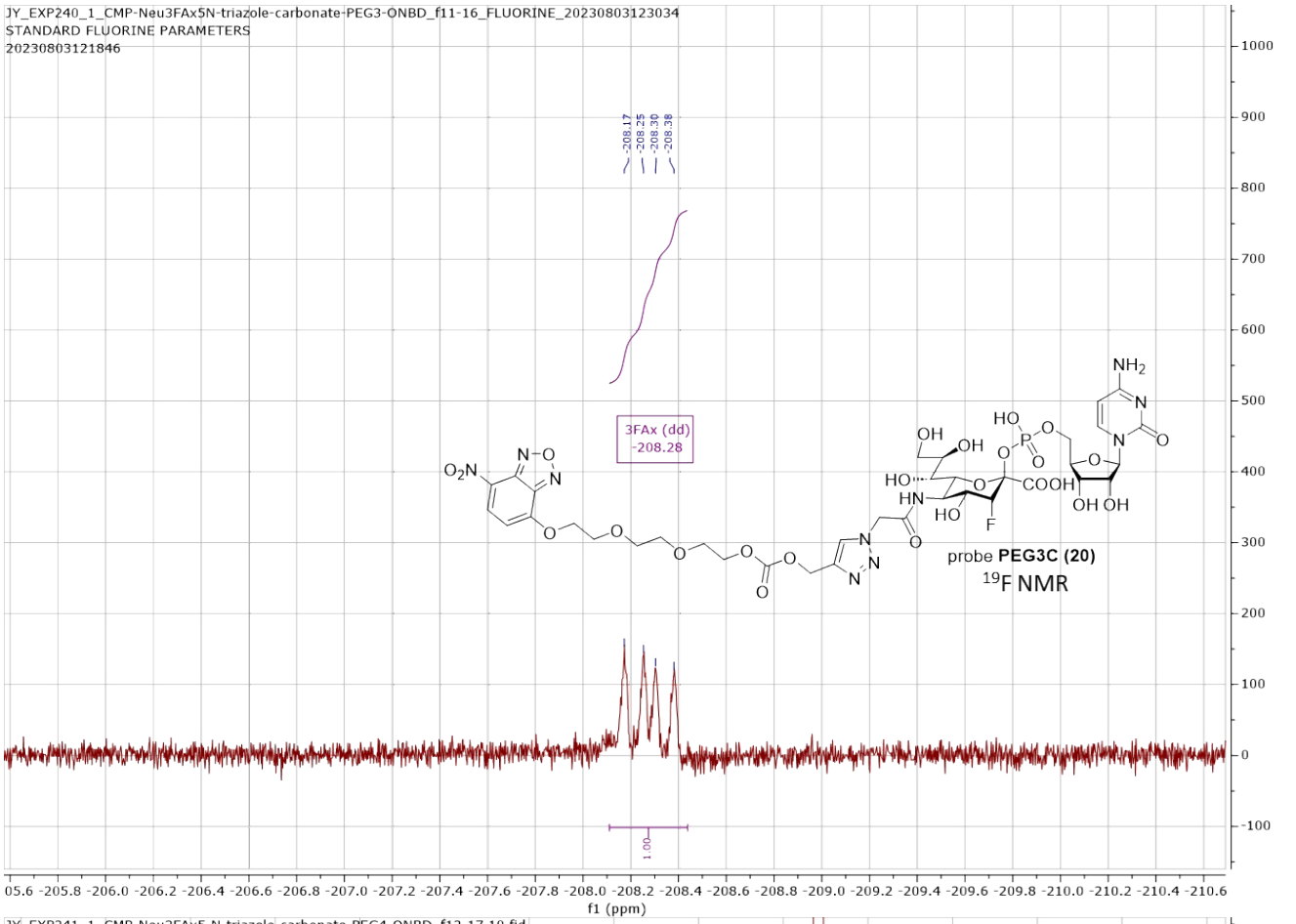


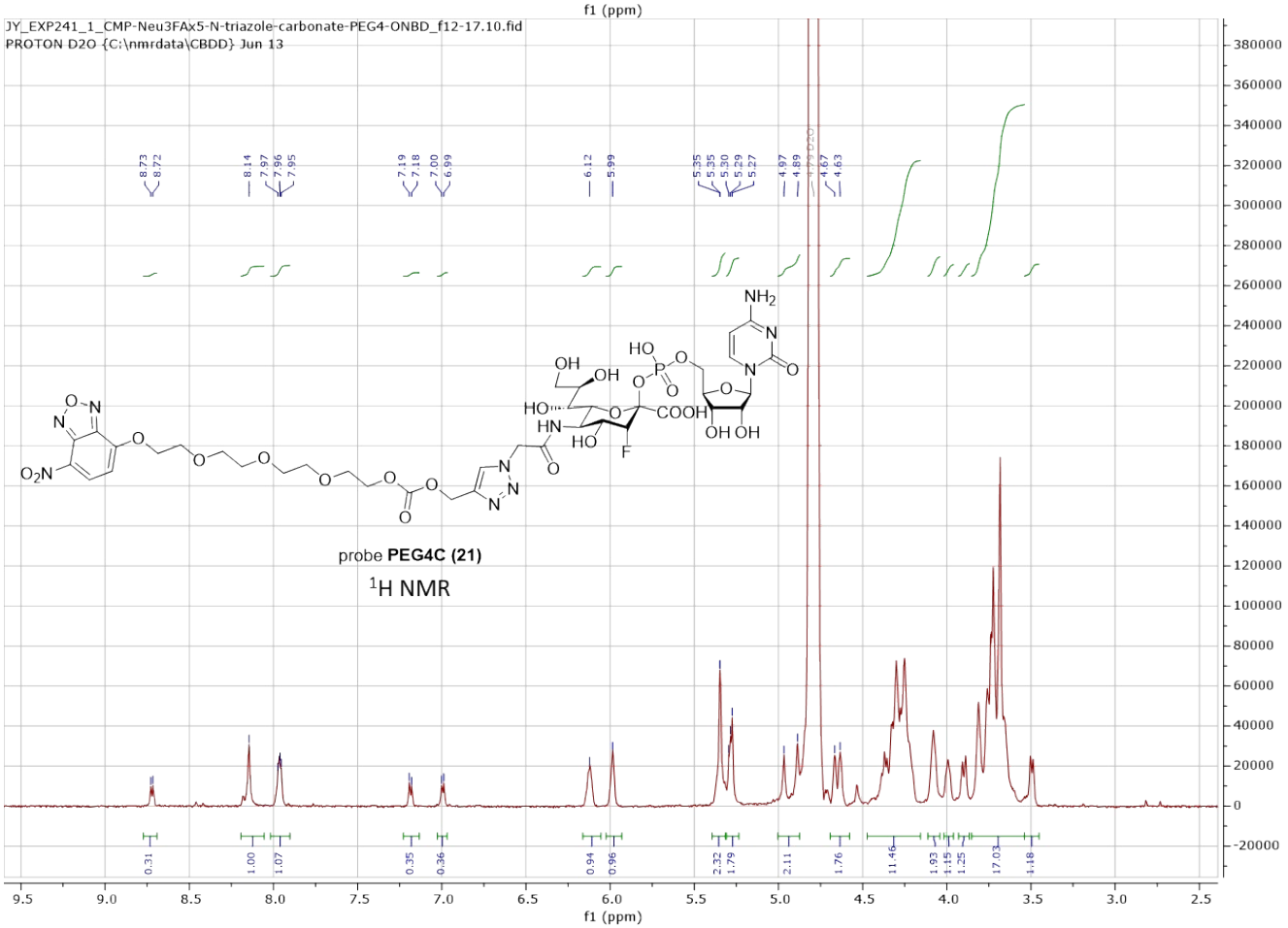
	f2 (ppm)	f1 (ppm)	Annotation
1	8.71	136.80	NBD H5
2	8.10	126.97	triazole CH
3	7.95	141.64	cytidine H6
4	6.99	106.34	NBD H6
5	6.10	96.51	cytidine H5
6	5.98	89.10	ribose H1
7	5.35	52.19	amide-CH2-triazole
8	5.21	60.41	carbonate-O-CH2-triazole
9	4.97	89.90	Sia H3
10	4.89	91.03	Sia H3
11	4.63	70.08	PEG CH2
12	4.38	67.50	PEG CH2
13	4.37	47.36	Sia H5
14	4.32	69.28	ribose H3
15	4.29	71.53	Sia H6
16	4.29	74.43	ribose H2
17	4.27	67.99	Sia H4
18	4.24	65.09	ribose H5
19	4.24	82.81	ribose H4
