Supplementary Material for:

^{68}Ga Chelating Bioorthogonal Tetrazine Polymers for the Multistep labeling of Cancer Biomarkers

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MATERIALS AND METHODS

Chemical reagents

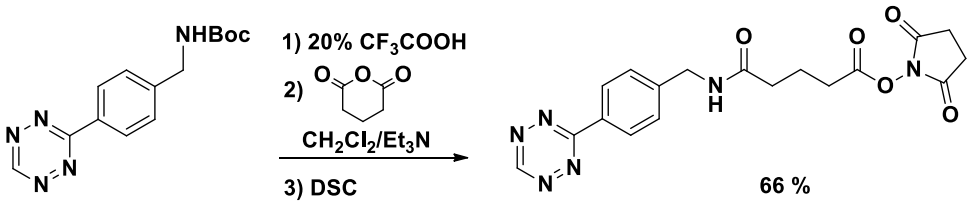
Chemical reagents were purchased from Sigma-Aldrich and used as received unless otherwise noted. Tert-butyl 4-(6-H-1,2,4,5-tetrazin-3- yl)benzylcarbamate and (E)-cyclooct-4-enol precursors were synthesized as previously described. [1, 3] Trypsin (0.05% T / 0.53mM EDTA) and L-Glutamine were purchased from Mediatech – VWR (San Diego, CA) and 10x PBS was purchased from Biomiga, Inc. (San Diego, CA). Penicillin/ streptomycin, Alexa Fluor 647 Carboxylic Acid Succinimidyl Ester, and Texas Red-X, Succinimidyl Ester, single isomer were all purchased from Life Technologies. Bisbenzimide H (Hoescht Stain) was purchased from Sigma Aldrich. Amino-terminated DTPA dextran was synthesized as previously described.[4]

Tissue culture/cell growth conditions

LS174T cells were grown in cDMEM media supplemented with 10% fetal bovine

serum, 1% L-glutamine, 1% penicillin/streptomycin. Cells were incubated in 5.0% carbon dioxide, 95% humidity at 37 °C. Generally, cells were grown in T-75 tissue culture flasks, seeded at densities between 500,000 and 750,000 cells per flask (cells were quantified with the Life Technologies Countess automated cell counter). The cells were trypsinized with TrypLE Express and resuspended in cDMEM. Cells were allowed to incubate for two days before incubating with probes as described below.

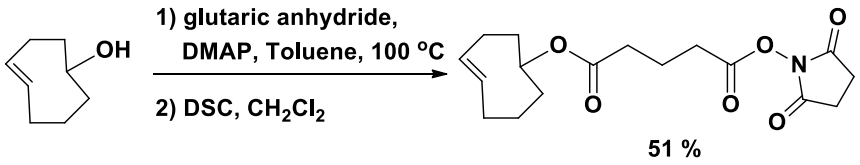
Synthesis of tetrazine NHS (2,5-dioxopyrrolidin-1-yl 5-((4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)-5-oxopentanoate)



CF_3COOH (0.25mL) was added to a stirred solution of tert-butyl 4-(6-H-1,2,4,5-tetrazin-3-yl)benzylcarbamate (10.0 mg, 0.033 mmol) in CH_2Cl_2 (1.0 mL) at room temperature. The resulting solution was stirred 2.0 hours at room temperature and then evaporated to afford (4-(6-H-1,2,4,5-tetrazin-3-yl)phenyl)methanamine TFA salt. The resulting salt was dissolved in CH_2Cl_2, after which Et3N (10.0 mg, 0.10 mmol) was added, followed by glutaric anhydride (4.0 mg, 0.033 mmol). This resulting solution was stirred for 1 hour at room temperature after which N,N'-disuccinimidyl carbonate (13.0 mg, 0.05 mmol) was added. The reaction solution was stirred at room temperature for 1 hour and then evaporated. The residue was purified by preparative TLC (Hexanes:EtOAc=3:1) to afford 9.0 mg product as a pink solid, in 66% yield. 1H NMR (400 MHz, CDCl3) 1H NMR (400

MHz, CDCl3) δ 2.15-2.18 (2H, m), 2.41 (2H, t, J = 8 Hz), 2.70 (2H, t, J = 8 Hz), 2.84 (4H, bs), 4.57 (2H, d, J = 8 Hz), 6.48 (1H, bs), 7.52 (2H, d, J = 8 Hz), 8.58 (2H, d, J = 8 Hz), 10.21 (1H, s); 13C (100 MHz, CDCl3) δ 21.15, 25.81, 30.10, 34.52, 43.49, 128.81, 128.83, 144.15, 158.01, 166.49, 168,55, 169.55. HRMS [M+Na]+ m/z calcd. for [C18H18N6O5Na]+ 421.1231, found 421.1229.

