A Bioorthogonal Turn-ON fluorescence Strategy for the Detection of Histone Acetyltransferase Activity

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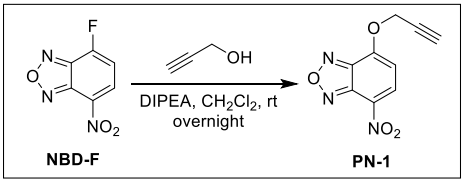
General methods

All the chemicals were purchased from Sigma-Aldrich and used as received unless otherwise specified. NBD-F was purchased from Dojindo Molecular Technologies, Inc. Human H3.1 protein was purchased from New England Biolabs. The progress of reactions was monitored by thin-layer chromatography (TLC) on silica gel plates (Selecto scientific, GA), and spots were visualized by UV, $KMnO_4$, and phosphomolybdic acid or other stains. Flash column chromatography was carried out with standard grade silica gel (Sorbent Technologies 60 Å, 230-400 mesh). The ^1H NMR and ^{13}C NMR spectra of compounds were recorded on either a Varian Unity INOVA 500 MHz or Varian Mercury Plus 400 MHz spectrometers using $CDCl_3$ as solvent and reported as follows. ^1H: br (broad singlet), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets). ^{13}C: chemical shift δ (ppm). Mass spectra were obtained on Bruker Autoflex (TOF) mass spectrometer or Orbitrap Elite system with ESI ion source. The purity of compounds was determined by Shimadzu HPLC system utilizing a column Aeris PEPTIDE 3.6u XB-C18, 250*4.6 mm, a flow of 1 mL/min, solvent A: H_2O with 0.1% trifluoroacetic acid, solvent B: acetonitrile with 0.1% trifluoroacetic acid, and UV-detection at 214 and 254 nm. In-gel fluorescence of SDS-PAGE gels was recorded on Typhoon TRIO+ Variable Mode Imager (GE Healthcare). Fluorescence spectra was recorded with a FluoroMax-4 fluorescence spectrofluorometer (HORIBA Scientific). Absorption spectrum were measured with a UV-VIS Spectrophotometer (Shimazu). The living cell fluorescence images were obtained on Observer. A1 (Carl Zeiss MicroImaging) microscope and Zeiss LSM 710 Confocal Microscope.

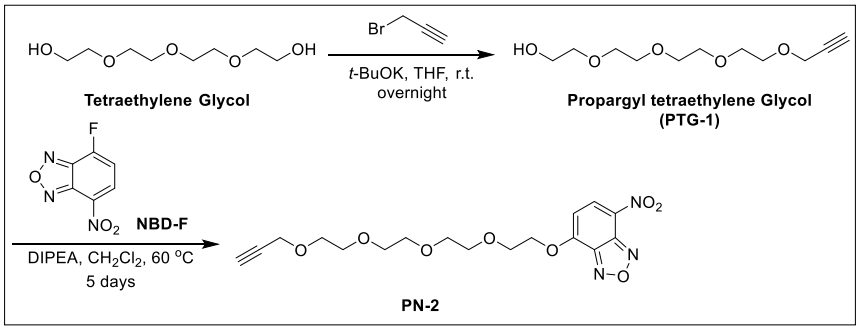
1. Experimental procedure for the synthesis of PN-1, PN-2, PN-3, 3AZ-CoA, H4(1-21) and H4(1-21)K16$_{3AZ}$; and the expression of p300 enzyme

Synthesis of PN-1 (Scheme S1)



NBD-F (9.1mg, 0.05mmol) was dissolved in 1mL of CH_2Cl_2, to this solution was added propargyl alcohol (3mg, 0.05mmol) and DIPEA (6.5mg, 0.05mL) at 0 °C, the resulted black mixture was allowed to stir at r.t. overnight, until TLC showed the reaction was completed. Then the solvent was evaporated and the resulting residue was subjected to flash chromatography (hexane/EtOAc 10/1 to 2/1), affording a dark green solid (m: 6mg). Yield 55%.[1] ^1H NMR (400 MHz, Chloroform-d) δ 8.57 (dd, J = 8.3, 0.8 Hz, 1H), 6.94 – 6.84 (m, 1H), 5.13 (dd, J = 2.5, 0.8 Hz, 2H), 2.72 (td, J = 2.4, 0.8 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.76, 145.24, 143.96, 133.51, 105.94, 78.89, 75.21, 58.18. MS (ESI): calcd. for $[C_9H_5N_3O_4+H]^+$ 220.0; found 220.2.

Synthesis of PN-2 (Scheme S2)

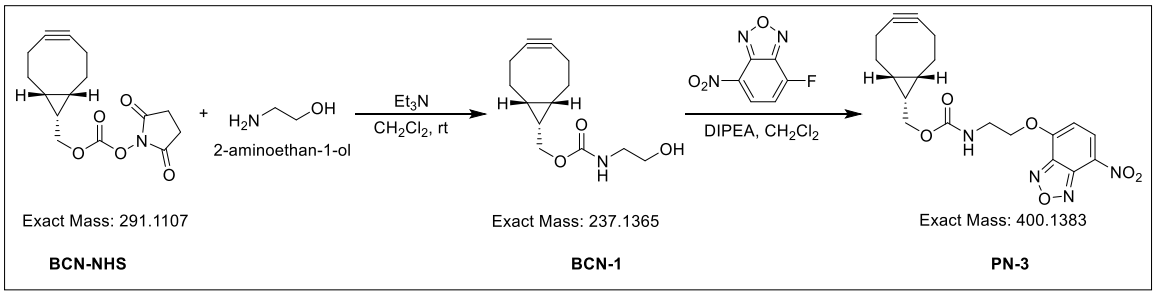


Step 1: To a solution of Tetraethylene glycol (1.94g, 10mmol) in 40mL of freshly distilled THF was added *t*-BuOK (0.56g, 5mmol) at 0°C, the mixture was stirred at r.t. for 30mins, then cooled to 0°C, and propargyl bromide (0.6g, 5mmol) dissolved in 5mL of THF was added dropwise. After that the reaction mixture was stirred at 0°C for 10min then warmed to r.t. and stirred at r.t. overnight. Then the reaction was filtered through celite, washed with THF, the filtrate was concentrated and purified by flash chromatography (EtOAc), the desired intermediate was obtained as a yellow oil. Yield 90%. ^1H NMR (400 MHz, Chloroform-*d*) δ 4.22 (d, *J* = 2.4 Hz, 2H), 3.78 – 3.69 (m, 6H), 3.68 (s, 8H),

3.62 (dd, *J* = 5.4, 3.7 Hz, 2H), 2.45 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 79.56, 74.63, 72.58, 70.59, 70.53, 70.50, 70.35, 70.26, 69.07, 61.69, 58.39. MS (ESI): calcd. for $[C_{11}H_{20}O_5+H]^+$ 233.1; found 233.3.

Step 2: Propargyl tetraethylene Glycol (8.7mg, 0.038mmol) was dis-solved in 1mL CH_2Cl_2 under a nitrogen atmosphere. To this solution was added NBD-F (9mg, 0.05mmol) in 1mL CH_2Cl_2, followed by the addition of DIPEA (6.46mg, 0.05mmol) at 0°C, the reaction solution turned into dark brown slowly. The reaction mixture was stirred at 60°C in a sealed tube for 5 days, then the solvent was removed under reduced pressure, the crude product was purified by column chromatography (Hexanes/EtOAc 2/1, then CH_2Cl_2/MeOH 40/1) to afford **PN-2** as a yellow solid (4.5mg), yield 30%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.49 (d, *J* = 8.3 Hz, 1H), 6.72 (d, *J* = 8.4 Hz, 1H), 4.54 – 4.44 (m, 2H), 4.13 (d, *J* = 2.4 Hz, 2H), 4.00 – 3.92 (m, 2H), 3.69 (dd, *J* = 6.1, 3.0 Hz, 2H), 3.64 – 3.57 (m, 10H), 2.35 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.80, 134.04, 105.08, 77.23, 74.56, 71.09, 70.68, 70.61, 70.41, 69.13, 69.00, 58.42. MS (ESI): calcd. for $[C_{17}H_{21}N_3O_8+Na]^+$ 418.1226; found 418.1225.

Synthesis of PN-3 (Scheme S3)



Step 1: 2-aminoethan-1-ol (3.15mg, 0.0515mmol) was dissolved in 1mL CH_2Cl_2 under a nitrogen atmosphere, followed by the addition of Et_3N (10μL), the mixture was stirred at r.t. for 30min, then **BCN-NHS** (10mg, 0.034mmol) dissolved in 0.5mL CH_2Cl_2 was added dropwise, the reaction mixture was stirred at r.t. overnight under darkness, then the solvent was removed under reduced pressure, the crude product was purified by column chromatography (Hexanes/EtOAc 5/1 to 1/1) to afford **BCN-1** as a colorless oil (7mg), yield 87.5%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.11 (s, 1H), 4.16 (d, *J* = 8.1 Hz, 2H), 3.73 (t, *J* = 5.1 Hz, 2H), 3.35 (q, *J* = 5.6 Hz, 2H), 2.41 – 2.13 (m, 6H), 1.58 (ddd, *J* = 12.6, 7.6, 5.0 Hz, 2H), 1.35 (q, *J* = 8.7 Hz, 1H), 0.96 (dtd, *J* = 9.5, 7.1, 6.2, 2.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 99.99, 98.83, 63.12, 62.55, 43.45, 29.05, 21.44, 20.14, 17.71. MS (ESI): calcd. for $[C_{13}H_{19}NO_3+H]^+$ 238.1; found 238.0.

