Supporting Information for:

Incorporation of Fluorine into an OBOC Peptide Library by Copper-Free Click Chemistry towards the Discovery of PET Imaging Agents

Emily Murrell[a], and Leonard G. Luyt[abc*]

a. Department of Chemistry, University of Western Ontario, 1151 Richmond Street, London, Ontario N6A 5B7, Canada
b. Departments of Oncology and Medical Imaging, University of Western Ontario, 1151 Richmond Street, London, Ontario N6A 5B7, Canada
c. London Regional Cancer Program, Lawson Health Research Institute, 790 Commissioners Road East, London, Ontario N6A 4L6, Canada

*lluyt@uwo.ca

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General Synthetic Experimental

All reactions were carried out under a nitrogen atmosphere using oven-dried glassware. Dichloromethane (DCM) was distilled over CaH_2. Other reagents and solvents were used as received from Sigma-Aldrich, Alfa Aesar, or Fisher Scientific. Amino acids and resins were received from Peptides International or Chem. Impex.. Flash chromatography was performed on a Biotage Isolera Prime automated flash purification system. Biotage SNAP KP-Sil 10 g, 25 g, or 50 g cartridges (45-60 micron) were used with flow rates of 12, 25, and 50 mL/min respectively for gradient solvent systems. Fractions were monitored and collected by UV absorbance using the internal UV detector set at 254 nm and 280 nm. NMR spectra were recorded on either an Agilent Mercury VX 400 or Bruker AvIII HD 400 MHz Spectrometer. Chemical shifts are recorded in parts per million. EI and CI HRMS was measured on a Thermo Scientific DFS (Double Focusing Sector) mass spectrometer. Peptides were purified by preparative reverse-phase HPLC-MS and analytical reverse-phase HPLC-MS was performed to assess purities. The system consists of a Waters 600 controller, Waters Prep degasser, Waters Quattro micro Mass, and Waters Mass Lynx software. The UV absorbance was detected using a Waters 2998 Photodiode array detector. A preparative column (Agilent Zorbax PrepHT SB-C18 Column 21.2 x 150 mm, 5 μm) or analytical column (Agilent Zorbax SB-C18 column 4.6 x 150 mm, 5 μm) was used. The solvent system runs gradients of 0.1 % trifluoroacetic acid (TFA) in ACN and 0.1 % TFA in water at a flow rate of 20 mL/min or 1.5 mL/min over 10 minutes with a 5 minute wash. After purification, the collected fractions were frozen at -78 °C and lyophilized.

Small Molecule Synthesis

5-[11,12-Didehydrodibenzo[*b*,*f*]azocin-5(*6H*)-yl]-4-oxobutanoic Acid (ADIBO-COOH) (1)

ADIBO-COOH **1** was prepared as described by Chadwick *et al.*[1] ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.67 (d, J = 7.5 Hz, 1H), 7.50 – 7.20 (m, 7H), 5.16 (d, J = 13.9 Hz, 1H), 3.70 (d, J = 13.9 Hz, 1H), 2.72 (ddd, J = 16.6, 8.9, 5.2 Hz, 1H), 2.60 (ddd, J = 17.0, 8.9, 5.2 Hz, 1H), 2.36 (ddd, J = 16.8, 6.4, 5.1 Hz, 1H), 1.99 (ddd, J = 16.6, 6.6, 5.2 Hz, 1H).^{13}C NMR (100 MHz, $CDCl_3$): δ 176.1, 172.6, 151.2, 147.8, 132.4, 129.3, 128.7, 128.6, 128.5, 128.0, 127.4, 125.7, 123.2, 122.8, 115.2, 107.6, 55.8, 29.7, 29.6. HRMS (EI): m/z calculated for $C_{19}H_{15}NO_3$: 305.1052; found: 305.1045.