Synthesis of *trans*-cyclooctene NHS ((E)-cyclooct-4-en-1-yl (2,5-dioxopyrrolidin-1-yl) glutarate)



DMAP (6.1 mg, 0.05 mmol) was added to a stirred solution of (E)-cyclooct-4-enol (5.0 mg, 0.040 mmol) in toluene (1.0 mL), followed by glutaric anhydride (6.0 mg, 0.05 mmol). The resulting reaction solution was heated to 100^{o}C and stirred at this temperature for 18 hours. After TLC indicated that the reaction had finished the solvent was evaporated and the residue was dissolved in CH_2Cl_2, followed by addition of N,N'-disuccinimidyl carbonate (13.0 mg, 0.05 mmol). After stirring at room temperature for 30 minutes, the reaction solution was evaporated and the residue was purified by preparative TLC (Hexanes:EtOAc=2:1) to afford 7.0 mg product as a colorless liquid, in 51 % yield. 1H NMR (500 MHz, CDCl3) δ 1.59-1.71 (2H, m), 1.89-2.05 (6H, m), 2.30-2.40 (6H, m), 2.68 (t, J = 10 Hz, 2H), 2.83 (4H, bs), 4.42-4.45 (1H, t, m), 5.46-5.60 (2H, m); 13C (100 MHz, CDCl3) δ 20.05, 25.80, 30.28, 31.18, 32.72, 33.30, 34.46, 38.81, 41.10, 80.64, 133.27, 135.13, 168.32, 169.27, 171.95; HRMS [M+Na]+ m/z calcd. for [C17H23NO6Na]+ 360.1418, found 360.1419.

Synthesis of Tetrazine DTPA Dextran used for ^{68}Ga labeling and in vivo experiments

The required aminated tetrazine DTPA Dextran precursor was prepared from amino-terminated Dextran DTPA (synthesized as previously described from dextran T10, final molecular weight approximately 16 kDa determined by dynamic light scattering)[4] and amine reactive tetrazine-NHS. One equivalent of DTPA Dextran was reacted with 10 equivalents of tetrazine-NHS for two hours at 21°C in 0.1M NaHCO3 buffer (pH 8.5). After reacting for 2 hours, the dextran was washed with Milli-Q deionized water 3 times using 3 kDa Amicon centrifuge filters. Tetrazine loading was quantified using the characteristic visible absorption band of tetrazine at approximately 530 nm. The tetrazine DTPA Dextran was subsequently reacted with an excess of acetic anhydride (1000 equivalents) in deionized water for 1 hour to ensure capping of any free amines to prevent nonspecific in vivo uptake.[5] The resulting solution was washed with 500 μL of Milli-Q water 3 times using 3 kDa Amicon centrifuge filters. After the final wash, the solvent was evaporated and the dried dextran stored at -20°C until needed. HPLC spectrum with UV detection indicated that the Tetrazine DTPA dextran was highly pure. The absorbance peak of tetrazine for the conjugate proves successful coupling. The polydispersity index 0.38 was measured by Dynamic Light Scattering with a Malvern Zetasizer Nano ZS.

Synthesis of Alexa Fluor 647 (AF 647) Tetrazine DTPA Dextran

Amino-terminated DTPA Dextran was reacted at room temperature for 1 hour with 1 equivalent of Alexa Fluor 647 (AF 647) in 0.1M NaHCO3 buffer (pH 8.5).

The product was rinsed with Milli-Q water 3 times using a 3 kDa Amicon centrifuge filter. The AF 647 DTPA Dextran was then reacted with tetrazine NHS as described above.