Step 2: **BCN-1** (7mg, 0.03mmol) was dissolved in 1mL CH_2Cl_2 under a nitrogen atmosphere. To this solution was added NBD-F (6.5mg, 0.036mmol) in 1mL CH_2Cl_2, followed by the addition of DIPEA (7.8mg, 0.06mmol) at 0°C, the reaction solution turned into dark green slowly. The reaction mixture was stirred at r.t. for 3 days, then the solvent was removed under reduced pressure, the crude product was purified by column chromatography (Hexanes/EtOAc 3/1 to 1/1) to afford **PN-3** as a yellow oil (2 mg), yield 25%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.48 (d, *J* = 8.3 Hz, 1H), 6.66 (d, *J* = 8.4 Hz, 1H), 5.21 (s, 1H), 4.40 (t, *J* = 5.1 Hz, 2H), 4.07 (dt, *J* = 21.4, 7.7 Hz, 2H), 3.71 (q, *J* = 5.1 Hz, 2H), 2.17 (q, *J* = 15.3 Hz, 4H), 1.37 – 1.23 (m, 3H), 0.98 – 0.70 (m, 4H). MS (ESI): calcd. for $[C_{19}H_{20}N_4O_6-H]^-$ 399.1; found 399.2.

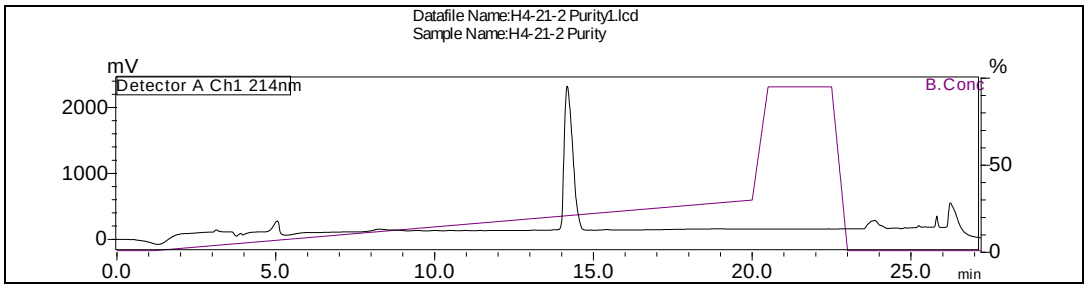
Synthesis of 3AZ-CoA

3-azidopropionyl CoA (3AZ-CoA) was synthesized by reacting 3-azidopropionyl anhydride with CoA-SH, following the procedures used in our previous study.[3]

Synthesis of H4 (1-21) peptide

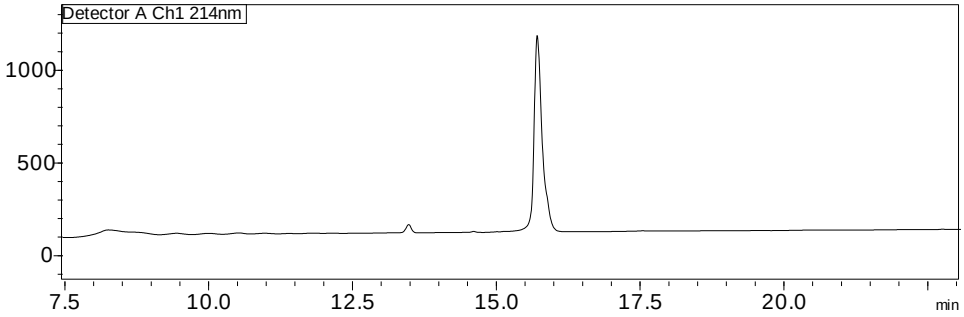
H4(1-21) peptide was synthesized using Fmoc [N-(9- fluorenyl) methoxycarbonyl]-based peptide synthesis protocol on a FOCUS XC peptide synthesizer (aapptec, Louisville, KY). The peptide sequence is as following: **H4(1-21)(Ac-SGRGKGGKGLGKGGAKRHRKV**). Each amino acid was coupled to the solid phase with 4 equiv. of amino acid/HCTU [O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3- tetramethyluronium hexafluorophosphate] (Chempep). The Fmoc group was deprotected with piperidine/DMF (20% v/v), and the N-terminal amino acid was acetylated with acetic anhydride. The peptide was cleaved from the Wang resin by a cleavage solution consisting of 95% trifluoroacetic acid (TFA), 2.5% H_2O, and 2.5% triisopropylsilane. It was then precipitated in cold ether and collected by centrifugation. Crude peptide was collected and purified using a Shimazu liquid chromatography instrument

equipped with a C18 RP-HPLC column (Agilent Polaris 150mm*21.2mm), where 0.05% TFA containing water (phase A) and 0.05% TFA-containing acetonitrile (phase B) were two mobile phases used in gradient purification. The identity of peptides were confirmed with MALDI-MS. The purity of peptides were checked by analytic HPLC using a C18 RP-HPLC column (phenomenex Aeris PEPTIDE 3.6u 250mm*4.6mm).



Synthesis of H4(1-21)-k16$_{3AZ}$ peptide

H4(1-21)-k16$_{3AZ}$ peptide was synthesized using Fmoc [N-(9-fluorenyl) methoxycarbonyl]-based peptide synthesis protocol on a FOCUS XC peptide synthesizer (aapptec, Louisville, KY). K16 lysine was instead using a Dde [1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl] protecting group. The peptide sequence is as following: H4(1-21)K16$_{3AZ}$ (N terminal to C terminal: **Ac-SGRGKGGKGLGKGGAK$_{3AZ}$RHRKV**). Each amino acid was coupled to the solid phase with 4 equiv. of amino acid/HCTU [O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (Chempep). After the solid phase synthesis of the Fmoc and Dde protected peptide, the N-terminal amino acid was capped with Ac group. The K16 Dde protecting group was then removed by 2% hydrazine in DMF. 3-azidopropanoic anhydride(1.2 equiv)[4] was then added together with pyridine (2 equiv) for the installation of 3AZ-group. Then the peptide was cleaved from the Wang resin by adding a cleavage solution consisting of 95% trifluoroacetic acid (TFA), 2.5% H_2O, and 2.5% triisopropylsilane. It was then precipitated in cold ether and collected by centrifugation. Crude peptide was collected and purified using a Shimazu liquid chromatography instrument equipped with a C18 RP-HPLC column (Agilent Polaris 150mm*21.2mm), where 0.05% TFA containing water (phase A) and 0.05% TFA-containing acetonitrile (phase B) were two mobile phases used in gradient purification. The identity of peptides were confirmed with MALDI-MS. The purity of peptides were checked by analytic HPLC using a C18 RP-HPLC column (phenomenex Aeris PEPTIDE 3.6u 250mm*4.6mm).



p300 protein expression and purification

The detailed procedure was described in the previous work of our lab.[4]

2. Turn-on fluorescence KAT assays using PN-1 or PN-2

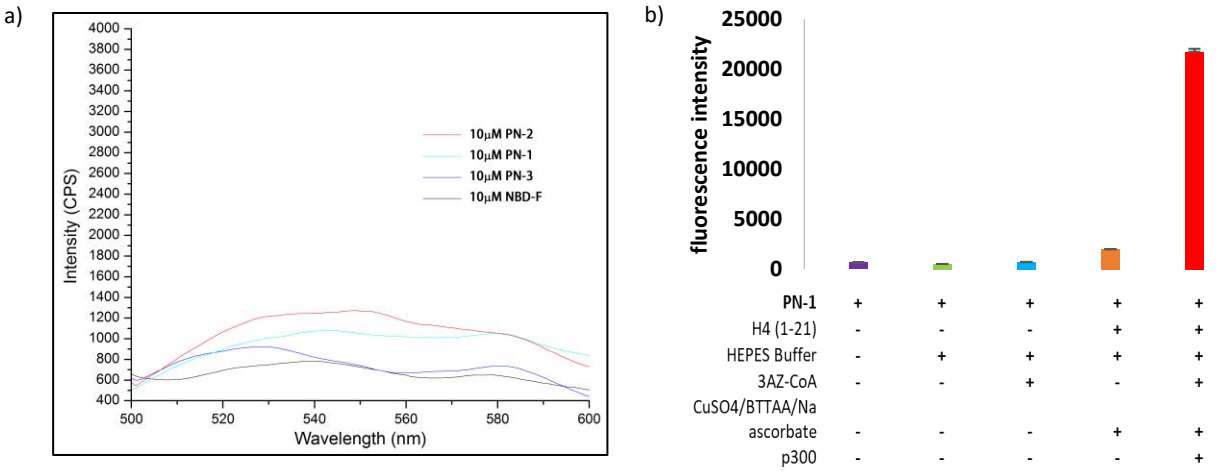


Figure S1 a) Fluorescence intensity comparison of O-NBD probes with NBD-F. These data showed that the synthesized O-NBD probes exhibited similar low fluorescence intensity to that of NBD-F. b) Fluorescence intensity of **PN-1** under different conditions (**PN-1** concentration: 10μM, incubation time: 1.5h, λ_{ex}=485nm, λ_{em}=545nm).