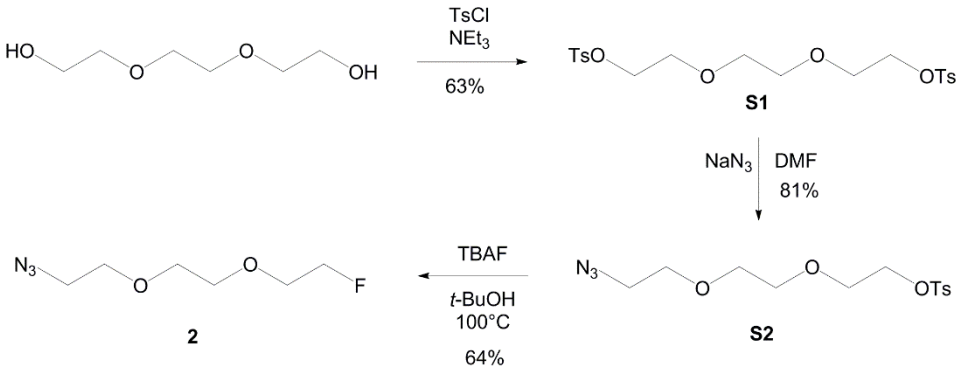

Figure S1. Scheme for synthesis of **2**.

2,2'-(ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl)bis(4-methylbenzenesulfonate) (S1)

Tosyl chloride (7.61 g, 39.9 mmol) was slowly added to a solution of triethylene glycol (2.00 g, 13.3 mmol) in triethylamine (50 mL). The reaction mixture was stirred overnight at room temperature, and the solvent was removed by rotary evaporation. The product was washed with water and extracted into EtOAc. The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The product was isolated by automated Isolera flash column chromatography (SNAP KP-Sil cartridge, 12-100% EtOAc/hexanes) yielding the ditosyl **S1** (2.77 g, 63%) as an orange solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.77 (d, J = 8.0 Hz, 4 H), 7.33 (d, J = 8.0 Hz, 4 H), 4.12 (t, J = 4.8 Hz, 4 H), 3.63 (t, J = 4.8 Hz, 4 H), 3.50 (s, 4 H), 2.42 (s, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 145.0, 133.0, 129.9, 128.0, 70.7, 69.3, 68.8, 21.7. HRMS (EI): *m*/*z* calculated for $C_{20}H_{26}O_8S_2$: 458.1069; found: 458.1079.

2-(2-(2-azidoethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (S2)

A solution of ditosyl **S1** (90.0 mg, 1.96 mmol) and sodium azide (113 mg, 1.72 mmol) in dry DMF (5 mL) was stirred at room temperature overnight. The reaction mixture was quenched with water and extracted into DCM. The organic fractions were washed with water, brine, and dried over $MgSO_4$. The combined organic layers were concentrated under reduced pressure and the product was isolated by automated Isolera flash column chromatography (SNAP KP-Sil cartridge, 17-100% EtOAc/hexanes), yielding the azide **S2** (57.0 mg, 81%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (d, J = 8 Hz, 2 H), 7.34 (d, J = 8 Hz, 2 H), 4.16 (t, J = 5 Hz, 2

H), 3.70 (t, J = 5 Hz, 2 H), 3.64 (t, J = 5 Hz, 2 H), 3.60 (s, 4 H), 3.36 (t, J = 5 Hz, 2 H), 2.44 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 144.9, 133.1, 129.9, 128.1, 70.9, 70.7, 70.2, 69.4, 68.9, 50.8, 21.8. HRMS (CI): m/z calculated for $C_{13}H_{20}N_3O_5S$ $[M+H]^+$: 330.1118; found: 330.1123.

1-azido-2-(2-(2-fluoroethoxy)ethoxy)ethane (2)

A commercially available solution of 1.0M TBAF in THF (6.94 mL, 6.94 mmol) was added to a solution of azide **S2** (1.14 g, 3.47 mmol) in *t*-BuOH (30 mL). The reaction mixture was stirred at 100 °C overnight. The reaction mixture was quenched with water and extracted into DCM. The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The product was purified by automated Isolera flash column chromatography (SNAP KP-Sil cartridge, 0-2% MeOH/DCM), yielding the product **2** (390 mg, 64%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.60 (t, J = 2.8 Hz, 1 H), 4.52 (t, J = 2.8 Hz, 1 H), 3.77 (t, J = 2.8 Hz, 1 H), 3.72 (t, J = 2.8 Hz, 1 H), 3.70-3.66 (m, 6 H), 3.38 (t, J = 3.2 Hz, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 83.3 (d, $^1J_{CF}$ = 169 Hz), 71.0, 70.9, 70.7 (d, $^2J_{CF}$ = 19.6 Hz), 70.3, 50.8. HRMS (CI): m/z calculated for $C_6H_{12}FN_3O_2$ $[M+H]^+$: 178.0986; found: 178.0990.