20	4.22	67.83	Sia H4
21	4.09	68.47	PEG CH2
22	3.99	69.76	Sia H7
23	3.93	68.63	PEG CH2
24	3.90	62.99	Sia H9a
25	3.66	62.99	Sia H9b
26	3.50	68.79	Sia H8

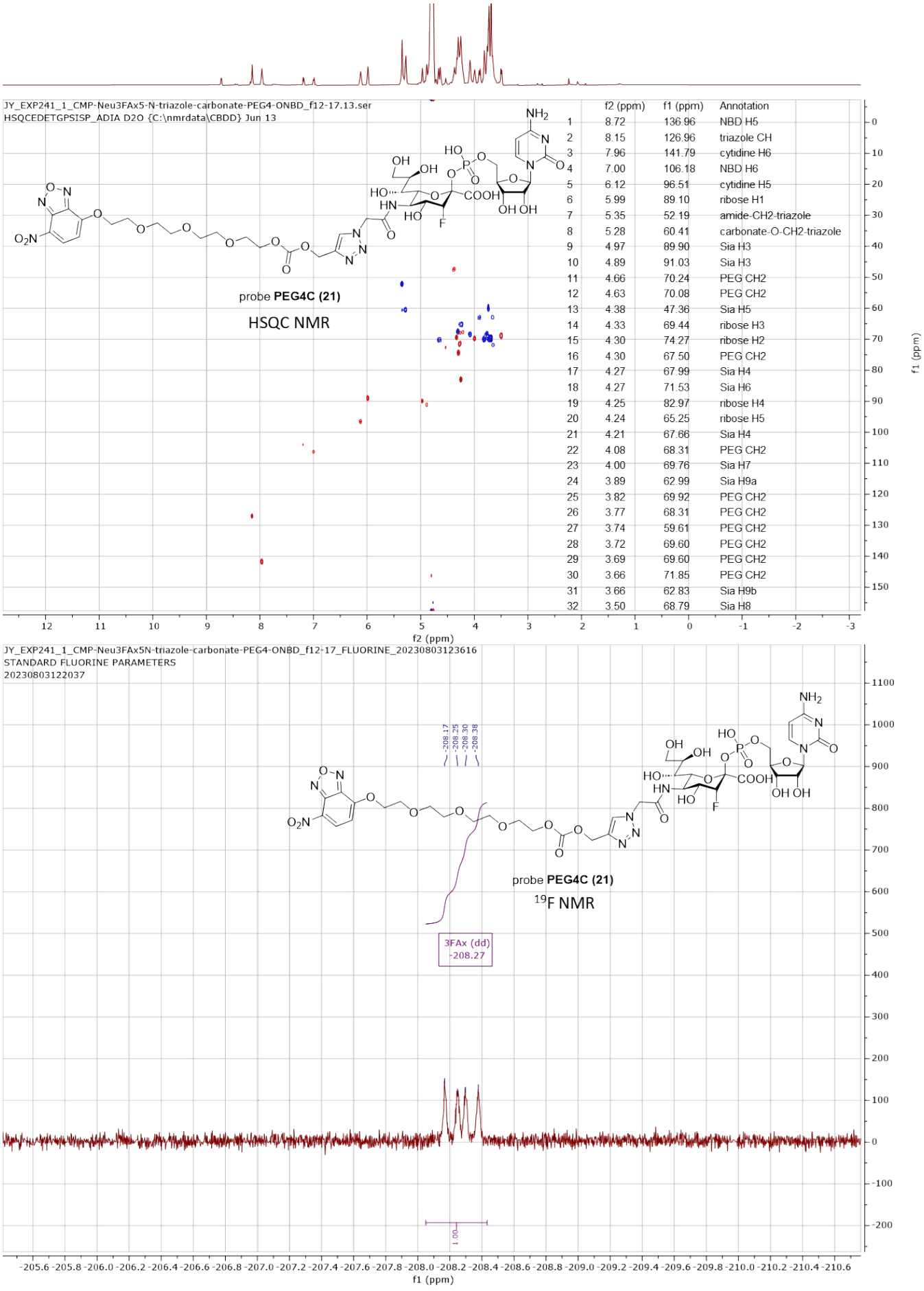




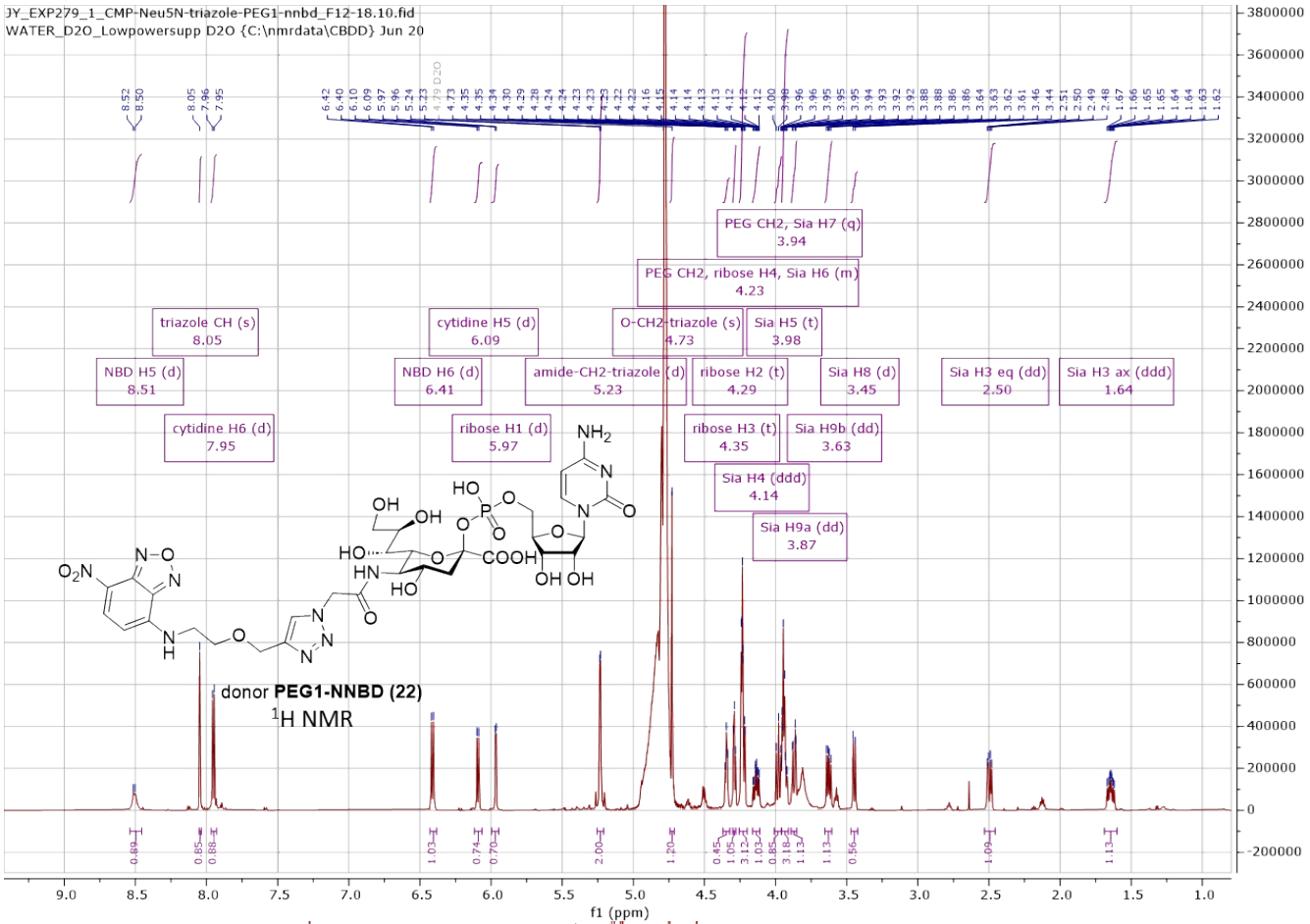
	f2 (ppm)	f1 (ppm)	Annotation
1	8.72	136.80	NBD H5
2	8.13	126.97	triazole CH
3	7.95	141.64	cytidine H6
4	6.99	106.18	NBD H6
5	6.11	96.51	cytidine H5
6	5.98	89.10	ribose H1
7	5.35	52.19	amide-CH2-triazole
8	5.26	60.41	carbonate-O-CH2-triazole
9	4.97	89.90	Sia H3
10	4.89	91.03	Sia H3
11	4.66	70.08	PEG CH2