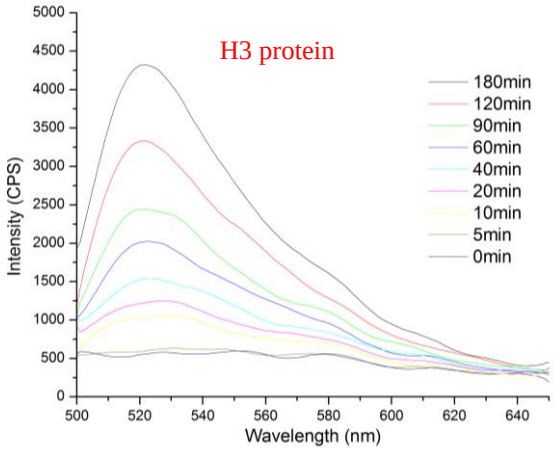


Fig. S2 Fluorescence spectra for click reaction time dependent KAT assay. Human H3 protein (2μM), 3AZ-CoA (5μM), p300 (0.2μM), **PN-2** (5μM). KAT enzymatic reaction time: 30min. After the reaction, a cocktail of click reagents which contained **PN-2** (5μM), $CuSO_4$ (50μM), BTTAA (50μM), and sodium ascorbate (500μM) in DMSO was added and the fluorescence was recorded as a function of click reaction time.

Fluorescence spectra of PN-2 with and without adding p300. 3AZ-CoA (10μM) and H4(1-21) peptide (5μM) were incubated with p300 (0.2 μM) in 50mM HEHEPS buffer (pH 8) for 30min for the transformation of 3AZ group. After the reaction, a cocktail of "click" reagents which contained **PN-2** (5μM), copper sulfate (50μM), BTTAA (50μM), and sodium ascorbate (500μM) in DMSO was added and incubated for 2h to allow for "click" reaction/intramolecular substitution to occur. Finally, the fluorescence spectra were measured with a fixed excitation wavelength of 485nm. The control group used the same conditions but without the addition of p300 at enzymatic reaction stage. For data see **Fig. 1b**.

3. Model study of O-NBD to N-NBD transformation

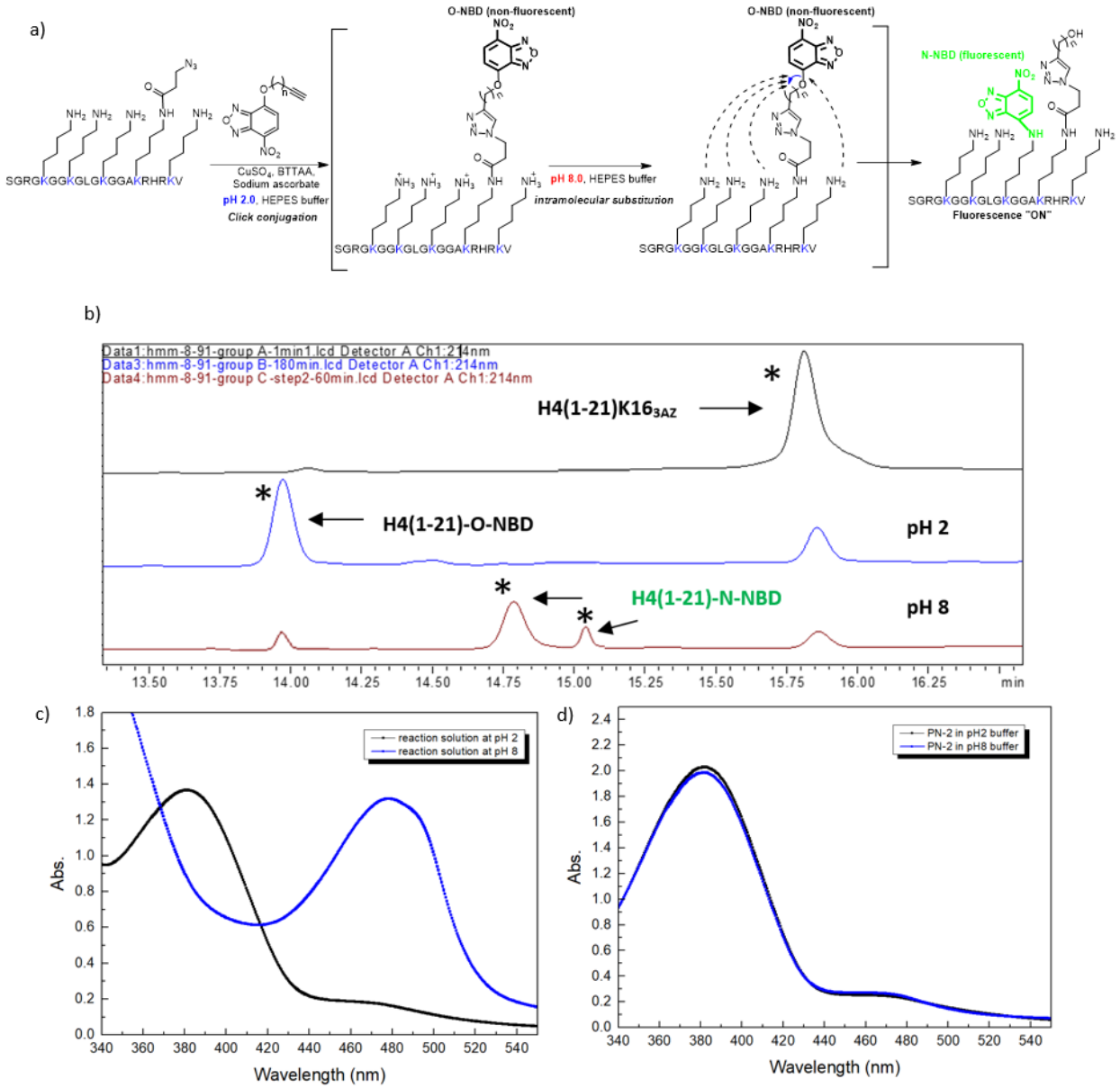


Figure S3 a) Illustration of click reaction of H4(1-21)K16$_{3AZ}$ with PN-2 at pH 2.0 and intramolecular N-substitution at pH 8.0. b) HPLC monitoring for click reaction of H4(1-21)K16$_{3AZ}$ with PN-2 at pH 2.0 (reaction time: 3h), then the reaction solution was adjusted to pH 8.0 for the intramolecular N-substitution reaction to occur. H4(1-21)K16$_{3AZ}$ 200μM, PN-2 200μM, $CuSO_4$ 2mM, BTTAA 2mM, sodium ascorbate 20Mm, HEPES buffer 100mM. c) Absorption spectra of click reaction solution (pH 2.0) and N-substitution reaction (pH 8.0). d) Absorption spectra of **PN-2** (200 μM) at pH 2.0 and pH 8.0.

