Supporting Information

Phosphine-Mediated Three-Component Bioconjugation of Amino-and Azidosaccharides in Ionic Liquids

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General information

Materials and reagents

All the chemicals including saccharides were purchased from commercial vendors unless otherwise noted. 1-Azido-2-(2-methoxyethoxy)ethane was synthesized according to previous reports.[6] Doxorubicin hydrochloride (D4193) and Disialylnonasaccharide-β-ethylazide (D4217) was purchased from Tokyo Chemical Industry. Valrubicin (AD-32) was purchased from Biovision (B1833) and Vancomycin HCl was purchased from APExBIO (B1223). Oritavancin was purchased from Carbosynth (AA16180). Toluidine blue (01804) and brilliant blue (6104-59-2) were purchased from ChemImpex. 7-Benzylamino-4-nitrobenz-2-oxa-1,3-diazole (NBD-amine) was purchased from CombiBlocks (QA-8328). Hyaluronic acid (HA101) and hyaluronate azide (HA-1901) were purchased from CreativePEGWorks. 5-TAMRA cadaverine (1248-25) and trans-cycloctyne (TCO)-amine HCl salt (1021-25) were purchased from ClickChemTools. FITC labeled vancomycin (SBR00028-1.5MG), chitosan practical grade shrimp (417963-25G), DEAE-dextran hydrochloride (D9885-10g), fluorescein isothiocyanate dextran 200k (FD2000S), and amino-peg4-alkyne (764248-10MG) were all purchased from Sigma-Aldrich.

Instrumentation

NMR was performed on Bruker AVANCE NEO 500, 600, and 700. For ^{15}N NMR, neat nitromethane (381.6 ppm, TCI N0209) was used as the external standard.

MALDI-MS was conducted on a Bruker Daltonics Autoflex-TOF. A sample (0.5 or 1µL) was mixed with an equal volume (0.5 or 1 µL) of matrix solution (20 mg/mL soln in 50:50:0.1 H_2O/MeCN/trifluoroacetic acid) on a ground-steel MALDI plate (Bruker 8280784). Super-DHB or gentisic acid (DHBA) was used as a matrix.

LC-MS analysis of saccharides and small molecule models were performed on Shimadzu LCMS-2020 with a 2.6 µm C18 column (50 × 2.1 mm). The flow rate was 1 mL/min with the gradient of acetonitrile (5-90%) in the presence of 0.1% formic acid. The analysis of the reactions was performed by the UV detection saccharide at 280 nm.

For the other saccharides (doxorubicin and vancomycin), LC-MS analysis was conducted on Agilent Technologies 1260 Infinity II series single quad instrument with 5 µm Luna C18 column (150 × 4.6 mm). The flow rate was 0.5 mL/min with the gradient of acetonitrile (10-90%) in the presence of 0.1% trifluoroacetic acid. The analysis of the reactions were performed by the UV detection of peptide peaks at 254 nm.

HRMS was conducted on a high resolution mass spectrometer – the *Thermo Fisher Scientific Exactive Plus MS*, a benchtop full-scan Orbitrap™ mass spectrometer – using Heated Electrospray Ionization (HESI). Samples were diluted in acetonitrile and analyzed via flow injection into the mass spectrometer at a flow rate of 200 µL/min and via NanoMate injection. The mobile phase was 90% acetonitrile with 0.1% formic acid and 10% water with 0.1% formic acid. The mass spectrometer was operated in positive ion mode.

LC-MS analysis of modified DNA was performed on Thermo Vanquish LC system and LTQ-XL linear ion trap MS system with a C18 column (Hpersil Gold 25003- 032130, particle size 3 µm, diameter: 2.1 mm, length: 30 mm). The flow rate was 0.4 mL/min. Triethylammonium acetate buffer (5 mM, pH 7.2) was used as eluent with the gradient of acetonitrile (5-40% for 3.5 min, and then 90% for 1.5 min). The analysis of the reactions were performed by the UV detection at 254 nm.

Gel fluorescence and western blot imaging was performed on Amersham ImageQuant 800 (Cytiva). Gel or blot imaging was conducted using 360-nm, 535-nm, and 635-nm light sources with correlating emission bandpass filters at 525 nm (±20 nm), 605 nm (±40 nm), and 705 (±40 nm), relatively. Anti-biotin western blot was conducted

with streptavidin-Cy5 conjugate (Jackson ImmunoResearch 016-170-084, 1:2,000 dilution) after blocking with 5% BSA in TBST buffer.

FT-IR was performed on Cary 630 FTIR spectrometer by Agilent technologies.

Cell culture

HEK 293T cells (ATCC, CRL-3216) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax, 10% fetal bovine serum (FBS) and penicillin/streptomycin (0.5 mg/mL) using 10 cm Petri dishes (Sigma-Aldrich 280721) under 5% CO_2 at 37 °C.

Experimental procedures

Post reaction clean-up processes for ionic liquid-based saccharide bioconjugation (procedures for Fig S24)

Extraction of hydrophilic saccharides. To BMPy OTF, potassium bicarbonate aqueous solution (20 mM final concn from 2-M stock solution), fluorescein isothiocyanate (FITC)-labeled dextran (1 mg/ mL final concn from 5 mg/ mL-stock solution) and 1X phosphate buffered saline (0.3 µL) was added (30 µL total volume) and subjected to liquid-liquid extraction using a water-immiscible ionic liquid (BMPy NTF_2, 30 µL) and H_2O (60 µL). FITC-dextran was recovered from the solution by transferring the aqueous layer to a new Eppendorf tube. Any ionic liquid left in the aqueous layer was further removed by washing with ethyl acetate (60 µL). Extraction was confirmed by visualization under a short UV wavelength (360 nm).

Acetone precipitation. To BMPy OTF, fluorescein isothiocyanate (FITC)-labeled vancomycin (3.0 mM final concn from 25-mM stock solution was added (30 µL total volume). Cold acetone (600 µL, –20 °C) was added in one portion to the mixture in a 1.7-mL Eppendorf tube. The mixture was mixed by flipping the tube upside-down multiple times and kept at –80 °C for 1 h. Once removed from the freezer, the mixture was centrifuged (15,000 rcf, 15 min, 4 °C) to obtain the precipitates. Then, the acetone was removed and the pellet air-dried on bench at rt for 15 min

Extraction of hydrophobic saccharides. To EMIM OAc, 1X phosphate buffered saline (0.3 µL) and valrubicin (0.5 mM final concentration from 10-mM stock solution in DMSO) were added (30 µL total volume). The mixture was subjected to liquid-liquid extraction using a 2:1 mixture of ethyl acetate/water. Valrubicin was obtained from the mixture by transferring the aqueous layer to a new Eppendorf tube. The aqueous layer was further washed with ethyl acetate (60 µL) to remove any ionic liquid in the solution.

Thin Layer chromatography of small saccharides and molecules. To a 1:1 mixture of BMPy OTf/MeOH in a 1.7-mL Eppendorf tube, a mixture of 3:3:1 valrubicin/ NBD-amine/brilliant blue (5 mM final concn from 50-mM stock solution of each) was added. The resulting solution (10 µL) was spotted on a reverse-phase TLC (MilliporeSigma #1156850001) and developed using 70% MeOH in H_2O and 1% trifluoroacetic acid. The portion of C18/silica gel containing valrubicin was extracted using hexafluoroisopropanol (750 uL) by sonicating the mixture for 10 min. The mixture was centrifuged (15,000 rcf, 15mins, room temperature) to obtain the supernatant containing valrubicin.

General procedure for acetone precipitation

Cold acetone (600–1200 µL, –20 ℃) was added in one portion to the reaction mixture (typically 20–40 µL) in a 1.7-mL Eppendorf tube. The mixture was mixed by flipping the tube upside-down multiple times and kept at –80 ℃ for 1 h to overnight. Once removed from the freezer, the mixture was centrifuged (15,000 rcf, 15 min, 4 ℃) to obtain the precipitates. Then, the acetone was removed and the pellet air-dried on bench at rt for 15 min. For MALDI-MS analysis, the pellet was further washed with an additional cycle of acetone and centrifugation before air-drying process. The dried pellet was reconstituted in 10–40 µL of water and analyzed by respective analytical methods.

Typical saccharide/small molecule model modification in ionic liquids

To ionic liquids (typically 10–40 µL for analytical scale), potassium bicarbonate aqueous solution (20 mM final concentration from 2-M stock solution in water), saccharide or small molecule (0.02–0.7 mM final concentration from 25–50-mM stock solution in water), alkyl azide (3–125 mM final concentration from 100–1000-mM stock solution in DMSO), and PAr_3 or $O{=}PPh_3$ (3–125 mM final concentration from 100–500-mM stock solution in DMSO) were added. The final concentration of H_2O was kept lower than 6% v/v. The reaction mixture was incubated at 37–50 °C for 2 h.

Pyrene amine modification with pyrene azide (a procedure for Fig 1b)

To DMF (30.25 uL), pyrene-amine (100 mM final concn from 0.4-M stock solution), pyrene-azide (20 mM final concn from 1-M stock solution), and PPh_3 (12.5 mM final concn from 1-M stock solution in 1:1 toluene/DMSO) were added (50 µL total volume). The reaction was quadruplicated in different Eppendorf tubes. The reaction mixtures were incubated at 50 °C overnight. The reaction mixtures were combined into one Eppendorf tube and placed at −20 °C for 1 week to afford crystals suitable for the X-ray analysis.

Pentanucleotide modification with azide

The urea forming reaction on pentanucleotide (5'-TTTTT-5' where T = thymidine with alkylamine containing 12 carbon linker) was performed following the same procedure as described in *Typical saccharide/small molecule model modification in ionic liquids* in 30-µL scale with the following conditions. 5'-TTTTT-5' (0.2 mM final concentration from 5-mM stock solution in H_2O), $^{12}C/^{13}C$-K_2CO_3 (20 mM final concentration from 2-M stock solution in H_2O), azide **1a** (7.5 mM final concentration from 250-mM stock solution in DMSO), and PPh_3 (20 mM final concentration from 500-mM stock solution in DMSO) in BMPy OTf at 50 °C for 2 h. The product was purified by adding cold acetone (600 µL, −20 °C) to the reaction mixture, mixed by upside-down shaking and allowed to sit at −80 °C overnight. The precipitates were collected by centrifugation (15,000 rcf, 15 min, 4 °C), and acetone was removed. The pellet obtained was further washed with acetone and the centrifugation process repeated before the pellet was air-dried on the bench at room temperature for 15 min. The dried pellet was reconstituted in 30 µL of ammonium bicarbonate (NH_4HCO_3) aqueous solution (5 mM) and analyzed using LC-MS.

Reaction of amine-containing anthracene compounds (a procedure for Figure 2b)

To a 1:1:1 mixture of DMF (10.53 uL), MeCN (10.53 uL), and BMPyOTf (10.53 uL), potassium bicarbonate aqueous solution (20 mM final concn from 2-M stock solution), anthracene derivatives (2.5 mM final concn from 50-mM stock solution in DMSO), **1a** (50 mM from 1000-mM stock solution in DMSO), and PPh_3 (50 mM final concn from 500-mM stock solution in DMSO) were added. The final concn of H_2O was kept at 1% v/v. The reaction mixture was incubated at a 50 °C incubator for 2 h. The reaction mixture was purified by thin layer chromatography (basic aluminum oxide 60, MilliporeSigma #1057130001) with 98:2 dichloromethane/MeOH as eluents. The product was recovered from alumina by addition of MeCN (750 uL) and sonication. This mixture was centrifuged (15,000 rcf, 15 min, rt) and the supernatant was analyzed by LC-MS.

Doxorubicin/valrubicin modification (a procedure for Figure 2d).

Aqueous solution of potassium bicarbonate (20mM final concentration prepared from 2-M stock solution in water), doxorubicin or valrubicin (0.3 mM final concentration prepared from 25-mM stock solution in DMSO), azide **1a** (7.5 mM final concentration from 250-mM stock solution in DMSO) and PPh_2(m-sulfophenyl) (3 mM final concentration prepared from 100-mM stock solution in DMSO) or DMSO used as a negative control were added to a 1:3 mixture of BMPy OTF and MeCN. The reaction mixture was incubated at 37 ^oC for 2 h. The product was purified by reverse-phase TLC plate (MilliporeSigma #1156850001) using 95:5 acetonitrile/water. The portion of silica/C18 containing the desired saccharides was extracted using hexafluoroisopropanol (750 uL) by sonicating the mixture of the solvent and silica/C18 for 10 min. The mixture was centrifuged (15,000 rcf, 15 mins at room temperature), and the supernatant was transferred to a 5-mL Eppendorf tube. The silica/C18 was washed with additional HFIP (200 uL) and transferred to the same Eppendorf tube. The content of the tube was diluted with MeCN (3 mL), syringe filtered, and the solvent was evaporated by gentle flow of the nitrogen gas. The obtained solution was analyzed by LC-MS.

Modification of vancomycin and its derivatives: FITC-vancomycin and oritavancin.

To BMPy OTf, potassium bicarbonate aqueous solution (20 mM final concn from 2-M stock solution), vancomycin and its derivatives (0.5 mM final concn from 25-mM stock solution in DMSO), carboxylic peg2azide (125 mM final concn from 1000-mM stock solution in DMSO), and tritolylphosphine (125 mM final concn from 500-mM stock solution in 1:1 DMSO/toluene) were added (30 µL total volume). The reaction mixture was incubated at 50 °C for 2 h and subjected to acetone precipitation as described in the *General procedure for acetone precipitation* section using 600µL cold acetone. The reconstituted samples were analyzed using LC-MS.

Chitosan modification (a procedure for Figure 3b)

To EMIM OAC (20 µL), chitosan or DEAE-dextran (1 mg/mL final concentration from 10 mg/mL stock solution in 1:1 BMIM: 1-M acetate buffer), biotin-peg3-azide (0.3-7.5mM final concentration from 15-375 mM stock solutions in DMSO), and PPh_3 or $O{=}PPh_3$ (0.3-7.5 mM final concentration from 15-375 mM stock solution in DMSO) were added. The final concn of H_2O was kept lower than 6% v/v. The reaction mixture was incubated in a 37 °C incubator for 2 h and subjected to *Post-reaction cleanup process for chitosan experiments*.

Post-reaction cleanup process for chitosan experiments

To the reaction mixture (20 µL) in a 1.7-mL Eppendorf tube, a mixture of 5:1 acetone/methanol (600 µL) was added in one portion. The mixture was mixed by upside-down shaking and sit at -80 °C for 1 h or overnight. The precipitates were collected by centrifugation (15,000 rcf, 15 min, 4 °C), and acetone/methanol was removed. The pellet was air-dried on the bench at room temperature for 15 min. The dried pellet was reconstituted in 20 µL of acetate buffer (20 mM, pH 5) and analyzed by dot blot methods.

Immunodetection of biotin tag of chitosan using anti-biotin antibodies

The reconstituted samples (0.5 µL) were heated for 1 min at 95°C and then spotted onto nitrocellulose membrane. Eosin Y solution (0.1 mM final concentration in water from 50-mM stock solution) was used for the total stain purpose for 5 min, and the membrane was rinsed with water twice. The stained membrane was imaged by ImageQuant 800 to obtain the colorimetric image. Then, the membrane was washed twice with TBST buffer for 5 min, blocked with 5% BSA in TBST buffer at rt for 20 min, incubated with streptavidin-Cy5 conjugate (1:2000) in the blocking buffer at rt for 40 min, washed with TBST buffer three times, and imaged by ImageQuant 800.

Pyrene-azide modification with amine reagent

To a 1:8:1 mixture of DMF (4.52 uL), MeCN (36.16 uL), and BMPyOTf (4.52 uL), potassium bicarbonate aqueous solution (20 mM final concn from 2-M stock solution), azidomethyl-pyrene (12.5 mM final concn from 250-mM stock solution in DMSO), 2-(2-methoxyethoxy)ethanamine (125 mM final concn from 1000-mM stock solution in DMSO), and PPh_3 (125 mM final concn from 500-mM stock solution in DMSO) were added. This reaction was replicated in 14 separate 1.7-mL Eppendorf tubes. The reaction mixtures were incubated at 50-°C incubator for 1 h. Once the reaction was complete, all the mixtures were combined into one tube. The solution was purified by thin layer chromatography (basic aluminum oxide 60, MilliporeSigma #1057130001) with 80:20 dichloromethane/hexane as eluents. This process was repeated two more times with 99:1 dichloromethane/MeOH as eluents for thin layer chromatography. The product was recovered from the alumina by addition of 9:1 MeCN/MeOH and sonication and analyzed by LC-MS and ^1H NMR after separating the insoluble alumina by centrifugation.

UndecaSaccharide-azide modification with alkylamine reagents (a procedure for Figure 4b)

BMPy OTf (16.8 uL), $KHCO_3$ (40 mM final concn from 2-M stock solution), Undecasaccharide-azide (0.2 mM final concn from 5-mM stock solution), alkyl amines with different degrees of substitution (20 mM final concn from 250-mM stock solution), and PPh_3 or $O{=}PPh_3$ (20 mM final concn from 1-M stock solution in 1:1 DMSO/toluene) were mixed and incubated at 50 ^oC for 2 hr. The rest of the procedure is the same as the acetone precipitation procedure from *General procedure for acetone precipitation.*

Typical procedure for modification of hyaluronic acid derivatives

To EMIM OAc (20 µL), Hyaluronic acid derivatives hyaluronic acid derivatives (2 mg/mL final concentration from 40 mg/mL-stock solution in H_2O), amine reagent (10–20 mM final concentration from 250-mM stock solutions in DMSO), and PPh_3 or $O{=}PPh_3$ (20 mM final concentration from 250-mM stock solution in DMSO) were added. The final concn of H_2O was kept lower than 6% v/v. The reaction mixture was incubated in a 37 °C incubator for 2 h and subjected to precipitation before analysis.

Hyaluronic acid derivative modification with TAMRA amine (a procedure for Figure 4c)

The modification procedure follows a protocol *Typical procedure for modification of hyaluronic acid derivatives* in 20 µL scale with following conditions. Hyaluronic acid derivatives (2 mg/mL final concentration from 40-mg/mL

stock solution in H_2O), TAMRA-NH_2 (20 mM final concentration from 250-mM stock solution in DMSO), $KHCO_3$ (20 mM final concentration from 2-M stock solution in H_2O), and PPh_3 or O=PPh_3 (20 mM final concentration from 500-mM stock solution in DMSO) were added into EMIM OAC and incubated for 2 h at 37 °C. To the reaction mixture (20 µL) in a 1.7-mL Eppendorf tube, a 5:1 mixture of cold acetone/methanol (600 µL) was added in one portion, mixed by upside-down shaking and placed overnight at –80 °C. The precipitates were collected by centrifugation (15,000 rcf, 15 min, 4 °C), and acetone/methanol was removed, and the pellet was air-dried on the bench at room temperature for 15 min. The dried pellet was reconstituted with 20 µL H_2O, spotted onto nylon membrane (MilliporeSigma #11209299001) (and washed overnight with 1:1 MeOH/DMSO, and the fluorescence intensity was quantified by ImageQuant 800.

The reconstituted samples were also spotted separately on a new membrane and stained with diluted Toluidine Blue solution (3 mM final concentration in water from 250-mM stock solution in DMSO) for 5 min and rinsed 4 times with water. The stained membrane was imaged by ImageQuant 800 to obtain the colorimetric image. For the quantification purpose, the experiment was done in triplicates but on different days and the fluorescence intensity was quantified by ImageJ software.

Hyaluronic acid derivative modification with Propargyl-NH_2 and Alkyne-Peg4-NH_2.

The modification procedure follows a protocol *Typical procedure for modification of hyaluronic acid derivatives*. Hyaluronic acid derivatives (2 mg/mL final concentration from 40-mg/mL stock solution in H_2O), propargyl amine or alkyne-peg4-amine (20 mM final concentration from 250-mM stock solution in DMSO), $KHCO_3$ (20 mM final concentration from 2-M stock solution in H_2O), and PPh_3 or O=PPh_3 (20 mM final concentration from 500-mM stock solution in DMSO) were added into EMIM OAc and incubated for 2 h at 37 °C. To the reaction mixture (20 µL), in a 1.7-mL Eppendorf tube, a mixture of acetone/methanol was added in one portion. The precipitation was performed with cold acetone/methanol (5:1 ratio, 600 µL,–20 °C). After the addition of acetone/methanol, the mixture was mixed by upside-down shaking and sit at –80 °C overnight. The precipitates were collected by centrifugation (15,000 rcf, 15 min, 4 °C), and acetone/methanol was removed and the pellet was air-dried on the bench at room temperature for 15 min. The dried pellet was reconstituted with 7 µL H_2O, spotted onto nylon membrane and rinsed with MeOH for 5 min, then membrane was washed with water twice and subjected to chemical blotting[5] for 30 min with the following conditions: THPTA (0.1 mM final concentration from 0.1-M stock solution in H_2O, sodium ascorbate (1.5 mM final concentration from 0.1-M stock solution in H_2O, $CuSO_4$ (0.1 mM final concentration from 100 mM stock solution in H_2O), and coumarin azide (10 µM final concentration from 20-mM stock solution in DMSO) in 5 ml of 1:1 H_2O/DMSO.

The reconstituted samples were also spotted separately on new nylon membrane and stained with the toluidine Blue solution (3mM final concentration in water from 250-mM stock solution in DMSO) for 5 min, and the membrane was rinsed 4 times with water. The stained membrane was imaged by ImageQuant 800 to obtain the colorimetric image. For the quantification purpose, the experiment was done in triplicates but on different days and the fluorescence intensity was quantified by ImageJ software.

Modification of hyaluronic acid derivatives with TCO-NH_2

The modification procedure follows a protocol *Typical procedure for modification of hyaluronic acid derivatives*. Hyaluronic acid derivatives (2 mg/mL final concentration from 40-mg/mL stock solution in H_2O) TCO-NH_2 (10 mM final concentration from 250-mM stock solution in DMSO), $KHCO_3$ (20 mM final concentration from 2-M stock solution in H_2O), and PPh_3 or O=PPh_3 (20 mM final concentration from 500-mM stock solution in DMSO) were added into EMIM OAC and incubated for 2 h at 37 °C To the reaction mixture (20 µL),a mixture of

acetone/methanol was added in one portion. The precipitation was performed with cold acetone/methanol (5:1 ratio, 600 µL,–20 °C). After the addition of acetone/methanol, the mixture was mixed by upside-down shaking and kept at –80 °C overnight. The precipitates were collected by centrifugation (15,000 rcf, 15 min, 4 °C), and acetone/methanol was removed and the pellet was air-dried on the bench at room temperature for 15 min. The dried pellet was reconstituted with 50-mM MES buffer containing sulfu-Cy5 tetrazine (20 µL,1mM) and incubated for 2 h at rt. Acetone (600 µL) was added to the reconstituted samples and precipitation was performed again at –80 °C overnight. After the centrifugation and removal of the supernatant, the samples were washed by two additional cycles of acetone addition and centrifugation. The pellet was air-dried on bench at rt for 15 min after removing the final acetone solution and then reconstituted with 20 µL H_2O, spotted on nylon membrane. The membrane was washed 4 times with 1:1 MeOH: DMSO and imaged by ImageQuant800 to obtain fluorescence image.

The reconstituted samples were spotted separately on a new nylon membrane and stained with the toluidine blue solution (3mM final concentration in water from 250-mM stock solution in DMSO) for 5 min and rinsed 4 times with water. The stained membrane was imaged by ImageQuant 800 to obtain the colorimetric image. For the quantification purpose, the experiment was done in triplicates but on different days and the fluorescence intensity was quantified by ImageJ software.

Cell Lysis

HEK 293T cultured cells without poly-D-lysine coating at 100% confluency (15 million) were taken out, washed three times with PBS, separated from the buffer by centrifugation (1000 rcf, 3 min, 4 °C), placed in –80 °C for 30 min, and lysed in PBS buffer (960 µL) containing 0.1% SDS, 0.1% triton and EDTA-free protease inhibitor (complete tablets, Roche #04-693-159-001). The cell lysate was transferred to 1.7-mL Eppendorf tube, and a homogenizer was used to assure the complete lysis of cells. The cell lysate was placed for 30 min in ice before centrifugation (15000 rcf, 15min, 4°C) and the supernatant was taken out to run the oxidation reaction.

