Electronic Supplementary Material (ESI) for Chemical Communications.


# Supplementary Information

# for

# A Trifunctional Cyclooctyne for Modifying Azide-Labeled Biomolecules with Photocrosslinking and Affinity Tags

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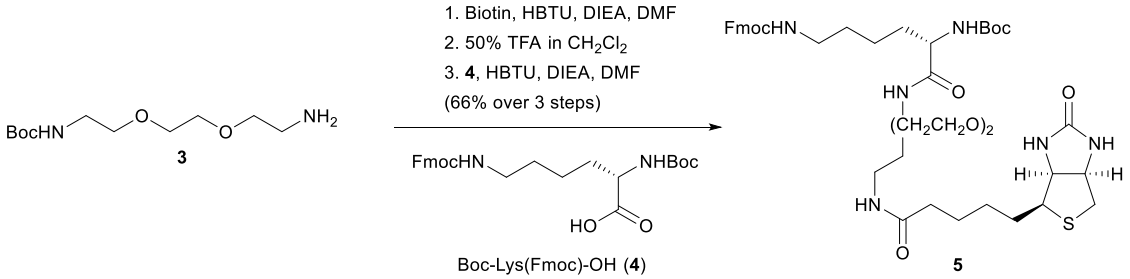
# I. Experimental

## Synthesis of BCN-DAz-Biotin

***General Methods for Synthesis.*** Materials and reagents were obtained from commercial sources without further purification unless otherwise noted. Anhydrous solvents were obtained either commercially or from an alumina column solvent purification system. All reactions were carried out in oven-dried glassware under inert gas unless otherwise noted. Analytical TLC was performed on glass-backed silica gel 60 Å plates (thickness 250 μm) from Dynamic Adsorbents and detected by charring with 5% $H_2SO_4$ in EtOH. Column chromatography was performed using flash-grade silica gel 32–63 μm (230–400 mesh) from Dynamic Adsorbents. $^1$H NMR spectra were recorded at 500 MHz with chemical shifts in ppm (δ) referenced to solvent peaks. $^{13}$C NMR spectra were recorded at 125 MHz. NMR spectra were obtained on a Varian Inova 500 instrument. Coupling constants (*J*) are reported in hertz (Hz). High-resolution electrospray ionization (HR ESI) mass spectra were obtained using a Waters LCT Premier XE using raffinose as the lock mass.

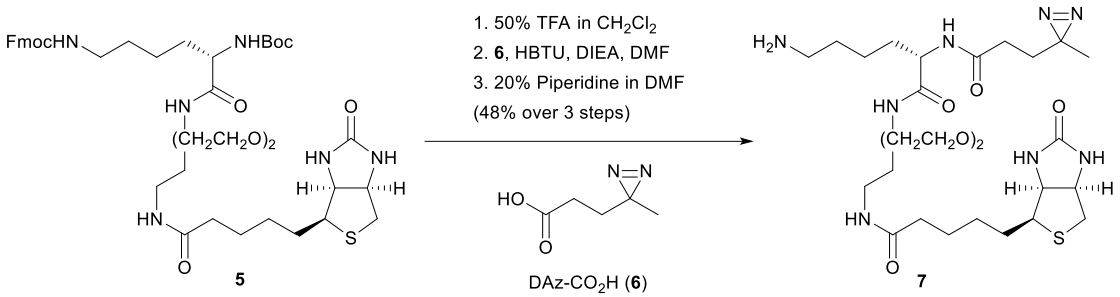
***Compound 5.***




A mixture of biotin (689 mg, 2.82 mmol), HBTU (1.43 g, 3.78 mmol), and DIEA (0.81 mL, 4.7 mmol) in anhydrous DMF (20 mL) was stirred for 20 min at room temperature under an argon atmosphere before being added dropwise to a solution of compound **3**[1] (600 mg, 2.41 mmol). The reaction mixture was stirred overnight at room temperature, after which the solvent was removed by rotary evaporation to give a red oil, which was subjected to silica gel column chromatography ($CH_2Cl_2$/$CH_3OH$/$Et_3N$, 100:10:1) to afford *N*-Boc-*N'*-biotinyl-3,6-dioxaoctane-1,8-diamine. All of the intermediate was dissolved in 50% TFA in $CH_2Cl_2$ (30 mL) and stirred at room temperature for 3.5 h. The solvents were removed by rotary evaporation to give a pale yellow oil, which was subjected to silica gel column chromatography ($CH_2Cl_2$/$CH_3OH$/$Et_3N$, 20:10:1) to afford *N*-