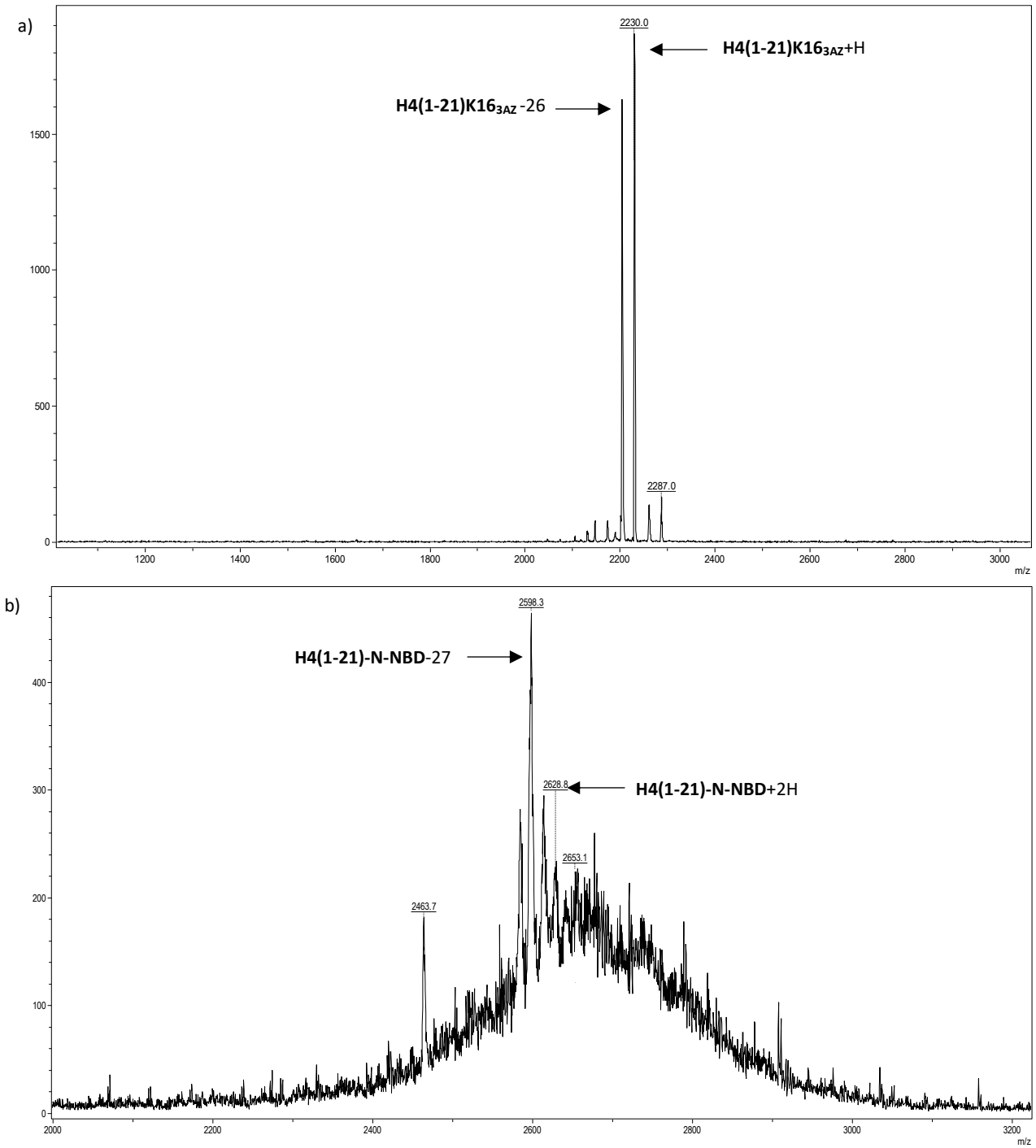


Figure S4 MALDI-MS of H4(1-21)K16$_{3AZ}$ (a) and H4(1-21)-N-NBD (b).

Enzyme reaction time dependent KAT assay using PN-2 as a fluorescence reporter. 3AZ-CoA (10μM) and H4(1-21) peptide (5μM) were incubated with p300 (0.2 μM) in 50mM HEHEPS buffer (pH 8) for a certain time (0-50min) for the transformation of 3AZ group. After the reaction, a cocktail of click reagents which contained **PN-2** (5μM), copper sulfate (50μM), BTTAA (50μM), and sodium ascorbate (500μM) in DMSO was added and incubated for 3h to allow for click reaction/intramolecular substitution to occur. Finally, the fluorescence emission spectra were measured with a fixed excitation wavelength of 485nm.

Enzyme concentration dependent KAT assay using PN-2 as a fluorescence reporter. 3AZ-CoA (10μM) and H4(1-21) peptide (5μM) were incubated with a certain concentration of p300 (from 0 to 0.32μM) in 50mM HEHEPS buffer (pH 8) for 30min for the transformation of 3AZ group. After the reaction, a cocktail of click reagents which contained **PN-2** (5μM), copper sulfate (50μM), BTTAA (50μM), and sodium ascorbate (500μM) in DMSO was added and incubated for 3h to allow for click reaction/intramolecular substitution to occur. Finally, the fluorescence emission spectra were measured with a fixed excitation wavelength of 485nm.

Quantification the inhibition potency of C646 using PN-2 as a fluorescence reporter. Certain concentration of C646 (0 to 16μM) was incubate with p300 (0.2μM) for 30 min in 50mM HEHEPS buffer (pH 8), then added to a reaction buffer containing 3AZ-CoA (10μM) and H4(1-21) peptide (5μM) for 1h. After the reaction, a cocktail of click reagents which contained **PN-2** (5μM), copper sulfate (50μM), BTTAA (50μM), and sodium ascorbate (500μM) in DMSO was added and incubated for 3h to allow for click reaction/intramolecular substitution to occur. Finally, the fluorescence emission spectra were measured with a fixed excitation wavelength of 485nm.

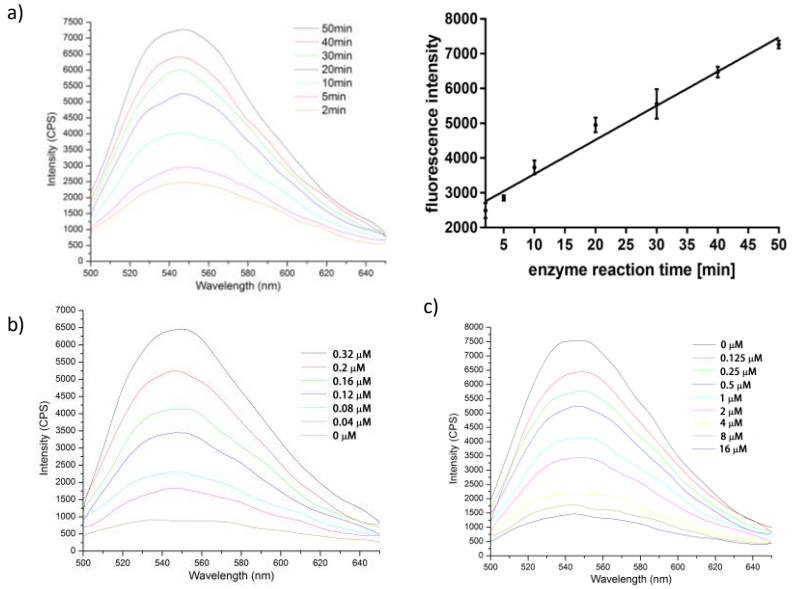


Fig. S5 Fluorescence response of the assay to enzymatic reaction time, enzyme concentration, and enzyme inhibitor. a) Fluorescence spectra for enzyme reaction time dependent KAT assay. H4(1-21) peptide (5μM) as the substrate, 3AZ-CoA (10μM) as the cofactor, with the catalysis of p300 (0.2μM); **PN-2** (5μM), click reaction time: 3h. b) Fluorescence spectra for enzyme concentration dependent KAT assay. H4(1-21) peptide (5μM) as the substrate, 3AZ-CoA (10μM) as the cofactor. Enzyme reaction time: 30mins; **PN-2** (5μM), click reaction time: 3h. c) Quantitation of C646 inhibition potency for p300 inhibition by fluorescence change. The reactions were performed in duplicate.

IC_{50} measurement:

Equation S1:

$$\frac{F-F_0}{F_{Max}-F_0} = \frac{1}{\left(1+\frac{[I]}{[IC_{50}]}\right)^h}$$

where F_{max}, F_0, h, and $[I]$ are maximum and minimum fluorescence signals on the Y-axis, hill coefficient, and inhibitor concentration, respectively.

4. In-gel fluorescence imaging

Extraction of cellular protein. Human embryonic kidney 293 cells (HEK293T cells) were cultured with DMEM medium supplemented with 10% (v/v) FBS at 37 ºC with 5% of CO_2. Cells were scraped after reaching to ~90% confluence and washed with ice-cold PBS for twice. Cells were collected by centrifugation at 12000 rpm at 4 ºC and were suspended with ice-cold M-PER® mammalian protein extraction reagent (ThermoFisher SCIENTIFIC) containing 1% protease inhibitor cocktail (ThermoFisher SCIENTIFIC). After sitting on ice for 15 min, cells were sonicated with 30% amplitude to release whole cellular proteins. Cellular proteins were collected by centrifugation for 20 min at 12000 rpm at 4 ºC and protein concentration was measured with Bradford assay. Cell lysates were aliquoted, flash frozen and stored at -80 ºC for future use.

Experimental procedure for in-gel fluorescence imaging by PN-2. 18 μL of mixture containing 20 μg of cell lysate, 25 μM of 3AZ-CoA and 1.2 μM of p300 in 50 mM of HEPES pH 8.0 and 0.1 mM of EDTA was incubated at 30 ºC for 1 h. Then 9 μL of click cocktail containing 150 μM of **PN-2**, 1.5 mM of ligand BTTP, 15 mM of sodium ascorbate and 1.5 mM of copper sulfate was added. Reaction mixture was incubated at room temperature for 3 h followed by the addition of 5 μL of SDS-PAGE loading dye. Samples were resolved on 15% SDS-PAGE gradient gel. The gel was then distained with 80% AcOH and scanned with Typhoon scanner (GE Healthcare Life Sciences) using fixed excitation and emission wavelengths at 488 nm and 522 nm.