Periodate oxidation and hydrazone formation on saccharide groups of glycoproteins in cell lysates.

Sodium periodate (30 mM final concentration from 1000-mM stock solution in H_2O) or H_2O (as a negative control) was added to a 1.7-mL Eppendorf tube containing cell lysates (310 µL total). The mixture was incubated for 1h at rt before quenching with 20 % v/v glycerol in TBST buffer (16.5 µL). 103-µL aliquots were transferred to three different tubes, and cold acetone (1200 µL,–20 °C) was added to each tube. After the addition of acetone, the mixture was mixed by inversion processes and kept at –80 °C overnight. The precipitates were collected by centrifugation (15,000 rcf, 15 min, 4 °C), acetone was removed, and the pellet was air-dried on the bench at room temperature for 15 min. The dried pellet was reconstituted with 50 µL of complete PBS buffer (0.1% v/v SDS, 0.1% v/v triton and EDTA-free protease inhibitor) followed by the addition of azido-PEG4-hydrazide (MedChemExpress HY-140814) (12 mM final concentration from 20-mM stock solution in DMSO) to both oxidized and control lysates. After an overnight reaction, acetone (600 µL) was added to the resultant mixture which was put at -80 °C overnight. Centrifugation (15,000 rcf, 15 mins, 4 °C) afforded the hydrazone azide pellets which were air-dried at rt for 15 min and reconstituted in 12.5-µL PBS buffer (0.1% v/v SDS, 0.1% v/v triton). The lysate concentration was determined by Bradford assay, and the concentration of the azide-tagged and non-tagged solutions were adjusted to the same.

Modification of azide containing saccharides in cell lysates.

To BMPy OTf (16.4 µL), $KHCO_3$ (20 mM final concentration from 2-M stock solution), cell lysates with and without hydrazone azide (0.91 mg/ mL), biotin amine (20 mM final concentration from 250-mM stock solution), and PPh_3/ $O{=}PPh_3$ (20 mM final concentration from 500-mM stock solution in DMSO) were added. The final concn of H_2O was kept lower than 6% v/v. The reaction mixture was incubated at 37 °C for 2 h and subjected to overnight precipitation using 1:1 toluene/acetone (600 µL) at – 80 °C. The resultant suspension was centrifuged (15,000 rcf, 15 mins, 4 °C), the air-dried pellet was reconstituted in 5 µL PBS buffer (0.1% v/v SDS, 0.1% v/v triton). To the reconstituted pellet (5 µL), water (2.5 µL) and 4X LDS sample buffer (2.5 µL) were added, mixed and used for SDS-PAGE gel electrophoresis for 40 min. The modified glycoproteins on the gel were transferred to a PVDF membrane (Biorad TransBlot Turbo PVDF membrane L002048A). The membrane was then activated with methanol and washed twice with water before Ponceu staining to obtain the colorimetric image using ImageQuant 800. The membrane was once again reactivated with methanol and washed twice with water followed by TBST buffer for 5 mins, blocked with BSA (50 mg/mL) in TBST buffer at rt for 1 h, incubated with streptavidin-Cy5 conjugate (1:2000) in the blocking buffer at rt for 40 min, washed with TBST buffer three times, and imaged by ImageQuant 800. Molecular weight marker (Thermo Scientific 26619) was used for the analysis.

Supporting figures

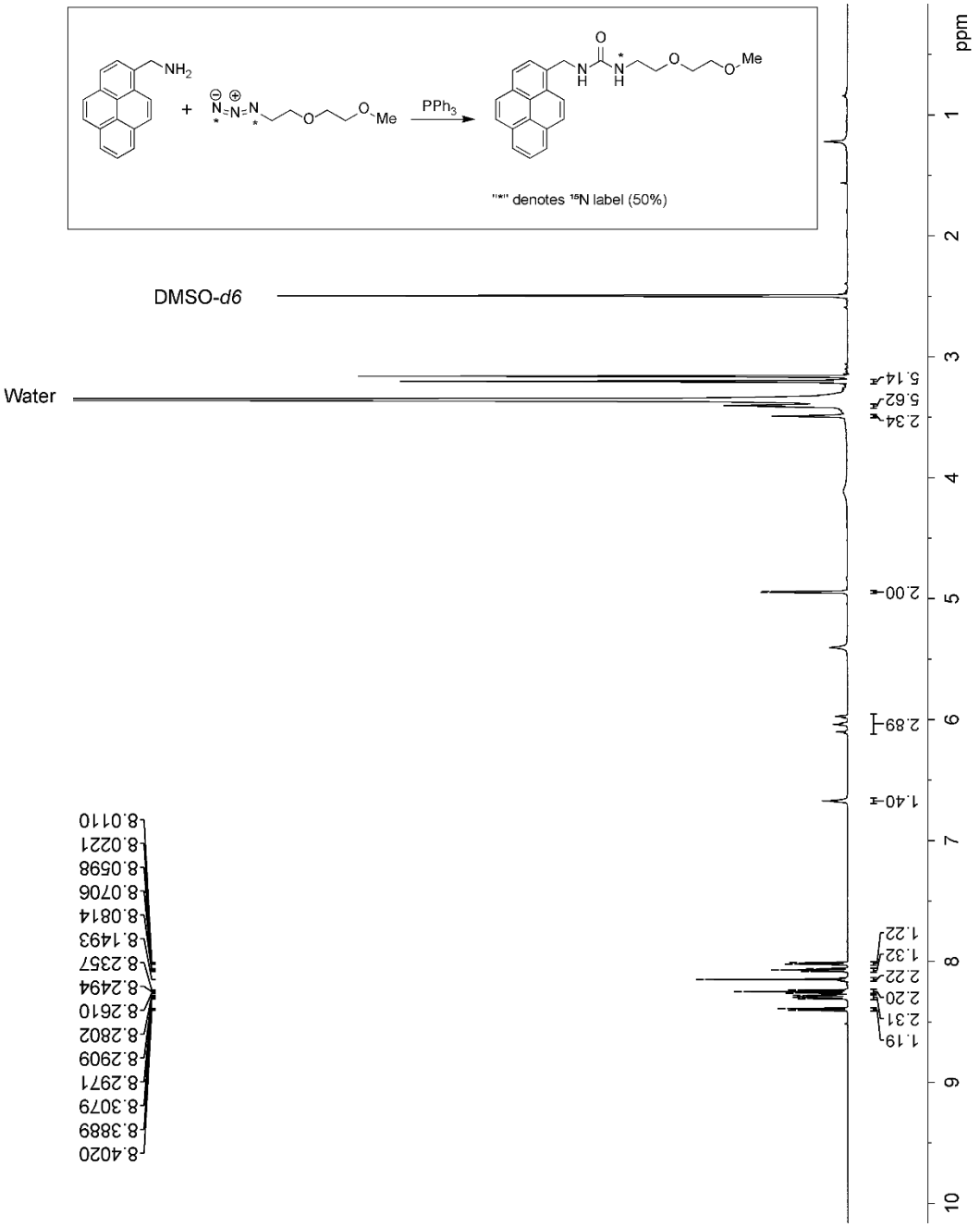


Figure S1. ^{1}H NMR spectrum of ^{15}N-enriched pyrene urea compound in DMSO-d_6 using terminal ^{15}N-enriched azide. Reaction conditions are described in *Preparative synthesis of small molecules* section

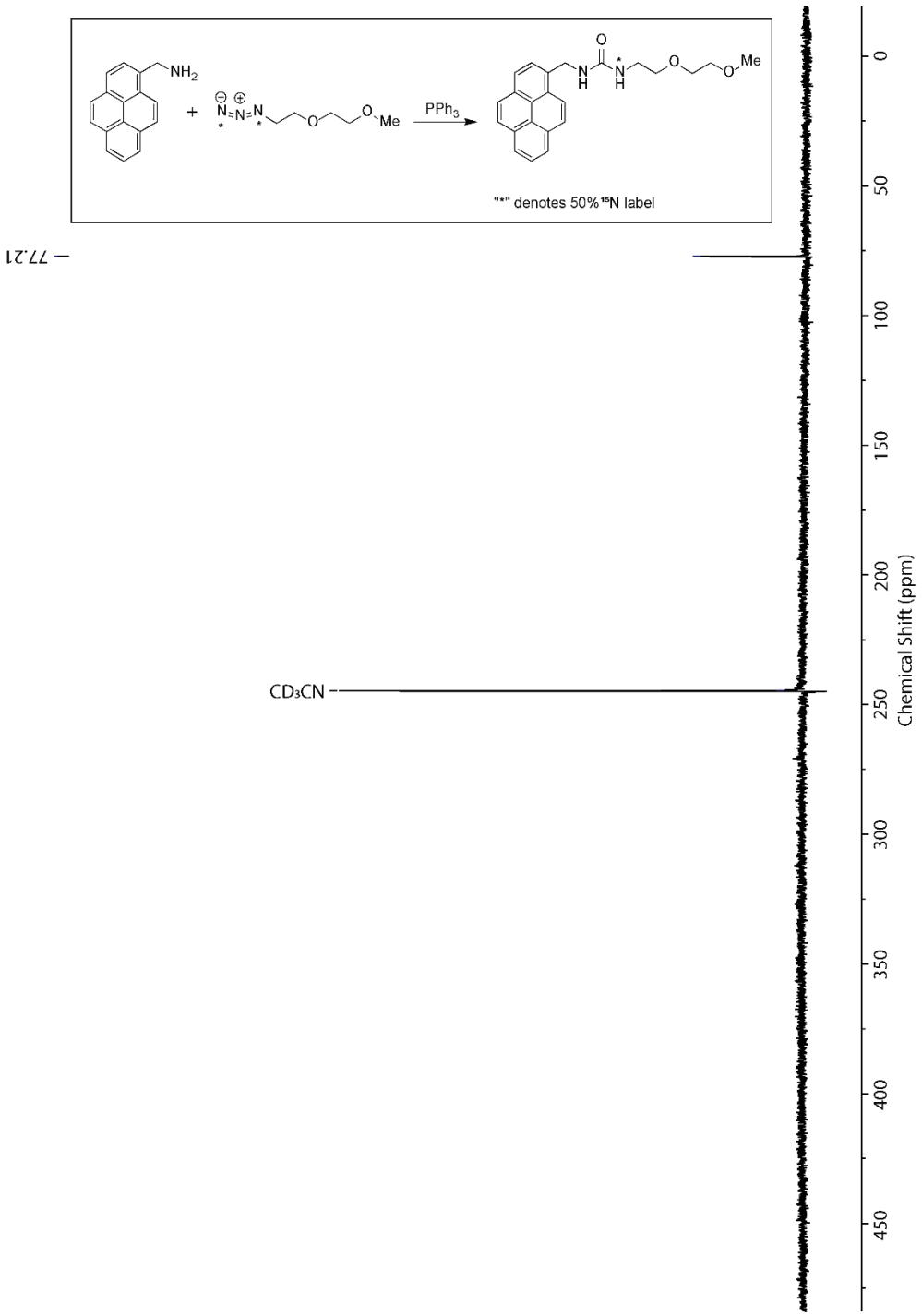


Figure S2. ^{15}N {^{1}H} NMR spectrum of ^{15}N-enriched pyrene urea compound in 95:5 CD_3CN/DMSO-d_6 using terminal ^{15}N-enriched azide

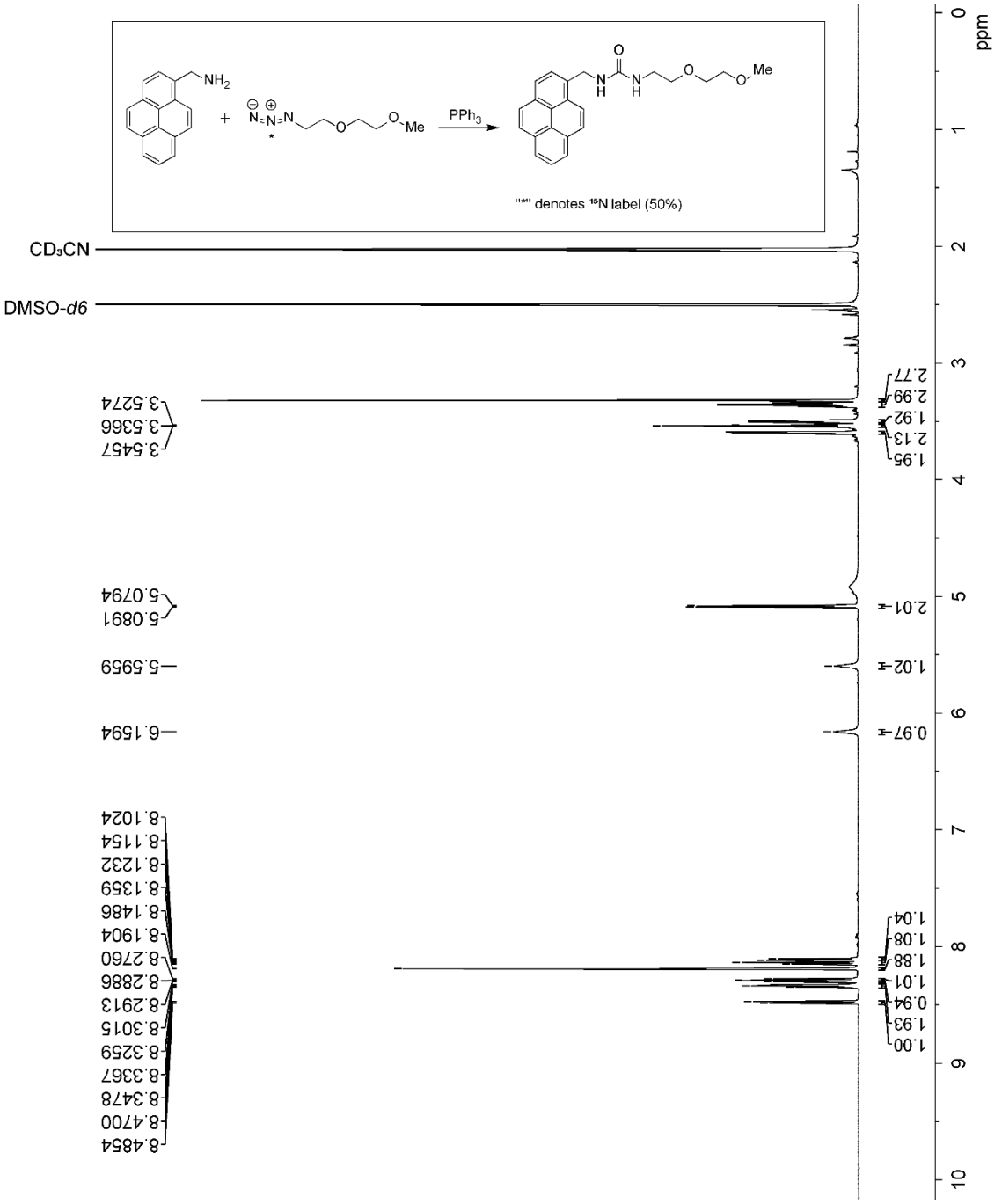


Figure S3. ^{1}H NMR spectrum of ^{15}N-enriched pyrene urea compound CD_3CN/DMSO-d_6 (95:5) using internal ^{15}N-enriched azide. Reaction conditions are described in *Preparative synthesis of small molecules* section.

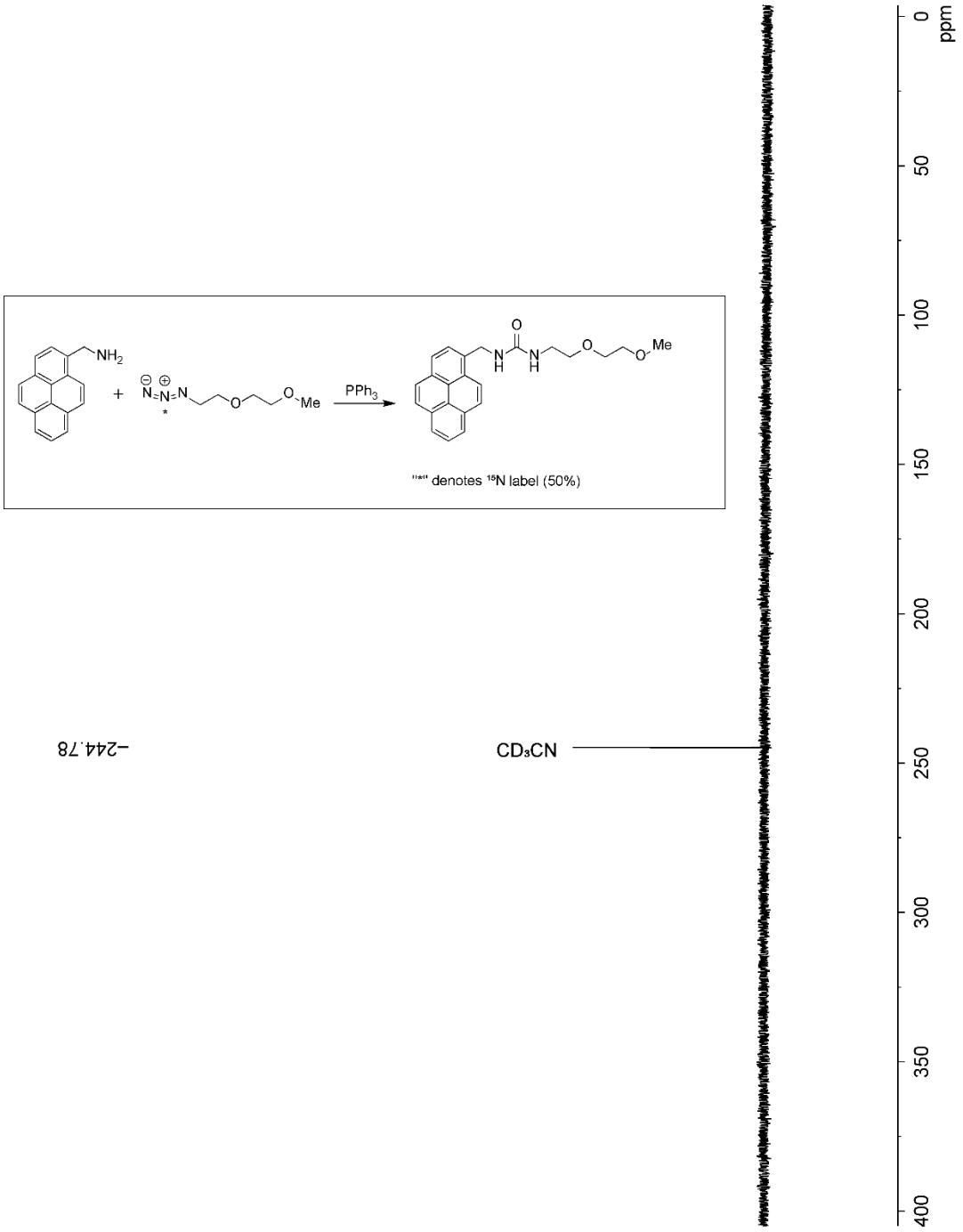


Figure S4. $^{15}N\{^{1}H\}$ NMR spectrum of ^{15}N-enriched pyrene urea compound in CD_3CN/DMSO-d_6 (95:5) using internal ^{15}N-enriched azide. Neat CH_3NO_2 (381.6ppm) was used as the external standard.

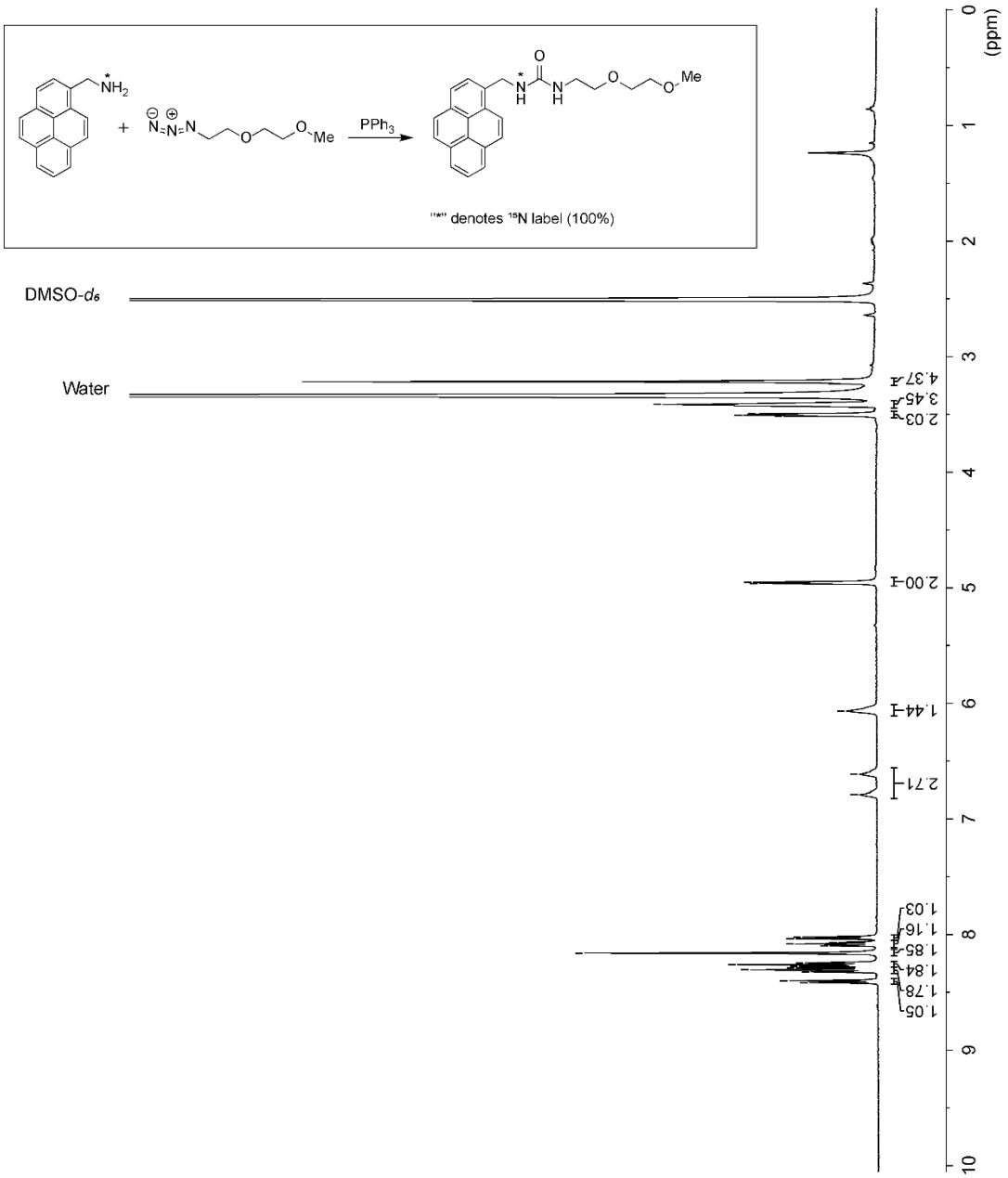


Figure S5. ^{1}H NMR spectrum of ^{15}N-enriched pyrene urea compound in DMSO-d_6. Reaction conditions are described in *Preparative synthesis of small molecules* section.

