Supporting Information

Diels-Alder Cycloaddition for Fluorophore Targeting to Specific Proteins inside Living Cells

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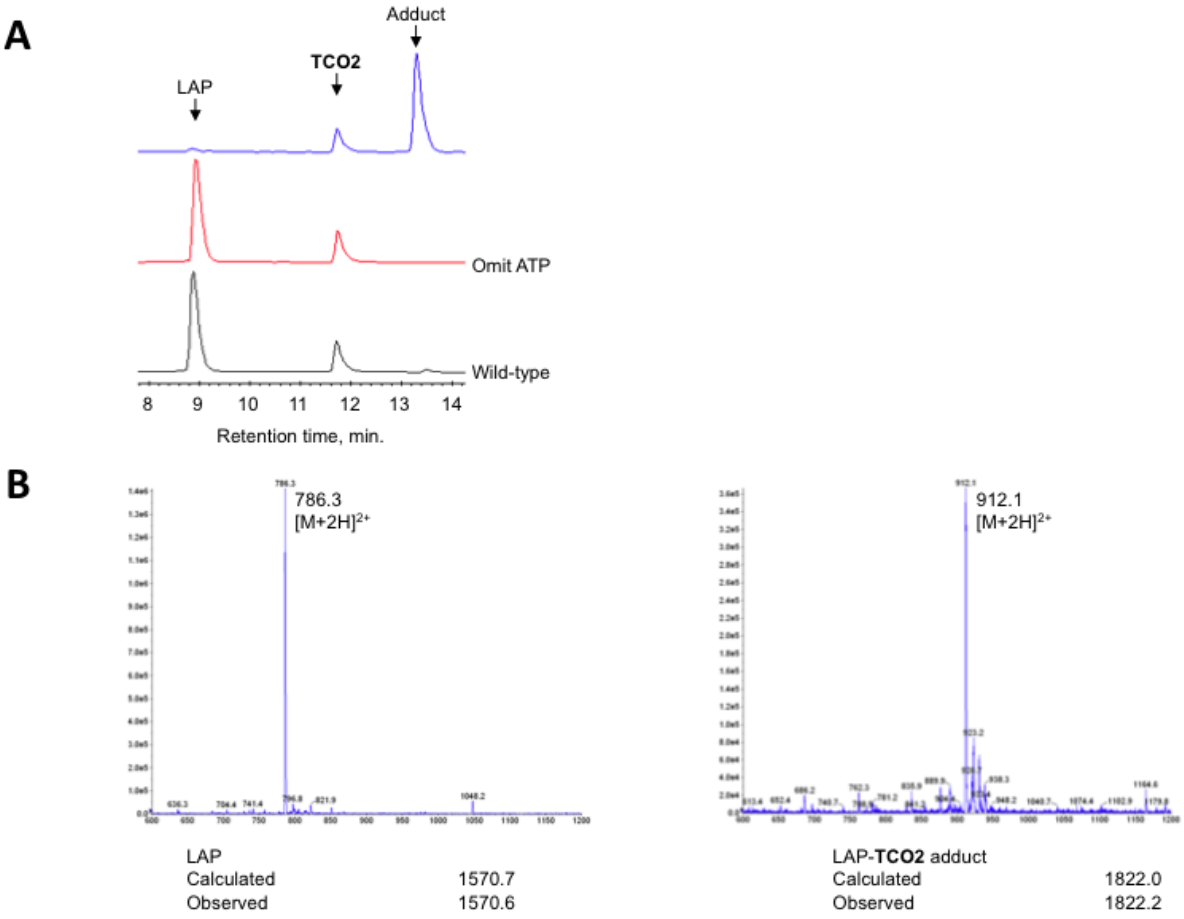
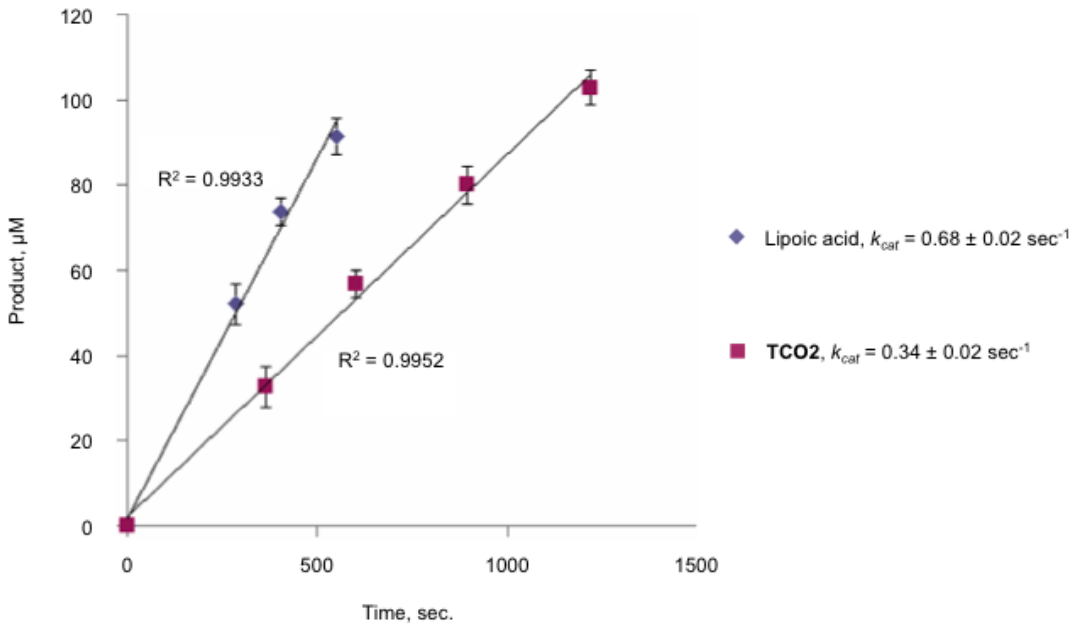
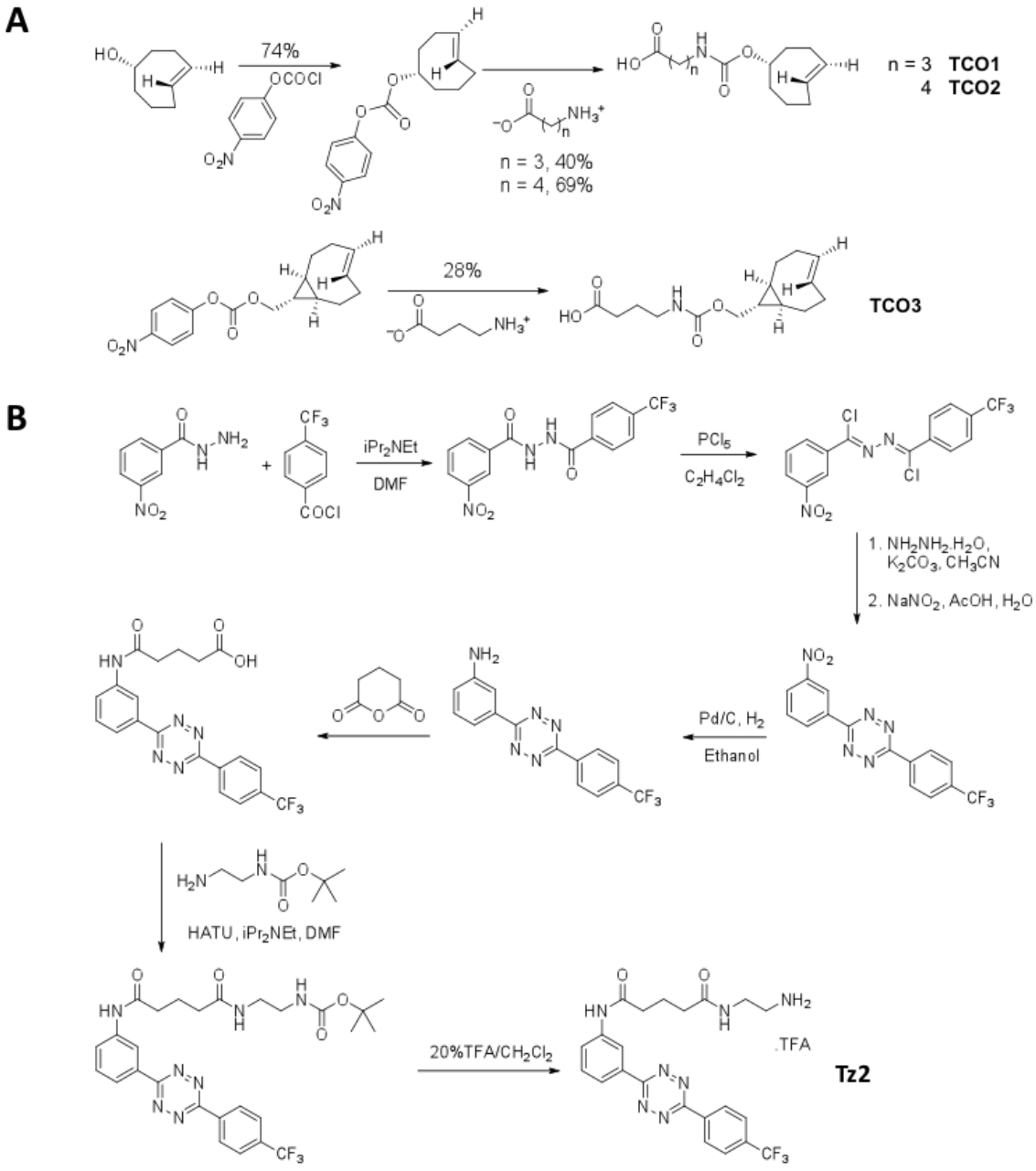
Supporting Figures



Supporting Figure 1 Analysis of LplA-mediated ligation of TCO2 onto LplA acceptor peptide (LAP) *in vitro.* (A) HPLC trace showing conversion of LAP to LAP-**TCO2** adduct by ^{W37V}LplA. No conversion was observed when adenosine triphosphate (ATP) was omitted or when wild-type ligase was used instead. **(B)** LAP and LAP-**TCO2** adduct annotated in (A) were confirmed by ESI mass spectrometry.

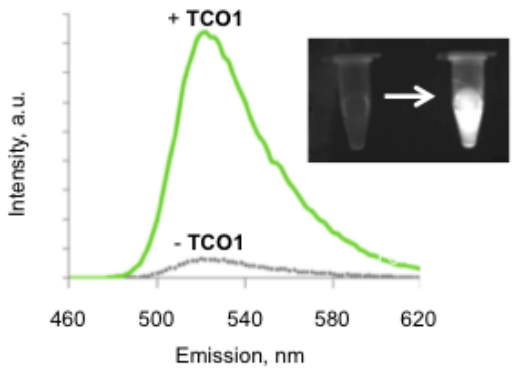


Supporting Figure 2 Estimation of Michaelis-Menten k_{cat} for TCO2 ligation onto LAP by ^{W37V}LplA *in vitro*. Ligation reactions were assembled with 500 µM **TCO2**, 500 µM LAP, and 250 nM ^{W37V}LplA. The amount of LAP-**TCO2** adduct formed at initial stages of the reaction was measured by HPLC. Assuming that the substrates are provided at concentrations greater than K_M, the k_{cat} for **TCO2** ligation is estimated to be 0.34 ± 0.02 s^{-1}. For comparison, the k_{cat} for lipoic acid ligation onto LAP, catalyzed by the same enzyme, is 0.68 ± 0.02 s^{-1}. This is ~ 3-fold higher than previously reported values for lipoic acid ligation by wild-type ligase[1], perhaps due to improvements in enzyme storage and assaying conditions (see Methods).



Suppporting Figure 3 Synthesis of *trans*-cyclooctenes and Tz2. (**A**) Syntheses of *trans*-cyclooctene substrates for LplA. (**B**) Synthesis of **Tz2**. Details and characterization under Specific Methods. DMF, dimethylformamide; HATU, (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate); TFA, trifluoroacetic acid.