biotinyl-3,6-dioxaoctane-1,8-diamine. All of the intermediate was taken forward to the next step. To a solution of (Boc)-Lys(Fmoc)-OH (2.00 g, 4.26 mmol) in anhydrous DMF (60 mL) was added DIEA (1.10 mL, 6.31 mmol) and HBTU (2.00 g, 5.27 mmol). After stirring for 30 minutes under an argon atmosphere, the mixture was added dropwise via syringe to *N*-biotinyl-3,6-dioxaoctane-1,8-diamine stirring in DMF (50 mL). After stirring for 2.5 h at room temperature under an argon atmosphere, the solvents were removed by rotary evaporation and the residue was purified by silica gel column chromatography ($CH_2Cl_2$/$CH_3OH$/$Et_3N$, 100:10:1) to afford compound **5** (1.30 g, 66% over three steps). $^1$H NMR (500 MHz, $CD_3OD$): δ 7.79 (d, *J* = 7.5 Hz, 2 H, Fmoc ArC*H*), 7.39 (t, *J* = 8.0 Hz, 2 H, Fmoc ArC*H*), 7.31 (t, *J* = 7.5 Hz, 2 H, Fmoc ArC*H*), 4.46 (dd, *J* = 5.0, 8.0 Hz, 1 H, biotin NH*CH*), 4.34 (d, *J* = 7.0 Hz, 2 H, Fmoc C$H_2$O), 4.26 (dd, *J* = 4.5 Hz, 8.0 Hz, 1 H, biotin NH*CH*), 4.20 (t, *J* = 6.0 Hz, 1 H, Fmoc benzylic C*H*), 4.10-3.96 (m, 1 H, Lys α-C*H*), 3.58 (s, 4 H, PEG OC$H_2$C$H_2$O), 3.54-3.51 (m, 4 H, PEG OC$H_2$), 3.37-3.34 (m, 4 H, PEG NHC$H_2$), 3.17-3.14 (m, 1 H, biotin SC*H*), 3.11 (t, *J* = 6.0 Hz, 2 H, Lys NHC$H_2$), 2.88 (dd, *J* = 4.5, 12.5 Hz, 1 H, biotin SC$H_{endo}$) 2.69 (d, *J* = 13 Hz, 1 H, biotin SC$H_{exo}$), 2.20 (t, *J* = 7.5 Hz, 2 H, biotin COC$H_2$), 1.75-1.40 (m, 12 H, biotin C$H_2$s, Lys C$H_2$s), 1.42 (s, 9 H, Boc C$H_3$s). $^{13}$C NMR (125 MHz, $CD_3OD$): *δ* 176.08, 175.27, 166.05, 158.86, 157.78, 145.36, 142.58, 141.38, 139.29, 129.87, 128.78, 128.19, 128.15, 126.16, 122.04, 120.94, 120.67, 108.27, 80.57, 71.28, 70.60, 70.50, 67.57, 63.33, 61.57, 56.98, 56.10, 49.84, 41.38, 41.02, 40.27, 36.72, 33.11, 30.49, 29.74, 29.48, 28.73, 26.81, 24.05. HRMS (ESI$^+$): *m/z* calcd. for $C_{42}H_{60}N_6O_9S$ [M + Na] $^+$: 847.4040; found: 847.4050.

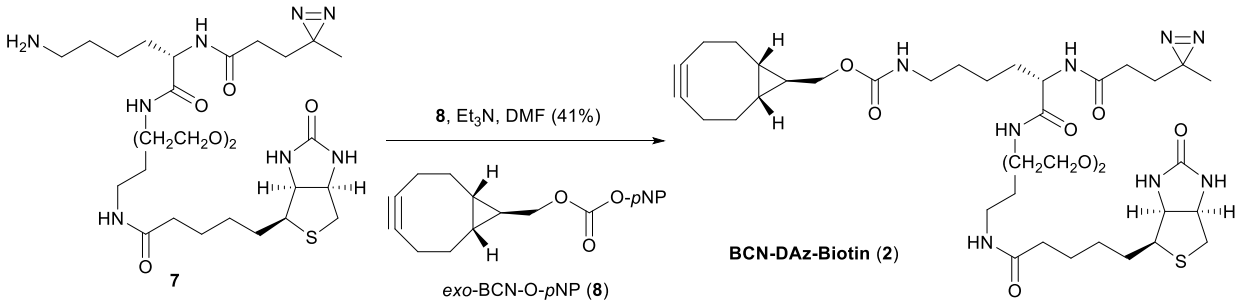
***Compound 7.***




Compound **5** (123 mg, 0.150 mmol) was dissolved in 50% TFA in $CH_2Cl_2$ (2.5 mL) and stirred under an argon atmosphere at room temperature for 2 h. The solvents were removed by rotary evaporation to give a crude syrup, which was taken forward to the next step. To a solution of **6**[2] (26.0 mg, 0.203 mmol) in anhydrous DMF (5.0 mL) was added DIEA (38 µL, 0.23 mmol) and

HBTU (69.0 mg, 0.182 mmol). After stirring this mixture under an argon atmosphere at room temperature for 30 minutes, it was added dropwise to a solution of the intermediate free amine (obtained above) in anhydrous DMF (3.0 mL). The reaction was stirred for 6 hours, then the solvents were removed by rotary evaporation and the crude residue was subjected to silica gel flash column chromatography ($CH_2Cl_2$/$CH_3OH$/$Et_3N$, 100:10:1) to afford the diazirine-modified intermediate. All of the intermediate was dissolved in 20% piperidine in DMF (2.50 mL) and stirred under an argon atmosphere at room temperature overnight. The solvents were removed by rotary evaporation and the crude material was purified by silica gel flash column chromatography ($CH_2Cl_2$/$CH_3OH$/$Et_3N$, 10:10:2) to give compound **7** (44 mg, 48% over three steps). $^1$H NMR (500 MHz, $CD_3OD$): δ 4.49 (dd, *J* = 4.0, 7.0 Hz, 1 H, biotin NHC*H*), 4.32-4.27 (m, 2 H, biotin NHC*H* and Lys α-C*H*), 3.61 (s, 4 H, PEG OC$H_2$C$H_2$O), 3.55 (t, *J* = 5.0 Hz, 4 H, PEG OC$H_2$), 3.38-3.35 (m, 4 H, PEG NHC$H_2$), 3.23-3.19 (m, 1 H, biotin SC*H*), 2.93 (dd, *J* = 5.5, 13.5 Hz, 1 H, biotin SC$H_{endo}$), 2.71 (d, *J* = 13.0 Hz, 1 H, biotin SC$H_{exo}$), 2.62 (t, *J* = 7.0 Hz, 2 H, Lys NHC$H_2$), 2.22 (t, *J* = 7.0 Hz, 2 H, biotin COC$H_2$), 2.16 (t, *J* = 7.5 Hz, 2 H, DAz COC$H_2$), 1.81-1.26 (m, 14 H, biotin C$H_2$s, Lys C$H_2$s, DAz C$H_2$), 1.01 (s, 3 H, DAz C$H_3$). $^{13}$C NMR (125 MHz, $CD_3OD$): δ 176.14, 174.55, 174.50, 166.12, 70.31, 70.61, 70.51, 63.49, 63.38, 61.62, 57.04, 54.84, 53.56, 42.28, 41.05, 40.33, 40.30, 36.76, 33.46, 32.99, 31.28, 31.05, 29.79, 29.51, 26.87, 26.39, 24.28, 19.76. HRMS (ESI$^+$): m/z calcd. for $C_{27}H_{48}N_8O_6S$ [M + Na] $^{+:}$ 635.3315; found: 635.3329.