Synthesis of anti-A33 *trans*-cyclooctene (TCO)

Antibody *trans*-cyclooctene conjugates were synthesized as previously described.[2] 100 μg of Anti-A33 monoclonal antibody (R&D Systems) was dissolved in 100 μL of a 90:10 mixture of 0.1 M NaHCO3 (pH 8.5) and anhydrous DMF. 50 equivalents of *trans*-cyclooctene NHS was added and the reaction mixture was gently shaken for two hours. The resulting solution was centrifuge filtered three times, using a 30 kDa Amicon filter and 90:10 $NaHCO_3$/ anhydrous DMF mixture as a wash. The antibody conjugate was resuspended in 0.1M $NaHCO_3$ at 1 mg/mL concentration (determined by monitoring the absorbance at 280 nm) and stored at 4°C. The equivalents of reactive TCO per anti-A33 antibody were quantified to be approximately 5.3 per antibody. This was determined by reaction with excess tetrazine Cy3 fluorescent probe (purchased from Click-Chemistry tools item #1018-1) , followed by purification by centrifugal filtration through a 30 kDa Amicon filter and determining the number of bound Cy3 probes using visible absorption spectroscopy (Figure S3). Attempts to characterize the TCO modification of this particular antibody using MALDI but were unsuccessful.

Fluorescence Microscopy

LS174T human colon cancer cells were incubated for two days in a Lab-Tek chamber slide maintained in cDMEM medium. Cells were treated with 200 nM

anti-A33 *trans*-cyclooctene in cell growth media and incubated for 30 minutes at 37°C. The media solution was aspirated and the cells were washed 3 times with cDMEM. After reincubation in cDMEM, the cells were treated with 10 μM of AF 647 tetrazine DTPA dextran and incubated for 30 minutes at 37°C. The media solution was aspirated, the cells were washed 3 times with cDMEM and reincubated in 200 μL cDMEM. To these cells, 200 μL of 2 μM Hoescht stain was added and incubated for 20 minutes at 37°C before imaging. All photos were collected with a 100x objective with a numerical aperature of 1.46 on a Zeiss AxioObserver Z1 inverted fluorescence microscope fitted with an ORCA-Flash 4.0 CMOS camera from Hamamatsu. The light source was a mercury arc-lamp from Sutter and images were processed using ImageJ 1.45j software package.

Radiochemistry

0.79 mg tetrazine DTPA dextran was dissolved in 300 μL 2M Sodium Acetate buffer solution (pH = 8.5). The dextran concentration for this stock solution was approximately 1.6×10^{-4} M. Caution: ^{68}Ga is radioactive and all procedures should be performed behind lead shields and by trained personnel equipped with radiation dosimetry monitoring badges. Radioactivity was measured on a Capintec CRC-15W dose calibrator. A ^{68}Ga generator (Eckert & Ziegler Isotope Products IGG100) was eluted with 5 mL 0.1 M HCl. A 1.5 – 3 mL portion (~800 μCi) of the eluate was collected into an 8 mL multipurpose polypropylene tube. 100 μL of tetrazine DTPA dextran stock solution was added to the ^{68}Ga containing vial. The mixture was shaken briefly and then incubated at room temperature for 15 minutes. The radiochemical purity (>99%) was confirmed by

instant thin layer chromatography. This value was calculated as RCY = RCP * Product Activity / Activity added, where RCP is the Radiochemical Purity. The RCP was calculated through standard Instant Thin Layer Chromatography technique. This RCY was corrected for decay.

Multistep labeling of LS174T colon cancer cells with ^{68}Ga tetrazine dextran

LS174T cells were checked for confluency, and then detached using trypsin/EDTA. The cells were split into 1 mL 130,000 cell aliquots in 1.7mL sterile eppendorf tubes. Cell aliquots were incubated with either 200 nM of transcyclooctene anti-A33 (n=3) or a control antibody consisting of 200 nM unmodified anti-A33 (n=3) for 1.5 hours at 37°C. After incubation the cells were pelleted and washed with 500 μL of PBS 3 times. The cells were dispersed in 100 μL of PBS and were treated with 40 μCi of 68Ga tetrazine dextran for 1 hour. The cells were pelleted and washed three times with 500 μL of PBS containing 2% FBS. The radioactive supernatant was removed via pipette and put into plastic scintillation vials behind lead shielding for decay. The radioactivity bound to the cells was determined on a Beckman Gamma 9000 well counter.

Animals

Four female Swiss Webster mice (27-33 g, 8 weeks old) and five female nude mice (19-21 g, 6 weeks old) were purchased from Charles River Labs. The mice were maintained at the animal facilities of John and Rebecca Moores Cancer Center upon arrival. All in

vivo procedures used in this study were approved by the University of California, San Diego, Institutional Animal Care and Use Committee review board. Mice were anesthetized with isoflurane prior to probe injection and imaging.