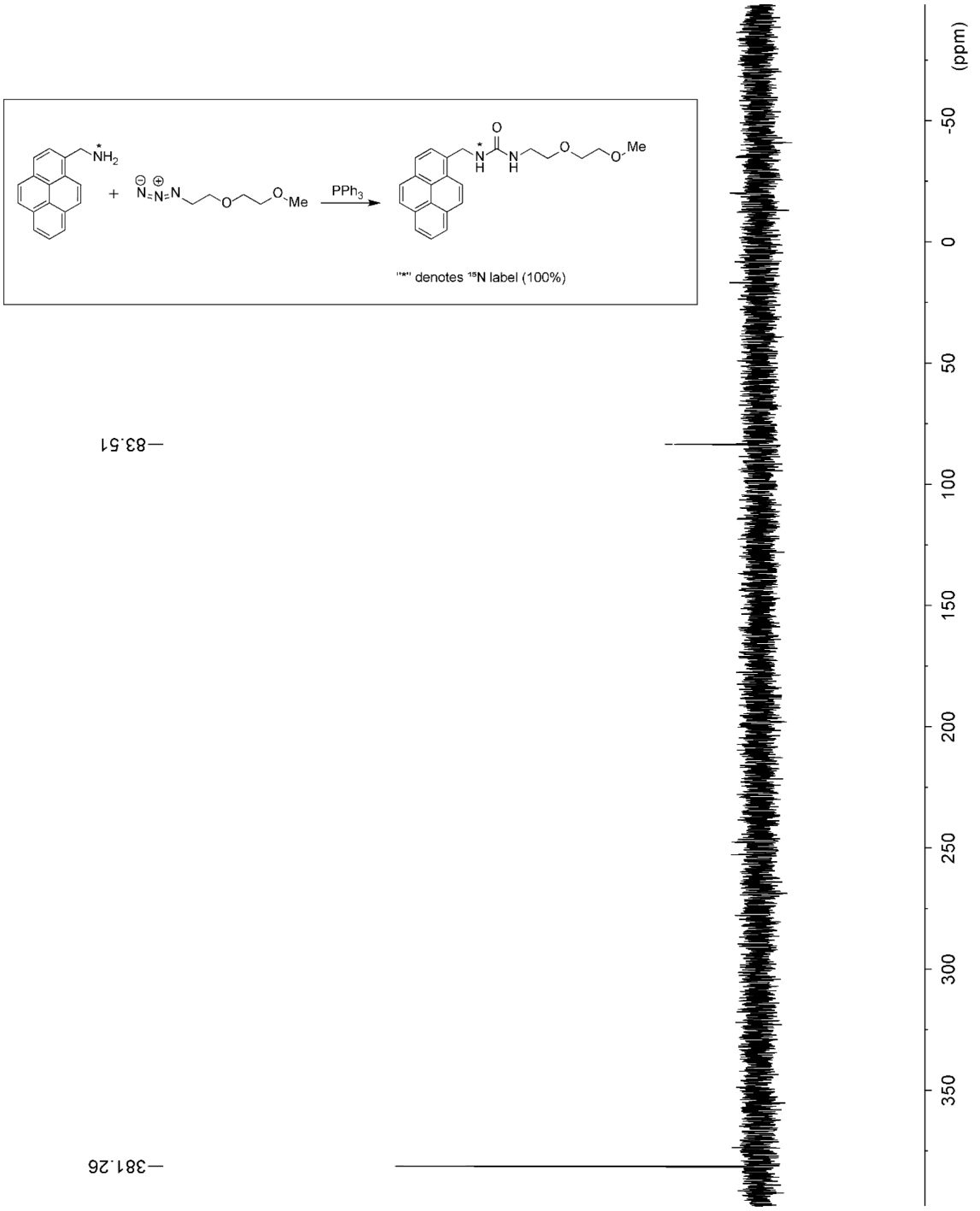


Figure S6. ^{15}N $\{^{1}H\}$ NMR spectrum of ^{15}N-enriched pyrene urea compound in DMSO-d_6. Neat CH_3NO_2 (381.6ppm) was used as the external standard.

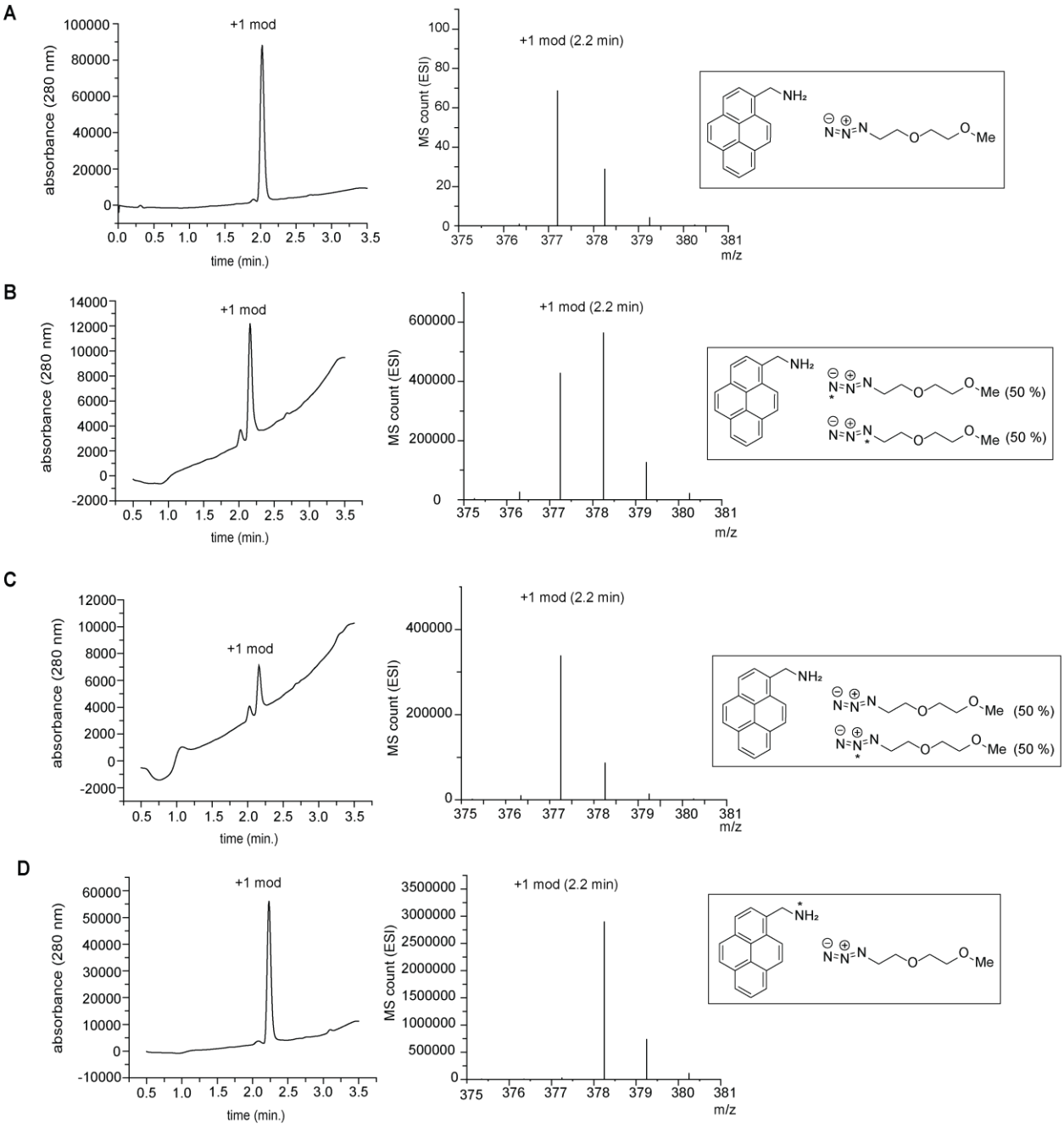


Figure S7. LC-MS analysis (Shimadzu LCMS-2020) of the reaction mixture of alkylazide **1a** with and pyrene amine (A), ^{15}N isotope on the terminal position of azide (B) and ^{15}N isotope on the internal position of azide (C).and ^{15}N-enriched pyrene amine with alkylazide **1a** Reaction conditions are described in the *Preparative synthesis of small molecules section*.

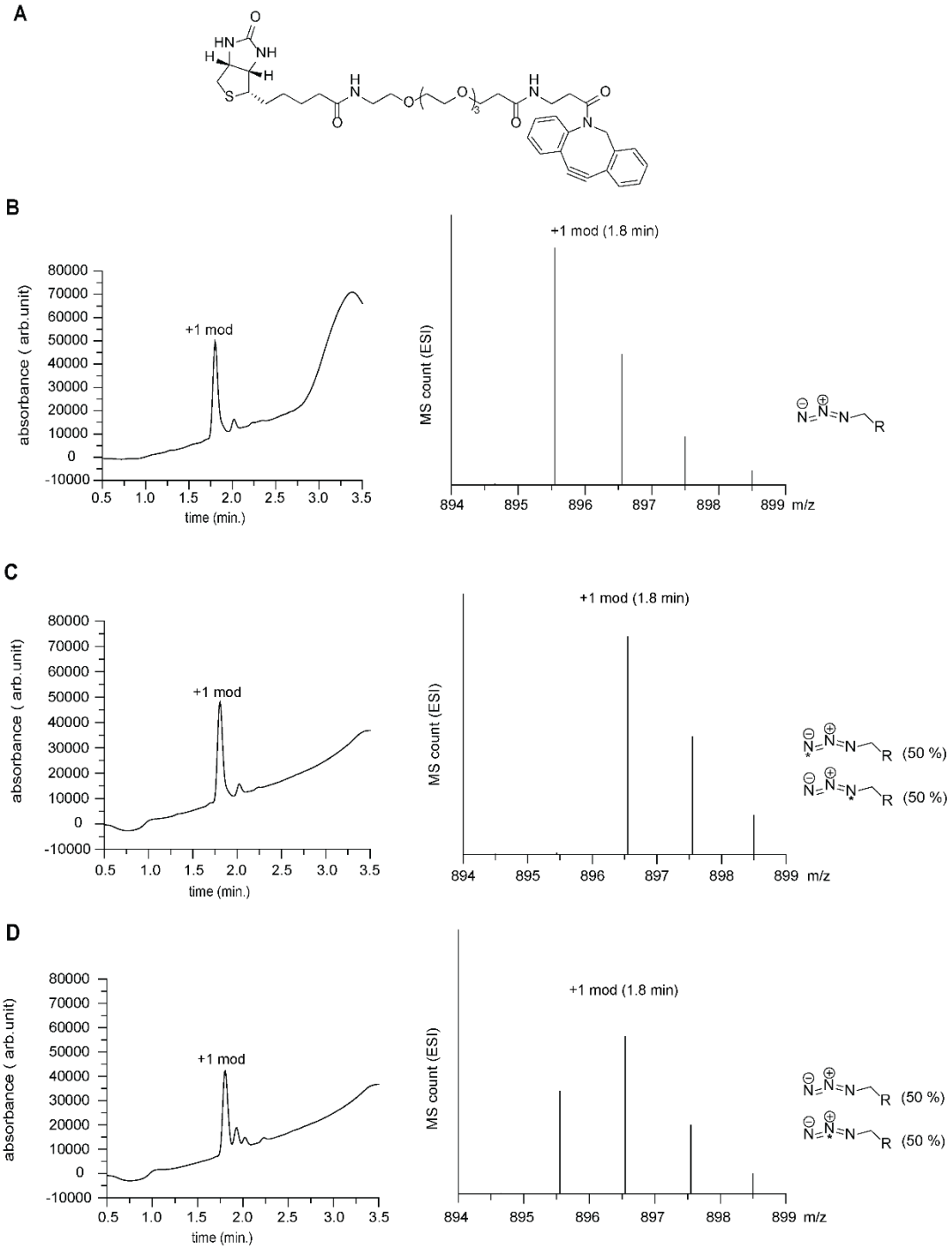


Figure S8. Chemical structure of cyclooctyne-biotin (A) and LC-MS analysis (Shimadzu LCMS-2020) of the reaction mixture of alkylazide **1a** and cyclooctyne-biotin with no isotope label (B), ^{15}N isotope on the terminal position (C) and ^{15}N isotope on the internal position (D). Reaction conditions: alkylazide **1a** (0.3 mM final concentration from 5-mM stock solution in DMSO), cyclooctyne biotin (0.1 mM final concentration from 5-mM stock solution in DMSO) in 1:1 H_2O/MeCN (40 µL) at room temperature, overnight.

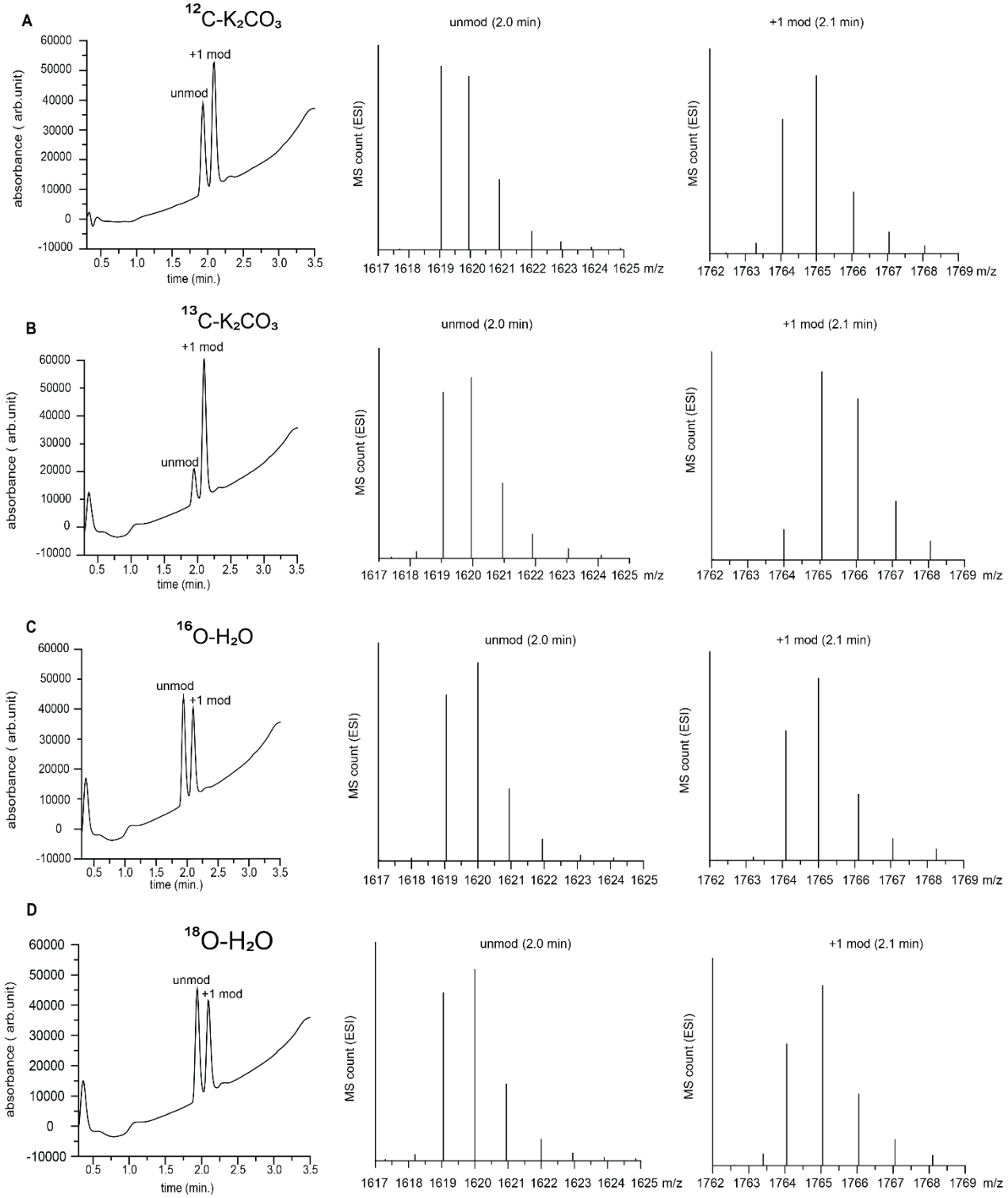


Figure S9. LC-MS analysis (Shimadzu LCMS-2020) of daptomycin modification using $^{12}C/^{13}C$-K_2CO_3 (A, B) and $^{16}O/^{18}O$-H_2O (C, D). Reaction conditions: Daptomycin (0.6 mM), $^{12}C/^{13}C$-K_2CO_3 (20 mM) or $^{16}O/^{18}O$-H_2O (0.3 µL, 1% v/v), azide **1a** (75 mM) and PPh_3 (75 mM) at 50 °C for 2 h.

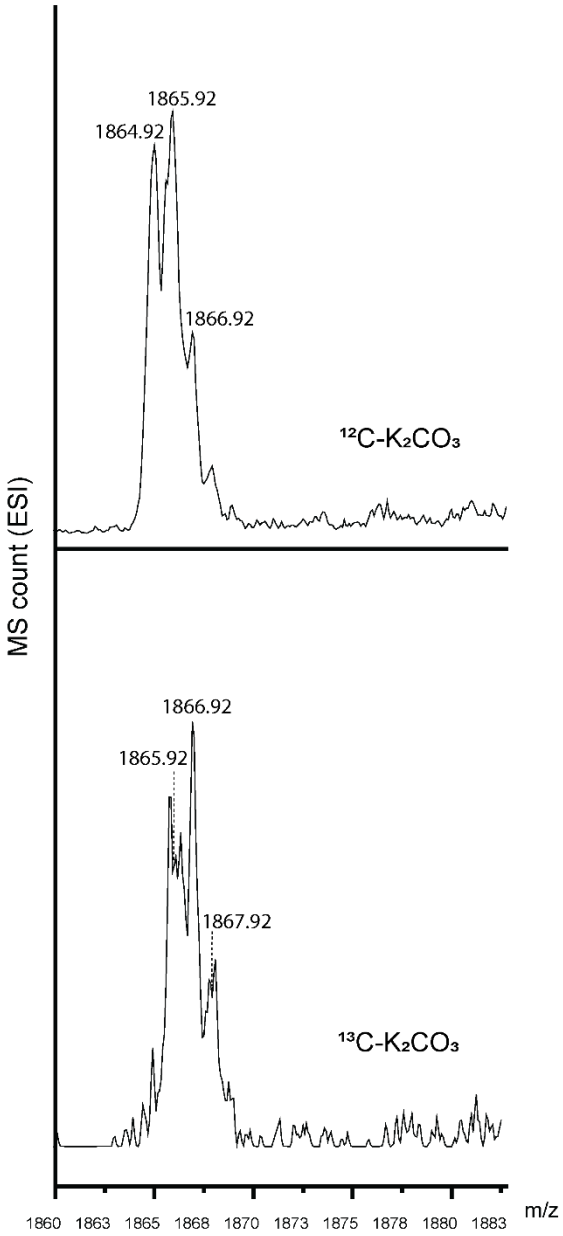


Figure S10. ESI-MS analysis (Thermo Scientific LTQ XL) of pentanucleotide (5'-TTTTT-5'-alkylamine) modification using ^{12}C-K_2CO_3 (upper spectrum) and ^{13}C-K_2CO_3 (lower spectrum). Reaction conditions are described in *Typical saccharide/small molecule model modification in ionic liquids* section.

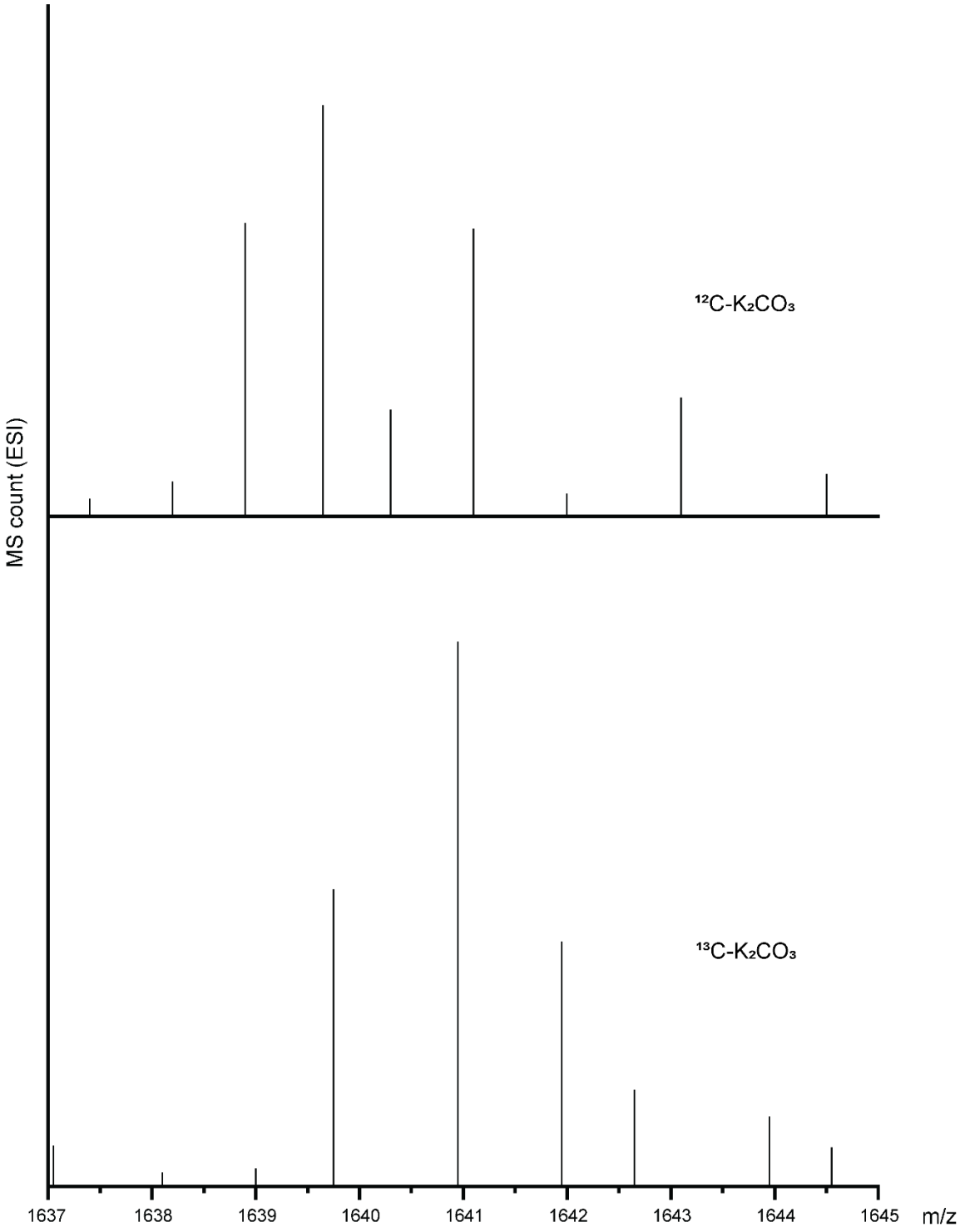


Figure S11. ESI-MS analysis (Shimadzu LCMS-2020) of vancomycin modification using ^{12}C-K_2CO_3 (upper spectrum) and ^{13}C-K_2CO_3 (lower spectrum). Reaction conditions are the same as vancomycin modification as described in *Typical saccharide/small molecule model modification in ionic liquids* section.

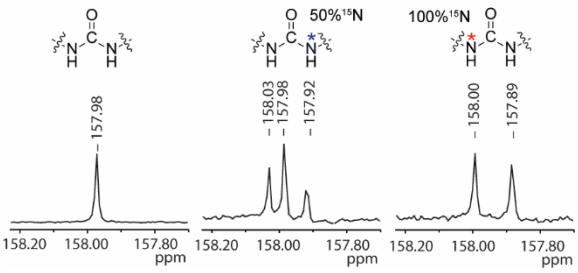


Figure S12. ^{13}C {^{1}H} NMR spectra of urea products with and without isotope labels. "*" sign in blue denotes 50% ^{15}N incorporation and the sign in red denotes its 100% incorporation.