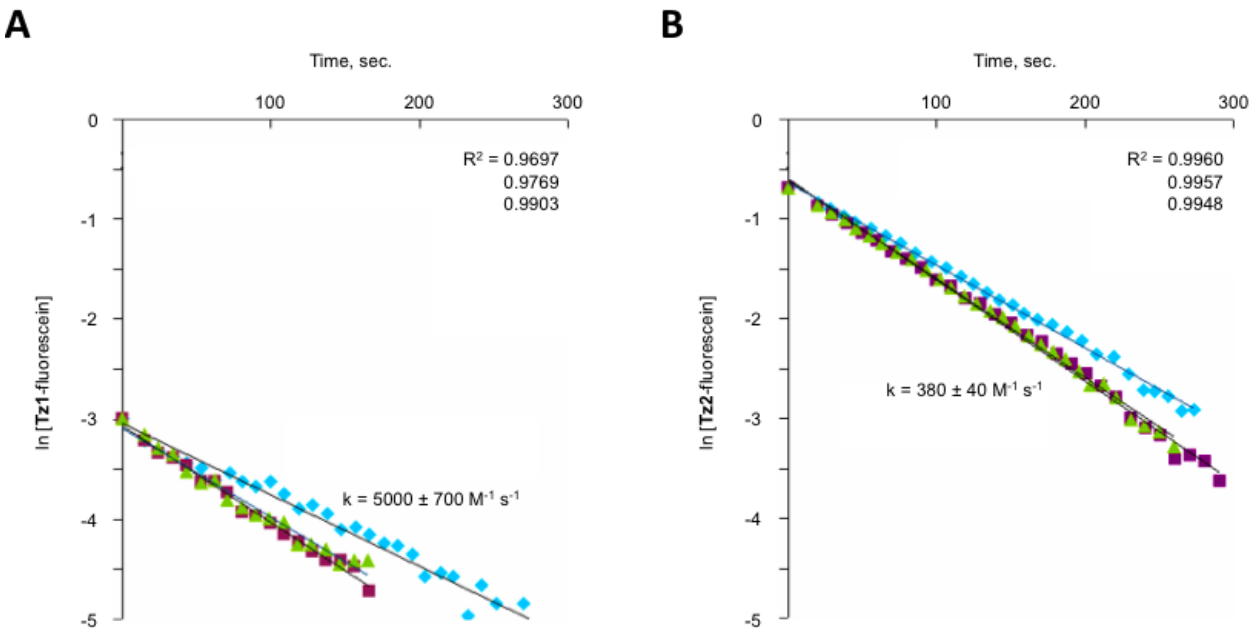
A



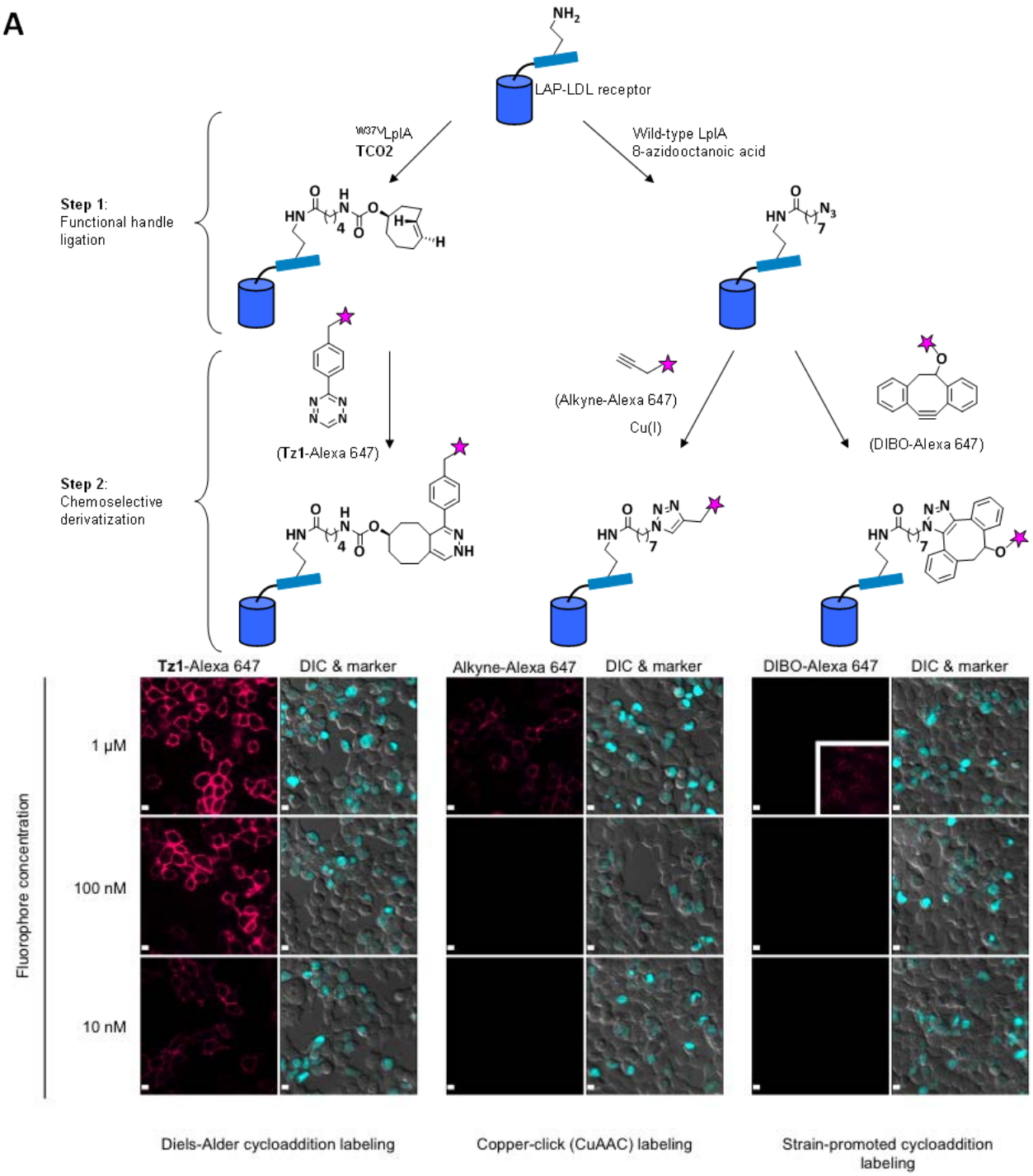
B

Probe	Fluorescence increase (fold)
Tz1-fluorescein	13.4
Tz2-fluorescein	16.7
Tz1-TMR	1.5
Tz1-Alexa 647	1.07

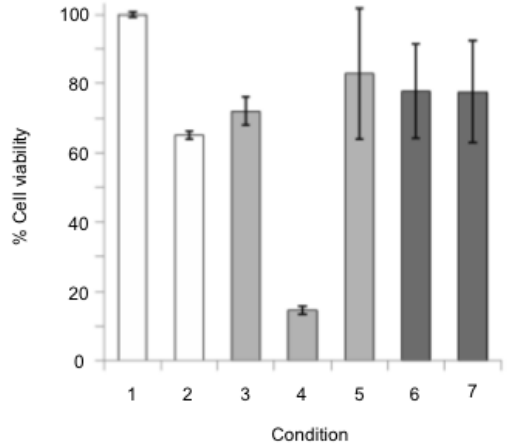
Supporting Figure 4 Fluorogenic response of tetrazine-fluorophore conjugates upon Diels-Alder cycloaddition with *trans*-cyclooctene. (A) Fluorescence emission of **Tz1**-fluorescein before (grey) and after (green) treatment with excess *trans*-cyclooctene **TCO1**. Inset: the same reaction visualized by camera with 354 nm excitation. **(B)** Fluorogenic responses of various tetrazine-fluorophore conjugates upon treatment with excess **TCO1**.



Supporting Figure 5 Measurement of second-order rate constants (*k*) for Diels-Alder cycloaddition between LAP-TCO2 and two tetrazine-fluorescein conjugates. (A) Measurement for **Tz1**-fluorescein. Product formation was measured as fluorescence output. Rate constant was calculated from triplicate runs, plotted here on a logarithmic scale and shown in three different colors. **(B)** The same measurement for **Tz2**-fluorescein. Rate constants are quoted to 2 significant figures.

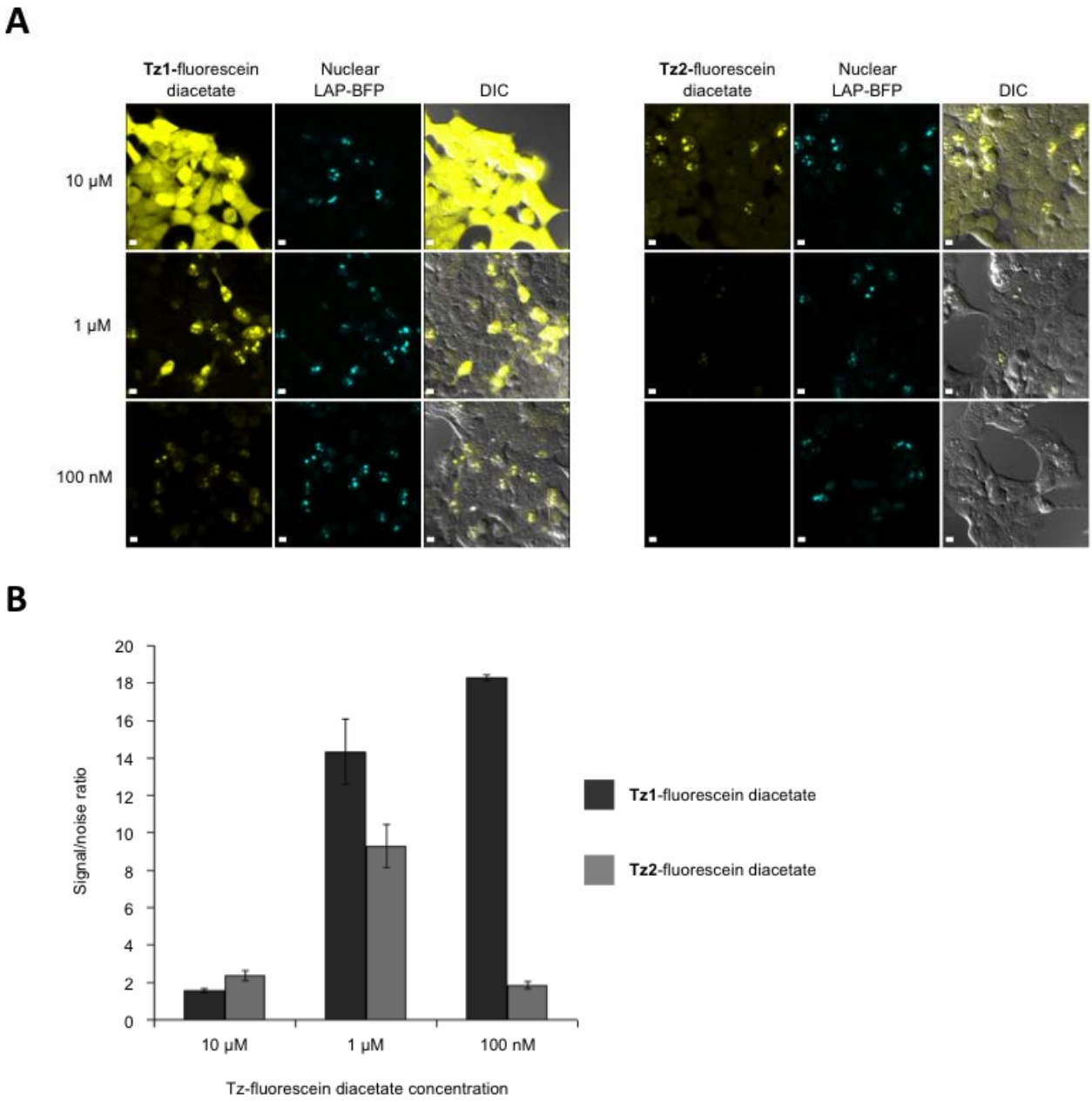

A
NH2
LAP-LDL receptor
W37V LplA
TCO2
Wild-type LplA
8-azidooctanoic acid
Step 1:
Functional handle
ligation
(Tz1-Alexa 647)
(Alkyne-Alexa 647)
Cu(I)
(DIBO-Alexa 647)
Step 2:
Chemoselective
derivatization
Tz1-Alexa 647
DIC & marker
Alkyne-Alexa 647
DIC & marker
DIBO-Alexa 647
DIC & marker
Fluorophore concentration
1 μM
100 nM
10 nM
Diels-Alder cycloaddition labeling
Copper-click (CuAAC) labeling
Strain-promoted cycloaddition
labeling

B

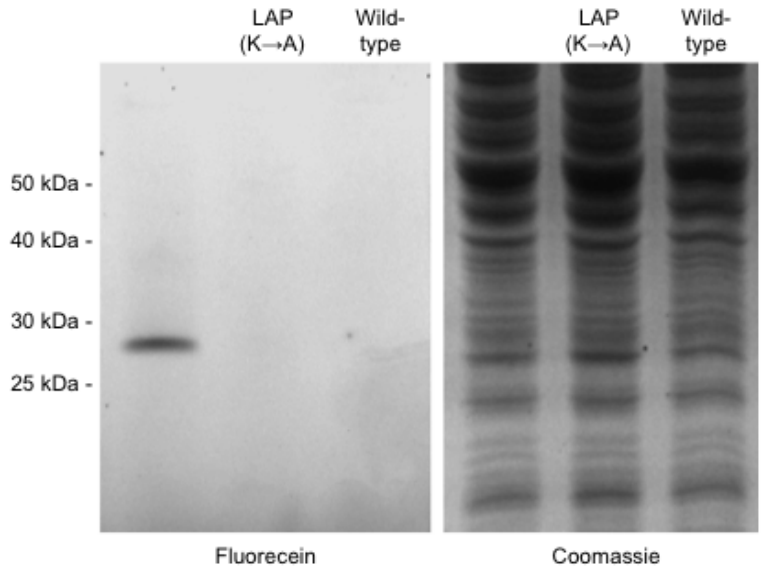


Condition	Transfection	CuAAC	Diels-Alder cycloaddtion
1	No	--	--
2	Yes	--	--
3	Yes	DMSO only	--
4	Yes	TBTA	--
5	Yes	THPTA	--
6	Yes	--	DMSO only
7	Yes	--	**TCO2** + **Tz1**-fluorescein

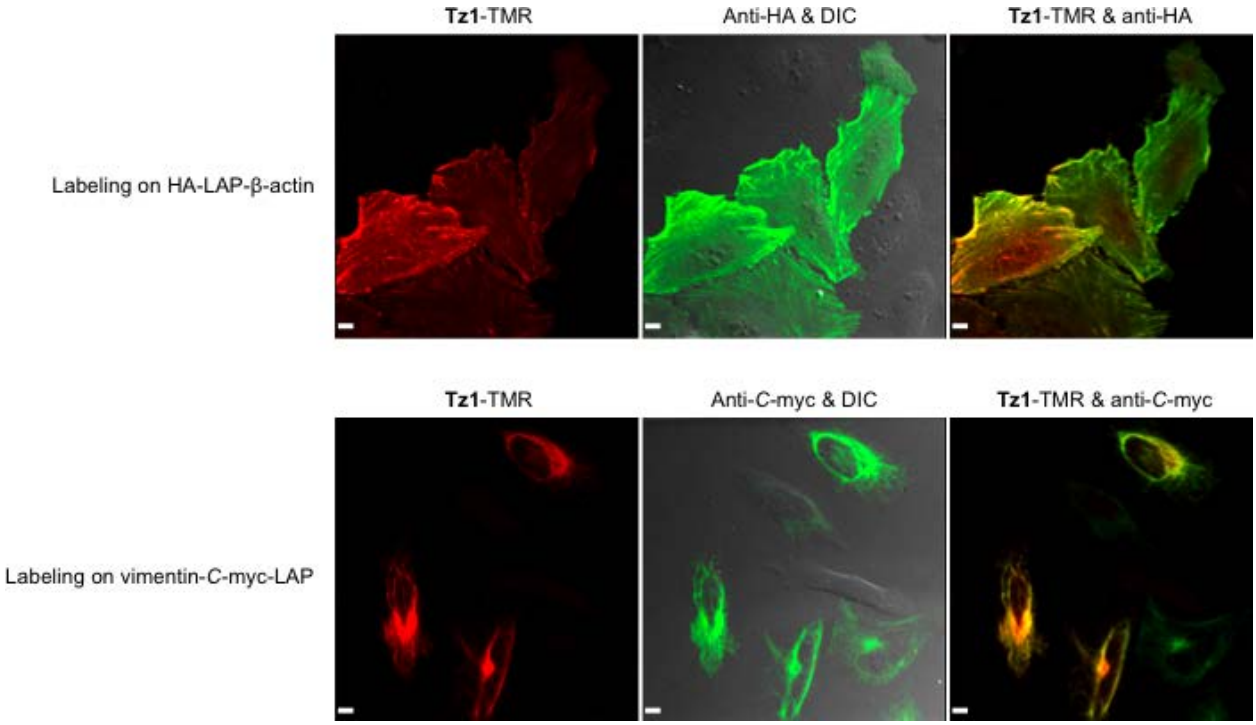
Supporting Figure 6 Comparison of Diels-Alder tetrazine-*trans*-cyclooctene cycloaddition, copper-catalyzed azide-alkyne cycloaddition (CuAAC), and strain-promoted azide-alkyne cycloaddition for cell surface fluorescence labeling. (A) HEK cells expressing LAP-LDL receptor and a nuclear cyan fluorescent protein transfection marker (shown in cyan, overlaid with DIC) were labeled in two steps, using three methodologies, as indicated by the scheme: Diels-Alder cycloaddition (left), CuAAC (middle), and strain-promoted cycloaddition (right)[2]. For the latter two, LAP was first derivatized with 8-azidooctanoic acid under conditions known to give quantitative yield (data not shown). DIBO is dibenzocyclooctyne. In all three cases, the second step was performed for 3 min., using the indicated Alexa 647 conjugates at the three indicated concentrations. Cells were imaged live after brief rinsing. Specific fluorescence staining with 1 µM DIBO-Alexa 647 was detectable (shown with enhanced contrast in inset). **(B)** Comparing cell viability after cell surface fluorescence labeling. Chinese hamster ovary cells expressing LAP-LDL receptor were labeled using Diels-Alder cycloaddition or CuAAC under the indicated conditions. Cell viability was then measured in triplicate, with untransfected and untreated cells defined as 100% viable. The tris(benzyltriazolylmethyl)amine (TBTA) ligand[3] was used at 100 µM. The tris(hydroxypropyltriazolyl)methylamine (THPTA) ligand[4] was used at 250 µM. Error bars, 2 s.d.