***Compound 2 (BCN-DAz-Biotin).***




To a solution of compound **7** (41.0 mg, 79.2 μmol) in anhydrous DMF (2.00 mL) was added *exo*-BCN-O-*p*NP **8**[3] (25.0 mg, 66.0 μmol) and $Et_3N$ (35 μL, 250 μmol). The reaction mixture was stirred under an argon atmosphere at room temperature for 18 hours, after which it was concentrated by rotary evaporation and purified by silica gel column chromatography

($CH_2Cl_2$/$CH_3OH$/$Et_3N$, 100:10:1) to afford BCN-DAz-Biotin **2** (26.0 mg, 50%). $^1$H NMR (500 MHz, $CD_3OD$): δ 4.50 (dd, *J* = 4.0, 7.0 Hz, 1 H, biotin NH*CH*), 4.31 (dd, *J* = 4.5, 7.5 Hz, 1 H, biotin NH*CH*), 4.28 (dd, *J* = 5.0, 8.5 Hz, 1 H, Lys α-C*H*), 3.94 (d, *J* = 7.5 Hz, 2 H, BCN C$H_2$O), 3.61 (s, 4 H, PEG OC$H_2$C$H_2$O), 3.55 (t, *J* = 5.0 Hz, 4 H, PEG OC$H_2$), 3.41-3.34 (m, 4 H, PEG NHC$H_2$), 3.23-3.19 (m, 1 H, biotin SC*H*), 3.09 (t, *J* = 8.5 Hz, 2 H, Lys NHC$H_2$), 2.93 (dd, *J* = 5.5, 13.5 Hz, 1 H, biotin SC$H_{endo}$), 2.71 (d, *J* = 12.5 Hz, 1 H, biotin SC$H_{exo}$), 2.38 (dd, *J* = 3.0, 13.5 Hz, 2 H, BCN propargylic C*H*s), 2.27-2.09 (m, 8 H, BCN propargylic C*H*s, BCN homopropargylic C*H*s, biotin COC$H_2$, DAz COC$H_2$), 1.77-1.29 (m, 16 H, BCN homopropargylic C*H*s, biotin C$H_2$s, Lys C$H_2$s, DAz C$H_2$), 1.01 (s, 3 H, DAz C$H_3$), 0.76-0.66 (m, 3 H, BCN cyclopropyl *H*s). $^{13}$C NMR (125 MHz, $CD_3OD$): δ 176.15, 174.52, 166.12, 159.37, 99.44, 71.32, 71.31, 70.61, 70.50, 69.95, 63.37, 61.62, 57.04, 54.78, 41.35, 41.07, 40.33, 40.30, 36.77, 34.40, 32.79, 31.31, 31.07, 30.55, 29.80, 29.51, 26.89, 26.41, 25.07, 24.17, 24.10, 21.94, 21.90, 19.76. HRMS (ESI$^+$): *m/z* calcd. for $C_{38}H_{60}N_8O_8S$ $[M + Na]^+$ : 811.4152; found: 811.4186.

## Metabolic Labeling and Analysis of *Mycobacterium smegmatis*

***Bacterial strains, media, and reagents.*** *M. smegmatis* mc$^2$155 was cultured at 37 °C in Middlebrook 7H9 liquid medium supplemented with ADC (albumin, dextrose, and catalase), 0.5% glycerol, and 0.05% Tween-80. A stock solution of synthetic BCN-DAz-Biotin was prepared in DMSO at a concentration of 10 mM and stored at –20 °C. A stock solution of 6-TreAz was prepared in ultrapure $H_2O$ at a concentration of 25 mM, sterile-filtered, and stored at –20 °C. A stock solution of avidin-488 (Life Technologies) was prepared in PBS 1x at a concentration of 1 mg/mL and stored at –20 °C. Prior to usage in bacterial labeling experiments, stock solutions were diluted to the desired concentration with the appropriate culture medium or buffer and temporarily stored at 4 °C.

***Procedures for bacterial labeling and analysis.*** Starter cultures of bacteria were generated by inoculating a single colony from a freshly streaked LB agar plate into 3 mL liquid medium in a

culture tube. Starter cultures were incubated at 37 °C with shaking until reaching mid-logarithmic phase and then diluted with liquid medium to the desired density for initiating experiments.

Labeling experiments were performed in 96-well plates. Bacteria were mixed with liquid medium and 6-TreAz in sterile flat-bottom 96-well plates to achieve the desired cell density and 6-TreAz concentration (25 μM) at a final volume of 200 μL. Plates were incubated at 37 °C with shaking in a Tecan plate reader (Infinite F200 PRO operated by Tecan iControl software) until the desired end-point (culture time 16 h, end-point $OD_{600}$ ~1.0-1.2).