F-PEG$_2$-ADIBO-COOH (3)

ADIBO-COOH **1** (0.158 g, 0.52 mmol) and **2** (0.115 g, 0.65 mmol) were dissolved in 5 mL of a 3:2 EtOH:H_2O mixture. The solution was shaken for two hours, diluted with water, frozen, and lyophilized. The product was purified as a combination of its two inseparable regioisomers by automated Isolera flash column chromatography (SNAP KP-Sil cartridge, 2-15% MeOH/DCM) to yield **3** as a yellow oily solid (0.200 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 – 7.07 (m, 8H), 6.12 (d, J = 16.7 Hz, 1H), 4.73-4.64 (m, 1H), 4.60-4.51 (m, 2H), 4.50 – 4.43 (m, 1H), 4.43 (d, J = 16.6 Hz, 1H), 4.02-3.91 (m, 2H), 3.79 – 3.69 (m, 2H), 3.69 – 3.55 (m, 4H) 2.58 – 2.26 (m, 2H), 2.0 (m, 1H), 1.99 – 1.62 (m, 1H). HRMS (EI): m/z calculated for $C_{25}H_{27}FN_4O_5$: 482.1965; found: 482.1972.

OBOC Library Synthesis

The library was synthesized using standard Fmoc solid phase peptide synthesis (SPPS) conditions on a Biotage SyroWave automated peptide synthesizer using a split-and-mix technique

in the dark. Briefly, 1 g of Tentagel S-NH_2 resin (90 μm, 0.29 meq/g) was swelled in DCM and manually coupled to Fmoc-ANP-OH linker (4 eq) using HCTU (4 eq) and *N*-*N*-diisopropylethylamine (DIPEA) (8 eq) in DMF for two hours. The resin was split into seventeen vessels. Fmoc removal was achieved using 40% piperidine in dimethylformamide (DMF) over two steps (5 min and 15 min). Each well was coupled to a different Fmoc-amino acid (4 eq) using HCTU (4 eq) and DIPEA (8 eq) in DMF for one hour. After each Fmoc-deprotection/coupling cycle, the resin was recombined into one pool and then divided into seventeen vessels again. This was repeated eight times to achieve peptides eight amino acids in length. To the combined library, F-PEG_2-ADIBO-COOH **3** (1.6 eq) was coupled using HATU (1.6 eq) and DIPEA (3.2 eq) in DMF for 5 hours. Full deprotection was done with 95% TFA, 2.5% triisopropylsilane (TIPS) and 2.5 % water for 5 hours. The library was rinsed thoroughly with DMF, DCM, MeOH, and stored in 70% EtOH/H_2O.

Hit Peptide Synthesis

Peptides were synthesized using standard Fmoc SPPS conditions on the Biotage SyroWave automated peptide synthesizer. Fmoc-protected Rink amide MBHA or Tentagel S- NH_2 resin was used as a solid support after swelling in DCM. Fmoc removal was achieved using 40% piperidine in dimethylformamide (DMF) over two steps (30 sec and 12 min). Amino acids were coupled with the Fmoc-protected amino acid (4 eq), HCTU (4 eq) and DIPEA (8 eq) in DMF/*N*-methyl pyrrolidinone (NMP**)** over 1 hour. The acid **3** was manually coupled to the N-terminus using 1 equivalent of **3**, 1 equivalent of HATU and 2 equivalents of DIPEA in DMF.

Tentagel peptides were deprotected using a solution of with 95% TFA, 2.5% TIPS and 2.5% water for 4 hours. Beads were rinsed thoroughly with DMF, DCM, MeOH, H_2O and stored in 70% EtOH/H_2O.