Suppporting Figure 7 Comparison of Tz1 and Tz2 for live cell fluorescence labeling. (A) Live cell labeling of intracellular proteins. Human embryonic kidney 293T cells expressing ^{W37V}LplA and a nuclear LAP-blue fluorescent protein construct (nuclear LAP-BFP, colored cyan) were treated with **TCO2** followed by the indicated concentrations of either **Tz1**- (left) or **Tz2**-fluorescein diacetate (right) for 5 minutes. Cells were then imaged live after dye washout for 1 hour. Scale bars, 10 µm. **(B)** Signal-to-noise ratio analysis of cells imaged in (A), measured from 24 cells under each condition. Noise is defined as fluorescence signal in BFP-negative cellular regions. Error bars, 2 SEM.



Suppporting Figure 8 Determination of Tz1-fluorescein diacetate labeling specificity by polyacrylamide gel electrophoresis and fluorescein in-gel fluorescence imaging. Human embryonic kidney 293T cells were transfected and labeled with **Tz1**-fluorescein diacetate exactly as in Figure 3B, except that the dye washout time was extended to 4 hours at 37 °C. Cells were then lysed, and the clarified lysate resolved on an SDS-polyacrylamide gel (12%). *Left:* Imaging of fluorescein in-gel fluorescence. Negative controls with a Lys→Ala mutation on LAP or with wild-type ligase are also shown. *Right:* The same gel was subsequently stained with Coomassie and re-imaged under white light.



Suppporting Figure 9 Visualization of actin and vimentin filaments by Tz1-TMR labeling and immunofluorescence staining. HeLa cells were transfected with LAP-β-actin (top) or vimentin-LAP (bottom) and labeled with **Tz1**-TMR exactly as in Figure 3C. After dye washout, cells were fixed with formaldehyde and permeabilized with methanol, then stained with anti-HA antibody (top) or anti-*C*-myc antibody (bottom). Subsequent staining with a secondary antibody Alexa Fluor 647 conjugate (colored green, and overlaid with DIC in the second panel) showed good colocalization with **Tz1**-TMR labeling (colored red, first panel). Scale bars, 10 μm.

General Methods

Synthesis and characterization of synthetic compounds

Unless otherwise stated, all reagents and solvents were purchased from commercial sources (Sigma-Aldrich, Acros Organics, Alfa Aesar, or TCI America) and used without further purification. Reactions were monitored using analytical thin-layer chromatography (0.25 mm silica gel 60 F_{254} plates, EMD Biochemicals). Desired products were purified on either flash column chromatography with normal phase silica gel or Varian Prostar preparatory reverse phase HPLC with a C-18 column (Varian Microsorb 300-5 C18 Dynamax). Synthetic products were characterized by electro-spray ionization mass spectrometry (Applied Biosystems 200 QTRAP) and by NMR (Bruker DRX-400).

Mammalian cell culture and transfection

Human embryonic kidney 293T (HEK), COS-7, and Chinese hamster ovary (CHO) cells were cultured as a monolayer in growth media: minimal essential medium (MEM, Mediatech) supplemented with 10% (v/v) fetal bovine serum (PAA Laboratories) at 37°C and under 5% CO_2. HEK and COS-7 cells for imaging were grown on 150 µm thickness glass cover slips pre-treated with 50 µg/ml fibronectin (Millipore). CHO cells for the cell viability assay were grown in plastic 96-well plates (Greiner Bio One). Cells were typically transfected at ~70% confluence using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions, then labeled 16 – 20 hours after transfection.

For hippocampal neuron cultures, Spague Dawley rat pups were sacrificed at embryonic day 18. Hippocampal tissue was digested with papain (Worthington) and DNaseI (Roche) and plated in MEM + L-glutamine (Sigma) supplemented with 10% (v/v) fetal bovine serum (PAA Laboratories) and B27 (Life Technologies) on glass cover slips pretreated with poly-D-lysine (Sigma) and mouse laminin (Life Technologies). At 3 days *in vitro*, half of the growth medium was replaced with Neurobasal (Life Technologies) supplemented with B27 and GlutaMAX (Life Technologies). Neuron transfection was performed at 5 days *in vitro*, using Lipofectamine 2000, using half the amount of the manufacturer's recommended reagent quantity. Cells were labeled and imaged at 12 days *in vitro*.

Genetic constructs

Constructs used in this study are summarized below with important features listed. Complete nucleotide sequences of all constructs can be found at: http://stellar.mit.edu/S/project/tinglabreagents/index.html

Name	Features	Notes
LplA in pYFJ16, for E. coli expression[5]	His_6-LplA	Trp37 mutants generated by QuikChange as previously reported
^{W37V}LplA in pcDNA3, for mammalian expression[6]	His_6-FLAG-LplA	FLAG = DYKDDDDK
LAP-LDL receptor in pcDNA4/TO	SS-LAP-HA-LDL receptor	SS = signal sequence LAP = GFEIDKVWHDFPA (modified Lys underlined) HA = YPYDVPDYA
LAP-neuroligin-1 in pCAG	SS-LAP-neuroligin-1	SS = signal sequence LAP = GFEIDKVWYDLDA
Nuclear LAP-BFP in pcDNA3	His_6-LAP-BFP-NLS	LAP = GFEIDKVWYDLDA Lys→Ala mutation in LAP prepared by QuikChange NLS = nuclear localization signal from Kalderon *et al.*[7]
LAP-β-actin	HA-LAP-β-actin	LAP = GFEIDKVWYDLDA HA = YPYDVPDYA
Vimentin-LAP	Vimentin-*C*-myc-LAP	*C*-myc = EQKLISEEDL LAP = GFEIDKVWYDLDA

Fluorescence microscopy

Cells placed in Tyrode's buffer or Dulbecco's phosphate buffered saline were imaged using a Zeiss AxioObserver.Z1 inverted confocal microscope with a 40X or 63X oil-immersion objective. The spinning disk confocal head was manufactured by Yokogawa. The following excitation sources and filter sets were used:

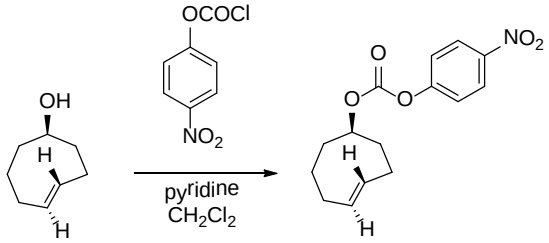
Fluorophore	Laser excitation (nm)	Emission (nm)	Dichroic (nm)
BFP	405	438/30	450
Fluorescein/GFP	491	525/30	502
Tetramethylrhodamine	561	605/20	585
Alexa Fluor 647	647	680/30	660

Images were acquired and processed using SlideBook software version 5.0 (Intelligent Imaging Innovations).

Specific Methods

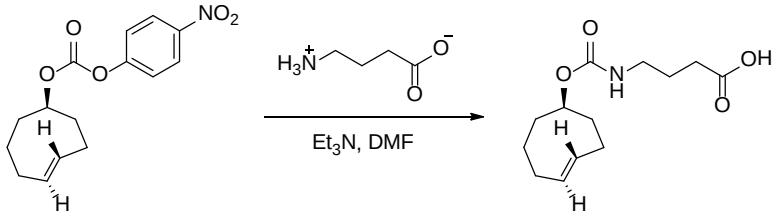
Synthesis of *trans*-cyclooctene probes

rel-(1R-4E-pR)-cyclooct-4-ene-1-yl (4-nitrophenyl) carbonate



The title compound was synthesized using an adaptation of our previously reported protocol[8]. To a stirring solution of *rel*-(1*R*-4*E*-p*R*)-cyclooct-4-enol[9] (0.732 g, 5.79 mmol) in anhydrous methylene chloride (100 mL) was added pyridine (1.20 mL, 14.5 mmol). A solution of 4-nitrophenylchloroformate (1.286 g, 6.38 mmol) in methylene chloride (20 mL) was added at room temperature and the resulting solution allowed to stir for 30 minutes. To the reaction was added NH_4Cl (aq), and the layers were separated. The aqueous layer was extracted twice with methylene chloride. The organic layers were combined, dried with $MgSO_4$, filtered, and concentrated onto silica gel using a rotary evaporator. Purification by column chromatography (5% ethyl acetate/hexanes) yielded 1.25 g (74%) of the title compound as a pale yellow solid. mp 74-75 °C. ^{1}H NMR (400 MHz, C_6D_6, δ): 7.66 (app d, *J* = 9.7 Hz, 2H), 6.74 (app d, *J* = 9.7 Hz, 2H), 5.29-5.12 (m, 2H), 4.40-4.35 (m, 1H), 2.13-1.98 (m, 4H), 1.86-1.73 (m, 2H), 1.71-1.57 (m, 3H), 1.40-1.31 (m, 1H). ^{13}C-NMR (100 MHz, C_6D_6, δ): 155.3 (u), 152.0 (u), 145.1 (u), 134.5 (dn), 132.7 (dn), 124.8 (dn), 121.2 (dn), 85.7 (dn), 40.5 (u), 38.2 (u), 33.9 (u), 32.2 (u), 30.9 (u). IR ($CHCl_3$, cm^{-1}): 3105, 3007, 2928, 2859, 1756, 1594, 1526, 1348 1261 1219, 993. Elem. Anal. Calcd: 61.85 C, 4.81 N, 5.88 H. Found: 61.99 C, 4.74 N, 5.94 H.

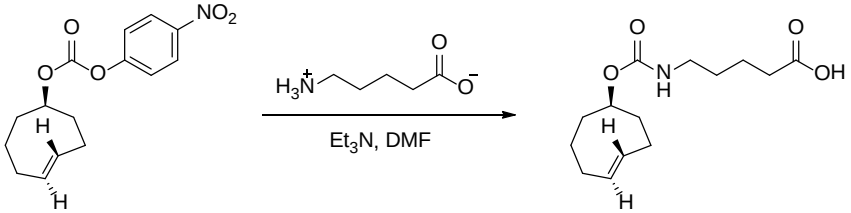
*rel-(1R-4E-pR)-cyclooct-4-ene-1-yl-N-butyric acid carbamate (**TCO1**)*



A round bottomed flask was charged with *rel*-(1*R*-4*E*-p*R*)-cyclooct-4-ene-1-yl (4-nitrophenyl) carbonate (30.0 mg, 0.103 mmol). The flask was evacuated and refilled with N_2. Anhydrous dimethylformamide (0.5 mL) was added, followed by triethylamine (44 µL, 0.31 mmol). 4-Aminobutyric acid (15.8 mg, 0.153 mmol) was added in a single portion. The flask was wrapped in foil and the reaction was allowed to stir for 22 h at room temperature. The reaction solution was diluted with water, and extracted three times with ethyl acetate. The aqueous layer was

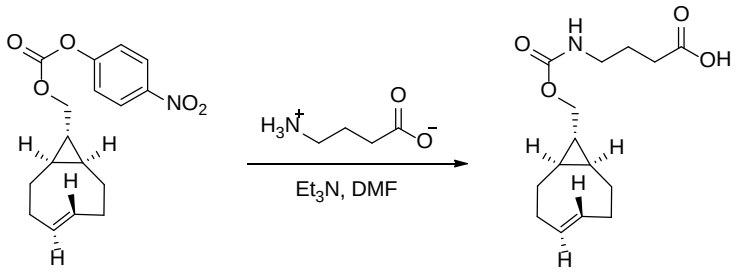
then acidified with 6% aq. acetic acid, and extracted three times with methylene chloride. The organic layers were combined and washed twice with water. The organic layer was dried with $MgSO_4$, filtered, and concentrated onto silica gel using a rotary evaporator. Purification by column chromatography (0-3% methanol/methylene chloride) yielded 9.9 mg (40%) of **TCO1** as a colorless oil. ^{1}H-NMR (400 MHz, CD_3OD): 5.62-5.54 (m, 1H), 5.50-5.42 (m, 1H), 4.35-4.14 (m, 1H), 3.09 (t, *J*= 6.9 Hz, 2H), 2.36-2.25 (m, 5H), 2.04-1.88 (m, 4H), 1.77-1.66 (m, 4H), 1.62-1.53 (m, 1H). ^{13}C-NMR (100 MHz, CD_3OD, δ): 177.2 (u), 158.9 (u), 136.3 (dn), 133.9 (dn), 81.8 (dn), 55.0 (u), 42.3 (u), 41.3 (u), 39.8 (u), 35.3 (u), 33.6 (u), 32.2 (u), 26.5 (u). IR ($CHCl_3$, cm^{-1}): 3448, 3408, 3007, 2938, 2859, 1707, 1648, 1510, 1442, 1255, 994. ESI-MS(+) calculated for $C_{26}H_{42}N_2NaO_8$, [2M+Na]: 533.3; found: 533.3.

*rel-(1R-4E-pR)-cyclooct-4-ene-1-yl-N-pentanoic acid carbamate (**TCO2**)*



A round bottomed flask was charged with *rel*-(1*R*-4*E*-p*R*)-cyclooct-4-ene-1-yl (4-nitrophenyl) carbonate (101 mg, 0.347 mmol). The flask was evacuated and refilled with N_2. Anhydrous dimethylformamide (1.7 mL) was added, followed by triethylamine (0.140 mL, 1.03 mmol). 5-aminopentanoic acid (60.6 mg, 0.517 mmol) was added in a single portion. The reaction was stirred for 20 hrs at room temperature. The reaction solution was diluted with water, and extracted twice with ethyl acetate. The aqueous layer was then acidified with 6% aq. acetic acid, and extracted three times with methylene chloride. The organic layers were combined and washed twice with water. The organic layer was dried with $MgSO_4$, filtered, and concentrated onto silica gel using a rotary evaporator. Purification by column chromatography (0-3% methanol/methylene chloride) yielded 64 mg (69%) of **TCO2** as a colorless oil. ^{1}H NMR (400MHz, CD_3OD, δ): 5.65-5.57 (m, 1H), 5.53-5.46 (m, 1H), 4.39-4.28 (m, 1H), 3.10 (t, *J*= 7.0 Hz, 2H), 2.40-2.29 (m, 5H), 2.07-1.90 (m, 4H), 1.80-1.69 (m, 2H), 1.65-1.57 (m, 3H), 1.55-1.47 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD, δ): 176.0 (u), 157.3 (u), 134.7 (dn), 132.4 (dn), 80.2 (dn), 40.8 (u), 39.8 (u), 38.3 (u), 33.8 (u), 33.1 (u), 32.1 (u), 30.7 (u), 29.0 (u), 21.8 (u). IR ($CHCl_3$, cm^{-1}): 3453, 3390, 3007, 2928, 2859, 1706, 1658, 1515, 1445, 1236, 995. ESI-MS(+) calculated for $C_{28}H_{46}N_2NaO_8$, [2M+Na]: 561.3; found: 561.2.