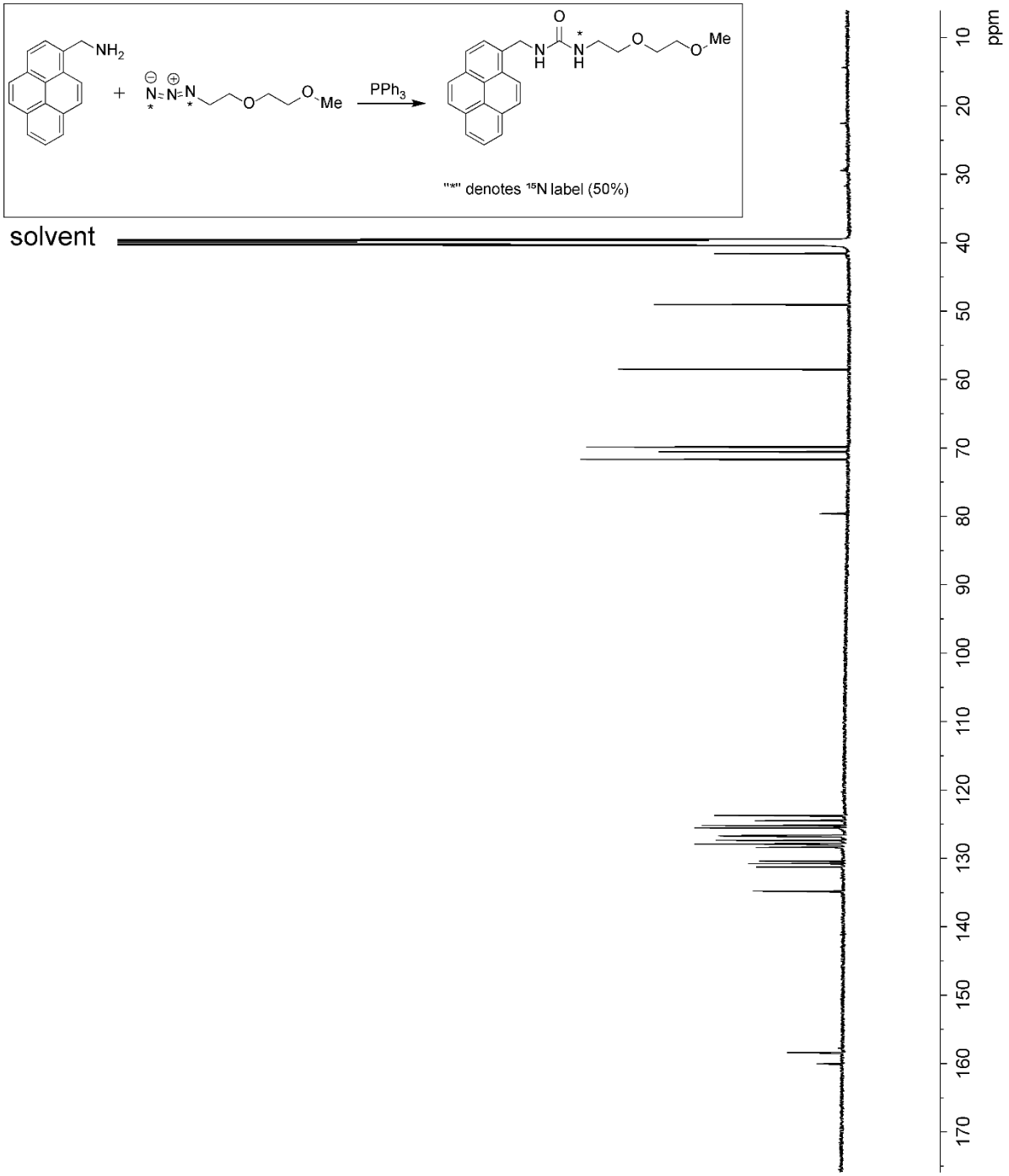


Figure S13. $^{13}C\{^{1}H\}$ NMR spectrum of ^{15}N-labeled pyrene urea compound in DMSO-d_6, using 50% terminal ^{15}N-enriched azide **1a**

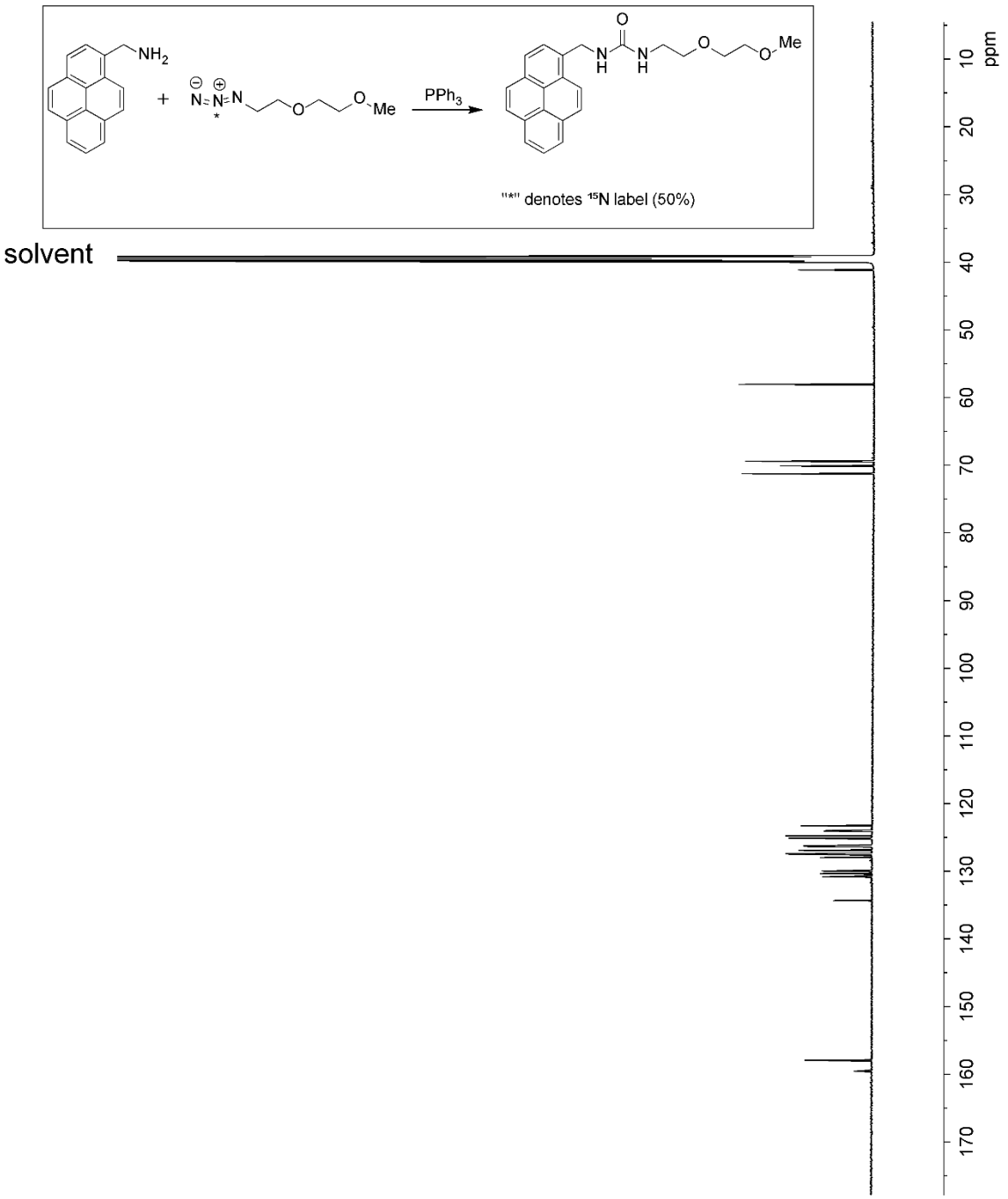


Figure S14. ^{13}C {^{1}H} NMR spectrum of ^{15}N-labeled pyrene urea compound in DMSO-d_6, synthesized using 50% internal ^{15}N-enriched azide **1a**.

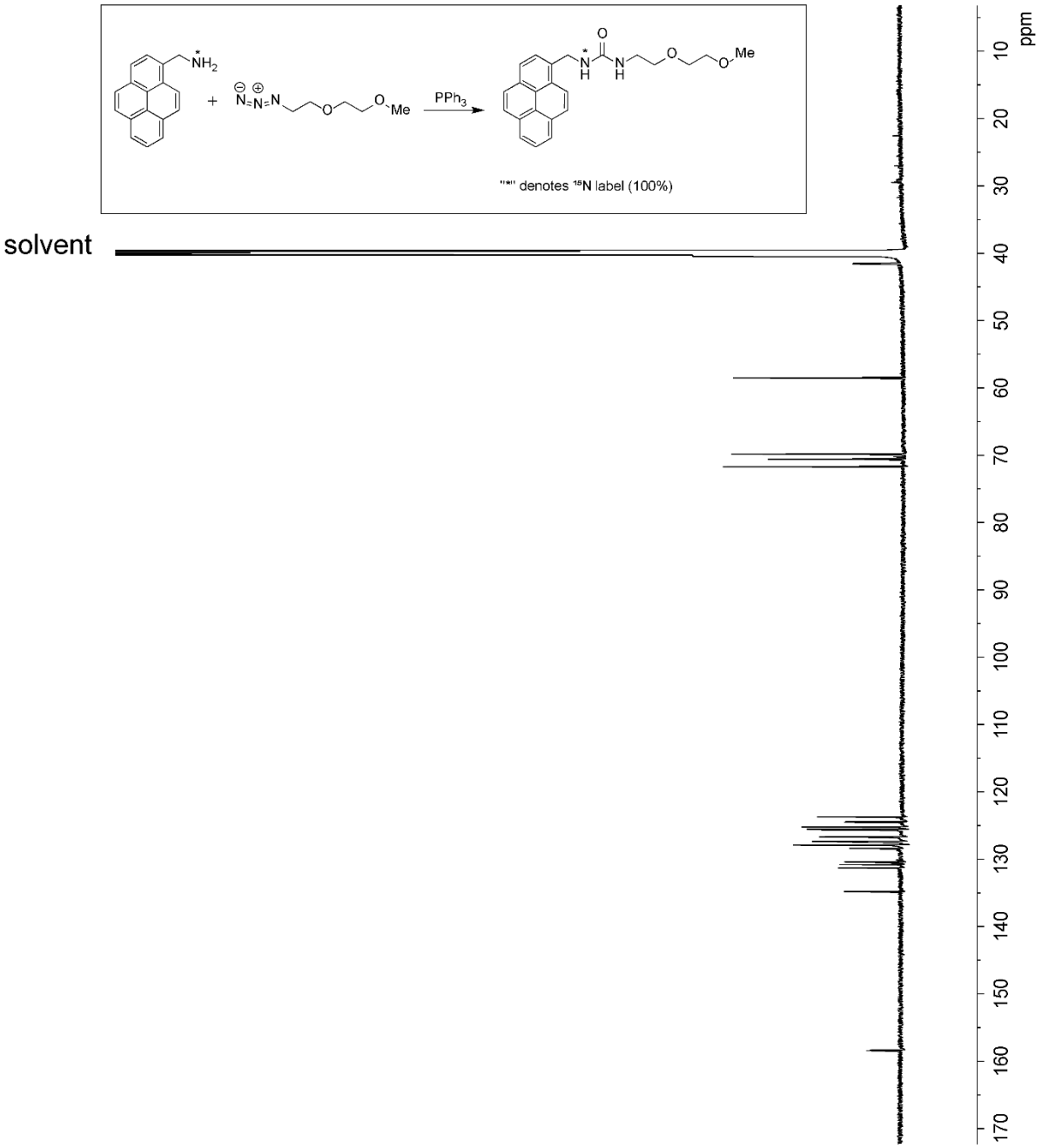


Figure S15. ^{13}C {^{1}H} NMR spectrum of ^{15}N-labeled pyrene urea compound in DMSO-d_6, synthesized using 100% ^{15}N-enriched pyrene amine.

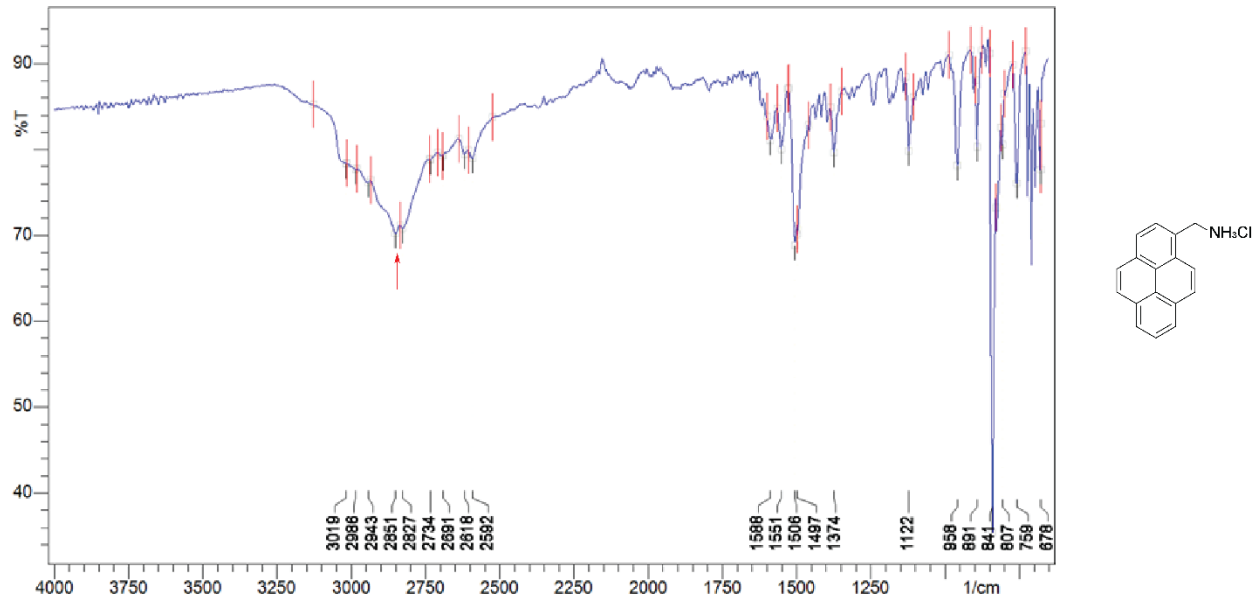


Figure S16. FT-IR spectrum of pyrene-NH_3Cl. Relevant peaks are labeled with a red arrow.

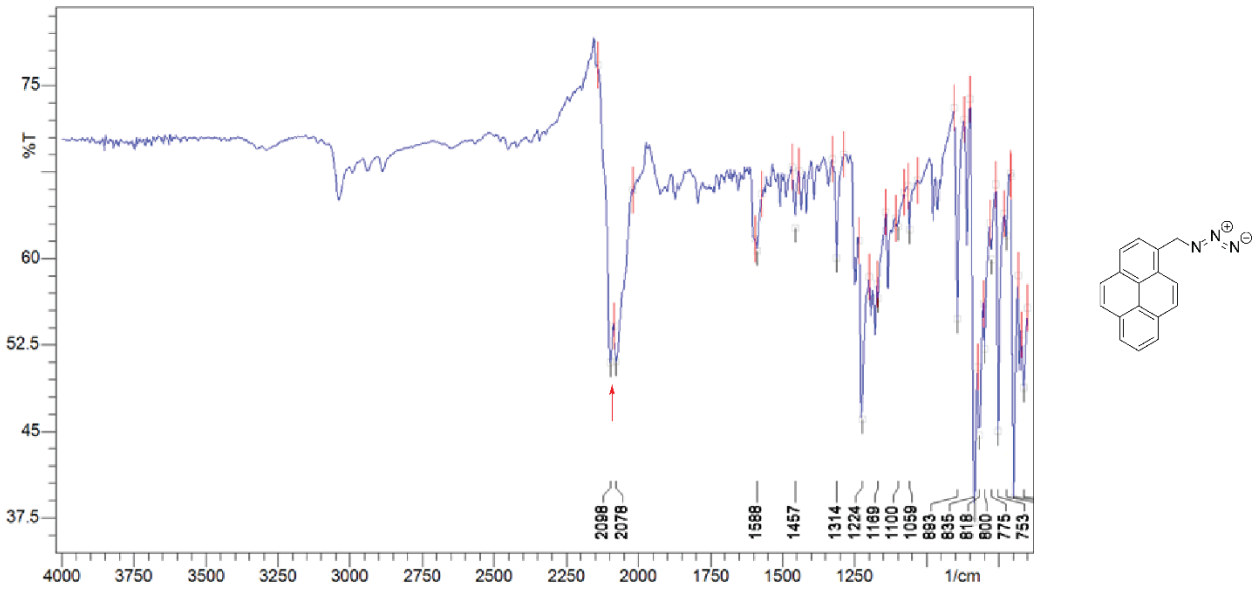
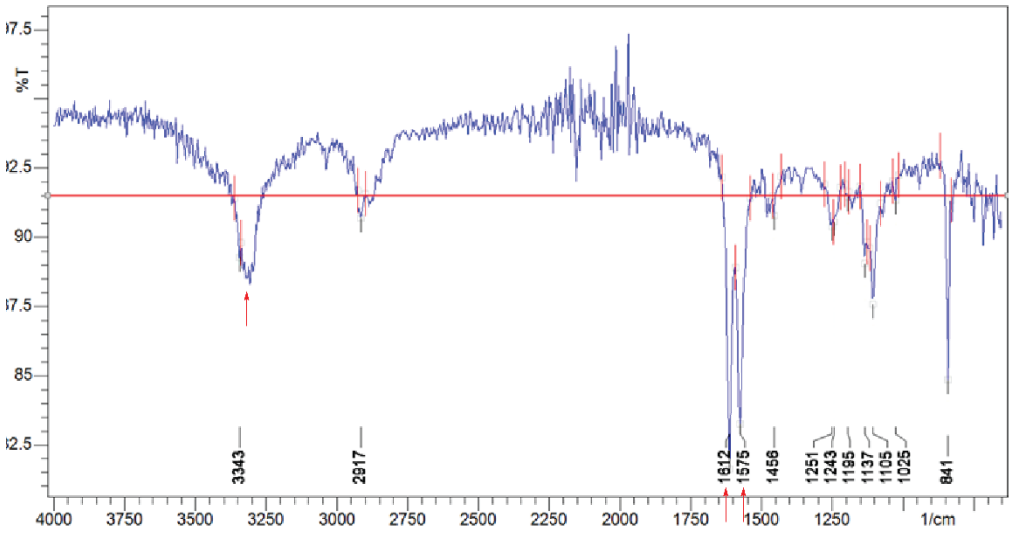


Figure S17. FT-IR spectrum of pyrene-azide. Relevant peaks are labeled with a red arrow.



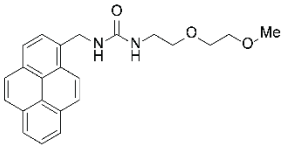


Figure S18. FT-IR spectrum of pyrene-urea. Relevant peaks are labeled with red arrow.[1]

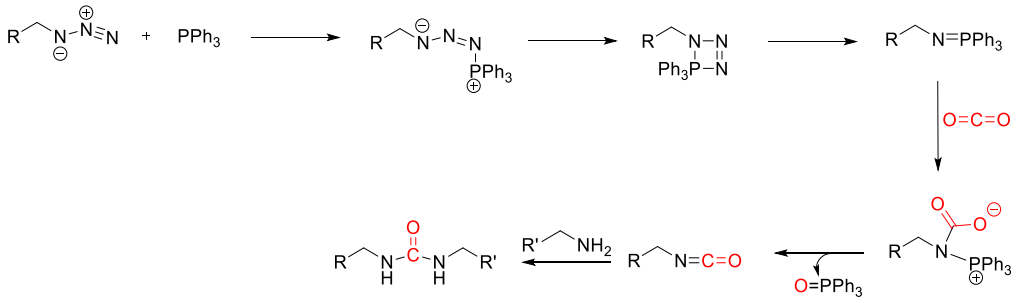


Figure S19. Proposed reaction mechanism of the urea formation mediated by triphenylphosphine.

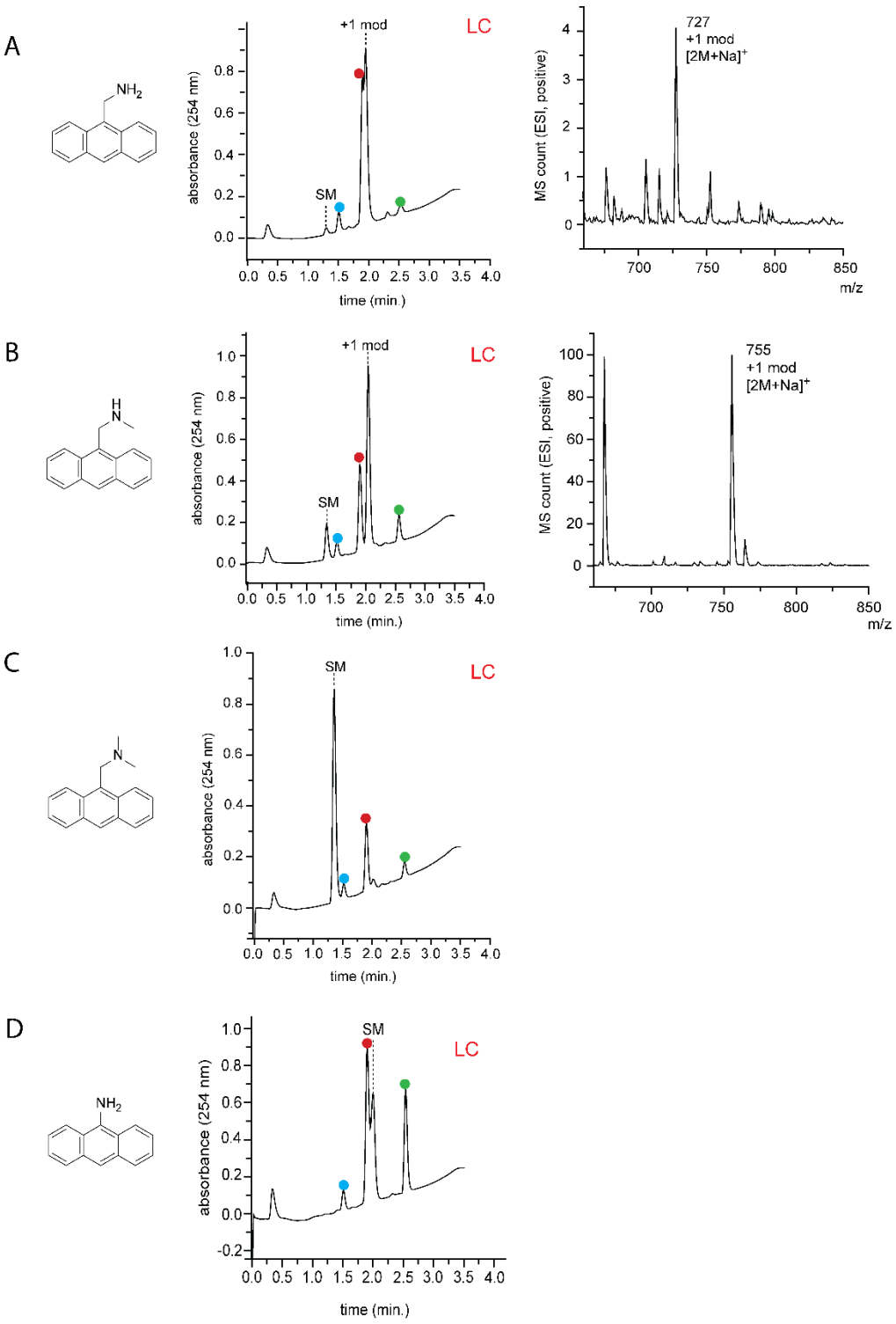


Figure S20. LC-MS analysis (Shimadzu LCMS-2020) of a crude reaction mixture of anthracene modification with azide mediated by PPh_3 after TLC purification. (A) alkyl primary amine, (B) alkyl secondary amine, (C) alkyl tertiary amine, and (D) aryl primary amine. Mass spectra (right) shows a peak corresponding to a modified compound (+1 mod in liquid chromatography). Based on the mass spectrometry, peaks at 1.5 (blue circle), 1.9 (red circle) and 2.6 (green circle) min prove to be PPh_3+azide-N2 (iminophosphorane), O=PPh_3, and PPh_3, respectively. Conversions shown in Figure 4B was obtained using the intensity of the starting material (SM) and product (+1 mod) peaks.