12	4.63	70.08	PEG CH2
13	4.37	47.36	Sia H5
14	4.33	69.44	ribose H3
15	4.32	67.50	PEG CH2
16	4.29	74.27	ribose H2
17	4.27	71.53	Sia H6
18	4.26	67.99	Sia H4
19	4.24	82.81	ribose H4
20	4.24	65.25	ribose H5
21	4.21	67.83	Sia H4
22	4.07	68.31	PEG CH2
23	3.99	69.76	Sia H7
24	3.90	62.99	Sia H9a
25	3.81	69.92	PEG CH2
26	3.79	68.31	PEG CH2
27	3.74	69.76	PEG CH2
28	3.72	59.61	PEG CH2
29	3.66	62.99	Sia H9b
30	3.49	68.79	Sia H8

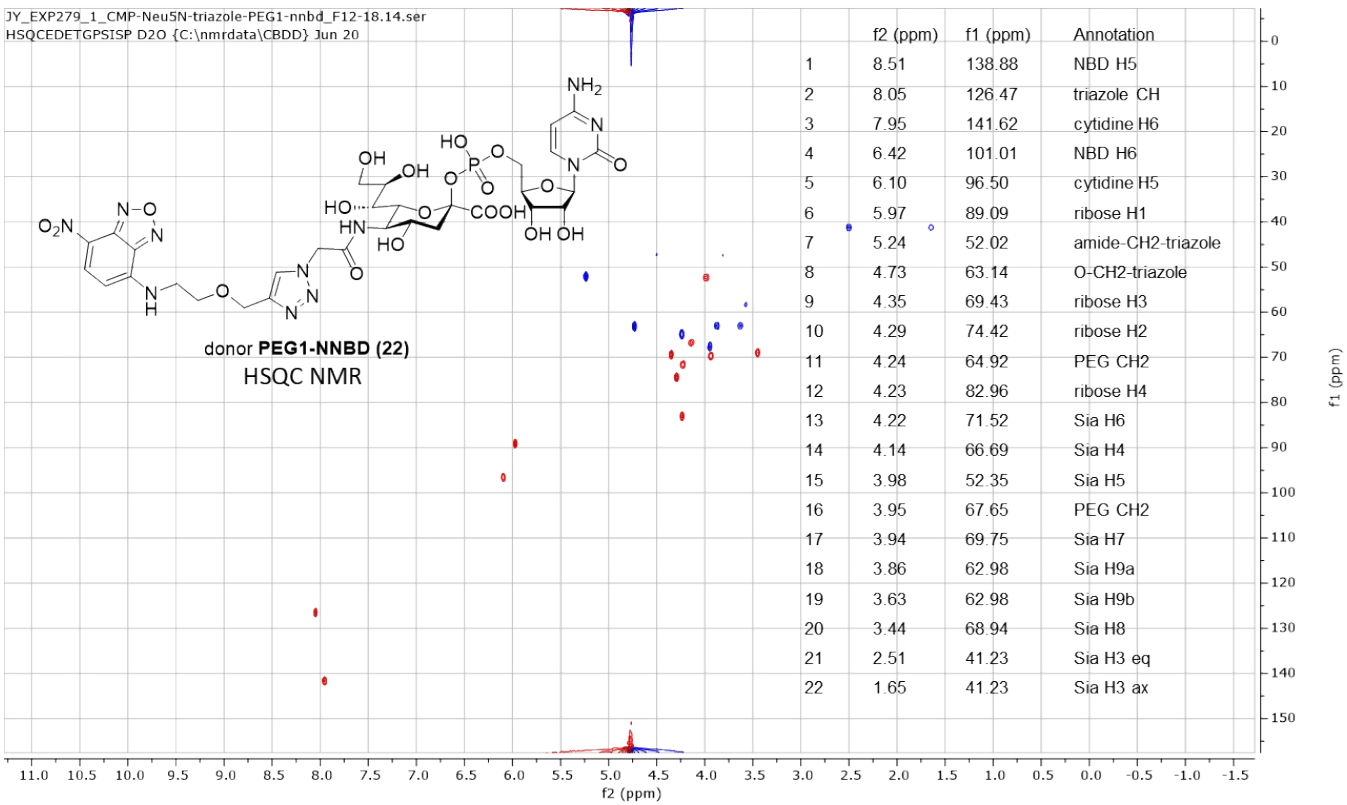

JY_EXP240_1_CMP-Neu3FAx5N-triazole-carbonate-PEG3-ONBD_f11-16_FLUORINE_20230803123034
STANDARD FLUORINE PARAMETERS