Human Plasma Stability

Human whole blood was centrifuged at 3200 rpm(2000 g) for 15min. The top layer of plasma was then immediately transferred to a conical vial. 100 μL DTPA-Tetrazine-T10 (0.14 nmol) was incubated with 1000 μl (~1mCi ^{68}Ga) at room temperature for 15 min. The product was centrifuged and washed with a 3K Amicon filter at 3000 rpm for 10 min. The retentate was brought to 1.1 ml with PBS and the the ^{68}Ga- DTPA-Tetrazine-T10 (200 μL) was incubated with 2.0 mL human blood plasma at 37°C. At 30 min, 1h, 2h, 3h post incubation, 0.1 mL of the mixture was removed for ITLC. (Solid phase: Whatman 31 ET strips; mobile phase: Acetone).

Positron Emission Tomography (PET) Imaging

Nuclear imaging was performed on a GE healthcare eXplore VISTA dual-ring small-animal PET Scanner. Doses of ^{68}Ga tetrazine DTPA dextran (2.2 nmol in 150 μL, approximately 30-70 μCi) were injected into mice through the tail vein. PET acquisitions were conducted in two beds static emission mode with a 400-700 keV energy window. All mice were scanned three times at 10, 30 and 60 minutes after injection. Immediately after their third scan, mice were euthanized with CO_2. Blood, liver, spleen, kidneys and intestine were harvested and weighed after which their radioactivity was determined on a

Beckman Gamma 9000 well counter. For A33/TCO in vivo conjugation experiments, PET acquisitions were performed approximately 1 hour after injection.

Adapting a previously published protocol[2], approximately 10^6 LS174T cells were injected subcutaneously in nude mice and allowed to grow for 2 weeks. For LS174T xenograft imaging a tail vein injection of 0.2 nmol (~30 μg) *trans*-cyclooctene modified fluorescent antibody (anti-A33 coupled with Alexa Fluor 750: Ex: 749 nm; Em: 775 nm) was administered using a 28 gauge insulin syringe. After 24 hours, 100 μL ($1.6x10^{-4}$ M) of tetrazine-DTPA dextran stock solution was mixed with 300 μL of Ga-68 eluate (~2 mCi) and the mixture incubated at RT for 15min. The solution was brought to 1.0 mL with 600 μL PBS. 2.2 nmol(~150μL, 300μCi)of ^{68}Ga-tetrazine-DTPA dextran was injected through tail vein. 1 hour post injection, the mouse was scanned in whole body static mode with 2 beds (6 min per bed) positioning. After scanning, the animal was sacrificed and dissected to determine %ID/gram for tumor versus muscle tissue. Finally, a Fluobeam 800 was used to obtain fluorescence images of the tumor and muscle tissue.

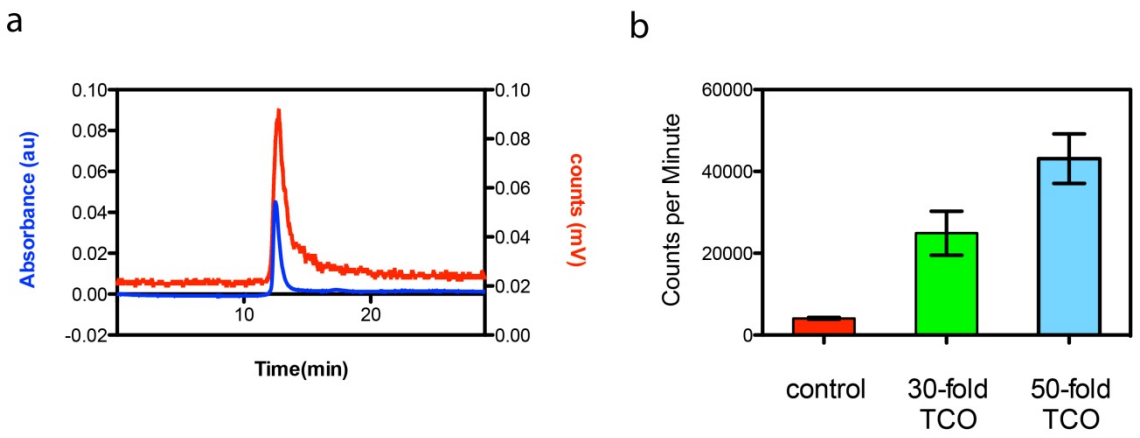


Figure S1. a) Size exclusion High Performance Liquid Chromatography of Ga-68 labeled tetrazine DTPA dextran. The blue trace shows the absorbance at 226 nm from a UV detector and the red trace shows the counts from a radioactivity detector. b) *In vitro* radiolabeling labeling of *trans*-cyclooctene (TCO) modified LS174T cells (n=3) with ^{68}Ga tetrazine DTPA dextran.