Full cleavage of the Rink-amide peptides from resin along with removal of side chain protecting groups was achieved with a solution of 95% TFA, 2.5% TIPS and 2.5% water over 5 h. The cleaved peptides were precipitated in cold *tert*-butyl methyl ether (TBME) and centrifuged at 3000 rpm for 15 min. The TBME was decanted, the peptide dissolved in water, frozen at -78 °C and lyophilized. The peptides were then purified by preparative reverse-phase HPLC-MS over

gradients of acetonitrile (0.1%TFA) in water (0.1 % TFA). The peptides were isolated in >98 % purity by analytical RP-HPLC. Peptides are a combination of the two inseparable regioisomers.

Table S1. Sample list of select peptide sequences resynthesized for *in vitro* validation. All are coupled to **3** on the N-terminal and are amidated on the C-terminal

Amino Acid Sequence	$[M+H]^+$ *m/z* theoretical	$[M+H]^+$ *m/z* found
-YKFKRLWP-	1600.86	1600.83
-HLTKYWET-	1540.74	1540.55
-HLTQYWET-	1540.70	1540.50
-AKEDNFRN-	1456.68	1456.54
-AQEDNFRN-	1456.64	1456.47
-NDEDEEYA-	1447.54	1447.39
-RLDSHDPK-	1430.70	1430.56
-RLDSHDPQ-	1430.66	1430.51
-NFKEFDHA-	1452.69	1452.53
-NFQEFPHA-	1452.65	1452.45
-FLFWGPAG-	1357.65	1357.52

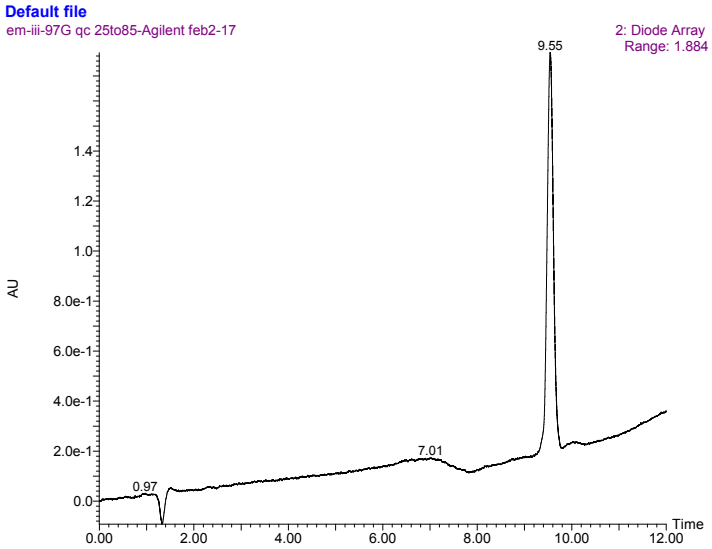

Figure S2. Sample HPLC chromatogram (UV trace, 25-85% ACN/H_2O with 0.1% TFA) of a purified peptide F-PEG_2-ADIBO-YKFKRLWP-NH_2.

Cell Culture

U87.CD4 and U87.CD4.CXCR4 cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Hong Kui Deng and Dr. Dan R. Littman. U87.CD4.CXCR4 cells were maintained in DMEM – high glucose (Sigma) containing 15% fetal bovine serum (FBS), 1 μg mL^{-1} puromycin, 300 μg mL^{-1} G418, and 1× penicillin–streptomycin. U87.CD4 cells were maintained in DMEM – high glucose (Sigma) containing 10% FBS, 300 μg mL^{-1} G418, and 1× penicillin–streptomycin. All cell lines were cultured at 37 °C in humidified atmosphere with 5% CO_2 and passaged 2 to 3 times per week.