For secondary labeling of bacteria with BCN-DAz-Biotin by SPAAC, a suspension of 6-TreAz-labeled cells (200 μL) was transferred to a v-bottom 96 well plate, centrifuged (3,600 rpm, 10 min, room temperature) and washed with PBS 1x containing 0.5% bovine serum albumin (PBSB) three times. Subsequently, cells were incubated with BCN-DAz-Biotin at the desired concentration for 30 minutes at room temperature in the dark, followed by washing three times with PBSB. Then, the cells were fixed with 4% paraformaldehyde in PBS 1x for 10 minutes and washed three times with PBS 1x as described above. Next, cells were incubated with avidin-488 conjugate (200:1 dilution of a 1 mg/mL stock solution in PBS 1x) for 15 minutes at room temperature in the dark. Finally, cells were washed with PBS 1x three times and prepared for analysis by flow cytometry or fluorescence microscopy.

***Flow cytometry.*** After fluorescent labeling of bacteria according to the above procedure, bacteria were transferred to 5 mL polystyrene Falcon tubes (BD Biosciences) and analyzed by flow cytometry. Flow cytometry was performed on a BD Biosciences FACSAria II flow cytometer. Fluorescence data was collected for 50,000 cells at an event rate of 500–1,000 events/sec and processed using BD FACSDIVA 8.0.1 software. All flow cytometry experiments were performed with three replicate samples, and data shown were representative of at least two independent experiments. Scatter-gated fluorescence analysis was used to obtain mean fluorescence intensities with doublet discrimination.

***Fluorescence microscopy.*** 10 μL of bacterial sample in PBS 1x were spotted onto a microscope slide, lightly spread into a thin layer using the edge of a coverslip, and allowed to air dry in the dark. Fluoromount-G mounting medium (SouthernBiotech) was applied, then cover slips were placed over the sample and immobilized with adhesive. Microscopy was carried out using an EVOS FL (Life Technologies) inverted microscope equipped with a 100 × 1.4 numerical aperture

Plan-Apochromat oil immersion lens. Fluorescence imaging was performed using a GFP (maximum excitation/emission = 470/510 nm) LED light cube. Images were captured with a Sony ICX445 CCD camera and processed using the FIJI distribution of ImageJ. Image acquisition and processing were performed identically for all test and control samples being compared. Imaging data shown were representative of at least two independent experiments.

**Photocrosslinking of BCN-DAz-Biotin and BSA**

***Reagents.*** Stock solutions of reagents used in the SPAAC step included 1 mM BCN-DAz-Biotin in DMSO, 1 mM DBCO-Biotin (Click Chemistry Tools) in DMSO, and 1 mM Az488 (Click Chemistry Tools) in DMSO. BSA (MP Biomedical) was defatted by washing with chloroform three times and drying under high vacuum. A stock solution of defatted BSA was prepared in ultrapure water at a concentration of 2 mg/mL.

***SPAAC and Photocrosslinking.*** SPAAC reactions were carried out by incubating Az488 (75 μM) with either BCN-DAz-Biotin (50 μM) or DBCO-Biotin (50 μM), or vehicle conditions, in the dark at 37 °C for 27 h with gentle shaking. Next, 20 μL aliquots of each reaction product were mixed with 10 μL of defatted BSA to achieve approximate final concentrations of 33 μM probe and 10 μM BSA. BSA-only vehicle controls were also prepared. These solutions were incubated in the dark at room temperature with shaking for 1 h, then split into equal volumes and either left non-irradiated or exposed to UV irradiation for 20 min. UV irradiation was performed using a 15-watt 365 nm UV bench lamp (UVP) with samples placed in a 96-well plate 30 cm from the lamp.

***Gel Electrophoresis and Western Blot Analysis.*** Samples were analyzed by SDS-PAGE and Western blot. Samples obtained as described above were mixed with sample loading buffer (0.25 M Tris-HCl pH 6.8, 8% SDS w/v, 30% Glycerol v/v, 0.02% Bromophenol Blue w/v, 10 mM dithiothreitol v/v), heated at 95 °C for 5 min, loaded into Mini-PROTEAN TGX 4–20% precast gels (Bio-Rad), and electrophoresed at 150 v for 90 min. In-gel fluorescence was detected with a Typhoon FLA 7000 (GE Healthcare Life Sciences) using fluorescein excitation/emission filters. For Western blotting, protein was transferred immediately following in-gel fluorescence to a nitrocellulose membrane at 100 v for 1 h. After transfer, the membrane was washed 3 times for 5 minutes each with TBST, blocked overnight with 5% non-fat dry milk (Bio-Rad) at 4 °C, washed 3 times for 5 minutes each with TBST, incubated with 1:1000 avidin-HRP conjugate (Bio-Rad) in