Experimental procedure for in-gel fluorescence imaging by PN-1 18 μL of mixture containing 20 μg of cell lysate, 25 μM of 3AZ-CoA and 1μM of p300 in 50 mM of HEPES pH 8.0 and 0.1 mM of EDTA was incubated at 30 ºC for 1 h. Then 9 μL of click cocktail containing 150 μM of **PN-1**, 1.5 mM of ligand BTTP, 15 mM of sodium ascorbate and 1.5 mM of copper sulfate was added. Reaction mixture was incubated at room temperature for 3 h followed by the addition of 5 μL of SDS-PAGE loading dye. Samples were resolved on 15% SDS-PAGE gradient gel. The gel was then distained with 80% AcOH and scanned with Typhoon scanner (GE Healthcare Life Sciences) using fixed excitation and emission wavelengths at 488 nm and 522 nm.

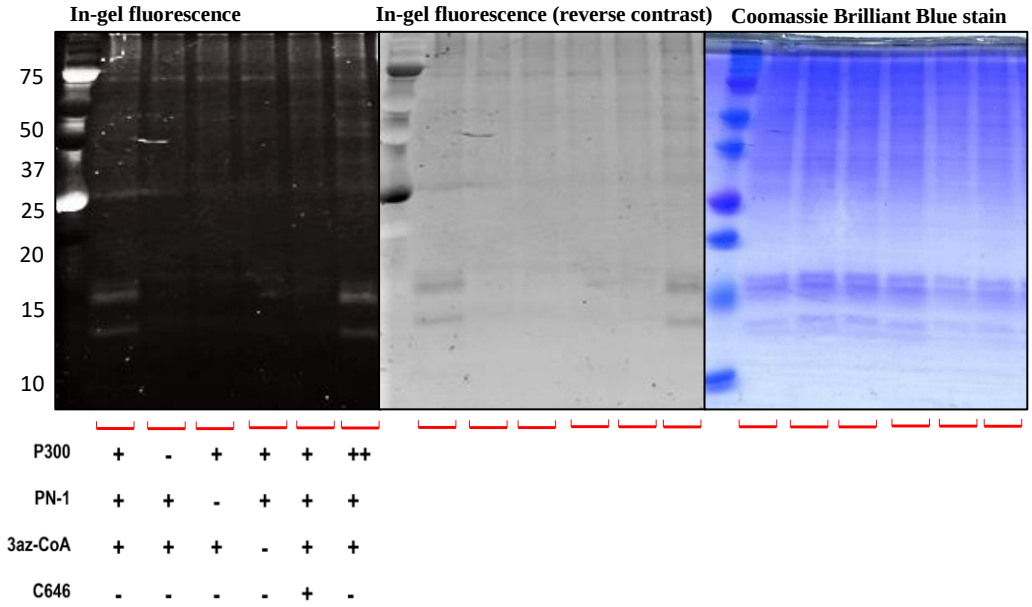
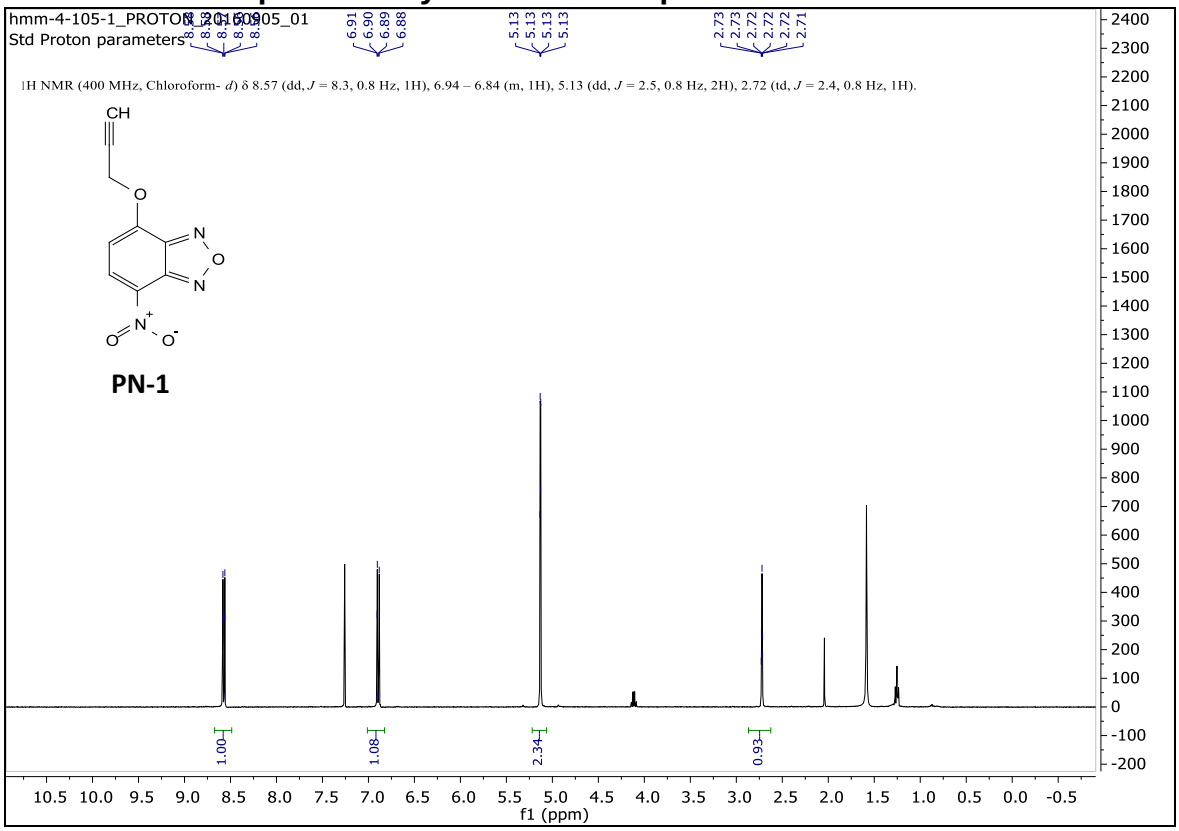
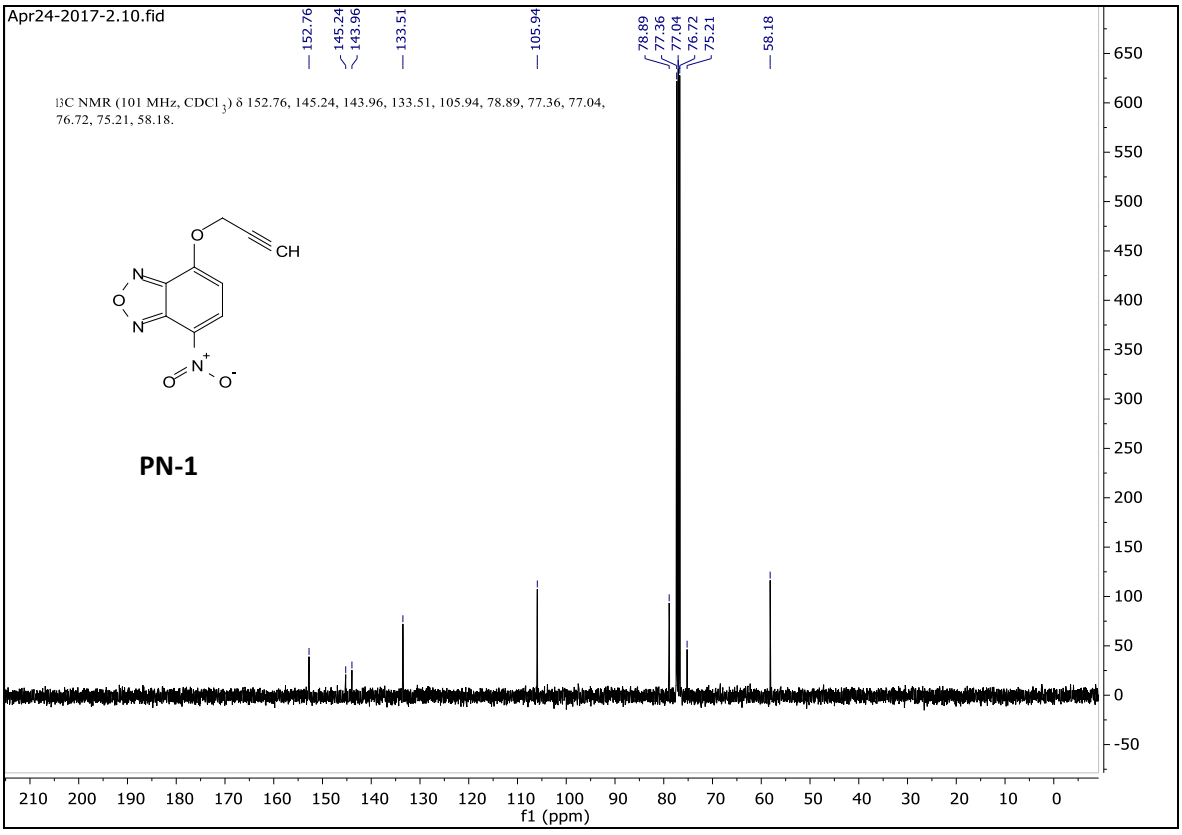