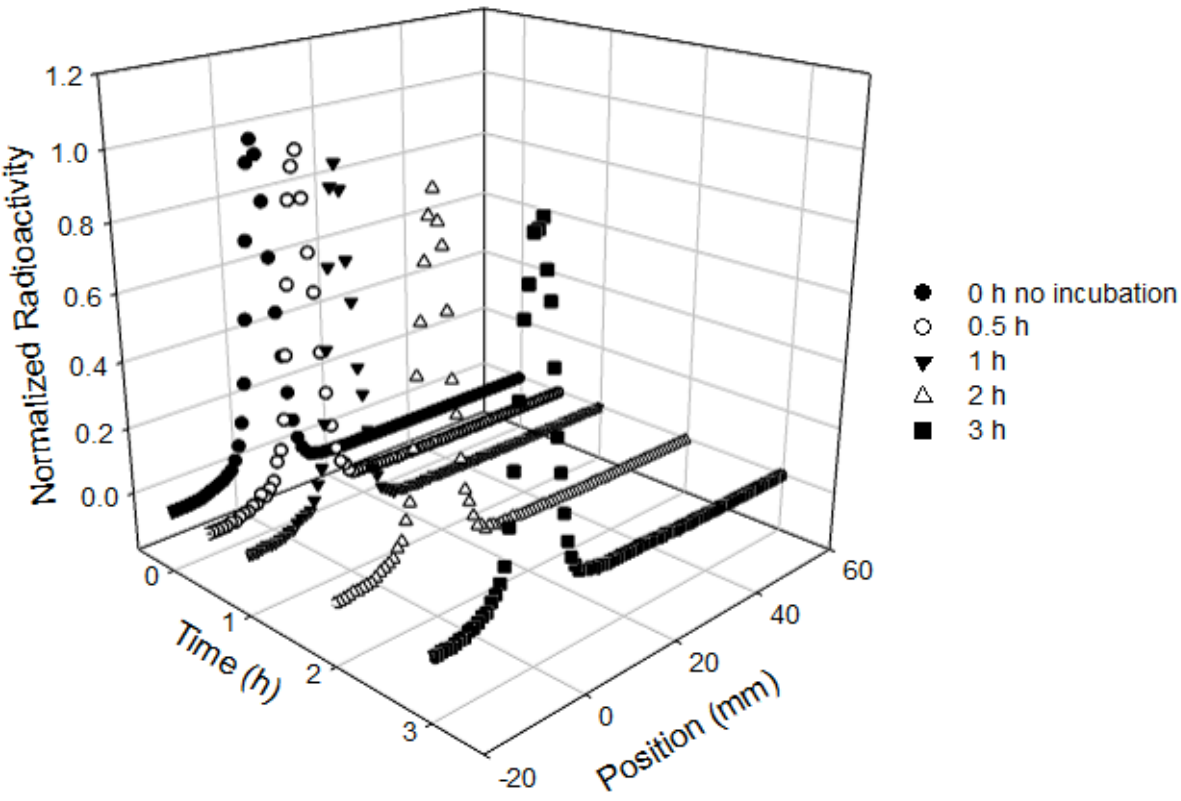


Figure S2. Blood stability time traces. Radioactity stayed at origin for samples obtained before incubation (solid circle) and 0.5 h(hollow circle), 1 h(solid triangle), 2 h(hollow triangle) and 3 h(solid square) after incubation with human plasma.