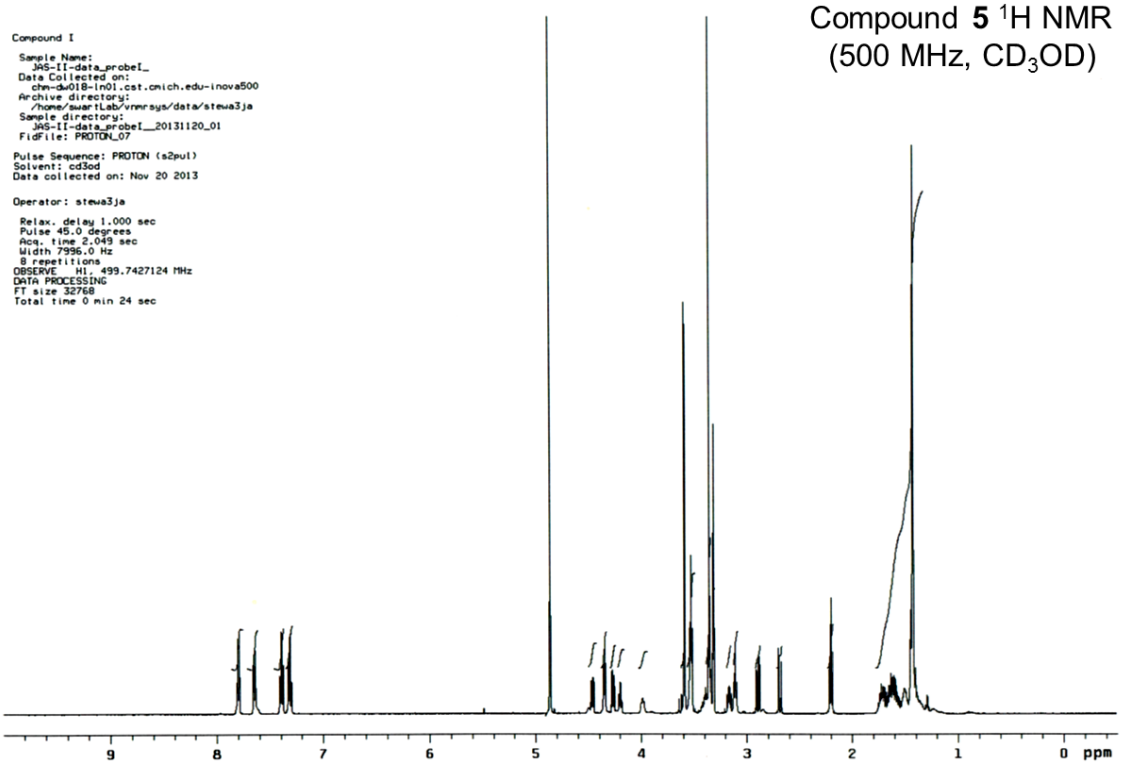
blocking solution for 1 h, washed 4 times for 15 minutes each with TBST, and incubated for 5 min with 1:1 luminol/enhancer solution:peroxide solution (Clarity Western ECL Substrate, Bio-Rad). Imaging of the membrane was performed using a ChemiDoc Touch Imaging System (Bio-Rad). For Ponceau staining of total protein, nitrocellulose membranes were exposed to Ponceau stain (0.5% Ponceau S. w/v, 1% acetic acid v/v) for 5 min and rinsed with water

## II. References for Supplementary Information

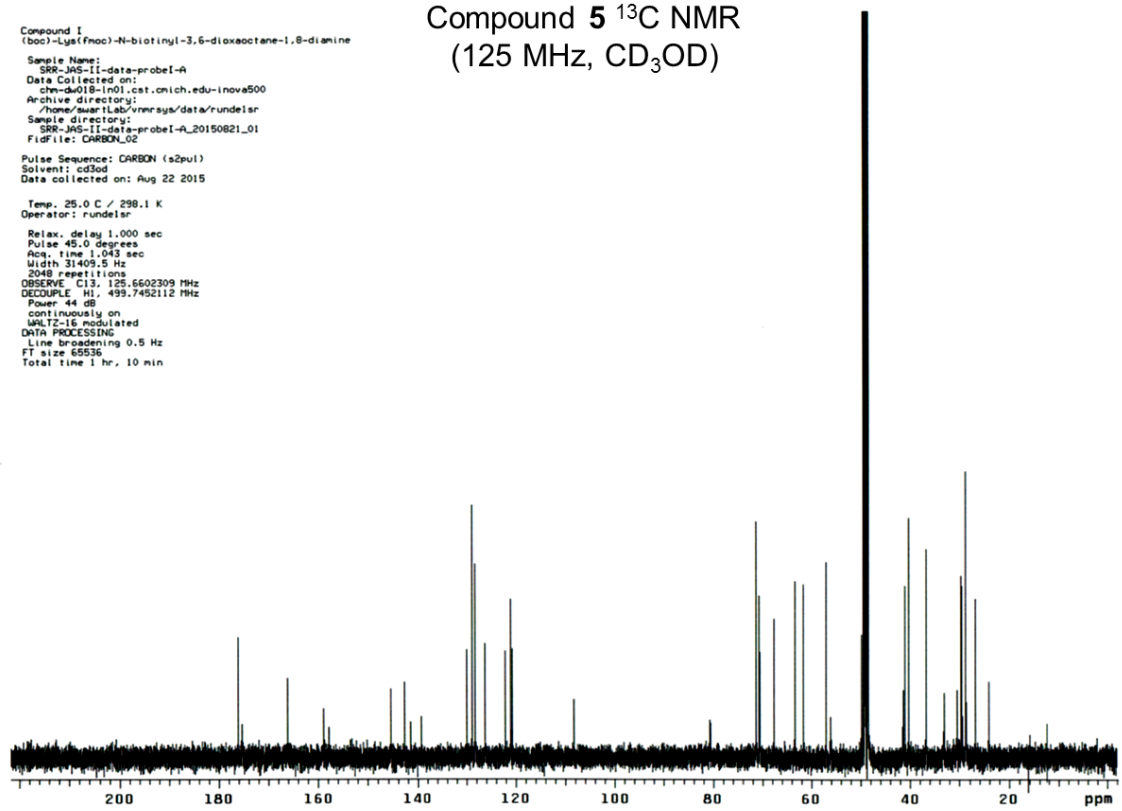

1. X. Ning, J. Guo, M. A. Wolfert and G.-J. Boons, *Angew. Chem. Int. Edit.*, 2008, **47**, 2253-2255.
2. M. R. Bond, H. Zhang, P. D. Vu and J. J. Kohler, *Nat. Protocols*, 2009, **4**, 1044-1063.
3. J. Dommerholt, S. Schmidt, R. Temming, L. J. A. Hendriks, F. P. J. T. Rutjes, J. C. M. van Hest, D. J. Lefeber, P. Friedl and F. L. van Delft, *Angew. Chem. Int. Edit.*, 2010, **49**, 9422-9425.