Library Screening

U87.CD4.CXCR4 cells at 80% confluency were incubated with 5 μM CellTracker™ Green CMFDA Dye (ThermoFisher) in serum-free DMEM media for 30 min at 37 °C. Dye was aspirated, and cells were lifted using an enzyme-free dissociation solution (Sigma). Cells were counted and resuspended in serum-free DMEM at 500,000 cells/mL. Library beads were suspended in phosphate-buffered saline (PBS) and dispersed in 6-well plates at ~20,000 beads/well (entire library is ~1.5 million beads). Cells were added to each well (1 mL, 500,000 cells) and total volumes were brought up to 3 mL with DMEM. Plates were shaken at 550 rpm for 1 hour at 37 °C. To each well, 0.33 mL of 37% paraformaldehyde was added for a final concentration of 4%. Fixing was quenched by the addition of 2 M glycine to a final concentration of 20%. The entire library of beads was recombined and rinsed with PBS repeatedly then suspended in PBS. Aliquots of this solution were added to the sample cup of a COPAS instrument (Union Biometrica) and diluted into the appropriate sheath reagent. Beads were sorted at ~20 events/s and excited by a 488 nm lamp to select for beads (via length (TOF) and optical density (Ext)) as well as to sort for fluorescence (green λ_{em} = 510±23 nm) (Figure S3). Beads were collected in bulk (~2500 events), rinsed, and then re-added to the sample cup and sorted with a higher set threshold of fluorescence for sorting (Figure S3). In this sort, hit beads were deposited into individual wells of a 96 well plate (~200 events). Wells were imaged by confocal fluorescent microscopy to remove any remaining false positive beads. Confocal fluorescence microscopy was performed on a Nikon A1R

Confocal Laser Microscope with a 488 nm laser for excitation and the emission range set to 500-550 nm.

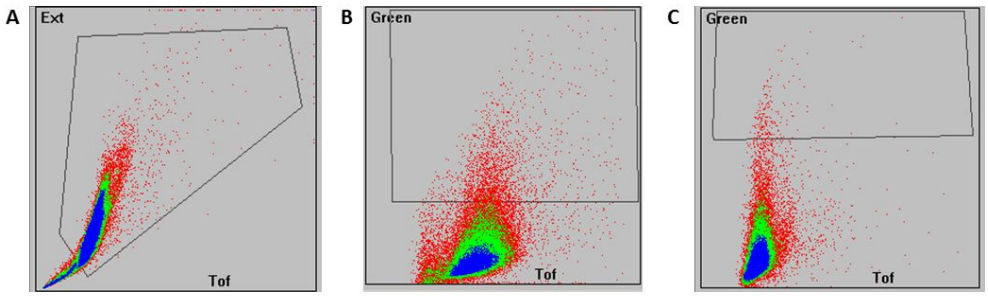

Figure S3. Screenshots of COPAS software showing A) gating selection for bead-sized objects based on size (TOF) and optical density (Ext); B) sorting selection for initial screen based on bead size and above-average green fluorescence (λ_{em}=510±23 nm); C) sorting selection for second round with higher threshold of green fluorescence.

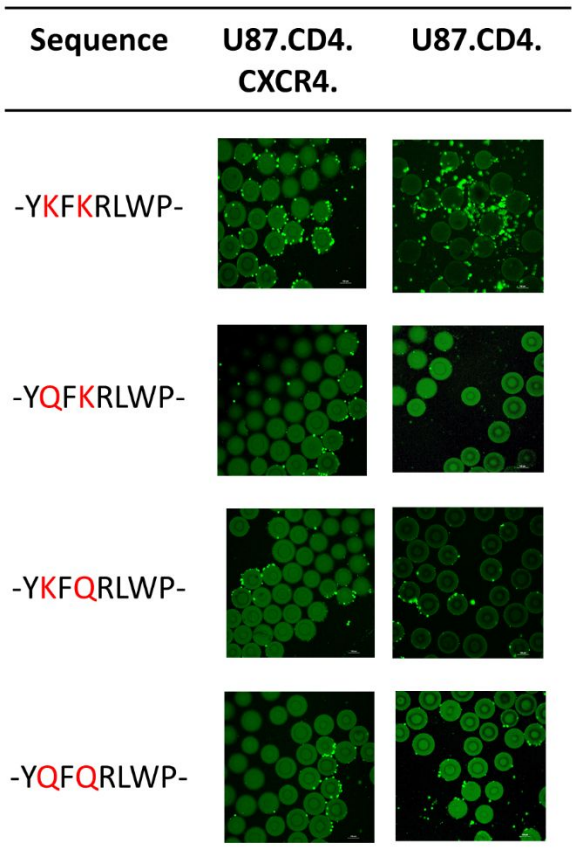

Figure S4. Uncropped images of Figure 4.