20230803121846
-208.17
-208.25
-208.30
-208.38
3FAx (dd)
-208.28
probe PEG3C (20)
19F NMR
f1 (ppm)


JY_EXP241_1_CMP-Neu3FAx5-N-triazole-carbonate-PEG4-ONBD_f12-17.10.fid
PROTON D2O {C:\nmrdata\CBDD} Jun 13
probe PEG4C (21)
1H NMR
f1 (ppm)

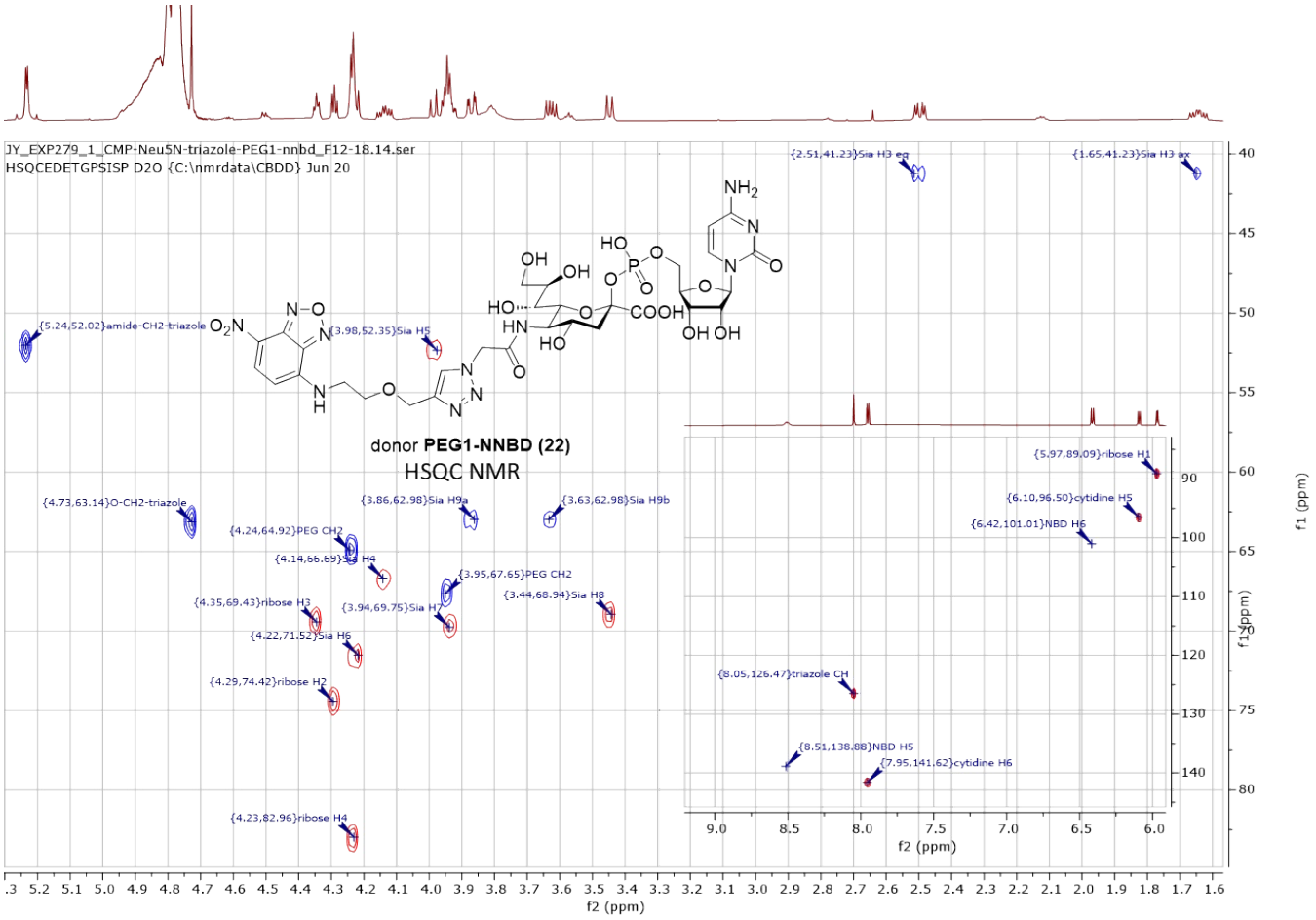


	f2 (ppm)	f1 (ppm)	Annotation
1	8.72	136.96	NBD H5
2	8.15	126.96	triazole CH
3	7.96	141.79	cytidine H6
4	7.00	106.18	NBD H6
5	6.12	96.51	cytidine H5
6	5.99	89.10	ribose H1
7	5.35	52.19	amide-CH2-triazole
8	5.28	60.41	carbonate-O-CH2-triazole
9	4.97	89.90	Sia H3