## III. NMR Spectra and HPLC Traces for Synthetic Compounds

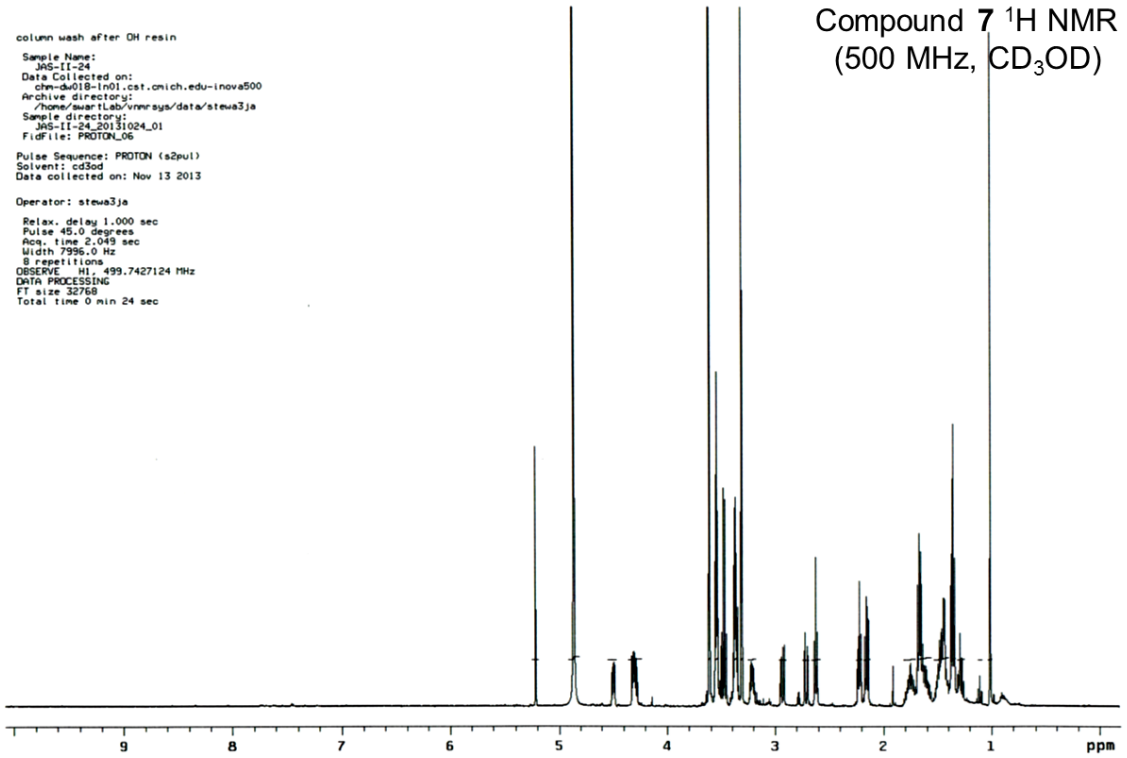
Compound **5** $^{1}$H NMR
(500 MHz, $CD_3OD$)