MALDI MS/MS

Hit beads from the library screen were suspended in 50 µL of water in individual wells of 96-well plates and placed under UV light (365 nm) for 2 hours to achieve cleavage from the bead due to the ANP linker. The aqueous solution was mixed with matrix α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile and placed onto the MALDI target. MALDI TOF/TOF was performed on an AB Sciex TOF/TOF 5800 by the facility manager of the MALDI Mass Spectrometry Facility at the London Regional Proteomics Centre.

Selectivity Analysis Assay

U87.CD4.CXCR4 or U87.CD4 cells at 80 % confluency were incubated with 5μM CellTracker™ Green CMFDA Dye (ThermoFisher) in serum-free DMEM media for 30 min at 37 °C. Dye was aspirated, and cells were lifted using an enzyme-free dissociation solution (Sigma). Cells were counted and resuspended in serum-free DMEM at 150,000 cells/mL. Tentagel beads expressing re-synthesized potential hit sequences were suspended in phosphate-buffered saline (PBS) and dispersed in 12-well plates at ~10,000 beads/well. Cells were added to each well (1 mL, 150,000 cells) and total volumes were brought up to 2 mL with DMEM. Plates were shaken at 550 rpm for 1 hour at 37 °C. Wells were directly imaged using confocal fluorescent microscopy (Nikon).

Competitive binding assays

In vitro CXCR4 affinity of final hit peptides and the lead peptide lacking any imaging moiety were determined through competitive binding assays using U87.CD4.CXCR4 cells with [^{125}I]-SDF-1 as the radioligand (Perkin Elmer). The compound of interest (30 μL, at concentrations ranging from 10^{-10} to 10^{-4} M) and 20,000 cpm of [^{125}I]-SDF-1 (20 pM) were mixed with the binding buffer (20 mM HEPES, 0.5% BSA in PBS, pH 7) in 1.5 mL Eppendorf Protein LoBind vials. A suspension of U87.CD4.CXCR4 cells (50,000 cells in 50 μL) was added to each vial to give a final volume of 300 μL. The vials were shaken at 550 rpm for 20 minutes at 37 °C. Immediately after the incubation, the vials were centrifuged at 13,000 rpm for 5 minutes and the supernatant removed. The cell pellet was washed with 500 μL of 50 mM Tris buffer (pH 7) and centrifuged again. The amount of [^{125}I]-SDF-1 bound to the cells was measured using a gamma counter (PerkinElmer). IC_{50} values were determined by non-linear regression analysis to fit a 4-parameter dose response curve using GraphPad Prism (Version 6.0c). All data points were obtained in triplicate.

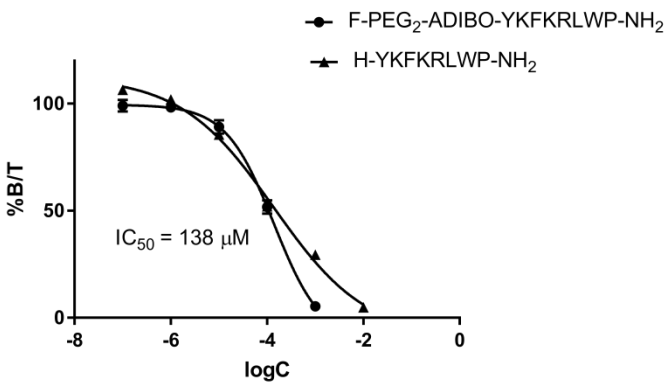

Figure S5. Competition binding curves for the lead compound imaging agent and its unmodified peptide sequence.

References

1. Chadwick, R. C.; Van Gyzen, S.; Liogier, S.; Adronov, A. Scalable Synthesis of Strained Cyclooctyne Derivatives. *Synthesis* **2014**, *46* (5), 669–677.