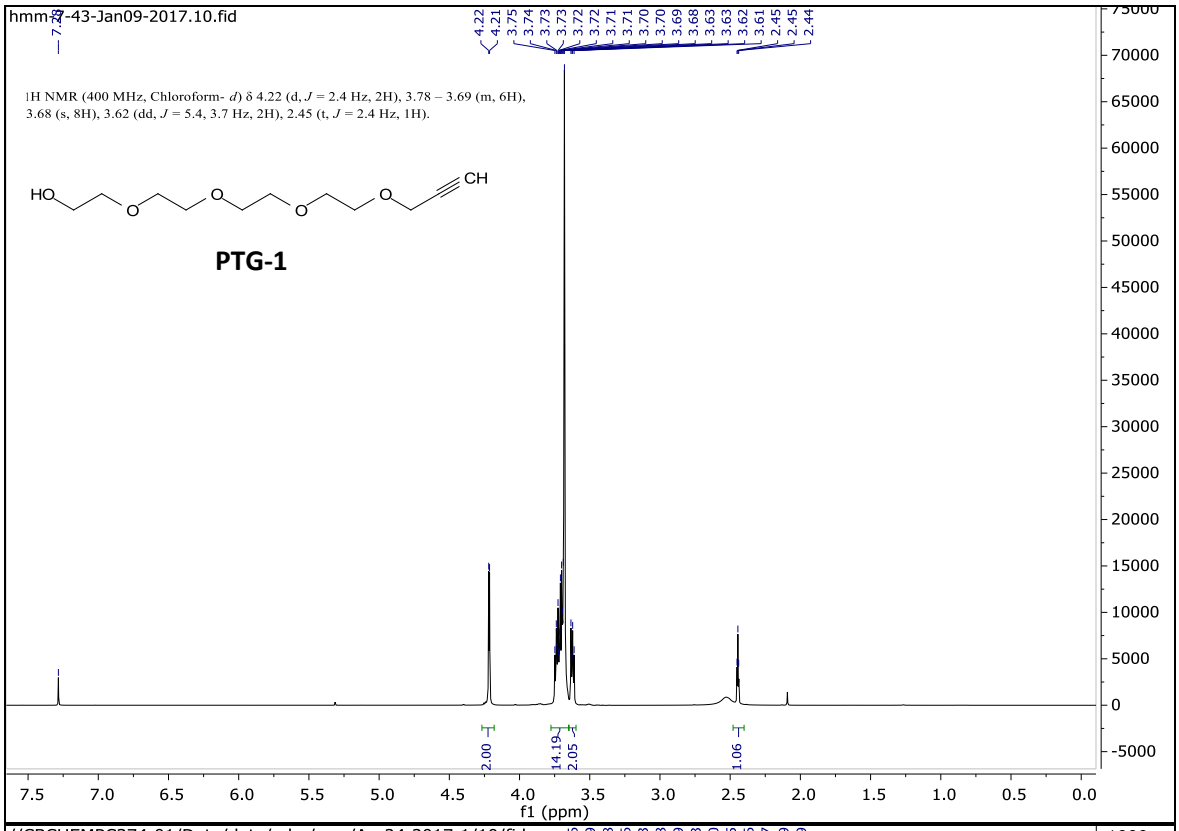
Fig. S6 Labeling of 293T cell lysate by PN-1. SDS-PAGE analysis of 293T cell lysate labeled with **PN-1** (in-gel fluorescence analysis: λ_{ex}=488nm, λ_{em}=520 nm). Labeling conditions: 293T cell lysate 16.5μg, 3AZ-CoA 25μM, p300 0.6 μM, HEPES buffer 50mM, pH 8. then $CuSO_4$ 1.5mM, BTTAA 1.5mM, sodium ascorbate 15mM, **PN-1** 150μM.

Experimental procedure for in-gel fluorescence imaging by PN-3. 18 μL of mixture containing 20 μg of cell lysate, 100 μM of 3AZ-CoA and 1.2 μM of p300 in 50 mM of HEPES pH 8.0 and 0.1 mM of EDTA was incubated at 30 ºC for 1 h. Then 9 μL of click cocktail containing 300 μM of **PN-3**, 1.5 mM of ligand BTTP, 15 mM of sodium ascorbate and 1.5 mM of copper sulfate was added. Reaction mixture was incubated at room temperature for 3 h followed by the addition of 5 μL of SDS-PAGE loading dye. Samples were resolved on 15% SDS-PAGE gradient gel. The gel was then distained with 80% AcOH and scanned with Typhoon scanner (GE Healthcare Life Sciences) using fixed excitation and emission wavelengths at 488 nm and 522 nm.