10	4.89	91.03	Sia H3
11	4.66	70.24	PEG CH2
12	4.63	70.08	PEG CH2
13	4.38	47.36	Sia H5
14	4.33	69.44	ribose H3
15	4.30	74.27	ribose H2
16	4.30	67.50	PEG CH2
17	4.27	67.99	Sia H4
18	4.27	71.53	Sia H6
19	4.25	82.97	ribose H4
20	4.24	65.25	ribose H5
21	4.21	67.66	Sia H4
22	4.08	68.31	PEG CH2
23	4.00	69.76	Sia H7
24	3.89	62.99	Sia H9a
25	3.82	69.92	PEG CH2
26	3.77	68.31	PEG CH2
27	3.74	59.61	PEG CH2
28	3.72	69.60	PEG CH2
29	3.69	69.60	PEG CH2
30	3.66	71.85	PEG CH2
31	3.66	62.83	Sia H9b
32	3.50	68.79	Sia H8





	f2 (ppm)	f1 (ppm)	Annotation
1	8.51	138.88	NBD H5
2	8.05	126.47	triazole CH
3	7.95	141.62	cytidine H6
4	6.42	101.01	NBD H6
5	6.10	96.50	cytidine H5
6	5.97	89.09	ribose H1
7	5.24	52.02	amide-CH2-triazole
8	4.73	63.14	O-CH2-triazole
9	4.35	69.43	ribose H3
10	4.29	74.42	ribose H2
11	4.24	64.92	PEG CH2
12	4.23	82.96	ribose H4
13	4.22	71.52	Sia H6
14	4.14	66.69	Sia H4
15	3.98	52.35	Sia H5
16	3.95	67.65	PEG CH2
17	3.94	69.75	Sia H7
18	3.86	62.98	Sia H9a
19	3.63	62.98	Sia H9b
20	3.44	68.94	Sia H8
21	2.51	41.23	Sia H3 eq
22	1.65	41.23	Sia H3 ax


JY_EXP279_1_CMP-Neu5N-triazole-PEG1-nnbd_F12-18.14.ser
HSQCEDETGPSISP D2O {C:\nmrdata\CBDD} Jun 20
donor PEG1-NNBD (22)
HSQC NMR
{5.24,52.02}amide-CH2-triazole
{3.98,52.35}Sia H5
{4.73,63.14}O-CH2-triazole
{3.86,62.98}Sia H9a
{3.63,62.98}Sia H9b
{4.24,64.92}PEG CH2
{4.14,66.69}Sia H4
{3.95,67.65}PEG CH2
{4.35,69.43}ribose H3
{3.94,69.75}Sia H7
{3.44,68.94}Sia H8
{4.22,71.52}Sia H6
{4.29,74.42}ribose H2
{4.23,82.96}ribose H4
{2.51,41.23}Sia H3 eq
{1.65,41.23}Sia H3 ax
{5.97,89.09}ribose H1
{6.10,96.50}cytidine H5
{6,42,101.01}NBD H6
{8.05,126.47}triazole CH
{8.51,138.88}NBD H5
{7.95,141.62}cytidine H6
f2 (ppm)
f1 (ppm)