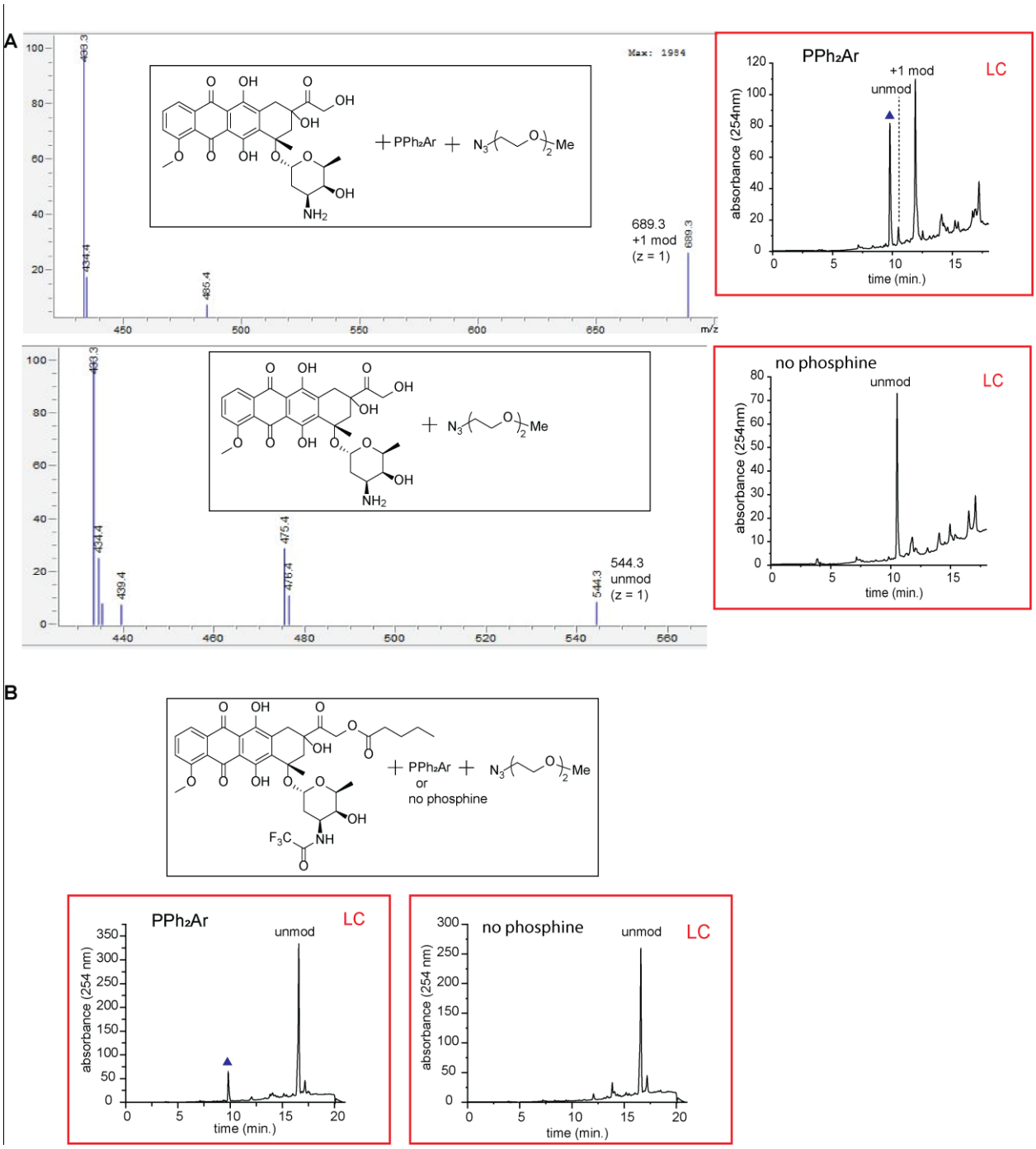


Figure S21. LC-MS analysis (Agilient Technologies 1260 Infinity II series) of a crude reaction mixture of doxorubicin (A) or valrubicin (B), and azide **1a** with or without PPh_2Ar (Ar = *m*-sulfophenyl). Mass spectra shown on the left corresponds to a peak for unmodified (unmod) and modified (mod) saccharides in the UV chromatograms (right). Peaks at 9.4 min (blue tringle) are found in saccharide-independent manner across different samples.

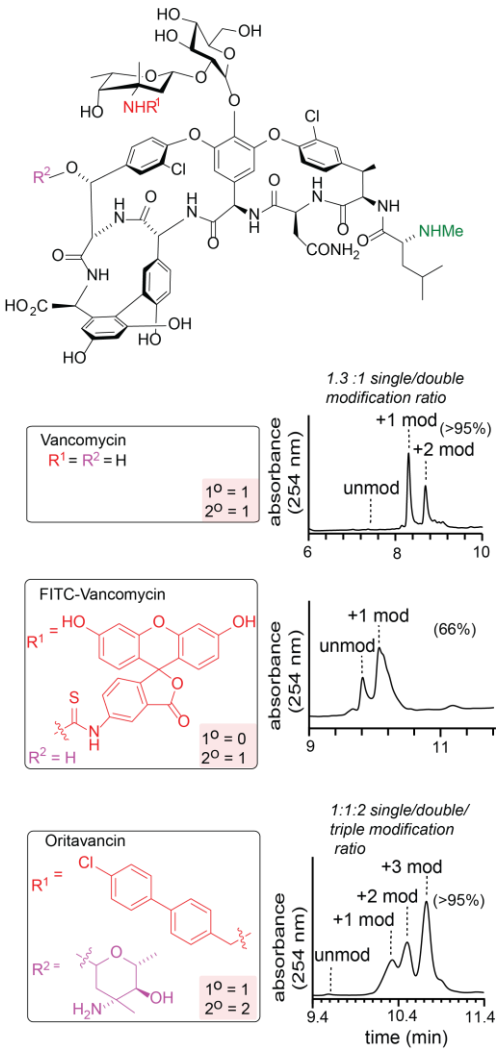
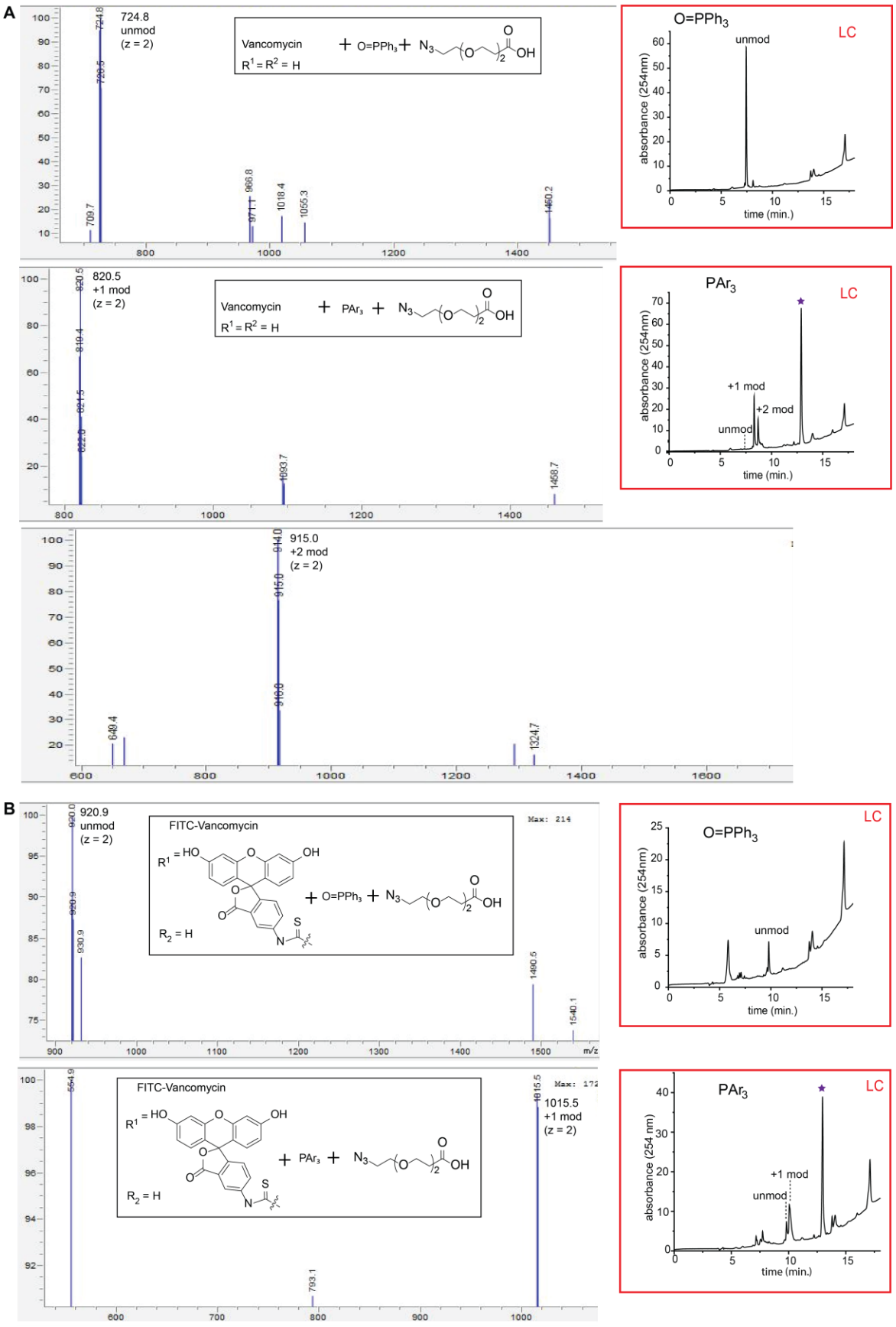


Figure S22. Chemical structure of vancomycin derivatives and LC analysis of the modification reaction with alkylazide containing a COOH group. The different substituents are highlighted in red and magenta while the monomethyl secondary amine is highlighted in green. The modification ratio and/or conversion were obtained by peak intensity (anthracene derivatives) or peak area integration (doxorubicin and vancomycin derivatives) in UV chromatograms. MS is shown in Figure S23 below.


A
724.8
unmod
(z = 2)
Vancomycin
R1 = R2 = H
+ O=PPh3 +
N3
OH
O=PPh3
LC
unmod
absorbance (254nm)
time (min.)
820.5
+1 mod
(z = 2)
Vancomycin
R1 = R2 = H
+ PAr3 +
N3
OH
PAr3
LC
+1 mod
+2 mod
unmod
absorbance (254nm)
time (min.)
915.0
+2 mod
(z = 2)
B
920.9
unmod
(z = 2)
FITC-Vancomycin
R1 = HO
R2 = H
+ O=PPh3 +
N3
OH
O=PPh3
LC
unmod
absorbance (254nm)
time (min.)
FITC-Vancomycin
R1 = HO
R2 = H
+ PAr3 +
N3
OH
1015.5
+1 mod
(z = 2)
PAr3
LC
+1 mod
unmod
absorbance (254 nm)
time (min.)

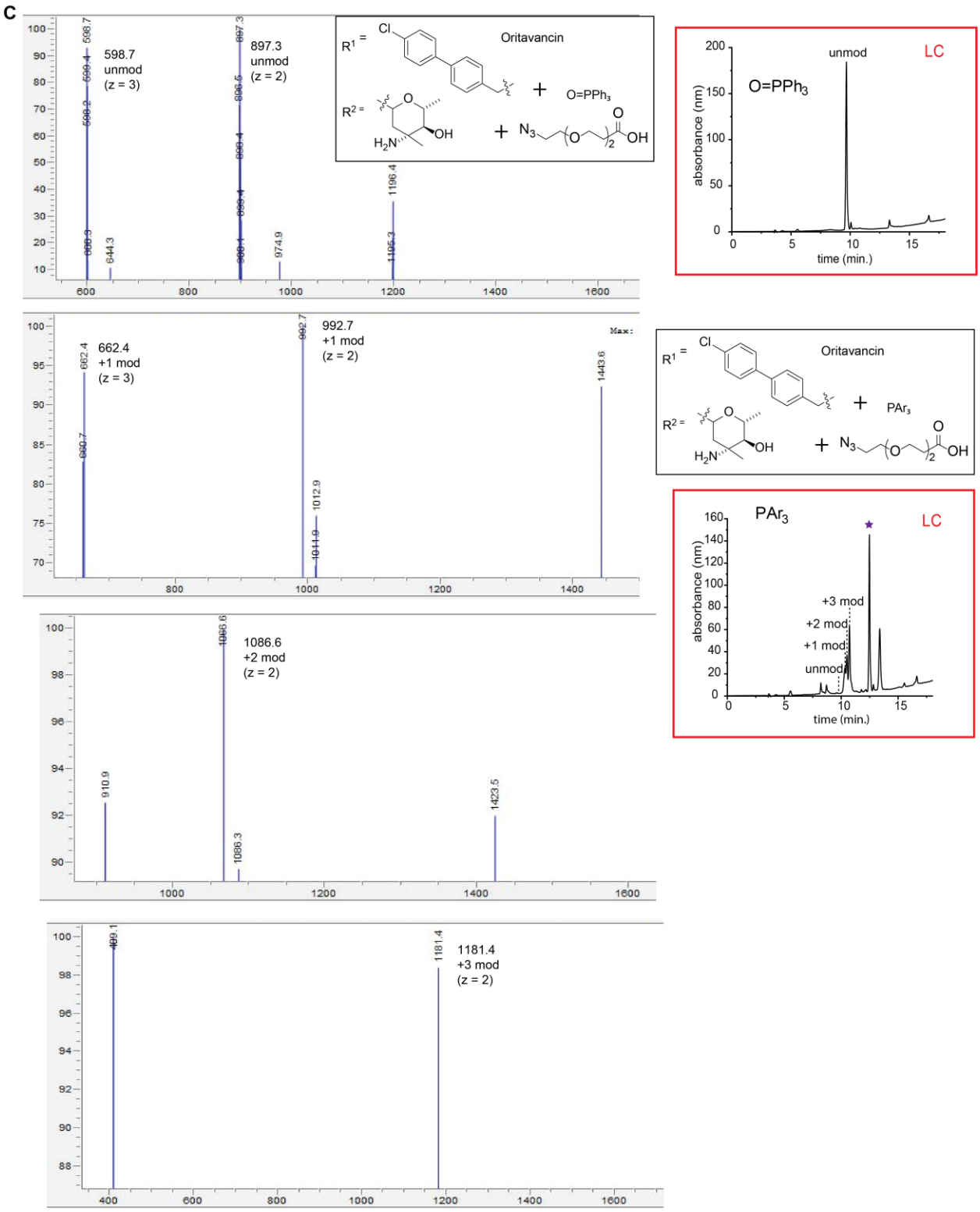


Figure S23. LC-MS analysis (Agilient Technologies 1260 Infinity II series) of a crude mixture of the reaction of vancomycin (A), FITC-vancomycin (B), or oritavancin (C) and alkylazide containing a COOH group with $O{=}PPh_3$ or P(*p*-tolyl)$_3$. Mass spectra shown on the left corresponds to a peak for unmodified (unmod) and modified (mod) saccharides in the UV chromatograms (right). Peaks at 13.7 min (purple star) are found in saccharide-independent manner across different samples.

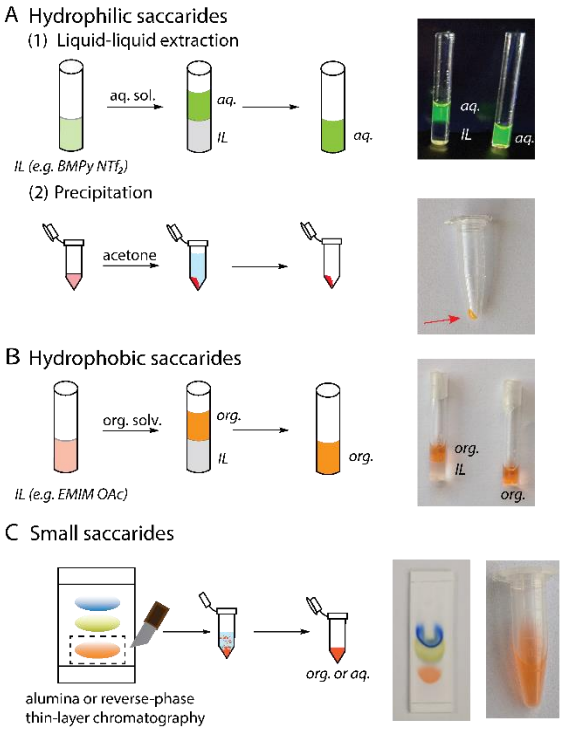


Figure S24. Post-reaction clean-up processes of ionic liquid-based carbohydrate bioconjugation prior to downstream analysis. IL: ionic liquid. Aq. sol.: aqueous solution. Org. solv.: organic solvent. (A) Schematic diagram and pictures/images for the liquid-liquid extraction and precipitation of hydrophilic saccharides. (1) Extraction processes for water-soluble saccharides. (Right) An image of extraction process of fluorescein isothiocyanate (FITC)-labeled dextran from a mixture of butylmethylpyrrolidinium triflate (BMPy OTf)/bistriflimide (BMPy NTf_2) by using 1X phosphate-buffered saline. (2) Scheme describing the precipitation process with acetone and a picture of pellets of FITC-labled vancomycin from a BMPy OTf reaction mixture. (B) A general scheme (left) and image (right) of extraction of hydrophobic carbohydrate valrubicin from ethylmethylimidazolium acetate (EMIM OAc) reaction mixture using ethyl acetate. (C) Thin layer chromatography (TLC)-based purification hydrophobic saccharide. A mixture of hydrophobic saccharide valrubicin (orange) with model compounds Coomassie Brilliant Blue (blue) and amine-containing nitrobenzofurazan (green) was subjected to reverse-phase TLC, followed by extraction of the target compound from stationary phase with hexafluorosiopropanol (the picture of the orange solution on the right).

Description of the purification protocol: To facilitate the ionic liquid-based bioconjugation of various saccharides with the diverse structure and solubility, we established multiple methods to separate the modified saccharides from the ionic liquid-containing reaction mixture. While a collective set of protocols for the compound extraction using ionic liquid has been available,[2] we faced difficulty separating the target saccharides from ionic liquids and excess chemical labeling reagents in microliter-scale reaction screening processes. To our knowledge, the majority of the previous efforts for the ionic liquid-based purification have been focused mainly on a large, industrial scale rather than small-scale reaction screening process, let alone bioconjugation process.[2] Precipitation of target biomolecules with organic solvent (e.g. acetone) was used for the previous reports of protein and DNA modification to remove ionic liquid and excess chemical reagents.[3,4] Although this precipitation approach was applicable to some relatively large saccharides, a significant loss of the samples was observed for many other carbohydrate derivatives.

To this end, we established a microliter-scale liquid-liquid extraction protocol for bioconjugation screening processes by using different combinations of the ionic liquid and phosphine reagent. For instance, hydrophilic

saccharides was successfully extracted through a bilayer system of water and water-immiscible ionic liquid such as bis(trifluoromethanesulfonyl)imide (NTf_2)-based ionic liquid, and the separated aqueous solution containing the desired saccharides can be further washed by organic solvent such as ethyl acetate. While water-immiscible (or relatively more hydrophobic) ionic liquid is inconvenient as a reaction medium for such hydrophilic carbohydrate due to their limited dissolution properties, we discovered that this extraction system can be applied for a reaction in water-miscible ionic liquid (e.g. butylmethylpyrrolidinium trifluoromethanesulfonate, BMPy OTf) by addition of water-immiscible ionic liquid after the reaction. For example, a product of a reaction performed in water-miscible BMPy OTf can be extracted with water or buffer by the addition of water-immiscible BMPy NTf_2, as the water-immiscible ionic liquid induces the phase separation from water even in the presence of the water-miscible ionic liquid. This process can be translated for hydrophobic saccharide targets by using ionic liquids immiscible with organic solvents during the reaction, and the desired hydrophobic compound can be extracted with organic solvent such as ethyl acetate. In addition to the extraction protocols, we also established a screening process for small saccharides using normal-phase (alumina) or reverse-phase (C18) thin-layer chromatography (TLC) as well. Those established protocols are not only conducive for our phosphine-mediated chemistry, but would future development of ionic liquid-based reaction screening processes also be facilitated by the protocols for biomolecules including carbohydrates, peptides/proteins, and nucleotides.

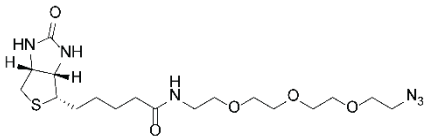


Figure S25. Chemical structure of biotin azide

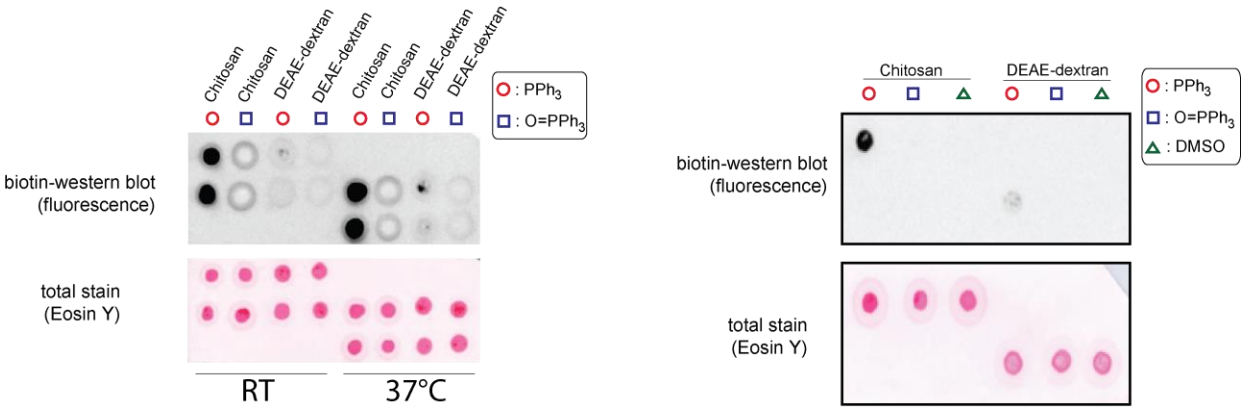


Figure S26. Representative blot membrane images for the anti-biotin western blot (Cy5-streptavidin) and total stain with eosin Y of chitosan and Diethylamino-ethyl (DEAE)-dextran modification. Reaction Conditions: chitosan (1 mg/mL final concentration from 10-mg/mL stock solution in 1:1 BMIM: 1-M acetate buffer), DEAE-dextran (1 mg/mL final concentration from 10-mg/mL stock solution in 1:1 BMIM: 1-M acetate buffer), biotin azide (3 mM final concentration from 100-mM stock solution in DMSO), and phosphines (3mM final concentration from 150-mM stock solution in DMSO) in EMIM OAC at room temperature or 37 °C for 2 h (left membrane images). The experiment was repeated three times to obtain the standard deviation (error bars, *n* = 3). Right membrane images include a negative control without phosphine reagents at 37 °C for 2 h

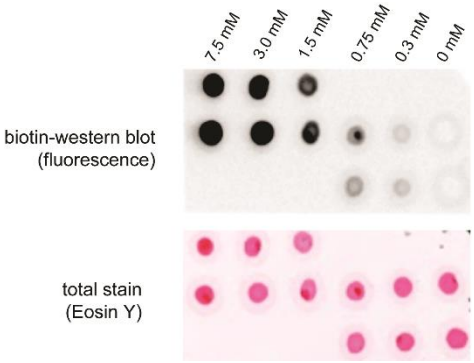


Figure S27. Representative blot membrane images for the anti-biotin western blot (Cy5-streptavidin) and total stain with eosin Y solution of chitosan modification with different biotin azide/phosphine concentration. Reaction Conditions: chitosan (1 mg/mL final concentration from 10 mg/mL stock solution in 1:1 BMIM: 1-M acetate buffer), biotin azide and phosphines in DMSO solution as 7.5 mM, 3 mM, 1.5 mM, 0.75 mM,0.3 mM, and 0 mM final concentration from 375 mM, 150 mM, 75 mM, 37.5 mM, and 15 mM, respectively. The reaction was done in EMIM OAC at 37 °C for 2 h. The experiment was done in triplicates to obtain the standard deviation (error bars, *n* = 3).