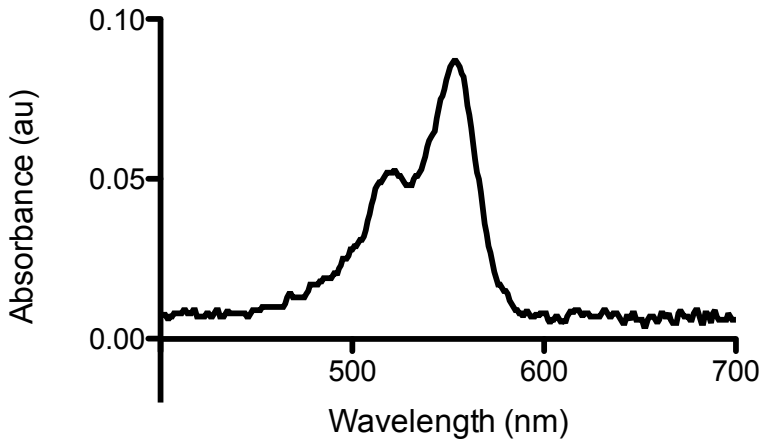


Figure S3. Absorption spectra of TCO modified anti-A33 monoclonal antibodies after subsequent tagging with a Cy3 labeled tetrazine fluorophore. The absorbance data was used to determine that there are approximately 5.3 tetrazine reactive TCO dienophiles per monoclonal antibody.