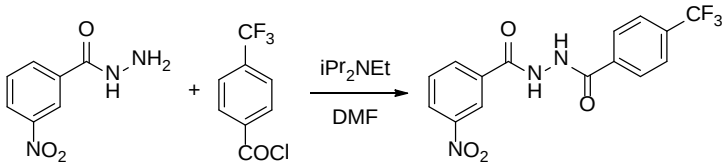
*(rel-1R,8S,9R,4E)-Bicyclo[6.1.0]non-4-ene-9-ylmethyl-N-butyric acid carbamate (**TCO3**)*



A round bottomed flask was charged with (1*R*,8*S*,9*R*,4*E*)-bicyclo[6.1.0]non-4-ene-9-ylmethyl (4-nitrophenyl) carbonate[10] (39.6 mg, 0.126 mmol). The flask was evacuated and refilled with N_2. Anhydrous dimethylformamide (0.6 mL) was added, followed by triethylamine (53 µL, 0.38 mmol). 4-aminobutyric acid (19.4 mg, 0.189 mmol) was added in a single portion. The flask was wrapped in foil and the reaction was stirred for 18 h at room temperature. The reaction solution was diluted with water, and extracted three times with ethyl acetate. The aqueous layer was then acidified with 6% aq. acetic acid and extracted three times with methylene chloride. The organic layers were combined and washed twice with water. The organic layer was dried with $MgSO_4$, filtered, and concentrated onto silica gel using a rotary evaporator. Purification by column chromatography (0-3% methanol/methylene chloride) yielded 10 mg (28%) of **TCO3** as a colorless oil. The ^{1}H NMR showed the title compound to be a ~6:1 mixture of carbamate rotamers, on the basis of intergration of the peaks at 3.96–3.85 ppm. ^{1}H-NMR (400 MHz, CD_3OD): 5.89-5.81 (m, 1H), 5.16-5.07 (m, 1H), 3.96–3.85 (d, *J*= 6.5 Hz, 2H), 3.12 (t, *J*= 6.5 Hz, 2H), 2.36-2.14 (m, 6H), 1.96-1.85 (m, 2H), 1.80-1.71 (m, 2H), 0.94-0.83 (m, 1H), 0.66-0.52 (m, 2H), 0.49-0.38 (m, 2H). ^{13}C-NMR (100 MHz, CD_3OD, δ): 174.1 (u), 156.4 (u), 136.2 (dn), 129.2 (dn), 67.3 (u), 38.0 (u), 36.7 (u), 31.7 (u), 30.7 (u), 29.1 (u), 25.6 (u), 23.4 (u), 23.1 (dn), 20.3 (dn), 19.2 (dn). IR ($CHCl_3$, cm^{-1}): 3449, 3292, 2997, 2928, 2859, 1708, 1658, 1515, 1447, 1255, 1014. ESI-MS(+) calculated for $C_{30}H_{47}N_2O_8$, [2M+H]: 563.3 ; found: 562.9.

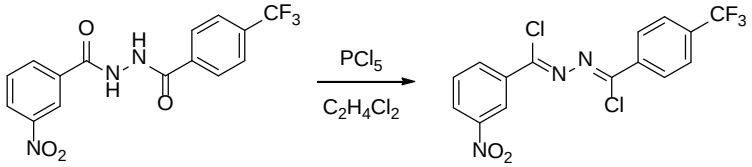
Synthesis of tetrazine **Tz2**

3-Nitro-2-[-(trifluoromethyl)benzoyl] hydrazide (1)



The following is a modification of the procedure of Blackman[10]. A stirring solution of 3-nitrobenzhydrazide (1.0 g, 5.5 mmol) and diisopropylethylamine (1.4 g, 11 mmol) in DMF (10 mL) was cooled to 0 °C under a nitrogen atmosphere. To this cold solution was slowly added 4-(trifluoromethyl)benzoyl chloride. The reaction mixture was allowed to stir for 3 h at rt. The mixture was diluted with 40 ml saturated bicarbonate solution and a solid was collected by filtration. The solid was rinsed with distilled water, suction dried, and rinsed then with hexane to give 1.6 g (84%) of the product as a pale yellow solid. The properties of the title compound matched those reported by Blackman[10], which are listed here: mp 223-225 ^{o}C. ^{1}H NMR (DMSO-d_6, 400 MHz, δ): 11.0 (s, 1H), 10.9 (s, 1H), 8.76 (t, *J* = 2.2 Hz, 1H), 8.47 (dd, *J* = 8.3 Hz, 2.4 Hz, 1H), 8.37 (dd, *J* = 7.9 Hz, 2.4 Hz, 1H), 8.13 (d, *J* = 8.3 Hz, 2H), 7.94 (d, *J* = 8.3 Hz, 2H), 7.87 (t, *J* = 7.6 Hz, 1H). ^{13}C NMR (DMSO-d_6, 100 MHz, δ): 164.7 (u), 163.8 (u), 147.9 (u), 136.1 (u), 133.8 (dn), 133.7(u), 131.7 (u) [q, ^{2}J(CF) = 35.2 Hz], 130.5 (dn), 128.4 (u), 126.6 (dn), 125.7 (dn) [q, ^{3}J(CF) = 4.0 Hz], 123.9 (u) [q, ^{1}J(CF) = 272 Hz], 122.2 (dn). HRMS (ESI+) [M+H] calcd. for $C_{15}H_9F_3N_3O_4$ 354.0702; found 354.0705.

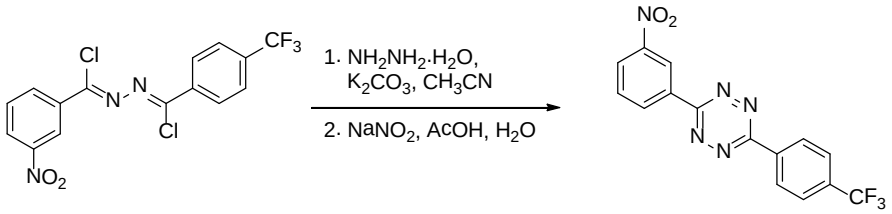
N'-(chloro(4-(trifluoromethyl)phenyl)methylene)-3-nitrobenzohydrazonoyl chloride (2)



The following is a modification of the procedure of Blackman[10]. A solution of 3-nitro-2-[-(trifluoromethyl)benzoyl] hydrazide (0.80 mg, 2.3 mmol) and anhydrous dichloroethane (15 mL) in round bottom flask was equipped with a stirbar and a reflux condenser, and PCl_5 (1.6 g, 7.7 mmol) was added to the stirring solution under nitrogen atmosphere. The reaction mixture was heated to reflux for 24 h. The reaction mixture was cooled to rt and slowly poured into ice water. The organic layer was separated from aqueous layer. The aqueous layer was extracted with with two 15 mL portions of CH_2Cl_2. The organics were combined, washed with saturated aq. $NaHCO_3$ (15 mL), dried over anhydrous MgSO4 and concentrated. The residue was purified by column chromatography (gradient of CH_2Cl_2/hexane) to give 0.55 g (62%) of the title compound as yellow solid. The properties of the title compound matched those reported by Blackman[10] which are listed here: mp 78-80 ^{o}C. ^{1}H NMR ($CDCl_3$, 400 MHz, δ): 8.93 (t, *J* = 2.0 Hz, 1H), 8.44 (dd, *J* = 8.0 Hz, 1.9 Hz, 1H), 8.38 (dd, *J* = 8.3 Hz, 2.3 Hz, 1H), 8.23 (d, *J* = 8.3 Hz, 2H),

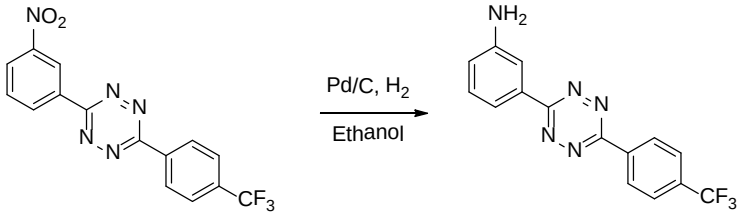
7.71 (d, *J* = 8.4 Hz, 2H), 7.66 (t, *J* = 8.1 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz, δ): 148.4 (u), 143.6 (u), 142.2 (u), 136.4 (u), 135.1 (u) , 133.9 (dn), 133.6 (u)[q, ^2J(CF) = 33.4 Hz], 129.8 (dn), 128.9 (dn), 126.4 (dn) [q, ^3J(CF) = 4.0 Hz], 125.6 (dn) 123.6 (u) [q, ^1J(CF) = 274 Hz], 123.5 (dn). HRMS (ESI+) [M+H] calcd. for $C_{15}H_9F_3N_3O_2Cl_2$ 390.0024; found 390.0064.

3-(3-nitrophenyl)-6-[4-(trifluoromethyl)phenyl]-s-tetrazine (3)



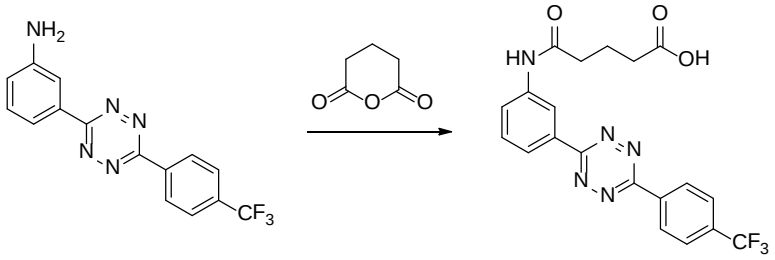
The following is a modification of the procedure of Blackman[10]. A round bottomed flask was charged with *N*'-(chloro(4-(trifluoromethyl)phenyl)methylene)-3-nitrobenzohydrazonoyl chloride (0.530 g, 1.36 mmol) and acetonitrile (10 mL), and was equipped with a reflux condenser. Hydrazine hydrate (0.068 mg, 1.36 mmol) was added, and the mixture was heated to reflux behind a blast shield for 1 h. Potassium carbonate (375 mg, 2.72 mmol) was added, and the mixture was heated to reflux for 24 h. Hydrazine hydrate (408 mg, 8.16 mmol) was added, and the mixture was heated to reflux for an additional hour. The mixture was cooled to rt, and diluted with CH_2Cl_2. The organics were washed with brine, dried over anhydrous $MgSO_4$, and concentrated. The crude residue was dissolved in acetic acid (4 mL) at 0 ^{o}C. The solution was stirred, and a solution of $NaNO_2$ (0.690 g, 10.0 mmol) in water (1 mL) was added dropwise. The mixture was allowed to stir for 3 h, and was then diluted with CH_2Cl_2 (50 mL). The organics were washed with sat. aq. $NaHCO_3$ (2 x 30 mL), dried over anhydrous magnesium sulfate and concentrated. The residue was the purified by column chromatography (gradient CH_2Cl_2 in hexane) to give **3** (260 mg, 55%) as pink solid. Anal. calculated for $C_{15}H_8F_3N_5O_2$: C, 51.88; H, 2.32; N, 20.17. Found: C, 51.48; H, 2.41; N, 19.81. The properties of the title compound matched those reported by Blackman[10], which are listed here: mp 217-219 ^{o}C. ^{1}H NMR ($CDCl_3$, 400 MHz, δ): 9.54 (t, *J* = 2.0 Hz, 1H), 9.01 (dd, *J* = 7.8 Hz, 1.6 Hz, 1H), 8.81 (d, *J* = 8.3 Hz, 2H), 8.51 (dd, *J* = 8.3 Hz, 2.3 Hz, 1H), 7.89 (d, *J* = 8.3 Hz, 2H), 7.84 (t, *J* = 8.1 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz, δ): 164.7 (u), 163.4 (u), 147.9 (u), 136.1 (u), 133.8 (dn), 133.7 (u), 131.5 (u) [q, ^{2}J(CF) = 34.5 Hz], 130.5 (dn), 126.6 (dn) [q, ^3J(CF) = 4.0 Hz], 125.6 (dn) 123.6 (u) [q, ^1J(CF) = 272 Hz], 122.2 (dn). HRMS (ESI) $[M+]^+$ calcd. for $C_{15}H_8F_3N_5O_2$ 347.0630; found 347.0622.

3-(3-aminophenyl)-6-[4-(trifluoromethyl)phenyl]-s-tetrazine (4)



A round bottom flask was charged with 10% Pd/C (100 mg), ethanol (15 mL) and 3-(3-nitrophenyl)-6-[4-(trifluoromethyl)phenyl]-*s*-tetrazine (247 mg, 0.712 mmol) under nitrogen atmosphere. The mixture was allowed to stir, and the flask was purged with hydrogen. Stirring continued under hydrogen (balloon pressure) for 12 h. The reaction mixture was diluted with methanol (25 mL), filtered, concentrated and purified by column chromatography to give the title compound (120 mg, 53 %) as a red solid, mp 214-216 ^{o}C. The properties of the title compound matched those reported by Blackman[10], which are listed here: ^{1}H NMR (DMSO-d_6, 400 MHz, δ): 8.71 (d, *J* = 8.3 Hz, 2H), 8.06 (d, *J* = 8.8 Hz, 2H), 7.81 (t, *J* = 2.0 Hz, 1H) 7.71 (m, 1H), 7.32 (t, *J* = 7.4 Hz, 1H), 6.89 (dd, *J* = 8.5 Hz, 1.9 Hz, 1H), 5.5 (m, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz, δ): 163.7 (u), 162.4 (u), 149.6 (u), 135.9 (u), 132.0 (u), 131.9 (u) [q, ^{2}J(C-F) = 34.5 Hz], 130.0 (dn), 128.2 (dn), 126.3 (dn) [q, ^{3}J(CF) = 4.0 Hz], 121.0 (u) [q, ^{1}J(CF) = 273 Hz], 118.2 (dn), 115.2 (dn), 112.4 (dn). HRMS (ESI) $[M+H]^{+}$ calcd. for $C_{15}H_{11}F_3N_5$ 318.0967; found 318.0966.