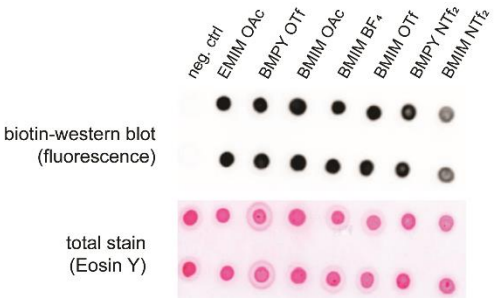


Figure S28. Representative blot membrane images for the anti-biotin western blot (Cy5-streptavidin) and total stain with eosin Y solution of the ionic liquid screening in chitosan modification. Reaction conditions: chitosan (1 mg/mL final concentration from 10 mg/mL stock solution in 1:1 BMIM OAC/1-M acetate buffer), biotin azide (3mM final concentration from 100 mM stock solution in DMSO), and phosphines (3 mM final concentration from 150 mM stock solution in DMSO) at 37 °C for 2 h. The experiment was replicated three times to get the standard deviation (error bars, *n* = 3).

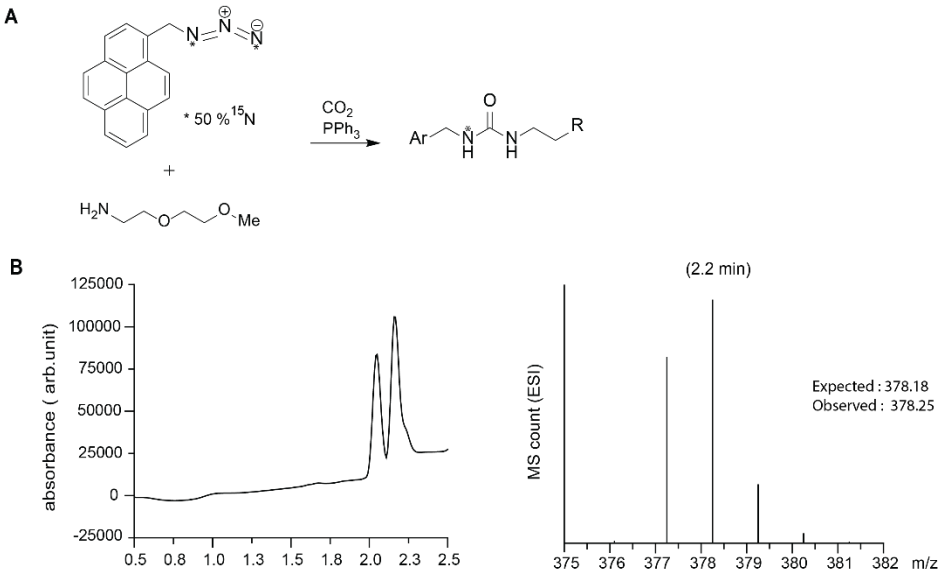


Figure S29. LC-MS analysis (Shimadzu LCMS-2020) of ^{15}N-labeled pyrene azide modification in ionic liquid. (A) Reaction scheme. (B) LC-MS analysis of the crude reaction mixture. Left: UV chromatogram (254 nm). Right Mass spectrum of the peak at 2.2 min. The reaction procedure is described in *Typical saccharide/small molecule modification in ionic liquids*.

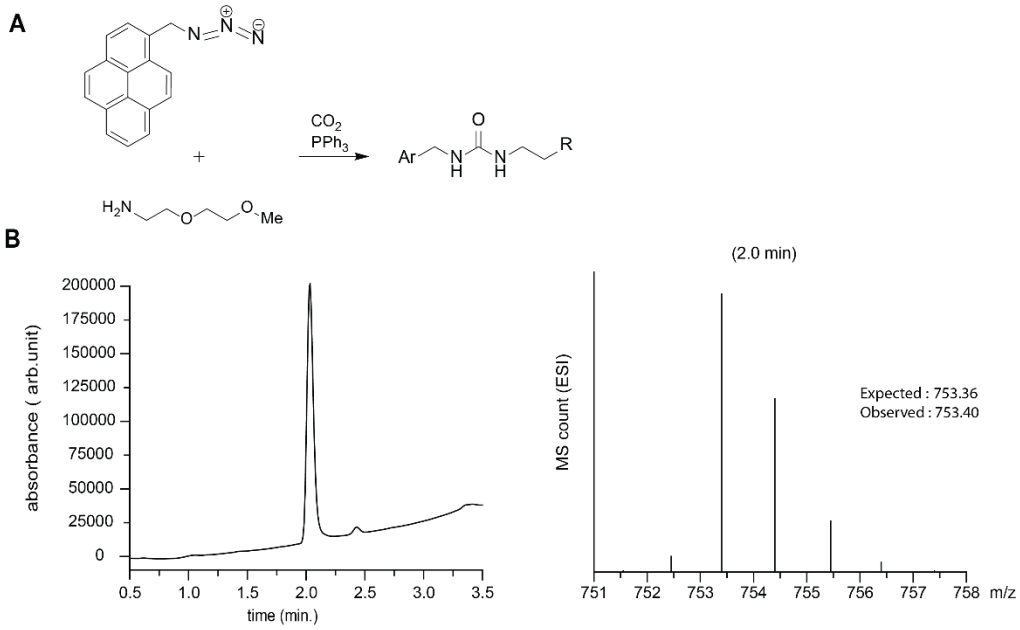


Figure S30. LC-MS analysis (Shimadzu LCMS-2020) of pyrene azide modification in ionic liquid. (A) Reaction scheme. (B) LC-MS analysis of the crude reaction mixture. Left: UV chromatogram (254 nm). Right Mass spectrum. Reaction procedure is described in *Typical saccharide/small molecule modification in ionic liquids*.

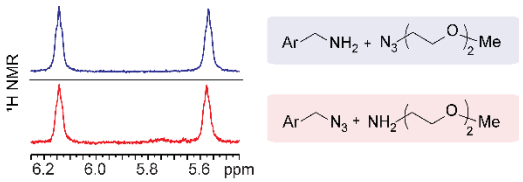


Figure S31. ^{1}H NMR analysis of the reaction of an azide substrate modified with excess alkylamine reagent (red/bottom), compared with the previously reported reaction of an amine substrate reacting with excess alkylazide reagent (blue/top).[3]

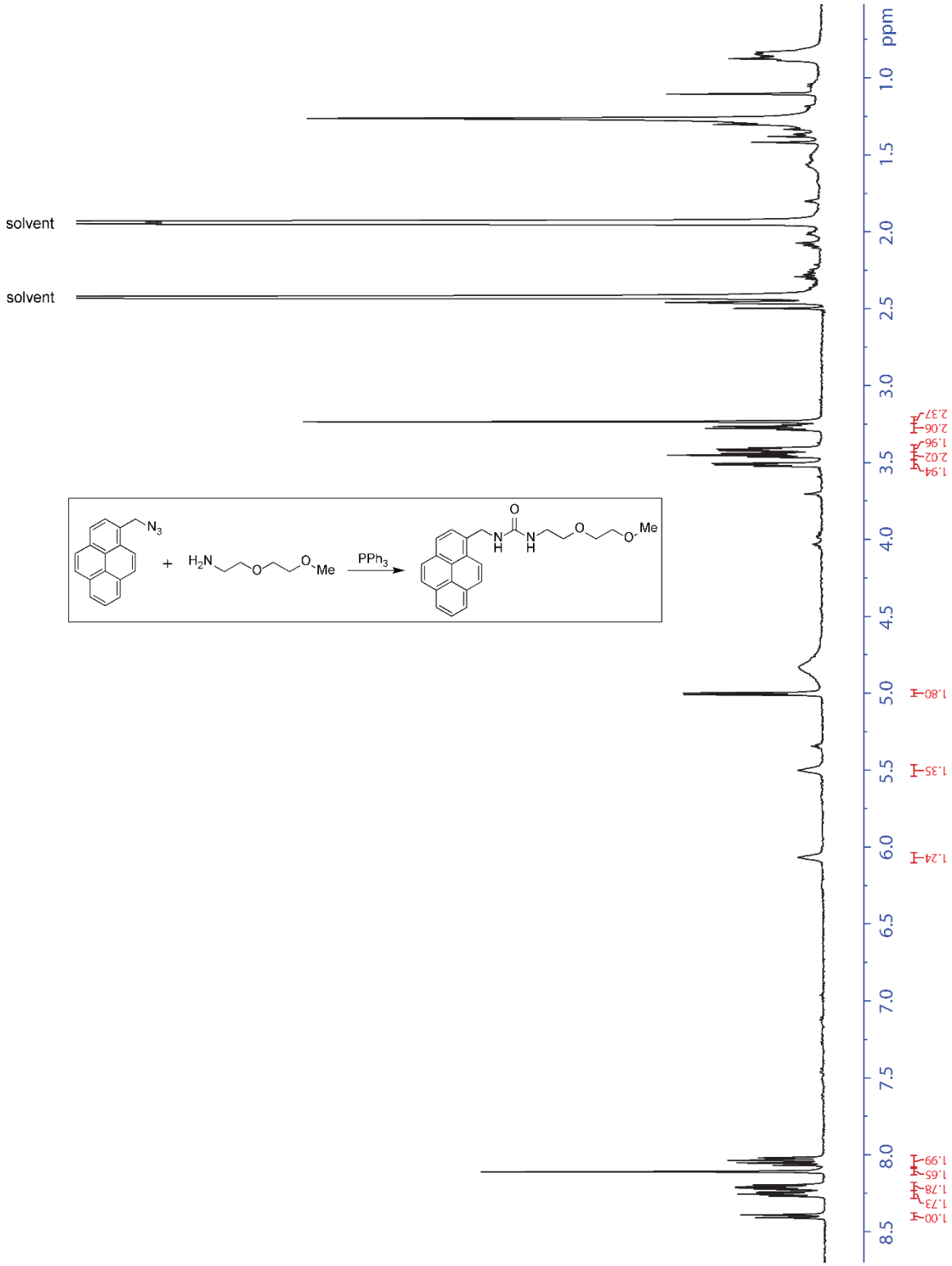


Figure S32. ^{1}H NMR spectrum of pyrene-urea compound in CD_3CN/DMSO-d_6 (95:5) produced by azide-pyrene with excess amine reagent. Reaction conditions and are described in typical saccharide/small molecule model modification in ionic liquid section.

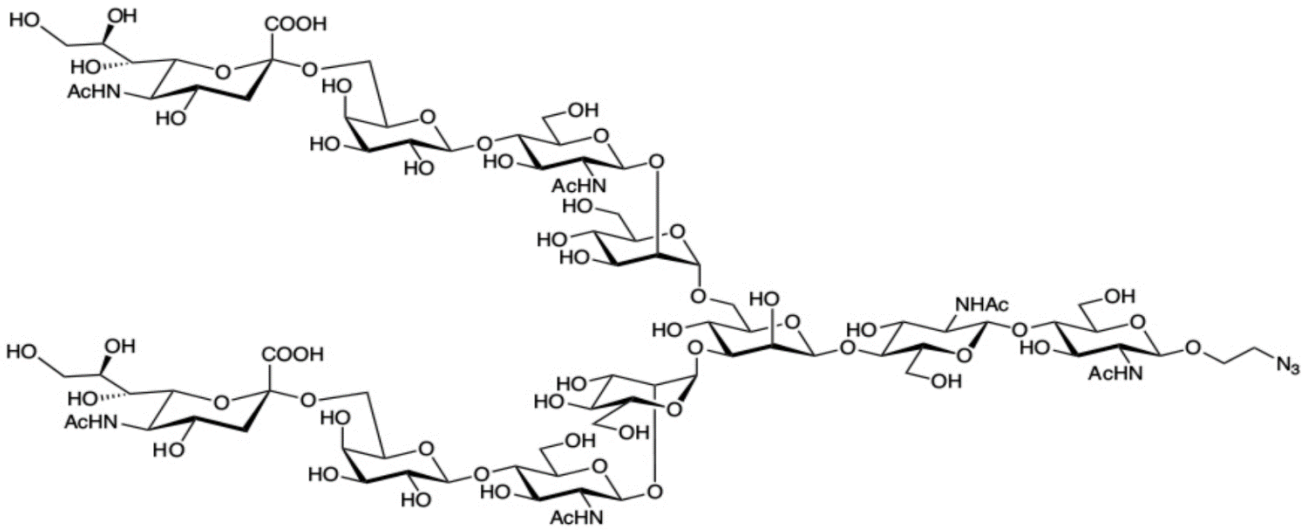


Figure S33. Chemical structure of disialylnonasaccharide-β-ethylazide used in Figure 6C.

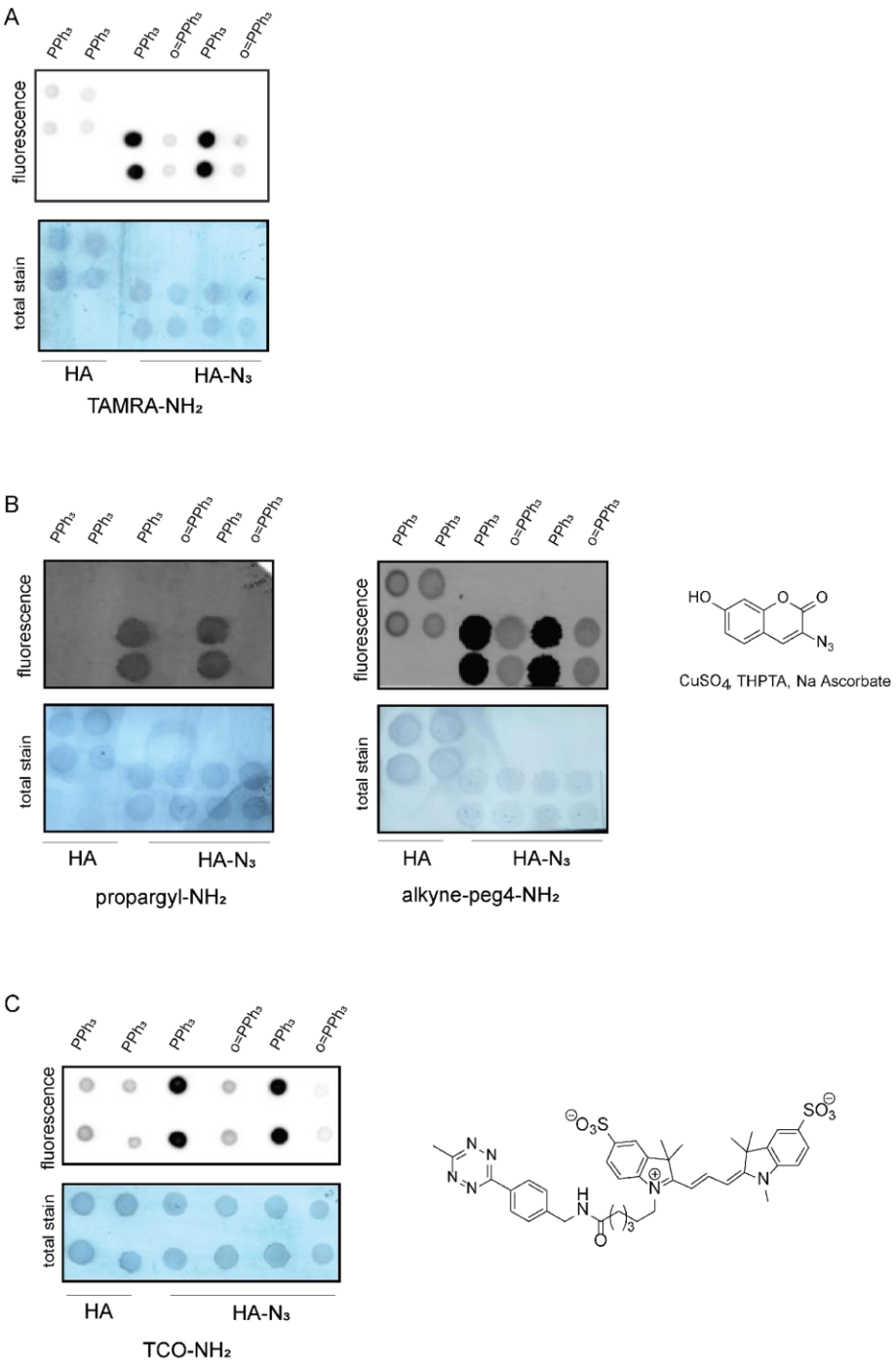


Figure S34. Representative blot membrane fluorescence (Cy3) and total stain (toluidine blue) images of phosphine-mediated hyaluronic acid (HA) and hyaluronic acid azide (HA-N_3) modification in ionic liquid. Reaction conditions: HYALURONIC ACID DERIVATIVES (2 mg/mL), amine reagent (10–20 mM), $KHCO_3$ (20 mM), phosphines: PPh_3 and O=PPH_3 (20 mM) in EMIM OAc at 37 °C for 2 h. (A) HA and HA-N_3 modification with TAMRA amine. (B) HA and HA-N_3 modification with propargyl amine (left) and alkyne-peg4 amine (right) using coumarine azide as a secondary label on blot membrane.[5] (C) HA and HA-N_3 modification with trans-cyclooctene amine using sulfo-cyanine5 tetrazine (1 mM in 50 mM MES buffer) as a secondary label. The experiment was replicated three times to get the standard deviation (error bars, *n* = 3).

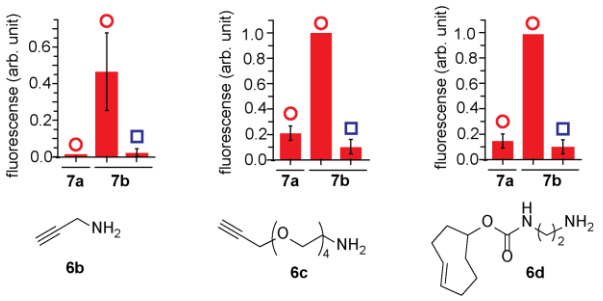


Figure S35. Modification of hyaluronic acid derivatives with amine reagent containing alkyne (left and middle) and trans-cyclooctene (right), which were subjected to the secondary modification with fluorophore bearing azide and tetrazine, respectively. Bar graphs represent quantification of dot blot analysis of the modification shown in Fig S34 B and C above.

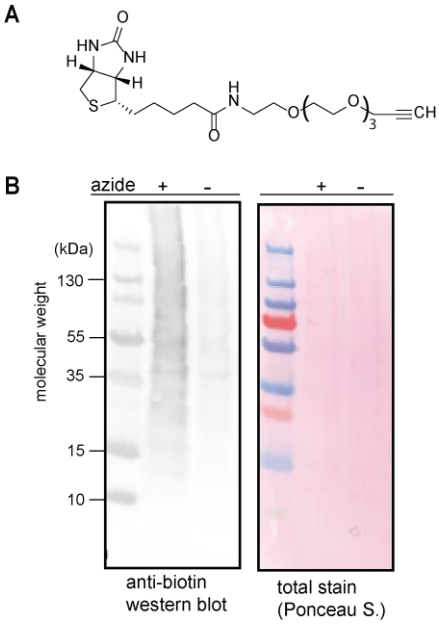


Figure S36. (A) Chemical structure of biotin-PEG4 alkyne. (B) Anti-biotin western blot and total stain (Ponceau S.) of the biotinylating reaction of human embryo kidney (HEK) 293T cell lysates with and without the azide treated with biotin-PEG4-alkyne. Reaction conditions: cell lysate with and without the azide tag (0.18 mg/mL), sodium ascorbate (2 mM final conc from 50-mM stock solution), THPTA (0.2 mM final conc from 25-mM stock solution), biotin PEG4-alkyne (0.6 mM final conc from 20-mM stock solution) and $CuSO_4$ (1 mM final concentration from 25-mM stock solution) in 1X phosphate buffered saline (30 µL total volume).

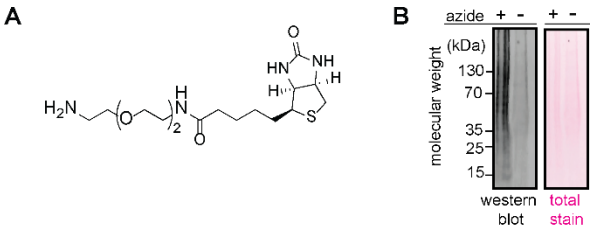


Figure S37. (A) Chemical structure of amine-functionalized biotin used in the cell lysate study. (B) Anti-biotin western blot and total stain (Ponceau S.) of the biotinylating reaction of human embryo kidney (HEK) 293T cells in BMPy OTf with and without the azide tag.

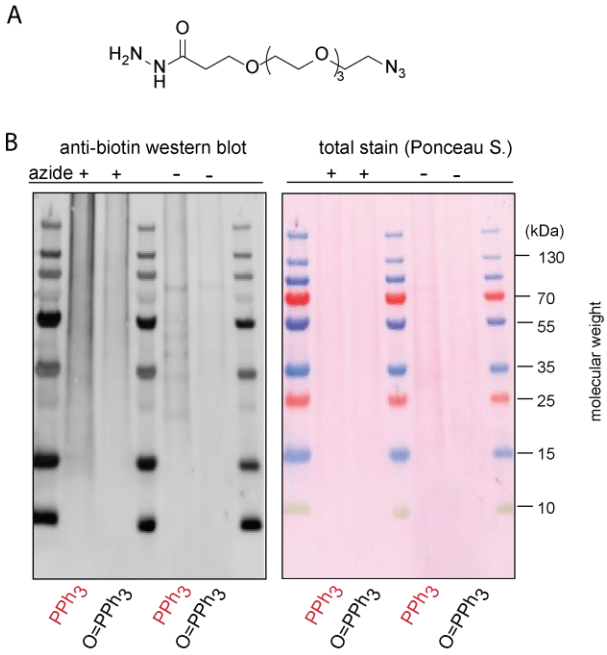


Figure S38. (A) Chemical structure of azido-PEG4-hydrazide. (B) Anti-biotin western blot and total stain (Ponceau S.) of the biotinylating reaction of human embryo kidney (HEK) 293T cell lysates with and without the azide tag. Reaction conditions: cell lysate with and without the azide tag (0.045 mg/mL), $KHCO_3$ (20 mM), biotin amine (20 mM), PPh_3 and $O{=}PPh_3$ (20 mM) in BMPy OTf at 37 ^oC.

Crystallographic data

Table 1. Crystal data and structure refinement for C35 H24 N2 O1.

Identification code	global	
Empirical formula	C35 H24 N2 O1	
Formula weight	488.59	
Temperature	100 K	
Wavelength	1.54180 Å	
Crystal system	Monoclinic	
Space group	C2/c	
Unit cell dimensions	a = 35.8398(12) Å	□= 90°.
	b = 4.60330(10) Å	□= 112.825(2)°.
	c = 15.1676(5) Å	□ = 90°.
Volume	2306.42(12) $Å^3$	
Z	4	
Density (calculated)	1.407 Mg/m^3	
Absorption coefficient	0.661 mm^{-1}	
F(000)	1024	
Crystal size	0.150 x 0.070 x 0.010 mm^3	
Theta range for data collection	2.675 to 66.554°.	
Index ranges	-42<=h<=42, -5<=k<=5, -16<=l<=17	
Reflections collected	17530	
Independent reflections	2025 [R(int) = 0.086]	
Completeness to theta = 65.223°	99.7 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	0.99 and 0.96	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	2020 / 4 / 210	
Goodness-of-fit on F^2	1.0249	
Final R indices [I>2sigma(I)]	R1 = 0.0643, wR2 = 0.1625	
R indices (all data)	R1 = 0.0792, wR2 = 0.1829	
Largest diff. peak and hole	0.41 and -0.37 $e.Å^{-3}$	

Weighting scheme

```
w = 1/[sigma**2(Fo**2 ) + ( 0.167 x P)**2 + 6.830 x P + 0.000 + 0.000 x sin theta]
P = 0.333 x max(Fo**2 ,0) + 0.667 x Fc**2
```

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3)

for C35 H24 N2 O1. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
N(1)	4810(1)	-1994(4)	2957(1)	29
C(18)	5000	-3563(6)	2500	27
O(3)	5000	-6250(5)	2500	39
C(1)	4610(1)	-3394(5)	3520(2)	30
C(2)	4350(1)	-1253(4)	3778(2)	28
C(3)	3996(1)	-68(4)	3072(2)	26
C(4)	3857(1)	-880(5)	2076(2)	29
C(5)	3522(1)	316(5)	1409(2)	30
C(6)	3286(1)	2429(4)	1656(2)	28
C(7)	3409(1)	3246(4)	2633(2)	26
C(8)	3763(1)	1991(4)	3339(2)	26
C(9)	3886(1)	2852(5)	4314(2)	28
C(10)	4235(1)	1616(5)	4997(2)	31
C(11)	4460(1)	-399(5)	4724(2)	31
C(12)	3645(1)	4951(5)	4561(2)	31
C(13)	3311(1)	6128(5)	3892(2)	32
C(14)	3179(1)	5322(4)	2899(2)	29
C(15)	2836(1)	6543(5)	2200(2)	32
C(16)	2718(1)	5738(5)	1250(2)	34
C(17)	2939(1)	3717(5)	977(2)	33

Table 3. Bond lengths [Å] and angles [°] for C35 H24 N2 O1.