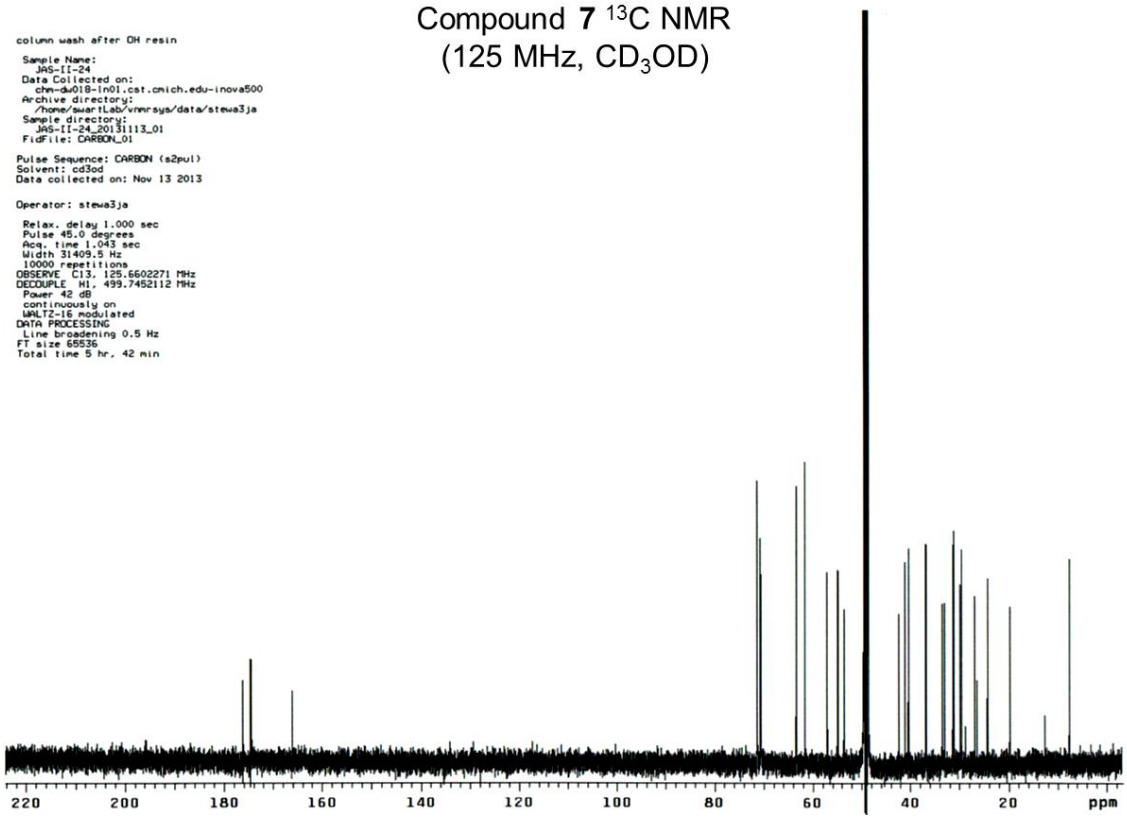

Compound **5** $^{13}$C NMR
(125 MHz, $CD_3OD$)



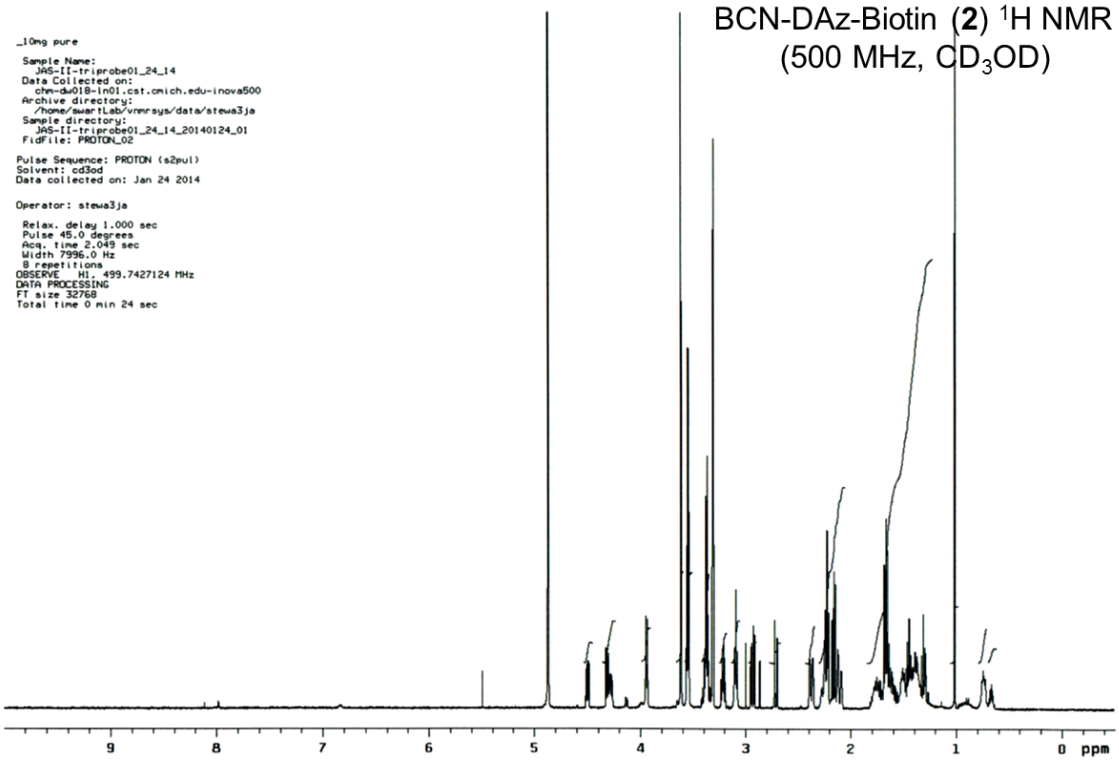
Compound **7** $^{1}$H NMR
(500 MHz, $CD_3OD$)