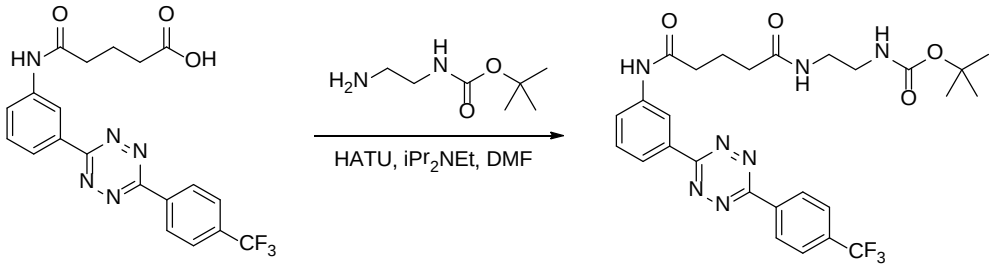
5-oxo-5-(3-(6-4-(trifluoromethyl)phenyl)-1,2,4,5-tetrazin-3-yl)phenylamino)pentanoic acid (5)



The following is a modification of the procedure of Blackman[10]. A 2 dram vial was charged with 3-(3-aminophenyl)-6-[4-(trifluoromethyl)phenyl]-*s*-tetrazine (100 mg, 0.315 mmol), glutaric anhydride (180 mg, 1.58 mmol) and THF (2 mL). The vial was flushed with nitrogen, capped, and heated with stirring at 80 ^{o}C for 4 h. The mixture was cooled to rt, centrifuged, and the supernatant decanted. The solid that was obtained was suspended in CH_2Cl_2 sonicated, centrifuged, supernatant decanted and dried to give the title compound (120 mg, 88%) as a pink solid. The properties of the title compound matched those reported by Blackman[10], which are listed here: mp 246-248 ^{o}C. ^{1}H NMR (DMSO-d_6, 400 MHz, δ): 10.3 (s, 1H), 8.92 (t, J = 1.8 Hz, 1H), 8.74 (d, *J* = 8.2 Hz, 2H), 8.23 (dd, *J* = 7.8 Hz, 1.8 Hz, 1H) 8.09 (d, *J* = 8.2 Hz, 2H), 7.92 (dd, *J* = 8.2 Hz, 2.3 Hz, 1H), 7.63 (t, *J* = 8.2, 1H), 2.43 (t, *J* = 7.1 Hz, 2H), 2.31 (t, *J* = 7.4 Hz, 2H), 1.85 (quin., *J* = 7.0 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz, δ): 174.3 (u), 171.3 (u), 163.5 (u), 162.6 (u), 140.4 (u), 135.9 (u), 132.0 (u), 132.0 (u) [q, ^{2}J(C-F) = 34.5 Hz], 130.1 (dn), 128.4 (dn), 126.4 (dn)

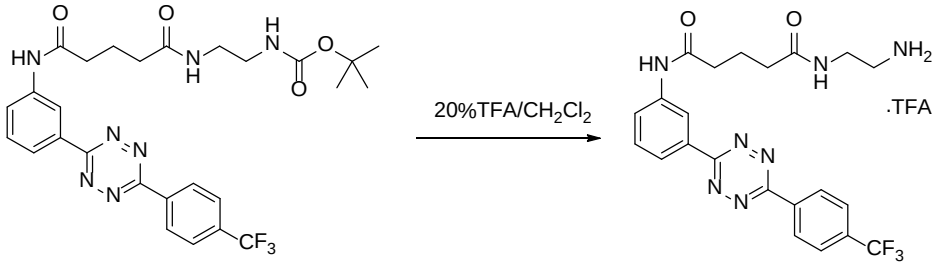
[q, ^3J(CF) = 4.0 Hz], 124.2 (u)[q, ^1J(CF) = 275 Hz], 123.1 (dn), 122.6 (dn), 118.0 (dn) ,35.5 (u) 33.1 (u), 20.4 (u).). HRMS (ESI) $[M+H]^+$ calcd. for $C_{20}H_{16}F_3N_5O_3$ 432.1283; found 432.1283.

tert-butyl (2-(5-oxo-5-((3-(6-(4-(trifluoromethyl)phenyl)-1,2,4,5-tetrazin-3-yl)phenyl)amino)pentanamido)ethyl)carbamate



A 2 dram vial was swept with nitrogen, and sequentially charged with 5-oxo-5-(3-(6-4-(trifluoromethyl)phenyl)-1,2,4,5-tetrazin-3-yl)phenylamino)pentanoic acid (75 mg, 0.17 mmol), HATU (172 mg, 0.46 mmol) and a solution of *ter*t-butyl (2-aminoethyl)carbamate (70 mg, 0.44 mmol) in anhydrous DMF (2 mL). The vial was capped, and the resulting mixture stirred for 20 h. The mixture was then diluted with CH_2Cl_2 (10 mL) and centrifuged. Residue was thrice suspended in CH_2Cl_2 (10 mL) sonicated, centrifuged, decanted supernatant and dried to give the title compound (70 mg, 70%) as a poorly soluble pink solid. ^{1}H NMR (DMSO-d_6, 400 MHz, δ): 10.3 (s, 1H), 8.94 (t, *J* = 2.0 Hz, 1H), 8.74 (d, *J* = 7.8 Hz, 2H), 8.23 (dd, *J* = 7.8 Hz, 2.0 Hz, 1H) 8.09 (d, *J* = 8.7 Hz, 2H), 8.01–7.83 (m, 2H), 7.63 (t, *J* = 7.8, 1H), 6.83 (br, s, 1H), 3.15–3.05 (m, 2H), 3.05–2.90 (m, 2H), 2.42-2.34 (m, 2H), 2.22-2.09 (m, 2H), 1.94-1.77 (m, 2H), 1.38 (s, 9H). LRMS (ESI) $[M+Na]^+$ calcd. for $C_{27}H_{30}F_3N_7O_4$ 596; found 596.

*N1-(2-aminoethyl)-N5-(3-(6-(4-(trifluoromethyl)phenyl)-1,2,4,5-tetrazin-3-yl)phenyl)glutaramide trifluoroacetic acid (**Tz2**)*

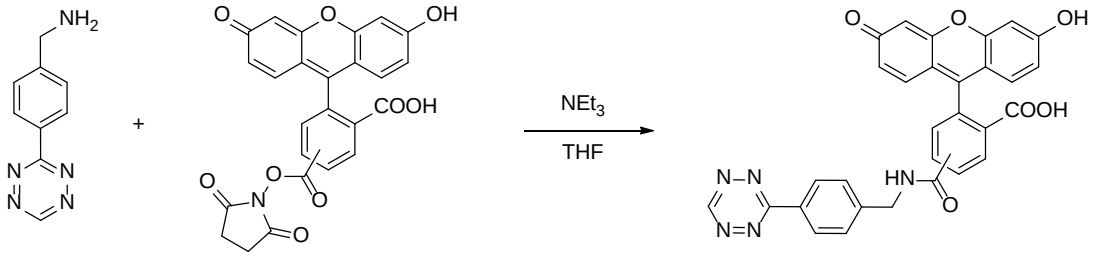


A 2 dram vial containing *tert*-butyl (2-(5-oxo-5-((3-(6-(4-(trifluoromethyl)phenyl)-1,2,4,5-tetrazin-3-yl)phenyl)amino)pentanamido)ethyl)carbamate (50 mg, 0.87 mmol) was flushed with nitrogen. A solution of 20% trifluoroacetic acid in CH_2Cl_2 (2 mL) was added, and the resulting mixture stirred for 2 h at rt. The mixture was concentrated to give 56 mg (92%, presuming a *bis*-TFA salt) of **Tz2** as red solid. ^{1}H NMR (DMSO-d_6, 400 MHz, δ): 10.3 (s, 1H), 8.93 (t, *J* = 2.2 Hz, 1H), 8.74 (d, *J* = 8.3 Hz, 2H), 8.24 (dd, *J* = 7.8 Hz, 2.0 Hz, 1H) 8.09 (d, *J* = 8.3 Hz, 2H), 8.03 (t, *J* = 5.0 Hz, 1H), 7.92 (m, 1H), 7.72 (br, s 3H), 7.63 (t, *J*= 7.8, 1H), 3.33–3.22 (m, 2H), 2.91–2.78 (m, 2H), 2.40 (t, *J* = 7.8 Hz, 2H), 2.20 (t, *J* = 7.8 Hz, 2H), 1.87 (quint, *J* = 7.8 Hz, 2H). ^{13}C NMR (DMSO-

d6, 100 MHz, δ): 173.1 (u), 171.8 (u), 164.0 (u), 163.1 (u), 140.8 (u), 136.4 (u), 132.6 (u) [q, ^2J(CF) = 32.3 Hz], 132.6 (u) 130.5 (dn), 128.8 (dn), 126.9 (dn) [q, ^3J(CF) = 3.6 Hz], 124.5(u) [q, ^1J(CF) = 281 Hz], 123.6 (dn), 122.9 (dn), 118.4(dn) ,36.9 (u) 36.2 (u), 35.1 (u), 21.4 (u). Peaks due to trifluoroacetate counterion were observed at: 158.6(u) [q, ^2J(CF) = 36.2 Hz], 116.4(u) [q, ^1J(CF) = 289 Hz]. LRMS (ESI) $[M+H]^+$ calcd. for $C_{22}H_{23}F_3N_7O_2$ 474; found 474.

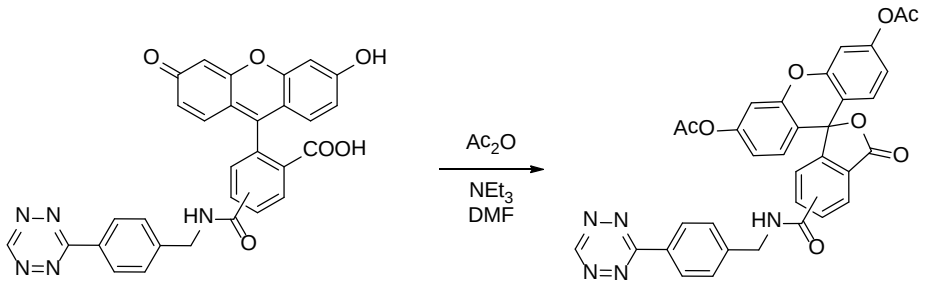
Synthesis of tetrazine-fluorophore conjugates

*Aminobenzyltetrazine carboxyfluorescein (**Tz1**-fluorescein)*



Tetrazine benzylamine (**Tz1**) was synthesized as previously described[11]. To a dried flask equipped with a stir bar was added **Tz1** (10.7 mg, 0.057 mmol) in 5 mL anhydrous THF followed by 5-(and 6-)carboxyfluorescein, succinimidyl ester (NHS-fluorescein; 13.2 mg, 0.028 mmol, Thermo Scientific) and Et_3N (11.9 µL, 0.085 mmol). The mixture was stirred overnight at room temperature under N_2 atmosphere. The solvent was removed under reduced pressure and the resulting solid was purified by normal phase silica gel column chromatography with 17% MeOH in CH_2Cl_2 + 0.1% (v/v) TFA. The eluate was dried under vacuum, then further purified by HPLC on a C-18 column (10-90% acetonitrile over 30 min. linear gradient). The product eluted at 19 min. and was freeze-dried to give **Tz1**-fluorescein as a dark orange solid. TLC R_f = 0.38 (17% v/v MeOH in CH_2Cl_2 + 0.1% v/v TFA). ESI (+) calculated for $[M-H]^-$: 544.13; found: 544.02.

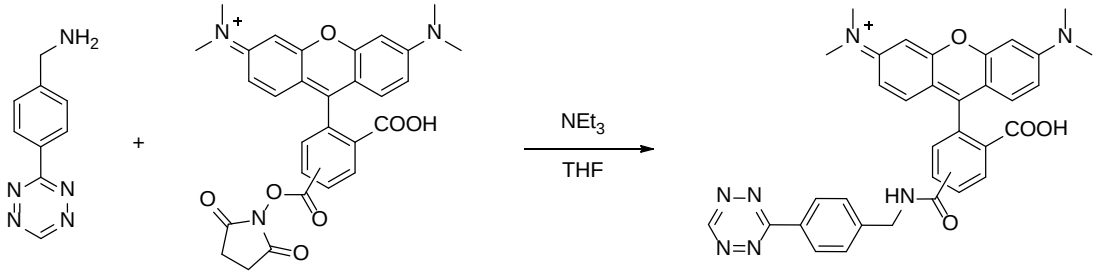
*Aminobenzyltetrazine carboxyfluorescein diacetate (**Tz1**-fluorescein diacetate)*



To a dried flask equipped with a stir bar was added **Tz1**-fluorescein (2 mg, 0.0037 mmol) in 2 mL anhydrous DMF, 3 eq. acetic anhydride, 5 eq. Et_3N. The mixture was stirred at room temperature under N_2 atmosphere for 2 hours, during which time the reaction mixture turned from orange to pink. For workup, the reaction mixture was diluted with 20 volumes of H_2O, and the product was extracted into EtOAc. After drying with sodium sulfate, the EtOAc solvent

was removed under reduced pressure to give a dark pink oil. The product was further purified by normal phase silica gel column chromatography (isocratic 100% ethyl acetate) to give a dark pink wax. ESI (+) calculated for $[M+H]^+$: 629.15; found: 629.82.

*Aminobenzyltetrazine tetramethylrhodamine (**Tz1**-TMR)*

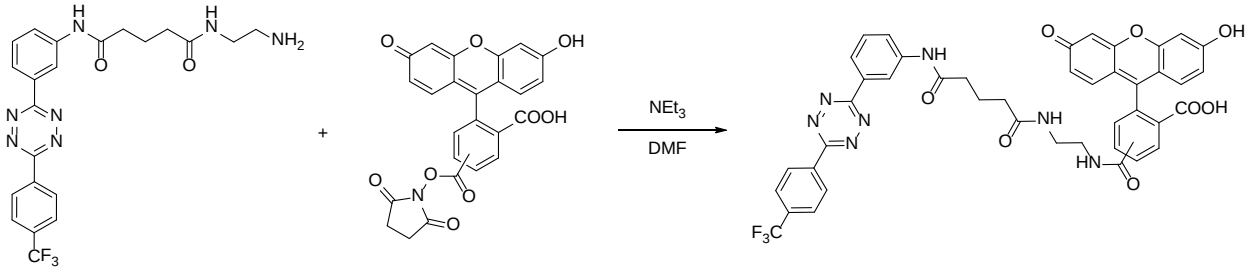


Synthesized under similar conditions as for **Tz1**-fluorescein, using 5-,6-carboxytetramethylrhodamine, succinimidyl ester (Thermo Scientific). After solvent removal, the resulting solid was purified by HPLC on a C-18 column (10-90% acetonitrile over 30 min. linear gradient). ESI (+) calculated for M^+: 600.24; found: 600.30.

*Aminobenzyltetrazine Alexa Fluor 647 (**Tz1**-Alexa 647)*

To a dried glass vial equipped with a stir bar was added 2 mg **Tz1** (10.6 µmol), Alexa Fluor 647 carboxylic acid, succinimidyl ester (0.3 mg, Live Technologies), and Et_3N (53.0 µmol) in 500 µL anhydrous DMSO. The reaction was stirred overnight at room temperature under N_2 atmosphere. The mixture was diluted with 10 volumes of H_2O, then freeze-dried into a dark blue solid. The solid was purified by HPLC on a C-18 column (10-90% acetonitrile over 20 min. linear gradient). The product eluted at 8 min. and was again freeze-dried to give a dark blue solid.

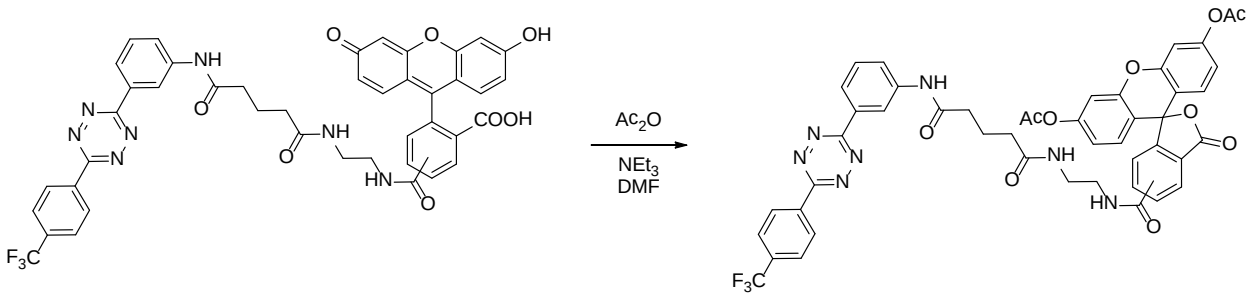
*Trifluoromethyl bisaryltetrazine amine, carboxyfluorescein conjugate (**Tz2**-fluorescein)*



To a dried flask equipped with a stir bar was added **Tz2** (20 mg, 0.034 mmol) in 1 mL anhydrous DMF followed by NHS-fluorescein (16 mg, 0.034 mmol) and Et_3N (24 µL, 0.17 mmol). The mixture was stirred overnight at room temperature under N_2 atmosphere. The solvent was removed under reduced pressure and the product was purified on normal phase silica gel column chromatography with 5-15% (v/v) MeOH in CH_2Cl_2. The eluate was dried under

vacuum, then further purified by HPLC on a C-18 column (10-90% acetonitrile over 30 min. linear gradient). The product eluted at 21 min. and was freeze-dried to give **Tz2**-fluorescein as a dark orange solid. TLC R_f = 0.40 (10% v/v MeOH in CH_2Cl_2). ESI (+) calculated for $[M+H]^+$: 832.4; found: 832.23.