N(1)-C(18)	1.355(2)
N(1)-C(1)	1.458(3)
N(1)-H(1)	0.862(17)
C(18)-O(3)	1.237(4)
C(1)-C(2)	1.510(3)
C(1)-H(11)	1.00(3)
C(1)-H(12)	1.05(3)
C(2)-C(3)	1.413(3)
C(2)-C(11)	1.390(3)
C(3)-C(4)	1.445(3)
C(3)-C(8)	1.420(3)
C(4)-C(5)	1.352(3)
C(4)-H(41)	0.98(2)
C(5)-C(6)	1.430(3)
C(5)-H(51)	1.00(3)
C(6)-C(7)	1.423(3)
C(6)-C(17)	1.401(3)
C(7)-C(8)	1.427(3)
C(7)-C(14)	1.419(3)
C(8)-C(9)	1.427(3)
C(9)-C(10)	1.398(3)
C(9)-C(12)	1.439(3)
C(10)-C(11)	1.394(3)
C(10)-H(101)	1.00(3)
C(11)-H(111)	1.04(3)
C(12)-C(13)	1.346(3)
C(12)-H(121)	0.98(3)
C(13)-C(14)	1.442(3)
C(13)-H(131)	1.03(3)
C(14)-C(15)	1.394(3)
C(15)-C(16)	1.385(4)
C(15)-H(151)	0.97(3)
C(16)-C(17)	1.386(3)
C(16)-H(161)	0.96(4)

C(17)-H(171)	1.02(3)
C(18)-N(1)-C(1)	121.49(18)
C(18)-N(1)-H(1)	118.6(11)
C(1)-N(1)-H(1)	119.6(11)
N(1)-C(18)-N(1)#1	115.6(3)
N(1)-C(18)-O(3)	122.21(13)
N(1)#1-C(18)-O(3)	122.21(13)
N(1)-C(1)-C(2)	110.83(17)
N(1)-C(1)-H(11)	110.7(14)
C(2)-C(1)-H(11)	109.3(14)
N(1)-C(1)-H(12)	105.8(13)
C(2)-C(1)-H(12)	113.4(13)
H(11)-C(1)-H(12)	106.6(19)
C(1)-C(2)-C(3)	121.3(2)
C(1)-C(2)-C(11)	119.6(2)
C(3)-C(2)-C(11)	119.0(2)
C(2)-C(3)-C(4)	122.36(19)
C(2)-C(3)-C(8)	119.7(2)
C(4)-C(3)-C(8)	117.9(2)
C(3)-C(4)-C(5)	121.5(2)
C(3)-C(4)-H(41)	120.1(14)
C(5)-C(4)-H(41)	118.3(14)
C(4)-C(5)-C(6)	121.7(2)
C(4)-C(5)-H(51)	117.2(18)
C(6)-C(5)-H(51)	121.1(18)
C(5)-C(6)-C(7)	118.5(2)
C(5)-C(6)-C(17)	122.8(2)
C(7)-C(6)-C(17)	118.8(2)
C(6)-C(7)-C(8)	120.0(2)
C(6)-C(7)-C(14)	119.7(2)
C(8)-C(7)-C(14)	120.3(2)
C(7)-C(8)-C(3)	120.4(2)
C(7)-C(8)-C(9)	119.5(2)
C(3)-C(8)-C(9)	120.1(2)
C(8)-C(9)-C(10)	118.9(2)

C(8)-C(9)-C(12)	118.8(2)
C(10)-C(9)-C(12)	122.4(2)
C(9)-C(10)-C(11)	120.4(2)
C(9)-C(10)-H(101)	119.6(17)
C(11)-C(10)-H(101)	120.0(17)
C(10)-C(11)-C(2)	121.9(2)
C(10)-C(11)-H(111)	118.9(16)
C(2)-C(11)-H(111)	119.0(16)
C(9)-C(12)-C(13)	121.5(2)
C(9)-C(12)-H(121)	115.9(18)
C(13)-C(12)-H(121)	122.3(18)
C(12)-C(13)-C(14)	121.2(2)
C(12)-C(13)-H(131)	121.1(15)
C(14)-C(13)-H(131)	117.5(15)
C(13)-C(14)-C(7)	118.7(2)
C(13)-C(14)-C(15)	121.7(2)
C(7)-C(14)-C(15)	119.6(2)
C(14)-C(15)-C(16)	120.5(2)
C(14)-C(15)-H(151)	117.0(18)
C(16)-C(15)-H(151)	122.2(18)
C(15)-C(16)-C(17)	120.7(2)
C(15)-C(16)-H(161)	118(2)
C(17)-C(16)-H(161)	121(2)
C(6)-C(17)-C(16)	120.8(2)
C(6)-C(17)-H(171)	119.4(18)
C(16)-C(17)-H(171)	119.8(18)

Symmetry transformations used to generate equivalent atoms:

#1 -x+1,y,-z+1/2

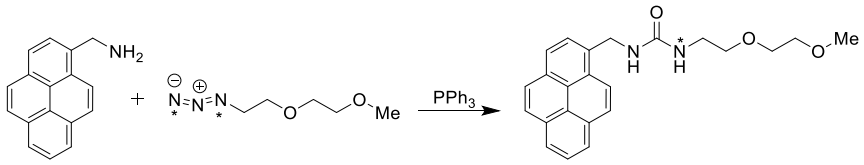
Table 4. Anisotropic displacement parameters ($Å^2 x\ 10^3$) for C35 H24 N2 O1. The anisotropic displacement factor exponent takes the form: $-2\pi^2[h^2 a^{*2} U^{11} + ... + 2 h k a^* b^* U^{12}]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
N(1)	32(1)	21(1)	40(1)	1(1)	21(1)	0(1)
C(18)	25(1)	25(1)	31(2)	0	13(1)	0
O(3)	48(1)	22(1)	60(2)	0	36(1)	0
C(1)	32(1)	25(1)	39(1)	6(1)	20(1)	1(1)
C(2)	29(1)	24(1)	35(1)	1(1)	17(1)	-3(1)
C(3)	28(1)	21(1)	34(1)	-1(1)	18(1)	-4(1)
C(4)	32(1)	24(1)	37(1)	-3(1)	19(1)	-4(1)
C(5)	34(1)	28(1)	31(1)	-2(1)	17(1)	-5(1)
C(6)	29(1)	25(1)	32(1)	-1(1)	14(1)	-7(1)
C(7)	29(1)	22(1)	31(1)	2(1)	16(1)	-4(1)
C(8)	29(1)	21(1)	32(1)	-1(1)	17(1)	-5(1)
C(9)	31(1)	23(1)	34(1)	0(1)	18(1)	-4(1)
C(10)	32(1)	32(1)	30(1)	1(1)	14(1)	-4(1)
C(11)	30(1)	29(1)	34(1)	2(1)	15(1)	-2(1)
C(12)	38(1)	26(1)	33(1)	-2(1)	18(1)	-4(1)
C(13)	37(1)	28(1)	38(1)	-2(1)	22(1)	0(1)
C(14)	31(1)	23(1)	37(1)	2(1)	18(1)	-4(1)
C(15)	31(1)	27(1)	42(2)	2(1)	19(1)	0(1)
C(16)	29(1)	32(1)	41(2)	5(1)	12(1)	-2(1)
C(17)	32(1)	32(1)	34(1)	1(1)	13(1)	-4(1)

Table 5. Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$x 10^3) for C35 H24 N2 O1.

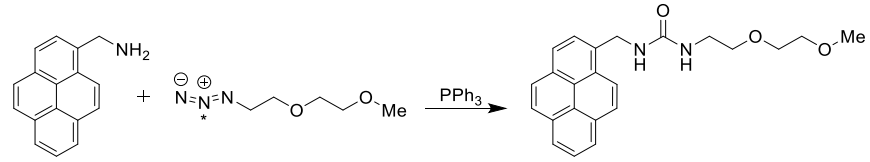
	x	y	z	U(eq)
H(11)	4814(8)	-4250(50)	4117(18)	27
H(12)	4447(7)	-5140(50)	3105(18)	25
H(41)	3999(7)	-2400(50)	1874(17)	23
H(51)	3446(10)	-330(70)	730(20)	49
H(101)	4329(9)	2250(60)	5680(20)	43
H(111)	4707(9)	-1410(60)	5250(20)	40
H(121)	3752(9)	5590(70)	5230(20)	49
H(131)	3153(8)	7750(50)	4064(19)	29
H(151)	2706(9)	8110(60)	2400(20)	44
H(161)	2490(11)	6720(70)	790(30)	60
H(171)	2850(9)	3160(70)	270(20)	49
H(1)	4828(7)	-130(40)	2959(17)	34(2)

Preparative synthesis of small molecules.



"*" denotes ^{15}N label (50%)

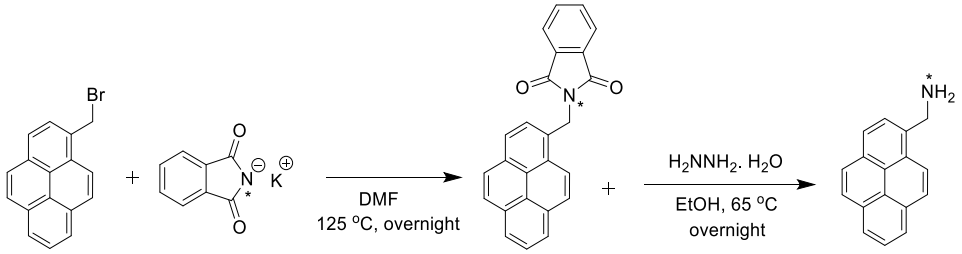
Synthesis of pyrene urea compound using alkylamine and terminal ^{15}N-enriched azide. Alkyl azide (25 mg, 0.165 mmol) was added to a 4-mL vial equipped with a magnetic stir bar. DMF (280 uL), DMSO (100 uL), and K_2CO_3 aq solution (13.6 uL, 2M, 0,034 mmol) were added. To the mixture, (aminomethyl)pyrene hydrochloride (9.1 mg, 0.039mmol) was added. The mixture was heated for 5 min at 50 °C, and then PPh_3 (90.2 mg, 0.327 mmol) solution in DMF (300 uL) was added dropwise. After the reaction mixture was heated at 50 °C overnight, the formation of the product was confirmed and purified by TLC (2:1 hexane/dichloromethane on basic aluminum oxide 60, MilliporeSigma #1057130001 The product was recovered from the alumina by addition of methanol (1 mL) and sonication for 3 minutes after which MeCN (9 mL) was added and sonicated for another 3 min. The suspension was passed through Celite followed by paper filtration to remove Celite. After concentrating the filtrate by nitrogen flow, the resulting solid was reconstituted in 1:1 MeOH/MeCN. TLC and Celite process was repeated using 99:1 dichloromethane/methanol (R_f = 0.3-0.4), and removal of the volatiles under vacuum afforded the pyrene urea compound as off-white solids (4.3 mg, 17%). NMR spectra of the product including ^{15}N-enriched compound are available in Figure S1 and S2 respectively.^1H NMR (700 MHz, DMSO-d_6): δ 8.40 (d, *J* = 9.2 Hz, 1H), 8.31 (dd, *J* = 11.9, 7.5 Hz, 2H) 8.25 (m, 2H), 8.15 (s, 2H), 8.07 (t, *J* = 7.6 Hz, 1H), 8.02 (d, *J* = 7.6 Hz, 1H), 6.67 (br, 1H), 6.04 (br, 1H), 4.94 (d, *J* = 5.9 Hz, 2H), 3.49 (m, 2H), 3.40 (m, 5H), 3.21 (m, 5H). ^{15}N {^1H} NMR (60 MHz, CD_3CN/DMSO-d_6 (95:5), ^{15}N-enriched sample): δ 77.2. HRMS-ESI (*m/z*) $[M + H]^+$ calcd for $C_{23}H_{25}O_3N_2$, 377.1860; found 377.1850, $[M + H]^+$ calcd for $C_{23}H_{25}O_3N^{15}N$, 378.1830; found 378.1816.



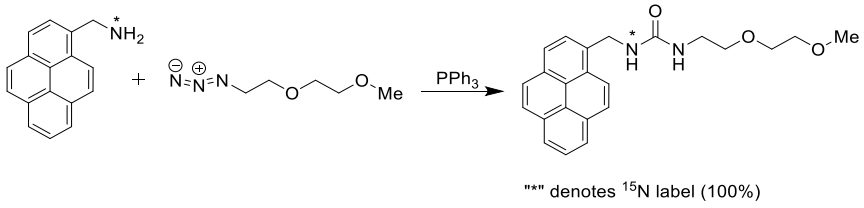
"*" denotes ^{15}N label (50%)

Synthesis of pyrene urea compound using alkylamine and internal ^{15}N-enriched azide. Azide (25 mg, 0.165 mmol) was added to a 4-mL vial equipped with a magnetic stir bar. DMF (280 uL), DMSO (100 uL), and K_2CO_3 aq solution (13.6 uL, 2M, 0,034 mmol) were added. To the mixture, (aminomethyl)pyrene hydrochloride (9.1 mg, 0.039mmol) was added. The mixture was heated for 5 min at 50 °C, and then PPh_3 (90.2 mg, 0.327 mmol) solution in DMF (300 uL) was added dropwise. After the reaction mixture was heated at 50 °C overnight, the formation of the product was confirmed and purified by TLC (2:1 hexane/dichloromethane on basic aluminum oxide 60, MilliporeSigma #1057130001 The product was recovered from alumina by addition of methanol (1 mL) and sonication for 3 minutes after which MeCN (9 mL) was added and sonicated for another 3 min. The suspension was passed through Celite followed by paper filtration to remove Celite. After concentrating the filtrate by nitrogen flow, the resulting

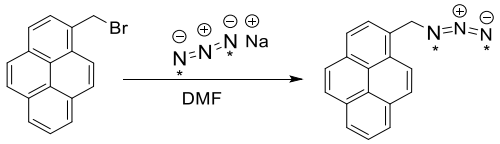
solid was reconstituted in 1:1 MeOH/MeCN. TLC and Celite process was repeated using 99:1 dichloromethane/methanol (R_f = 0.3-0.4), and removal of the volatiles under vacuum afforded the pyrene urea compound as off-white solids (3.6 mg, 14%). NMR spectra of the product including ^{15}N-enriched compound are available in Figure S3 and S4 respectively. ^{1}H NMR (700 MHz, CD_3CN/DMSO-d_6 (95:5): δ 8.48 (d, *J* = 10.8 Hz, 1H), 8.34 (t, *J* = 7.7 Hz, 2H) 8.30 (d, *J* = 7.1 Hz, 1H), 8.28 (d, *J* = 8.8 Hz, 1H), 8.19 (s, 2H), 8.14 (t, *J* = 8.9 Hz, 1H), 8.03 (d, *J* = 8.9 Hz, 1H), 6.16 (br, 1H), 5.60 (br, 1H), 5.08 (d, *J* = 6.8 Hz, 2H), 3.60 (m, 2H), 3.54 (t, *J* = 6.4, 2H), 3.50 (m, 2H) 3.36 (m, 2H), 3.32 (s, 3H). ^{15}N {^{1}H} NMR (60 MHz, CD_3CN/DMSO-d_6 (95:5), ^{15}N-enriched sample): δ 244.78. HRMS-ESI (*m/z*) $[M + H]^+$ calcd for $C_{23}H_{25}O_3N_2$, 377.1860; found 377.1850.



Synthesis of ^{15}N-enriched pyrene amine: The synthetic procedure was adopted from previous reports using ^{15}N-enriched potassium phtalimide (Millipore-Sigma #299243).[7] Pyrene bromide (48.6mg, 0.165 mmol) was dissolved in DMF (350 uL) in a 4 mL vial equipped with a magnetic stir bar. To the mixture, ^{15}N-enriched potassium phtalimide (50 mg, 0.165 mmol) was added, the reaction vial was sealed with cap and vinyl tape and covered with aluminum foil. After the reaction mixture was heated overnight at 125 °C, cold H_2O (2 mL) was added to the resulting mixture and transferred to a .1.7 mL eppendorf tube. The precipitates were collected by centrifugation (15,000 rcf, 15 mins, 4 °C), the pellets were washed again with H_2O and centrifuged using the same conditions as stated previously. Evaporation of the volatiles by gentle flow of nitrogen gas afforded the phthalimide intermediate. The ^{15}N-enriched pyrene phtalimide intermediate was dissolved in EtOH (2 mL) in a 4 mL vial, and hydrazine hydrate (54.6 mg, 1.09 mmol) was added. After the reaction mixture was heated overnight at 65 °C, EtOH (2 x 2 mL) was added to the resulting mixture which was then centrifuged (15,000 rcf, 15 mins, 4 °C) to obtain the supernatant. A gentle flow of nitrogen gas over the supernatant gave the product as a mixture with phthalimide-derived impurity (28.5 mg). This crude material was used without further purification ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.41-8.06 (m, 9H), 4.47 (s, 2H). HRMS-ESI (*m/z*) $[M + H]^+$ calcd for $C_{17}H_{14}{}^{15}N$, 233.1091; found 233.1085.

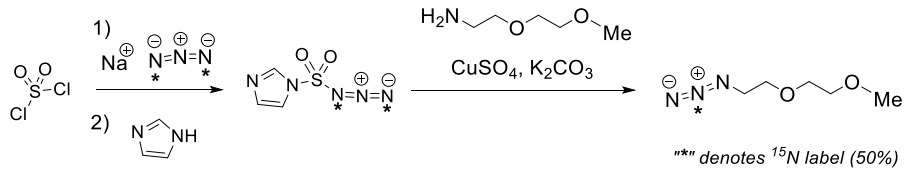


Synthesis of pyrene urea compound using ^{15}N enriched alkylamine and unlabeled azide. Azide (25 mg, 0.165 mmol) was added to a 4-mL vial equipped with a magnetic stir bar. DMF (280 uL), DMSO (100 uL), and K_2CO_3 aq solution (13.6 uL, 2M, 0,034 mmol) were added. To the mixture, ^{15}N enriched aminomethyl pyrene (9.1 mg, 0.039mmol) was added. The mixture was heated for 5 min at 50 °C, and then PPh_3 (90.2 mg, 0.327 mmol) solution in DMF (300 uL) was added dropwise. After the reaction mixture was heated at 50 °C overnight, the formation of the product was confirmed by TLC, and the reaction mixture was also purified by TLC (2:1 hexane/dichloromethane on basic aluminum oxide 60, (MilliporeSigma #1057130001). The product was recovered from the alumina by addition of methanol (1 mL) and sonication for 3 minutes, and then MeCN (9 mL) was added and sonicated for another 3 min. The suspension was passed through Celite followed by paper filtration to remove Celite. After concentrating the filtrate by nitrogen flow, the resulting solid was reconstituted in 1:1 MeOH/MeCN. TLC and Celite process was repeated using 99:1 dichloromethane/methanol (R_f = 0.3-0.4), and removal of the volatiles under vacuum afforded the pyrene urea compound as off-white solids (2.2 mg, 9%). NMR spectra of the product including ^{15}N-enriched compound are available in Figure S5 and S6 respectively. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.41 (d, *J* = 9.3 Hz, 1H), 8.31 (t, *J* = 8.2 Hz, 2H) 8.26 (t, *J* = 7.3 Hz, 2H), 8.16 (s, 2H), 8.08 (t, *J* = 7.6 Hz, 1H), 8.03 (d, *J* = 7.6 Hz, 1H), 6.69 (br, 1H), 6.07 (br, 1H), 4.96 (d, *J* = 5.6 Hz, 2H), 3.51 (m, 2H), 3.41 (m, 3H), 3.21 (m, 4H). ^{15}N {^{1}H} NMR (60 MHz, DMSO-d_6, ^{15}N-enriched sample): δ 83.51. ESI-MS: calcd for $C_{23}H_{25}N_2O_3$ $[M+H]^+$ 378, found 378. HRMS-ESI (*m/z*) $[M + H]^+$ calcd for $C_{23}H_{25}O_3N^{15}N$, 378.1830; found 378.1823.



"*" denotes ^{15}N label (50%)

Synthesis of ^{15}N-enriched pyrene azide: The synthetic procedure was adopted from previous reports using ^{15}N-enriched sodium azide (Millipore-Sigma #609374).[8] Pyrene bromide (50.8mg, 0.172 mmol) was added to DMF (300 uL) in a 4-mL vial equipped with a magnetic stir bar. To the mixture, powder of NaN_3 (17.0 mg, 0.258mmol) was added, the reaction vial was sealed with cap and electrical tape immediately. After the reaction mixture was heated overnight at 60 °C, the formation of the product was confirmed by thin layer chromatography. The volatiles were removed by the gentle flow of N_2 gas, and then the resulting solid was reconstituted in H_2O (2 mL). The product was extracted from the aqueous phase with diethyl ether (2 x 3 mL), dried with Na_2SO_4 and filtered into a 20-mL vial. The mixture was dried under vacuum to afford a yellow waxy solid (29.1 mg, 57%). ^{1}H NMR spectra of the product including ^{15}N-enriched compound are available in Figure S33 and S34 respectively. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.23 (d, *J* = 10.0 Hz, 1H), 8.15 (m, 4H), 7.98 (m, 4H), 4.99 (s, 2H). ^{15}N {^{1}H} NMR (60 MHz, $CDCl_3$, ^{15}N-enriched sample): δ 78.5. HRMS-ESI (*m/z*) $[M + H]^+$ calcd for $C_{17}H_{12}{}^{15}NN_2$, 259.0996; found 259.0996.



Synthesis of ^{15}N-enriched alkylazide at the internal nitrogen: The synthetic procedure was adopted from previous reports using ^{15}N-enriched sodium azide (Millipore-Sigma #609374).[9,10] To a 4-mL vial, ^{15}N-enriched sodium azide (69.9 mg, 1.08 mmol) was added. Dry MeCN (1 mL) was added to the vial in air, and the suspension was cooled down in an ice bath for 2 min. Sulfuryl chloride (87.4 µL, 1.08 mmol) was added to the vial in air with a mechanical pipette, and the mixture was stirred at rt overnight. The reaction mixture was cooled in an ice bath for 2 min, and imidazole (147.9 mg, 2.16 mmol) was added in two portions. After the reaction mixture was stirred at rt for 3 h, the formation of the imidazolyl sulfonylazide product was confirmed by silica gel thin layer chromatography using 1:1 EtOAc/hexane as an eluent (Rf ~0.4). EtOAc (3 mL) was added to the reaction mixture, and the organic layer was washed with water (1 mL) and saturated $NaHCO_3$ solution (1 mL), dried over Na_2SO_4, filtered, and dried by the gentle flow of nitrogen gas. Shortly after the removal of the solvents by the nitrogen gas flow, the sulfonyl azide intermediate was dissolved in methanol (1.5 mL) for the azide transfer reaction to prevent its decomposition. In a 4-mL vial, 2-(2-methoxyethoxy)ethanamine (103.0 mg, 0.864 mmol) and K_2CO_3 (113.0 mg, 0.818 mmol) were suspended in methanol (1 mL), and $CuSO_4{\cdot}5H_2O$ (2.6 mg, 0.010 mmol) was added. To the blue suspension, the solution of the sulfonyl azide in methanol was added in two portions (i.e. 2 × 0.75 mL). After the mixture was stirred at rt overnight, the resulting light gray suspension was concentrated to ~0.25 mL by the gentle flow of nitrogen gas, and the formation of the alkylazide product as well as the disappearance of the amine starting material were confirmed by silica gel thin layer chromatography (4:1 EtOAc/hexane) using anisaldehyde and ninhydrin stain, respectively. Water (6 mL) and HCl aqueous solution (1 mL, 6 M stock) was added, and the product was extracted with EtOAc (3 × 5 mL). The combined organic layer was dried over Na_2SO_4, filtered, and dried under vacuum to afford the crude product of the alkylazide as a light yellow oil (84.0 mg, 67%). The crude material was used without further purification for the phosphine-mediated reaction. ^{1}H NMR spectra of the product including ^{15}N-enriched compound are available in Figure S35 and S36 respectively. ^{1}H NMR (500 MHz, $CDCl_3$): δ. 3.66 (m, 4H), 3.56 (m, 2H), 3.39 (m, 5H). ^{15}N {^{1}H} NMR (60 MHz, $CDCl_3$, ^{15}N-enriched sample): δ 248.2.