5. NMR and mass spectra of synthesized compounds



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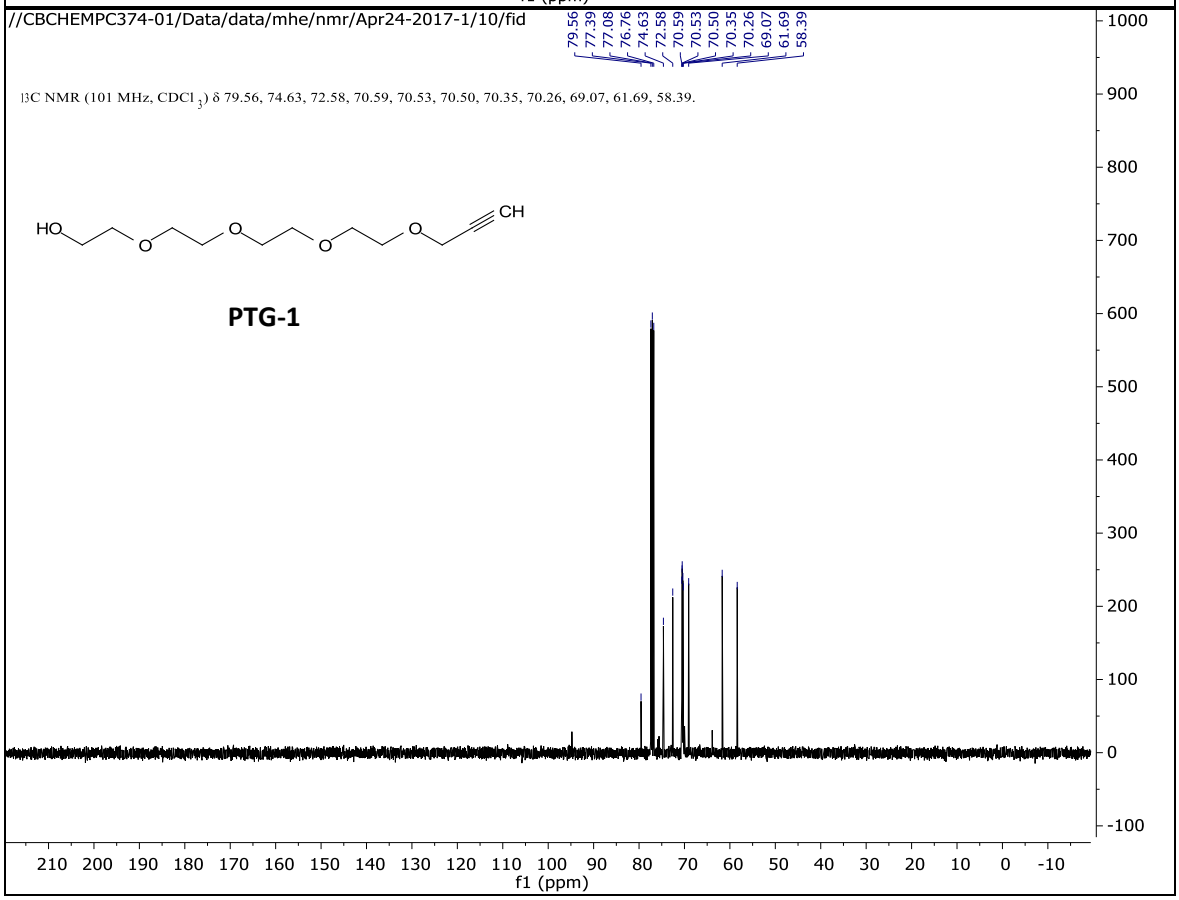
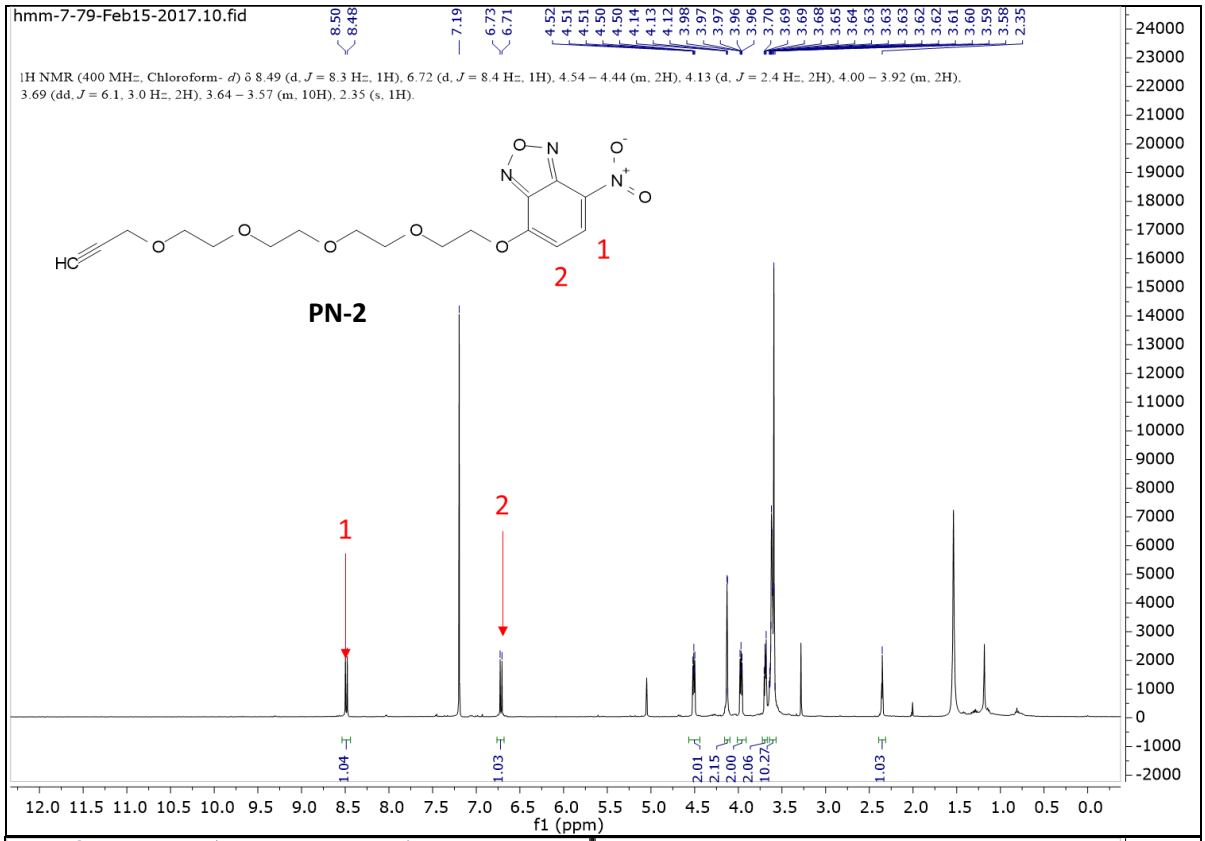
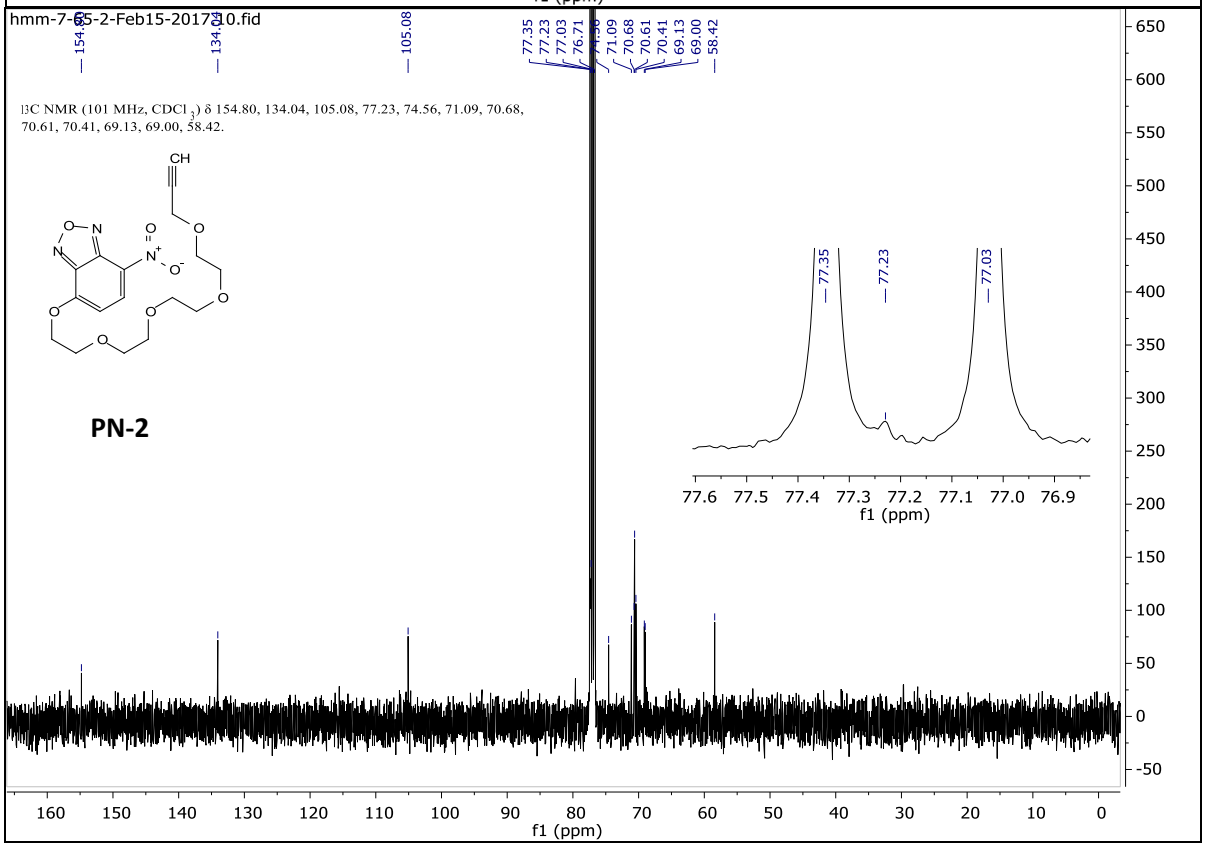
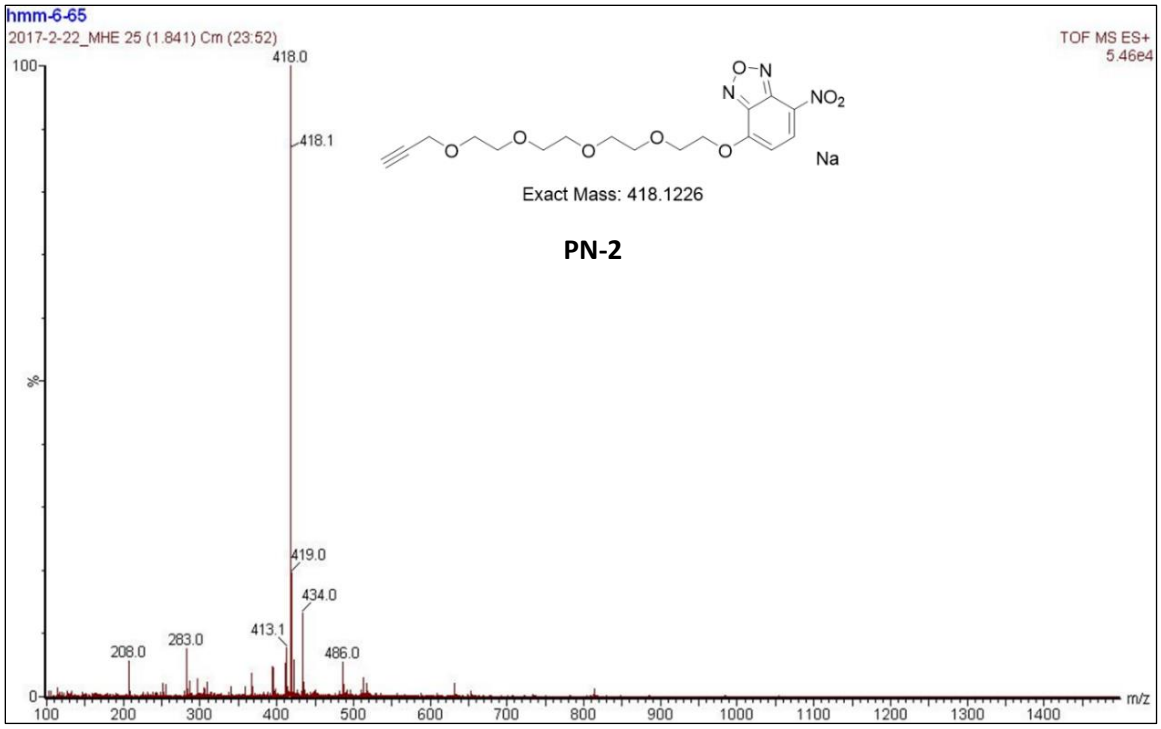
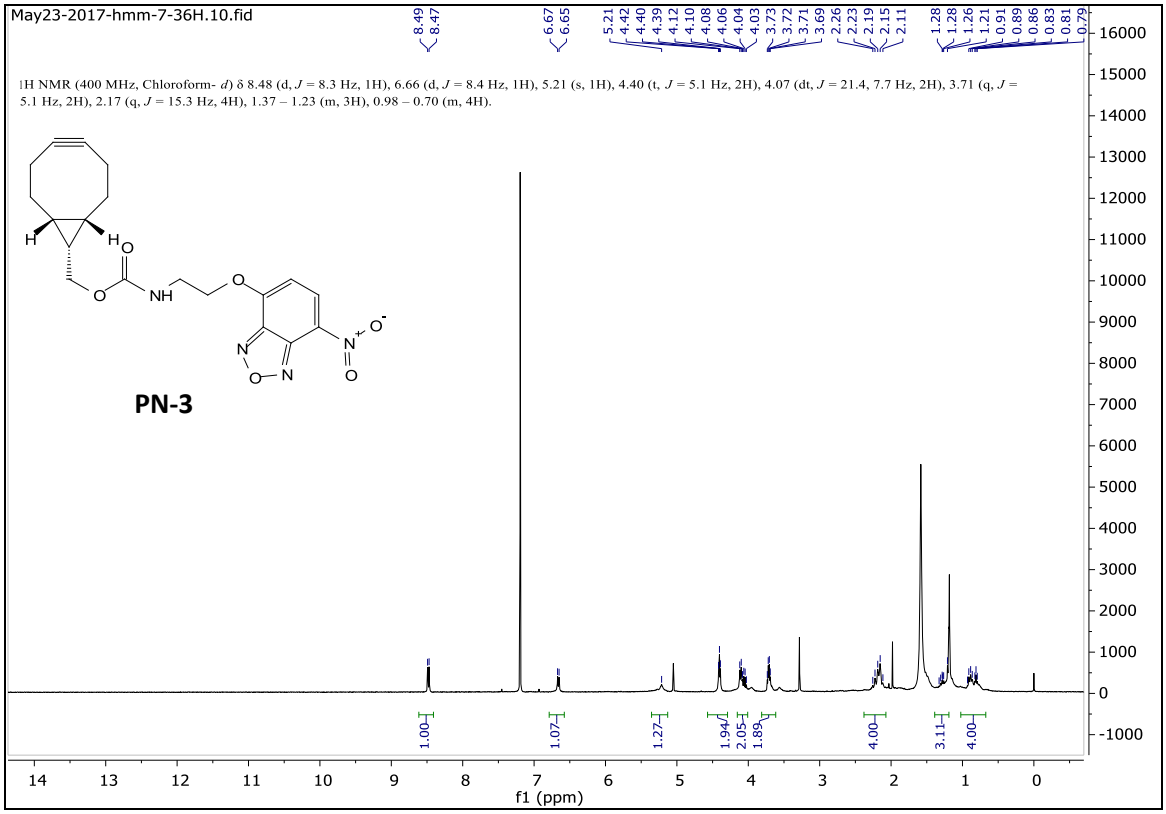
1H NMR (400 MHz, Chloroform- d) δ 4.22 (d, J = 2.4 Hz, 2H), 3.78 – 3.69 (m, 6H), 3.68 (s, 8H), 3.62 (dd, J = 5.4, 3.7 Hz, 2H), 2.45 (t, J = 2.4 Hz, 1H).
HO
O
O
O
O
CH
PTG-1
f1 (ppm)