*Trifluoromethyl bisaryltetrazine amine, carboxyfluorescein diacetate conjugate (**Tz2**-fluorescein diacetate)*



2 mg (0.0024 mmol) **Tz2**-fluorescein was used to synthesize **Tz2**-CFDA in the same protocol as for **Tz1**-CFDA. The extracted product was further purified by normal phase silica gel column chromatography (isocratic 100% ethyl acetate) to give a dark pink wax. ESI (+) calculated for $[M+H]^+$: 916.25; found: 916.44.

HPLC assay for *in vitro* LplA-mediated *trans*-cyclooctene probe ligation onto LAP (**Table 1 and Supporting Figure 1A**)

Reactions were assembled with 250 nM (or 1 μM ^{W37V}LplA for **Supporting Figure 1A**), 200 μM LAP (GFEIDKVWYDLDA), 500 μM *trans*-cyclooctene (**TCO1**, **TCO2**, or **TCO3**), 2 mM ATP, and 5 mM $Mg(OAc)_2$ in Dulbecco's phosphate buffered saline with 10% (v/v) glycerol and incubated at 30 °C for 30 min. (or 60 min. for **Supporting Figure 1A**). LplA protein was purified as previously described[2] and stored at - 80 °C in 20 mM Tris-HCl, pH 7.5 supplementated with 10% v/v glycerol. Reactions were quenched with 30 mM EDTA (final concentration) and resolved by HPLC (Varian ProStar) on a C-18 column using a linear gradient of 25 – 60% acetonitrile in H_2O (with 0.1% v/v trifluoroacetic acid) over 14 minutes. Species were detected at 210 nm absorbance. Peaks corresponding to LAP and its *trans*-cyclooctene adducts were confirmed by ESI mass spectrometry, and shown in **Supporting Figure 1B** for **TCO2**. The extent of conversion was calculated from ratios of peak areas, neglecting minor extinction coefficient changes to LAP due to *trans*-cyclooctene ligation.

Live cell surface fluorescence labeling with dye washout (**Figures 2A** and **2D**)

HEK cells were rinsed twice with Tyrode's buffer (145 mM NaCl, 1.25 mM $CaCl_2$, 3 mM KCl, 1.25 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 10 mM glucose, 10 mM HEPES, pH 7.4), then treated with 5 μM ^{W37V}LplA, 100 μM **TCO2**, 1 mM ATP and 1 mM $Mg(OAc)_2$ in the same buffer for 15 minutes at room temperature. Cells were rinsed 3 times before further treatment with 100 nM **Tz1**-fluorescein in Tyrode's buffer for 5 minutes at room temperature. Imaging was performed live after another 2 rinses. LAP-LDL receptor and nuclear cyan fluorescent protein marker were transfected at a 1:1 ratio, with altogether 400 ng plasmid per 1 cm^2 culture.

Hippocampal neurons were labeled in the same way, except that the **TCO2** ligation step was shortened to 10 minutes and performed at 37 °C. 100 nM **Tz1**-Alexa 647 was used for **Figure 2D**. LAP-neuroligin-1 and Homer1b-GFP were transfected at a 1:1 ratio, with altogether 2 μg plasmid per 2 cm^2 culture. We routinely observed that the **Tz1**-Alexa 647 conjugate bound non-specifically to cellular debris in a *trans*-cyclooctene independent manner, contributing some punctate background in imaging. This problem can be alleviated by having healthy neuron cultures with minimal debris.

Live cell surface fluorogenic labeling without dye washout (**Figures 2B and 2C**)

HEK cells grown in a monolayer on #1.5 Lab-Tek II chambered coverglass (Nalge Nunc International) were treated with **TCO2** as in **Figure 2A**. After 5 rinses with Tyrode's buffer, the chamber was placed on the microscope objective covered with 200 μL of the same buffer. Image acquisition sequence was initiated immediately after 200 μL of 100 nM **Tz1**-fluorescein in Tyrode's buffer was added to the chamber, and briefly mixed by pipeting. Final concentration of **Tz1**-fluorescein was therefore 50 nM after mixing. LAP-LDL receptor and a mCherry fluorescent protein transfection marker were transfected at a 1:1 ratio, with altogether 400 ng plasmid per 1 cm^2 culture.

To quantify the imaging signal/noise ratio, 17 cells with obvious surface fluorescence (by eye) at the 180 sec. time point were chosen and separate masks created automatically by the Slidebook software over the fluorescent rims. The averaged pixel intensity was defined as "signal". To measure noise, 10 cells with no obvious surface fluorescence (by eye) at the 180 sec. time point were chosen, and rectangular masks created manually over the interiors of these cells. The averaged (over all 10 masks) pixel intensity was defined as "noise". Both "signal" and "noise" had a background subtraction from averaged pixel intensity corresponding to non-cellular regions.

Live intracellular fluorescence labeling with dye washout (**Figure 3**)

HEK cells were rinsed once with MEM, then treated with 200 µM **TCO2** in the same medium for 30 min. at 37 °C. Cells were rinsed twice, then left in complete medium (MEM with 10% v/v fetal bovine serum) for a further 30 min. at 37 °C to allow excess unligated **TCO2** to wash out of cells. 500 nM **Tz1**-fluorescein diacetate or 1 µM **Tz1**-TMR in MEM was then added to cells for 5 min. at 37 °C. Cells were then rinsed twice with complete medium and kept at 37 °C for excess dye to wash out. Complete medium was replaced twice more at 20 and 40 minutes later to improve washout. Cells were imaged live after altogether 2 hours in complete medium. HEK cells were transfected with 300 ng nuclear LAP-blue fluorescent protein and 50 ng ^{W37V}LplA per 1 cm^2 culture.

We have separately determined that the 30 min. **TCO2** washout step is necessary for achieving strong labeling signal (data not shown). If this step is omitted, excess, unligated **TCO2** could presumably compete for reaction with the tetrazine-fluorophore conjugates to reduce labeling yield.

In Figure 3C, COS-7 cells expressing cytoskeletal proteins were labeled similarly to HEK cells, except that 100 µM **TCO2** was used, **Tz1**-fluorescein diacetate loading concentration was reduced to 100 nM, and tetrazine-dye washout time was reduced to 1 hour before cells were imaged live. COS-7 cells were transfected with 200 ng LAP-actin or 200 ng vimentin-LAP along with 50 ng ^{W37V}LplA per 1 cm^2 culture.

Measurement of k_{cat} for *in vitro* ^{W37V}LplA mediated ligation of **TCO2** and lipoate onto LAP (**Supporting Figure 2**)

Reactions were assembled with 500 µM **TCO2** or lipoic acid, 500 µM LAP (GFEIDKVWYDLDA), 2 mM ATP, 5 mM $Mg(OAc)_2$ and 250 nM ^{W37V}LplA and kept in a 30 °C waterbath. After 5, 10, 15 and 20 minutes, an aliquot was drawn from the reaction vial, quenched with 30 mM EDTA (final concentration) and the product quantified by HPLC as in **Table 1**. The plot of product concentration against time was fitted to a linear line whose slope corresponds to the initial velocity. The value of k_{cat} was calculated from the Michaelis-Menten equation $V_{max} = (k_{cat})([Enzyme])$ at substrate-saturating conditions. Measurements were performed in triplicate.

Measurement of tetrazine-dye fluorescence turn-on after Diels-Alder cycloaddition (**Supporting Figure 4**)

Tetrazine-fluorophore conjugates were dissolved in Dulbecco's phosphate buffered saline, pH 7.4 at approximately 100 nM concentration. Solutions with > 100-fold excess **TCO1** in DMSO or DMSO vehicle alone added were transferred into an opaque, flat-bottom 96-well plate (Greiner Bio One) and their fluorescence emission scanned with a Safire Tecan fluorescence microplate reader. Excitation was fixed at 430 nm for fluorescein, 530 nm for TMR, and 610 nm for Alexa 647. Fold-changes in fluorescence turn-on are reported at respective fluorescence emission maximum wavelengths.

Measurement of *in vitro* second-order Diels-Alder cycloaddition rate constant between LAP-**TCO2** and tetrazine-fluorescein conjugates (**Supporting Figure 5**)

LAP-**TCO2** adduct was prepared by mixing 500 µM LAP with 1 mM **TCO2**, 2 µM ^{W37V}LplA, 2 mM ATP, and 5 mM $Mg(OAc)_2$ in Dulbecco's phosphate buffered saline (DPBS), pH 7.4 supplemented with 10% v/v glycerol. Ligation reaction was allowed to proceed at 30 °C for 4 hours to maximize ligation yield. The mixture was then resolved by preparatory HPLC on a C-18 column (25-45% acetonitrile over 30 min. linear gradient, supplemented with 0.1% v/v trifluoroacetic acid), where the product eluted at 19 min. and its identity confirmed by ESI mass spectrometry. The eluate was freeze-dried into a white powder and dissolved in DPBS for subsequent measurements.

To measure second-order rate constant by pseudo-first-order approximation, 100 µL **Tz1**- or **Tz2**-fluorescein (100 nM in DPBS) was loaded into an opaque, flat-bottom 96-well plate (Greiner Bio One), then mixed with 100 µL LAP-**TCO2** (3.3 µM in DPBS). The fluorescence intensity at 520 nm was immediately recorded at 9-second intervals until the reaction reached completion in approximately 5 minutes. The fluorescence intensity was then converted to [tetrazine-fluorescein], assuming that initial fluorescence corresponded to 50 nM and final fluorescence corresponded to 0 nM tetrazine-fluorescein. The plot of ln[tetrazine-fluorescein] against time was fitted to a linear line whose slope corresponds to the pseudo-first order rate constant, which was then converted to the second-order rate constant. Measurements were performed in triplicate.

Comparing Diels-Alder cycloaddition, copper catalyzed azide-alkyne cycloaddition (CuAAC), and copper-free "click" chemistries for cell surface fluorescence labeling and toxicity analysis (**Supporting Figure 6**)

HEK cells were rinsed twice with Tyrode's buffer, then treated with 1 mM ATP, 5 mM $Mg(OAc)_2$, and either 10 µM ^{W37V}LplA / 100 µM **TCO2** (for subsequent Diels-Alder staining) or 10 µM wild-type LplA / 100 µM 8-azidooctanoic acid (for subsequent CuAAC and strain-promoted cycloaddition staining)[2] in the same buffer for 30 min. at room temperature. These were previously determined, by subsequent lipoic acid pulse labeling, to give almost quantitative yield of 8-azidooctanoic acid ligation (data not shown). Cells were then rinsed and treated with

Tz1-Alexa 647, alkyne-Alexa 647 with 50 μM $CuSO_4$/2.5 mM sodium ascorbate/250 μM THPTA ligand[12] (a gift from Chayasith Uttamapinant), or DIBO-Alexa 647 (Life Technologies) in Tyrode's buffer for 3 minutes at room temperature and imaged live after further rinsing. HEK cells were transfected with LAP-LDL receptor and nuclear cyan fluorescent protein marker in a 1:1 ratio, with altogether 400 ng per 1 cm^2 culture.

To assess the toxicity of labeling conditions, HEK cells grown in flat-bottom 96-well plates (Greiner Bio One) were transfected and treated as in **Supporting Figure 6A**, except that the LplA concentration was reduced to 1 μM, and the **TCO2**/8-azidooctanoic acid ligation and fluorescence staining steps were changed to 15 minutes and 5 minutes, respectively. Afterward, 100 μL of premixed CellTiter-Glo reagent (Promega) was added into each well. The plate was shaken in a 30°C orbital shaker for 10 minutes and the luminescence from each well was recorded by a SPECTRAmax dual-scanning microplate spectrofluorometer. Measurements were performed in triplicate.

Quantification of labeling signal/noise ratio for **Tz1**- and **Tz2**-fluorescein diacetate (**Supporting Figure 7B**)

Masks over the nuclear regions were generated automatically in the Slidebook software by gating the BFP fluorescence. 24 gates of a wide range of BFP intensities over 3 fields of view for each condition were randomly chosen. The fluorescein intensities within these gates were defined as "signal". Rectangular gates in the perinuclear regions of these chosen cells were drawn manually and their corresponding fluorescein intensities defined as "noise". Both "signal" and "noise" were background-adjusted from the averaged fluorescence intensity in non-cellular regions.

Determination of Tz1-fluorescein diacetate labeling specificity by polyacrylamide gel electrophoresis and fluorescein in-gel fluorescence imaging (**Supporting Figure 8**)

HEK cells grown in 6-well plates (Greiner Bio One) were transfected with 3 μg nuclear LAP-blue fluorescent protein and 500 ng ^{W37V}LplA, then treated with **TCO2** followed by **Tz1**-fluorescein diacetate in the same way as for Figure 3B, except that the dye washout in complete medium at 37 °C was lengthened to 4 hours. Cells were then rinsed twice with DPBS and scraped off the surface. Cells were lysed by 3 rounds of freezing and thawing in hypotonic lysis buffer (1 mM HEPES, 5 mM $MgCl_2$, pH 7.5) supplemented with protease inhibitor cocktail (Sigma Aldrich) and phenylmethanesulfonyl fluoride. The lysate was clarified by centrifuging at 10,000 g for 5 min. at 4 °C and the supernatant resolved on a 12% SDS polyacrylamide gel. Fluorescein in-gel fluorescence was imaged on a FUJIFILM FLA-9000 gel imager with a 473 nm laser using a blue long-pass filter. After fluorescence imaging the same gel was stained with Coomassie and re-imaged under white light after destaining.

Visualization of actin filaments and vimentin intermediate filaments by Tz1-TMR labeling and immunofluorescence staining (**Supporting Figure 9**)

HeLa cells grown on glass coverslips were transfected and labeled with **Tz1**-TMR in the same way as for Figure 3C. Cells were then fixed with 3.7% (v/v) formaldehyde in DPBS for 15 min. at room temperature and subsequently permeabilized with methanol for 5 min. at - 20 °C. Samples were blocked with 0.5% (w/v) casein in DPBS for 4 hours at room temperature, then treated with a 1:300 dilution of rabbit-anti-HA antibody (Life Technologies) or mouse-anti-*C*-myc antibody (Life Technologies) followed by a 1:300 dilution of goat-anti-rabbit or goat-anti-mouse antibody Alexa Fluor 647 conjugate (Life Technologies) for 15 min. each step in the blocking buffer.