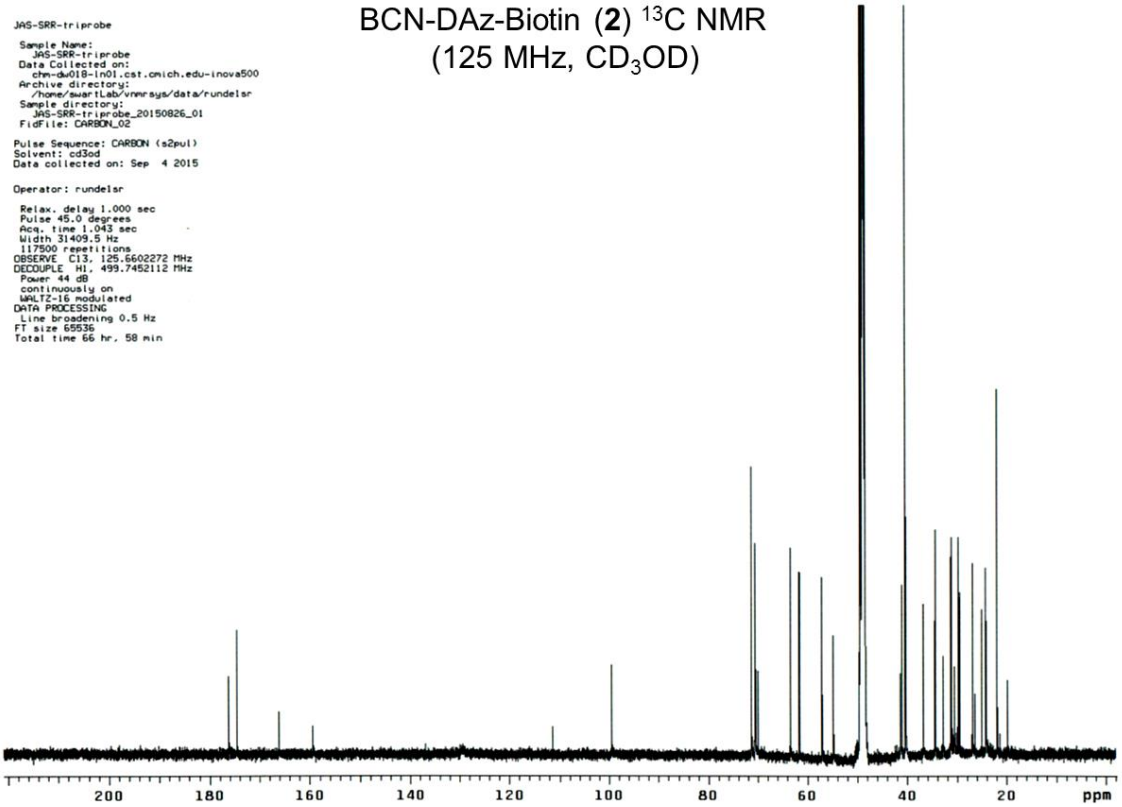

Compound **7** $^{13}$C NMR
(125 MHz, $CD_3OD$)

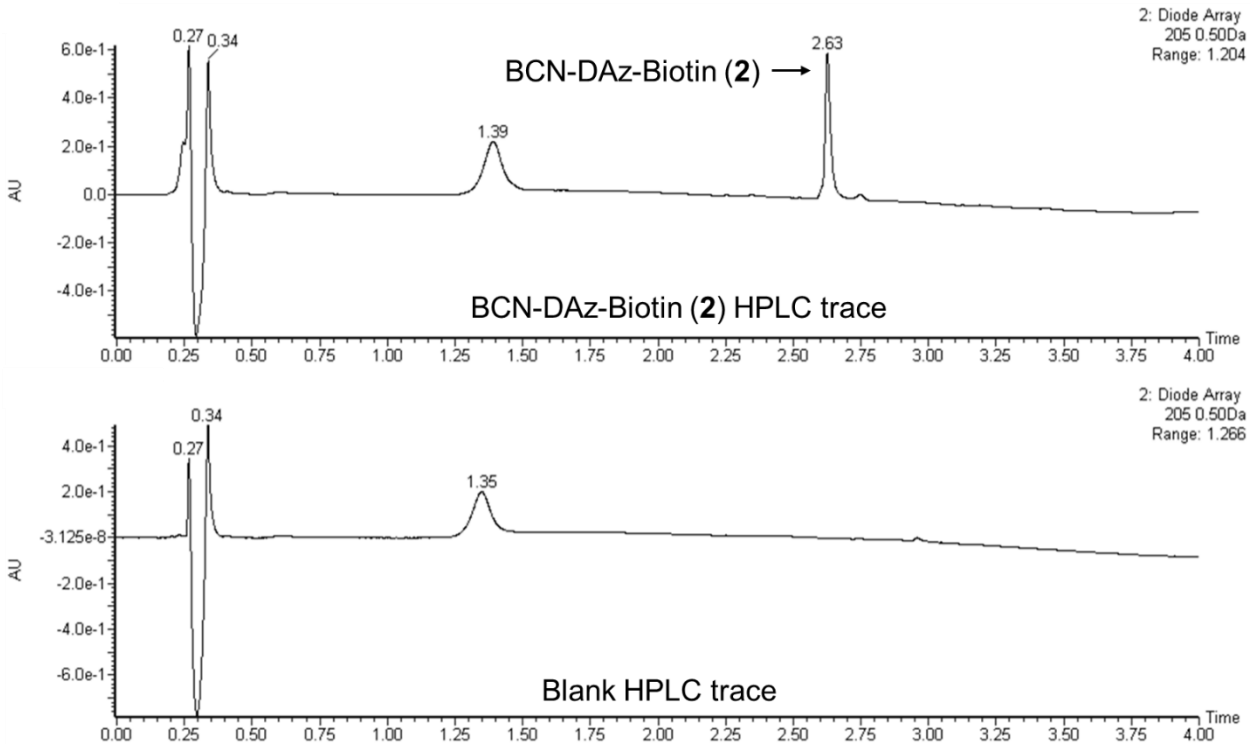


BCN-DAz-Biotin (**2**) $^{1}$H NMR
(500 MHz, $CD_3OD$)




BCN-DAz-Biotin (**2**) $^{13}$C NMR
(125 MHz, $CD_3OD$)






HPLC analysis of BCN-DAz-Biotin (**2**) was performed on a Waters Acquity UPLC equipped with a Waters Acquity UPLC BEH C18 column (2.1 x 50 mm, 1.7 μm particle size) and a photodiode array detector. The separation was run at a flow rate of 0.5 mL/min using a gradient elution of 0% solvent B (0.0 to 0.5 min) → 80% solvent B (0.5 to 3.9 min) → 0% solvent B (3.9 to 4.0 min). Solvent A, 10% acetonitrile in water with 0.1% formic acid; Solvent B, 100% acetonitrile with 0.1% formic acid. Absorbance at 205 nm is shown.