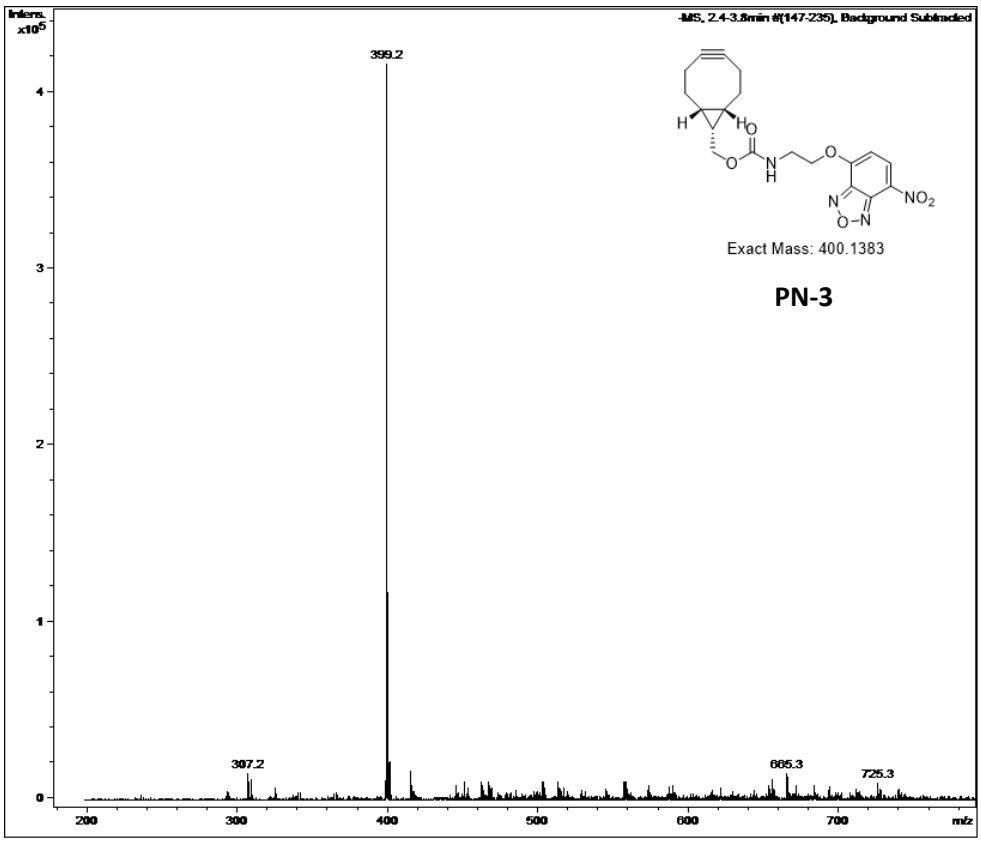

//CBCHEMPC374-01/Data/data/mhe/nmr/Apr24-2017-1/10/fid
13C NMR (101 MHz, CDCl3) δ 79.56, 74.63, 72.58, 70.59, 70.53, 70.50, 70.35, 70.26, 69.07, 61.69, 58.39.
HO
O
O
O
O
CH
PTG-1
f1 (ppm)


hmm-7-79-Feb15-2017.10.fid
1H NMR (400 MHz, Chloroform- d) δ 8.49 (d, J = 8.3 Hz, 1H), 6.72 (d, J = 8.4 Hz, 1H), 4.54 – 4.44 (m, 2H), 4.13 (d, J = 2.4 Hz, 2H), 4.00 – 3.92 (m, 2H), 3.69 (dd, J = 6.1, 3.0 Hz, 2H), 3.64 – 3.57 (m, 10H), 2.35 (s, 1H).
PN-2
f1 (ppm)


hmm-7-65-2-Feb15-2017.10.fid
13C NMR (101 MHz, CDCl3) δ 154.80, 134.04, 105.08, 77.23, 74.56, 71.09, 70.68, 70.61, 70.41, 69.13, 69.00, 58.42.
PN-2
f1 (ppm)


hmm-6-65
2017-2-22_MHE 25 (1.841) Cm (23:52)
TOF MS ES+
5.46e4
418.0
418.1
419.0
434.0
413.1
283.0
208.0
486.0
486.0
Exact Mass: 418.1226
Na
PN-2
m/z


May23-2017-hmm-7-36H.10.fid
1H NMR (400 MHz, Chloroform- d) δ 8.48 (d, J = 8.3 Hz, 1H), 6.66 (d, J = 8.4 Hz, 1H), 5.21 (s, 1H), 4.40 (t, J = 5.1 Hz, 2H), 4.07 (dt, J = 21.4, 7.7 Hz, 2H), 3.71 (q, J = 5.1 Hz, 2H), 2.17 (q, J = 15.3 Hz, 4H), 1.37 – 1.23 (m, 3H), 0.98 – 0.70 (m, 4H).
PN-3
f1 (ppm)



References

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