References

(1) Puthenveetil, S.; Liu, D. S.; White, K. A.; Thompson, S.; Ting, A. Y. *J. Am. Chem. Soc.* **2009**, *131*, 16430-16438.

(2) Fernandez-Suarez, M.; Baruah, H.; Martinez-Hernandez, L.; Xie, K. T.; Baskin, J. M.; Bertozzi, C. R.; Ting, A. Y. *Nat. Biotechnol.* **2007**, *25*, 1483-1487.

(3) Chan, T. R.; Hilgraf, R.; Sharpless, K. B.; Fokin, V. V. *Org. Lett.* **2004**, *6*, 2853-2855.

(4) Presolski, S. I.; Hong, V.; Cho, S. H.; Finn, M. G. *J. Am. Chem. Soc.* **2010**, *132*, 14570-14576.

(5) Jiang, Y. F.; Cronan, J. E. *J. Biol. Chem.* **2005**, *280*, 2244-2256.

(6) Uttamapinant, C.; White, K. A.; Baruah, H.; Thompson, S.; Fernandez-Suarez, M.; Puthenveetil, S.; Ting, A. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 10914-10919.

(7) Kalderon, D.; Roberts, B. L.; Richardson, W. D.; Smith, A. E. *Cell* **1984**, *39*, 499-509.

(8) Taylor, M. T.; Blackman, M. L.; Dmitrenko, O.; Fox, J. M. *J. Am. Chem. Soc.* **2011**, *133*, 9646-9649.

(9) Royzen, M.; Yap, G. P. A.; Fox, J. M. *J. Am. Chem. Soc.* **2008**, *130*, 3760-3761.

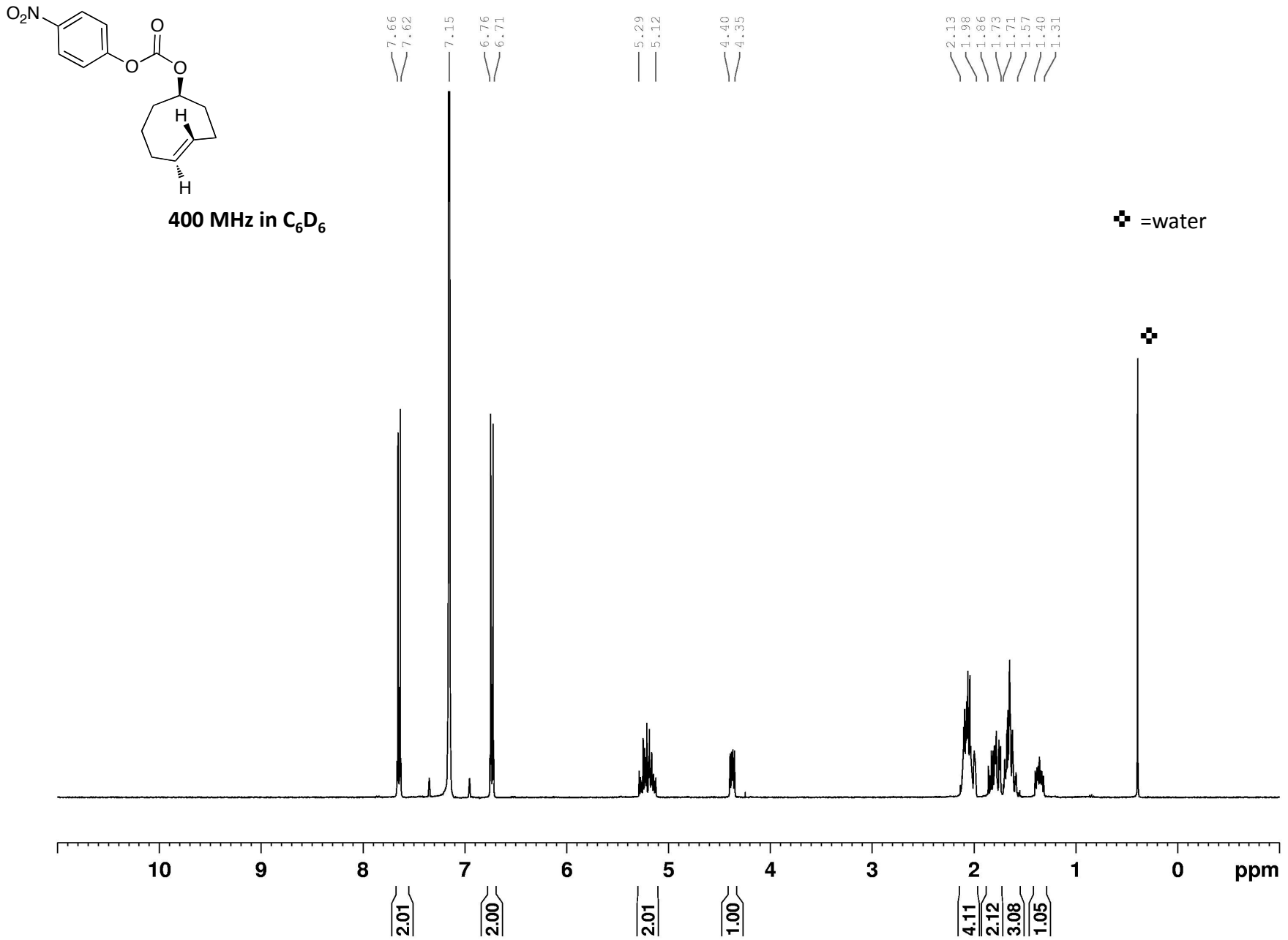
(10) Blackman, M. L. Ph.D. thesis, **2011**. University of Delaware, Newark, DE.

(11) Devaraj, N. K.; Weissleder, R.; Hilderbrand, S. A. *Bioconjugate Chem.* **2008**, *19*, 2297-2299.

(12) Hong, V.; Steinmetz, N. F.; Manchester, M.; Finn, M. G. *Bioconjugate Chem.* **2010**, *21*, 1912-1916.

Full reference 5 in main text:

(5) Los, G. V.; Encell, L. P.; McDougall, M. G.; Hartzell, D. D.; Karassina, N.; Zimprich, C.; Wood, M. G.; Learish, R.; Ohane, R. F.; Urh, M.; Simpson, D.; Mendez, J.; Zimmerman, K.; Otto, P.; Vidugiris, G.; Zhu, J.; Darzins, A.; Klaubert, D. H.; Bulleit, R. F.; Wood, K. V. *ACS Chem. Biol.* **2008**, *3*, 373-382.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1.86
1.73
1.71
1.57
1.40
1.31
400 MHz in C6D6
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9
8
7
6
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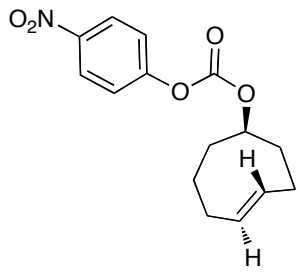

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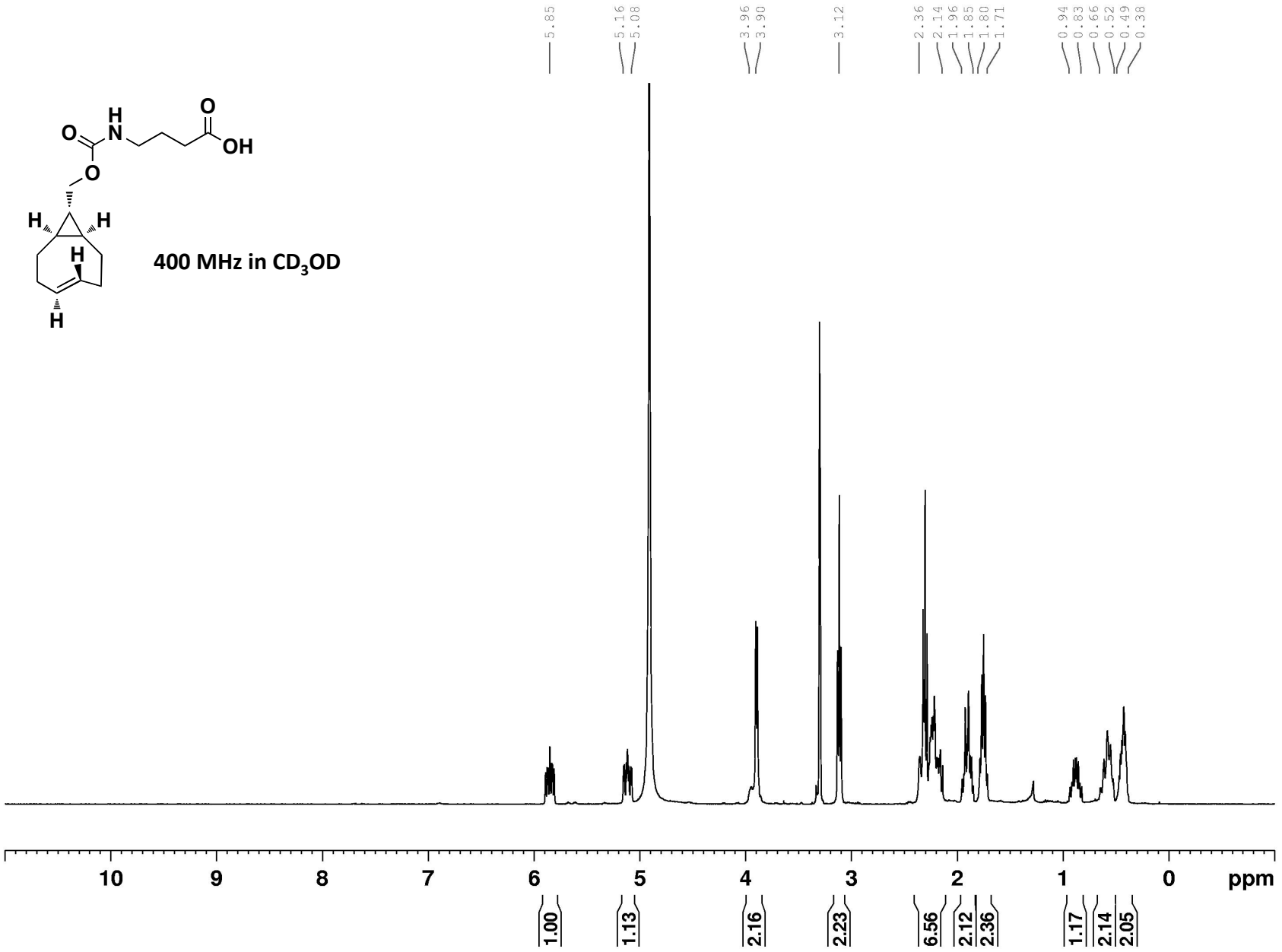
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0.83
0.66
0.52
0.49
0.38
400 MHz in CD3OD
10
9
8
7
6
5
4
3
2
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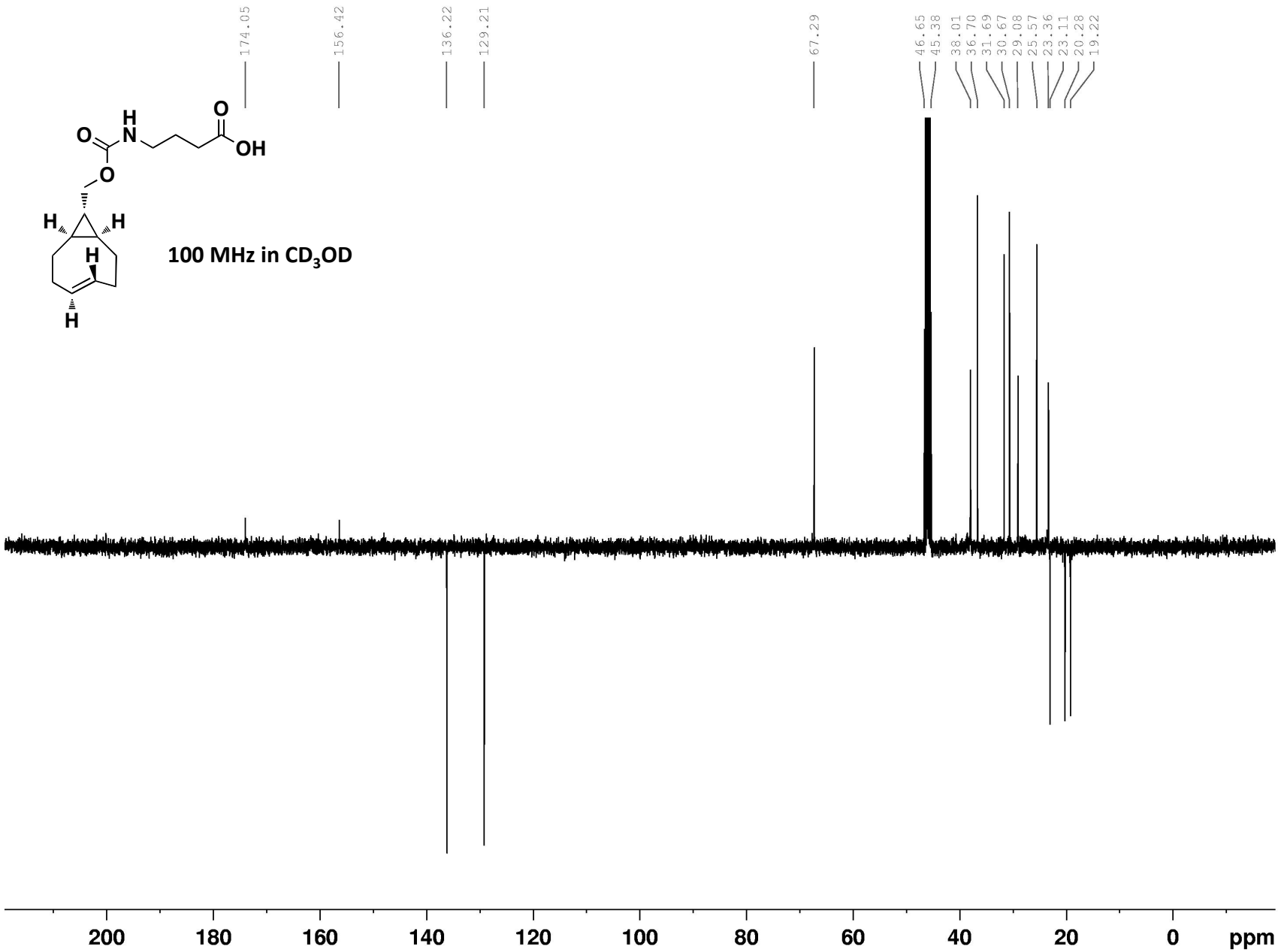
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23.36
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100 MHz in CD3OD
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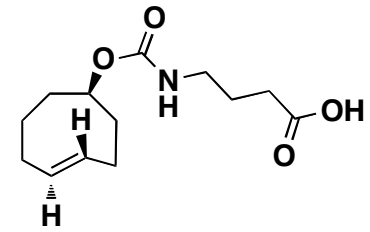