High-resolution mass spectrometry (HRMS) data

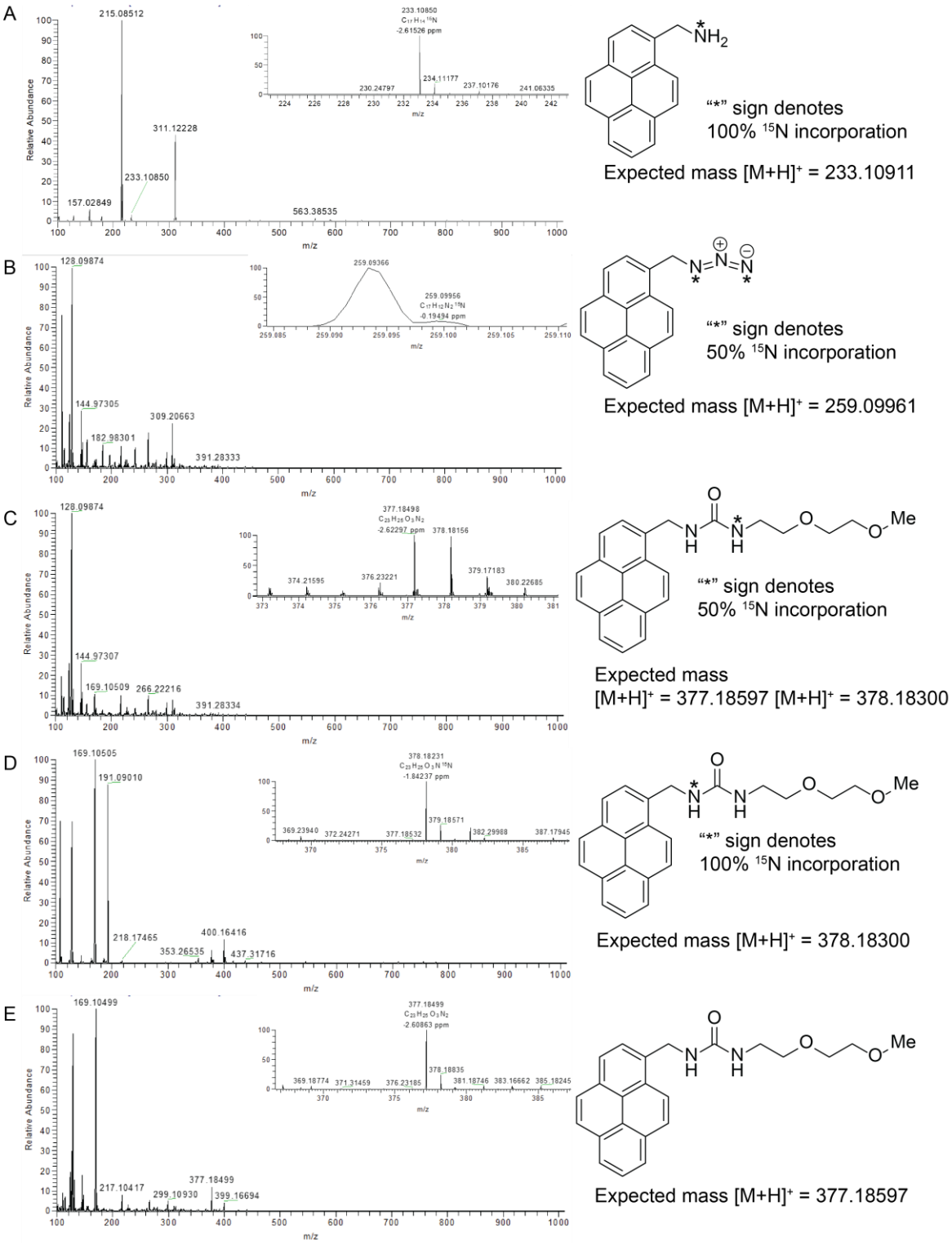


Figure S39. HRMS-ESI spectra of synthesized compounds. (A) ^{15}N-enriched pyrene amine. (B) ^{15}N-enriched pyrene azide. (C) Pyrene urea compound synthesized using alkylamine and terminal ^{15}N-enriched azide. (D) Pyrene urea compound synthesized using ^{15}N enriched alkylamine and unlabeled azide. (E) Pyrene urea compound synthesized using alkylamine and internal ^{15}N-enriched azide.

NMR Spectra of synthesized compounds

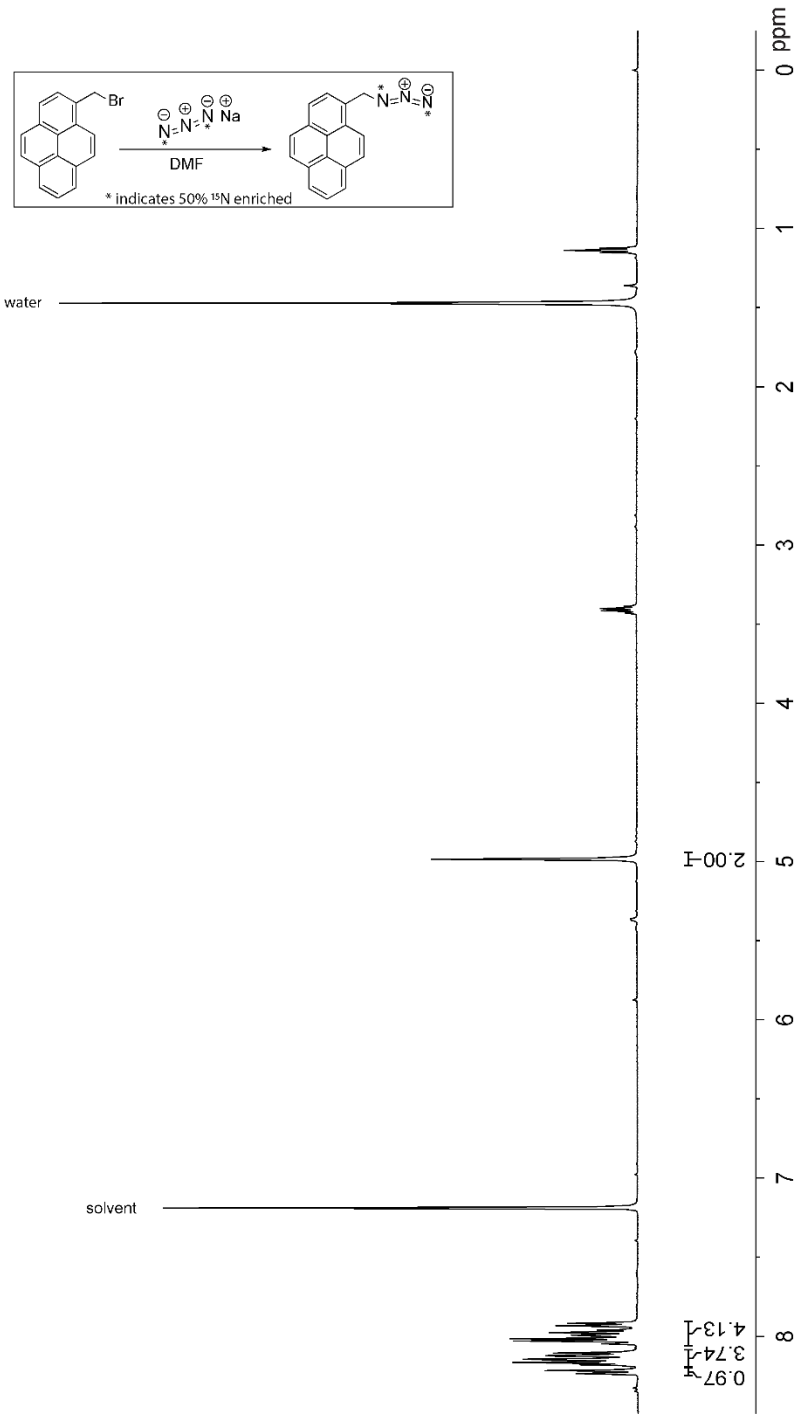


Figure S40. ^{1}H NMR spectrum of 50% ^{15}N-enriched pyrene azide in $CDCl_3$. Reaction conditions and preparations are described in preparative synthesis of small molecule procedures.

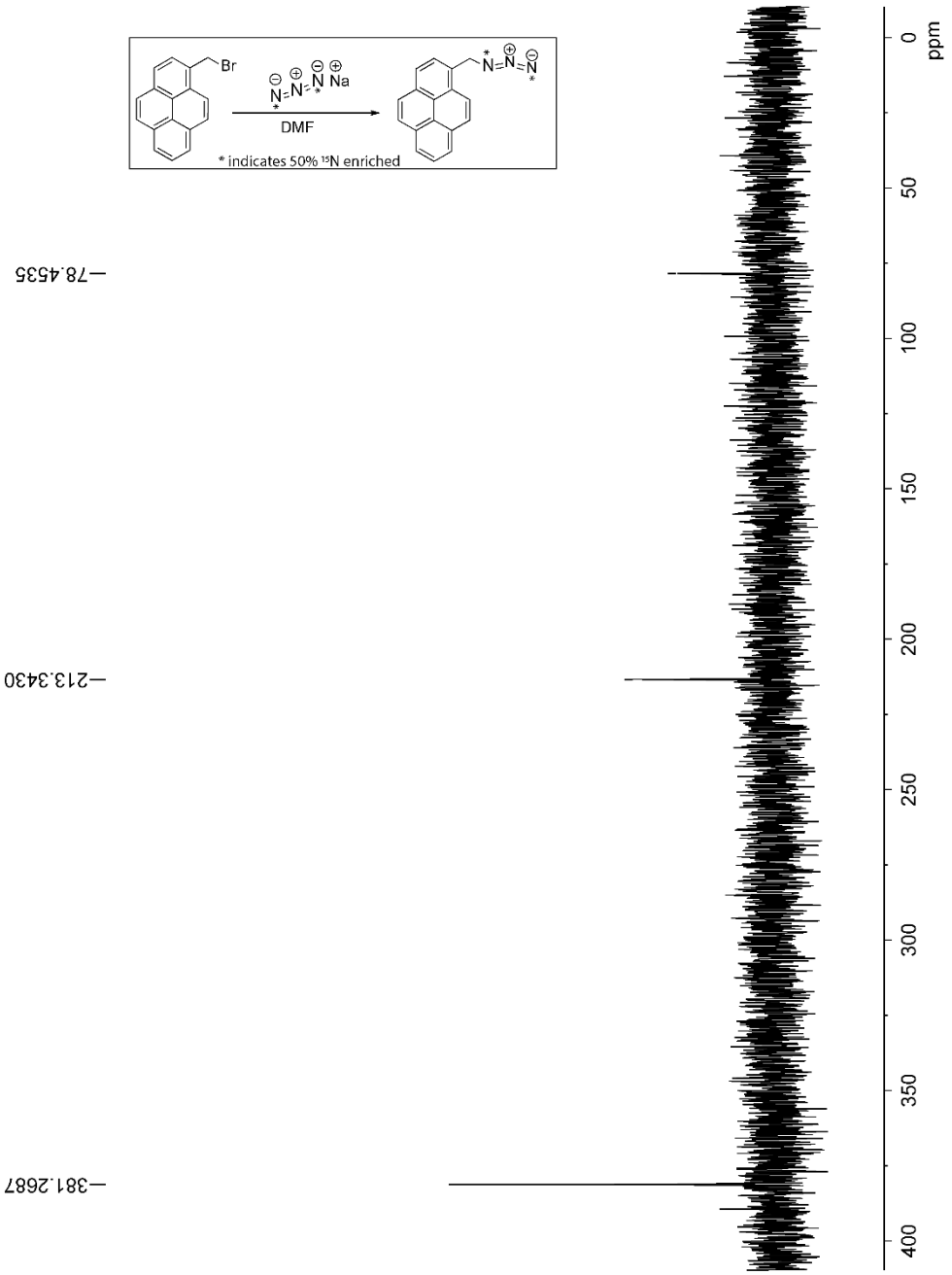


Figure S41. ^{15}N NMR for pyrene azide with ^{15}N enrichment in DMSO-d_6.

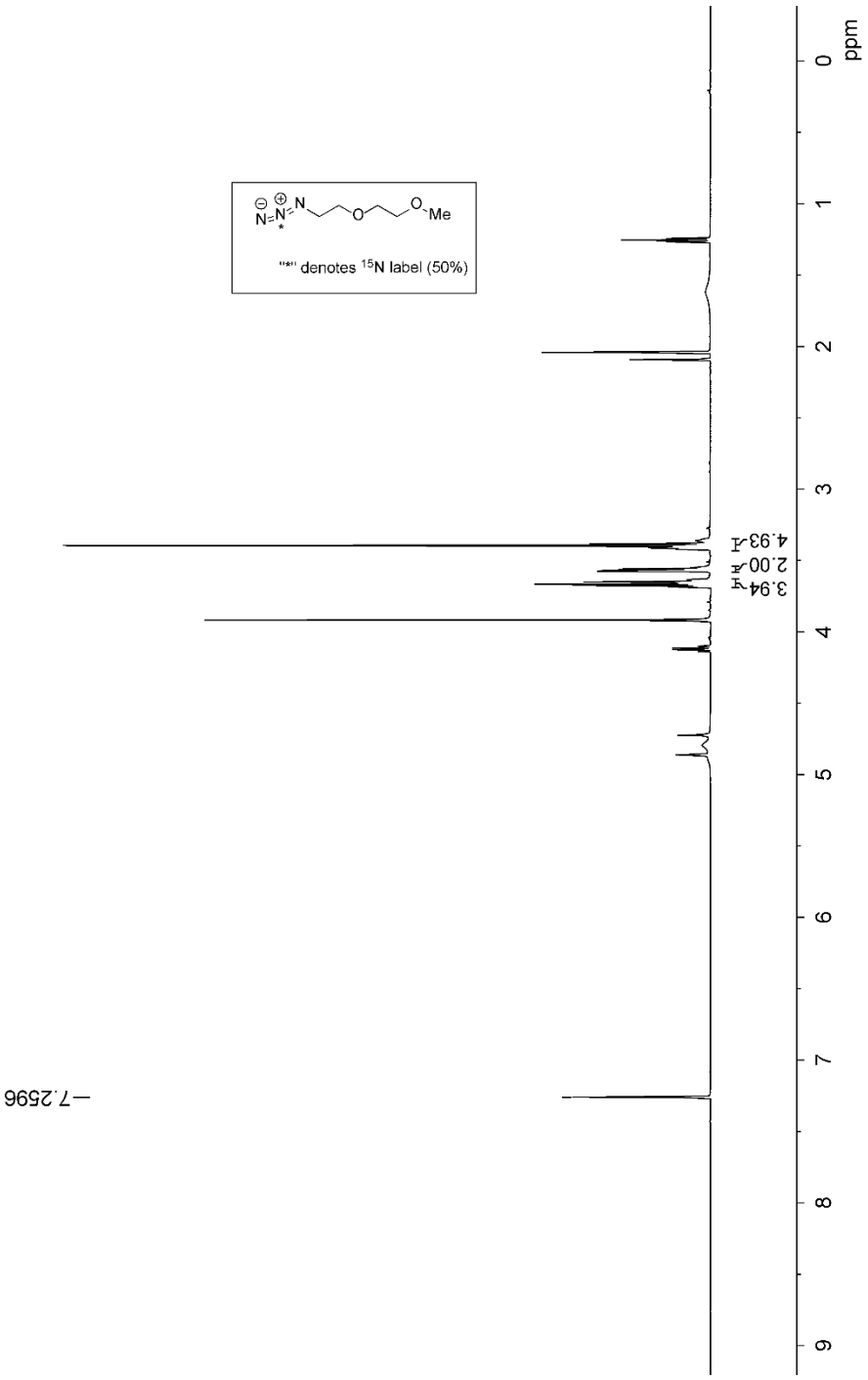


Figure S42. ^{1}H NMR spectrum of internal ^{15}N enriched azide in $CDCl_3$

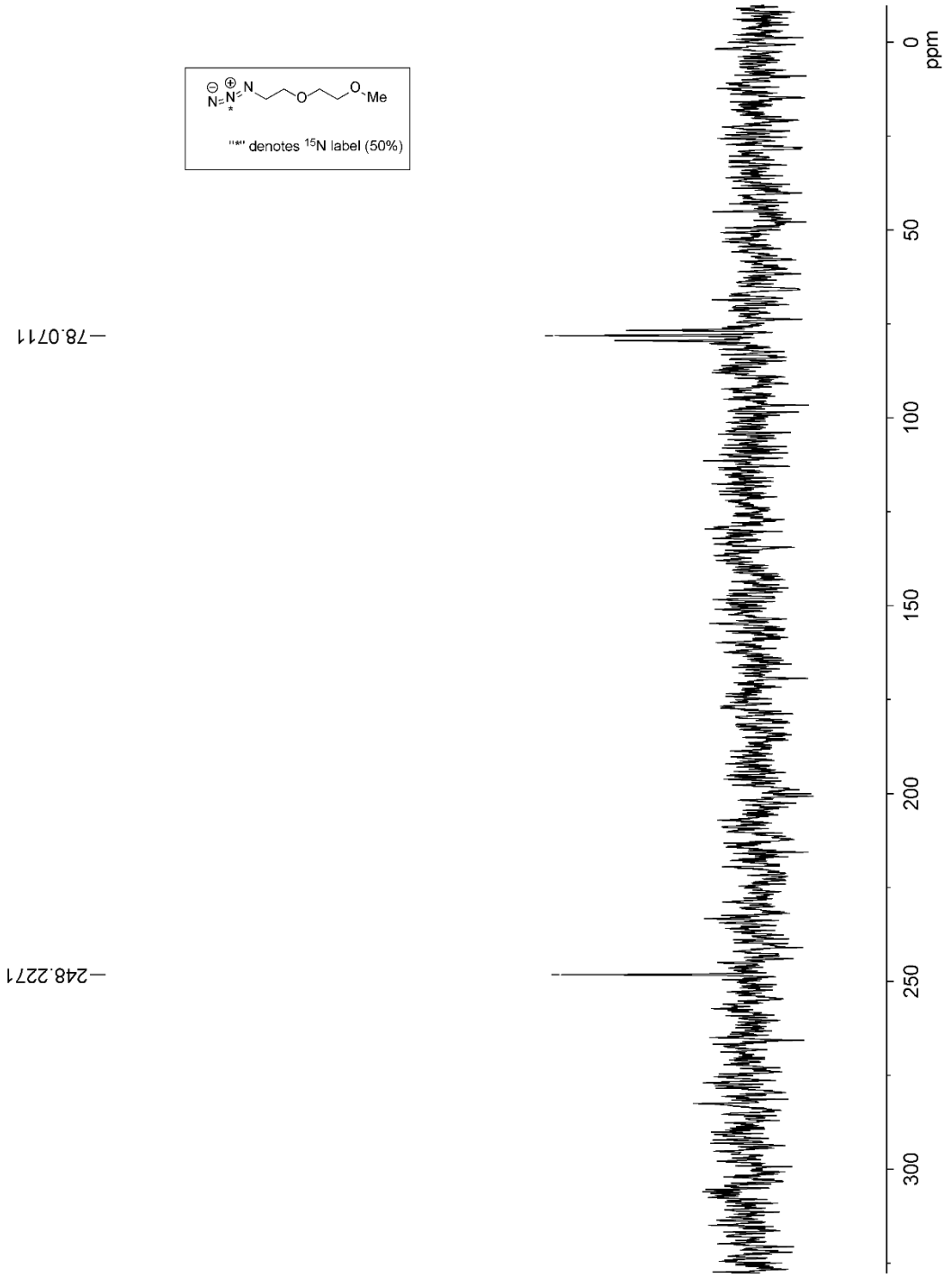


Figure S43. ^{15}N NMR spectrum of internal ^{15}N enriched azide in $CDCl_3$. Neat CH_3NO_2 (381.6 ppm) was used as the external standard.

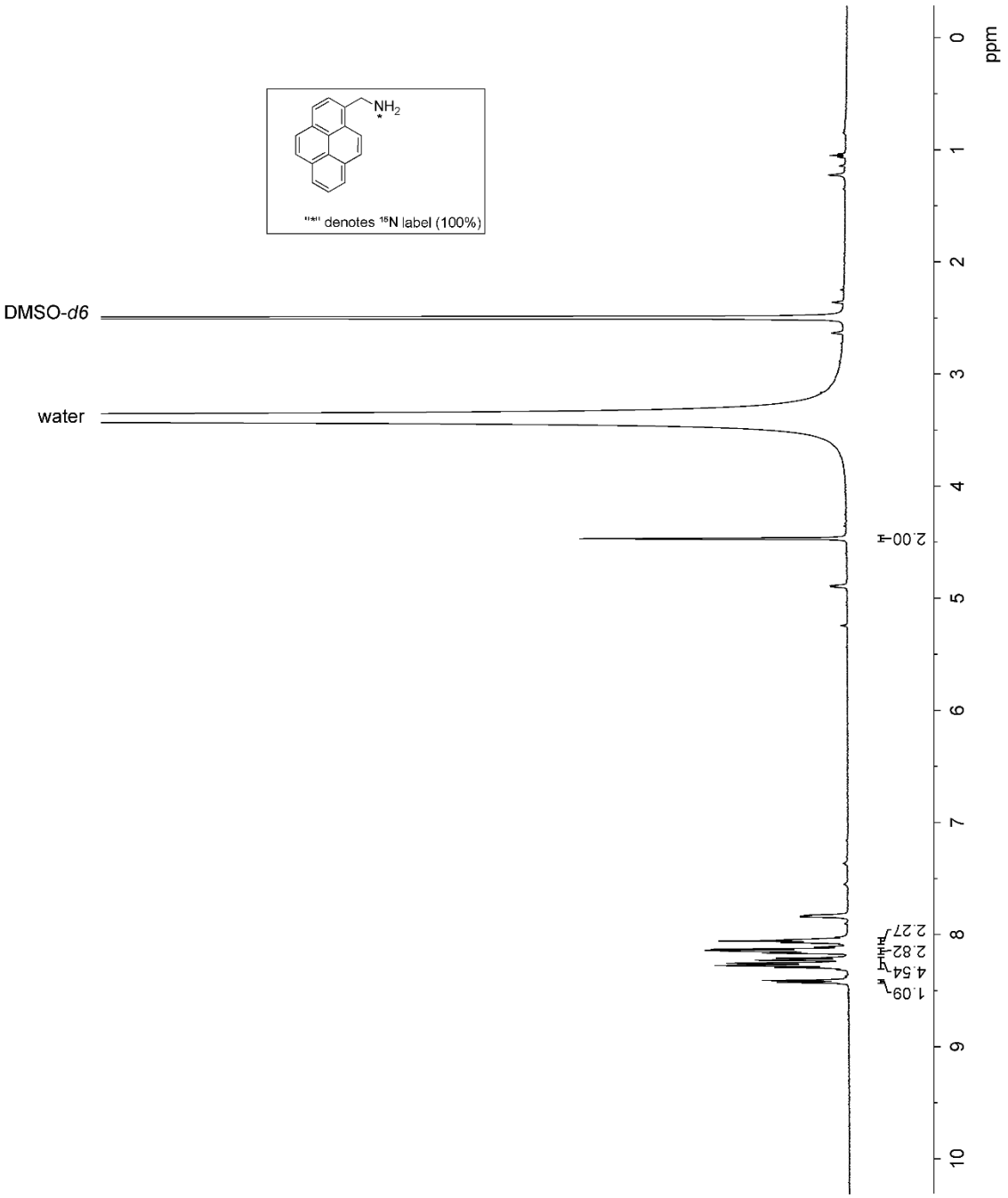


Figure S44. ^{1}H NMR spectrum of ^{15}N enriched pyrene amine in DMSO-d_6

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