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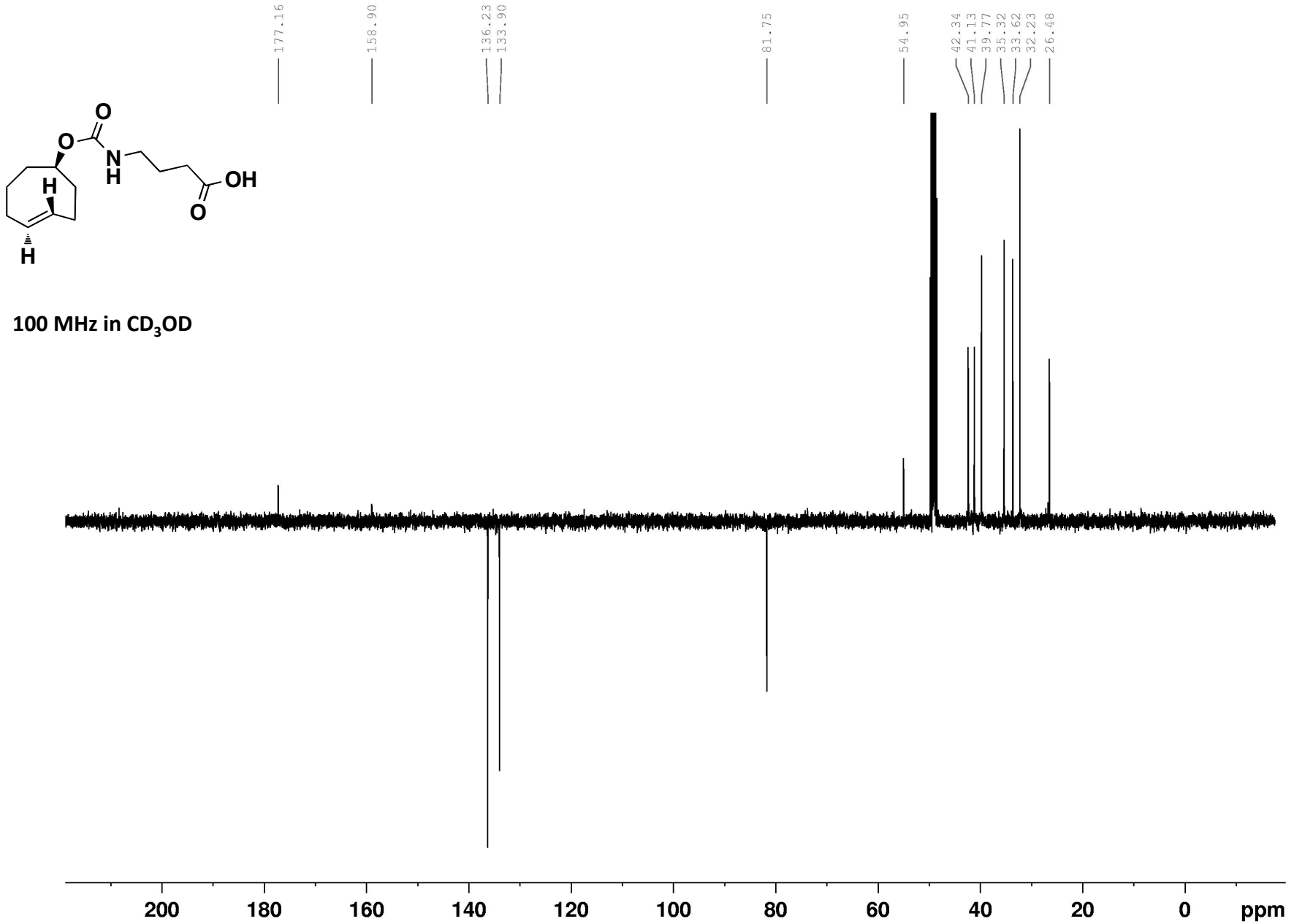
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O
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100 MHz in CD3OD
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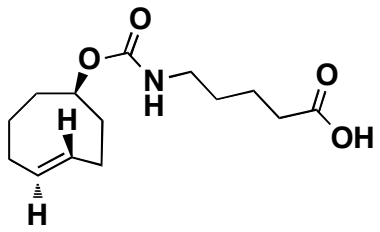

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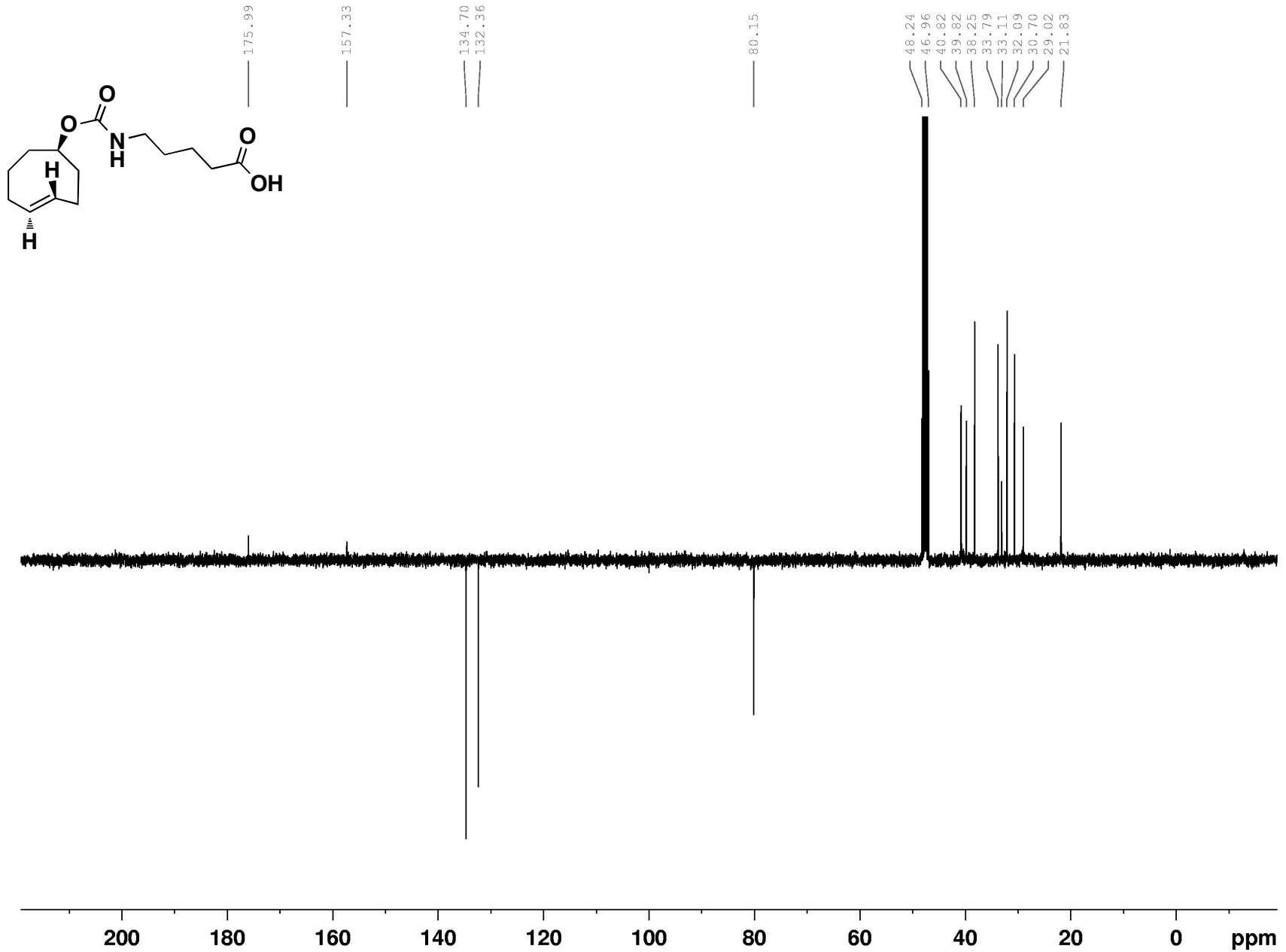
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O
O
N
H
O
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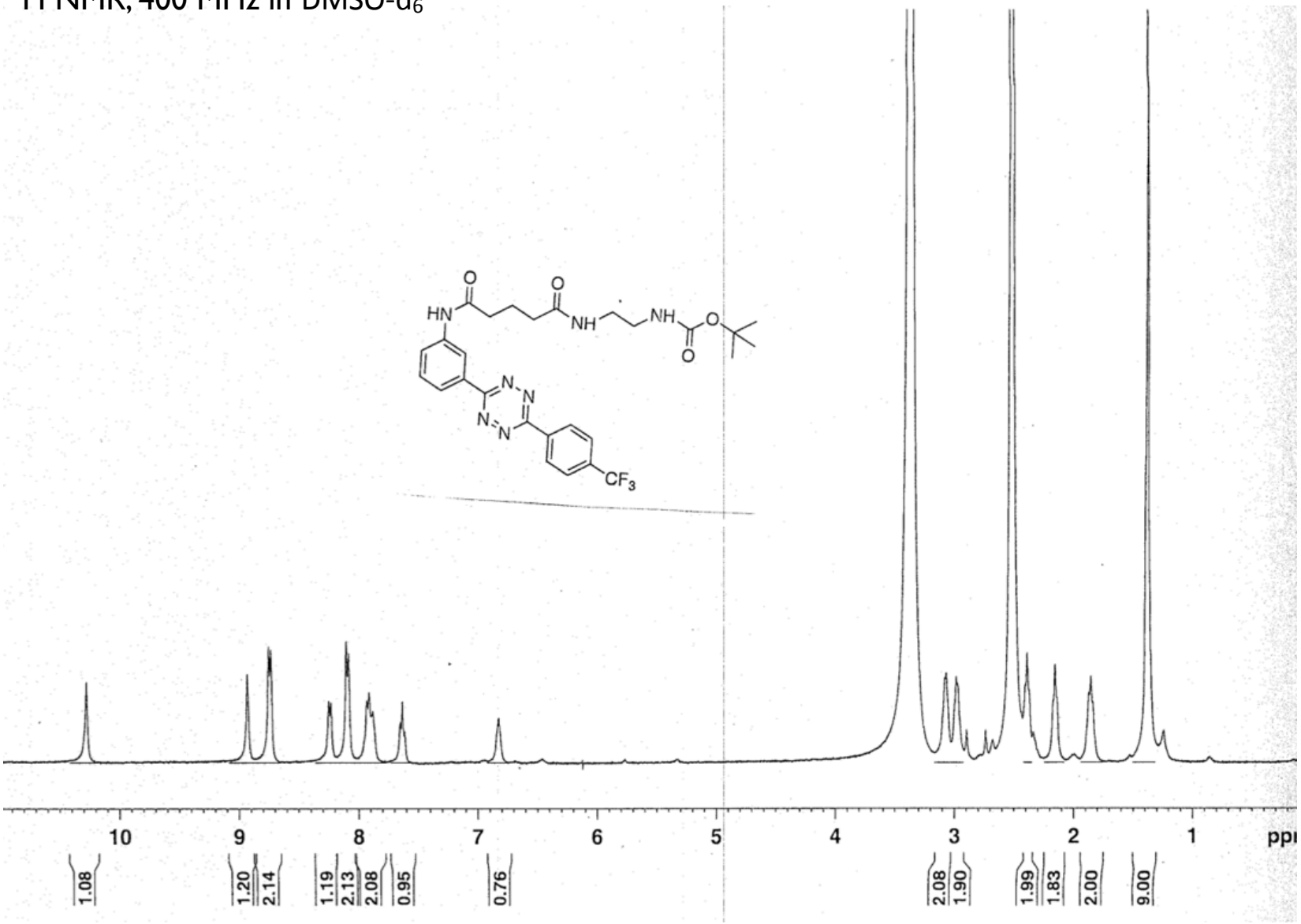
^{1}H NMR, 400 MHz in DMSO-d_6



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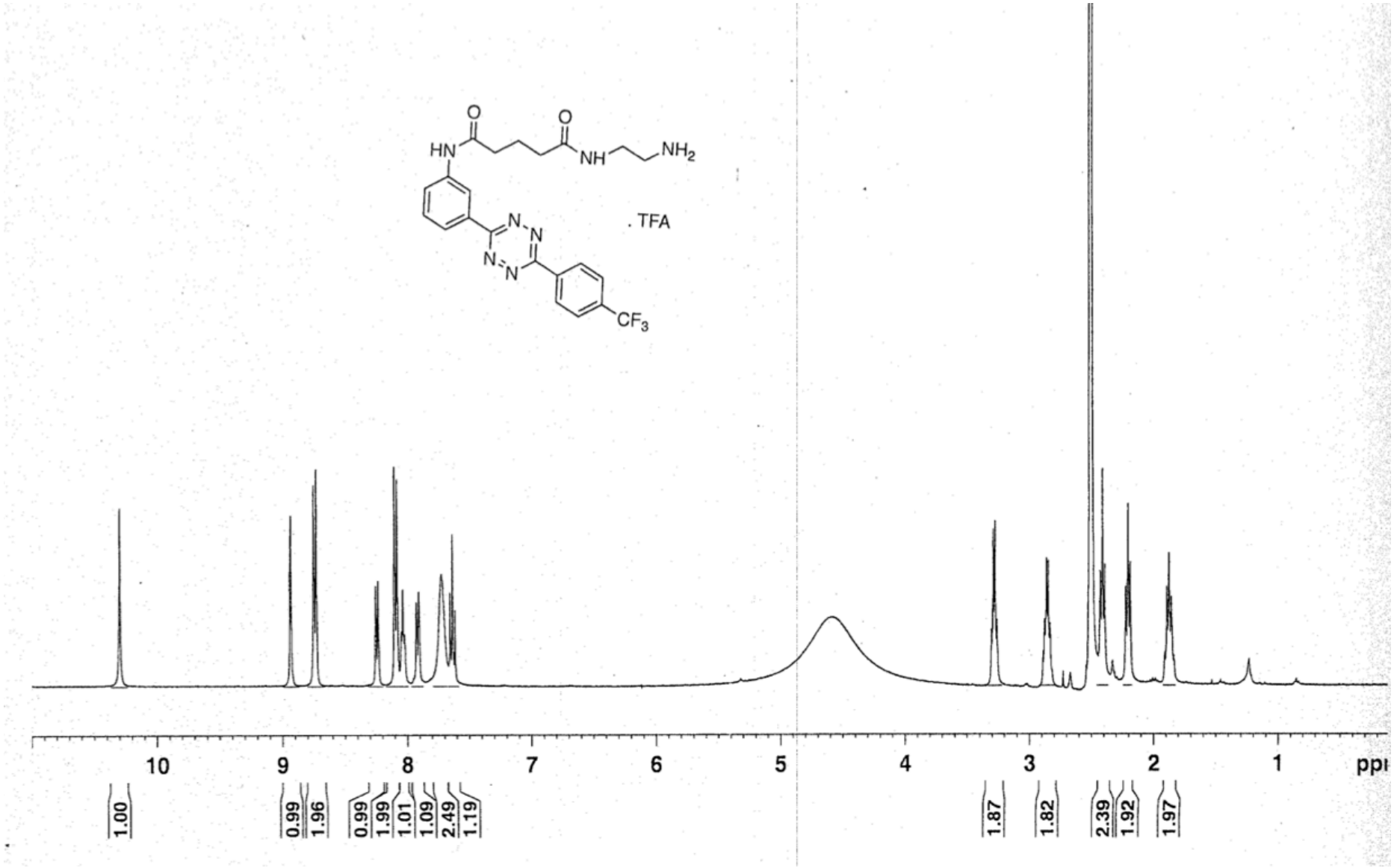

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