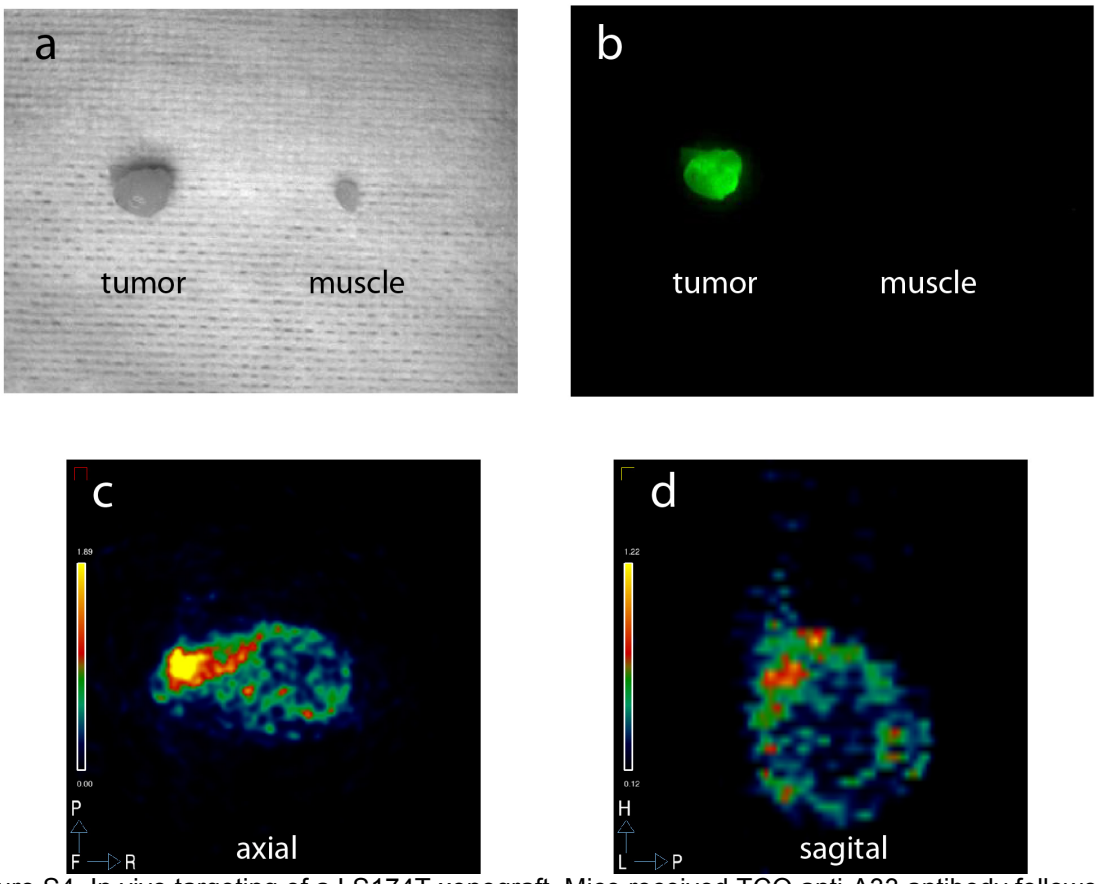
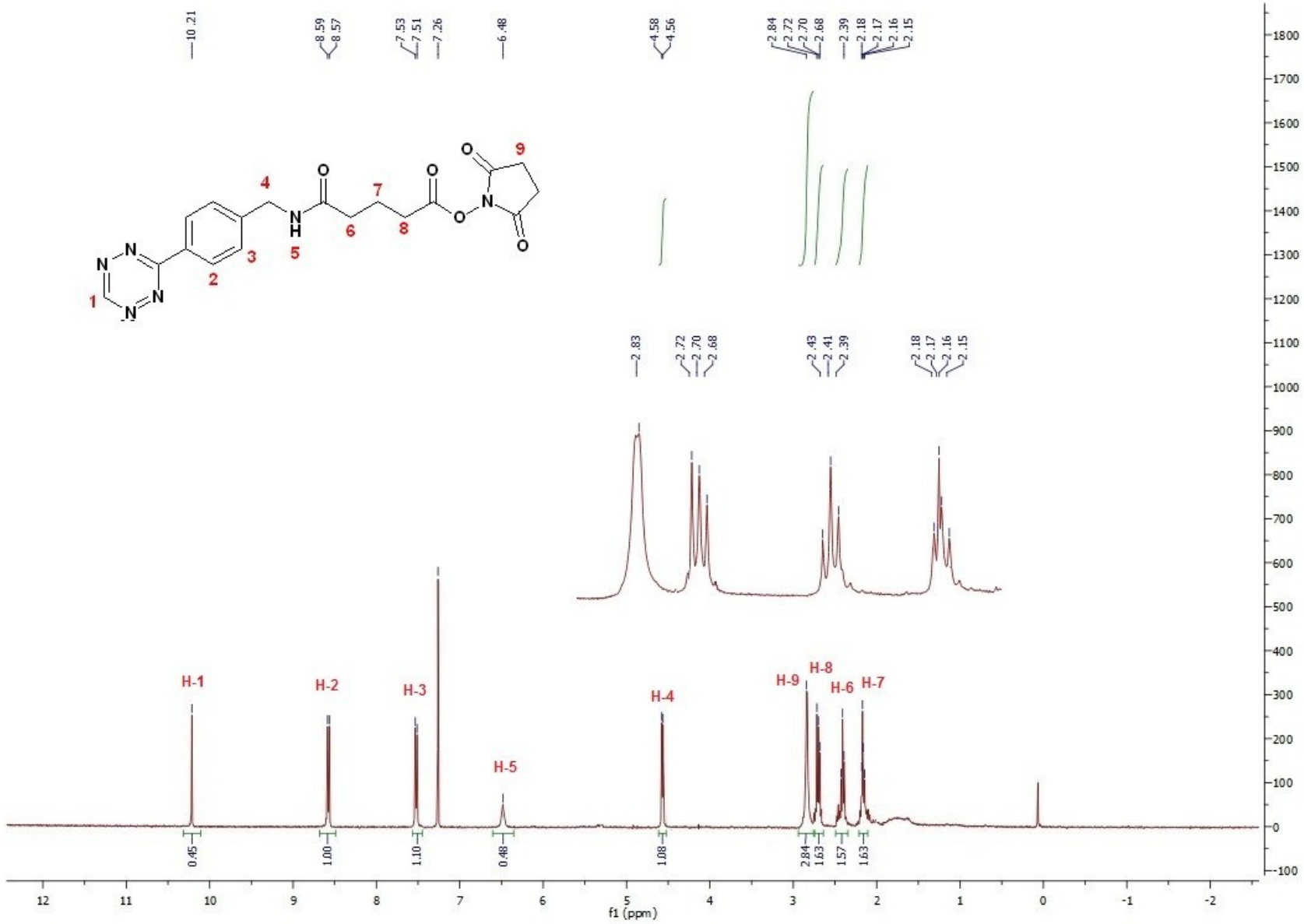


Figure S4. In vivo targeting of a LS174T xenograft. Mice received TCO anti-A33 antibody followed by ^{68}Ga dextran tetrazine (see above for experimental details). Fluorescence images were taken after sacrifice and excision of tissue. PET images were taken in vivo. A) Brightfield image of excised tumor and muscle tissue. B) Fluorescence (ex. 780 nm em. 800 nm) image of the same tumor and muscle tissue demonstrating significant uptake of TCO antibody into the LS174T xenograft. C) Axial slice of xenograft in a live mouse after injection of ^{68}Ga dextran tetrazine. D) Sagital slice of xenograft in a live mouse after injection of 68Ga dextran tetrazine. Tumor/Muscle %ID/g ratio was 3.9 ± 1.8.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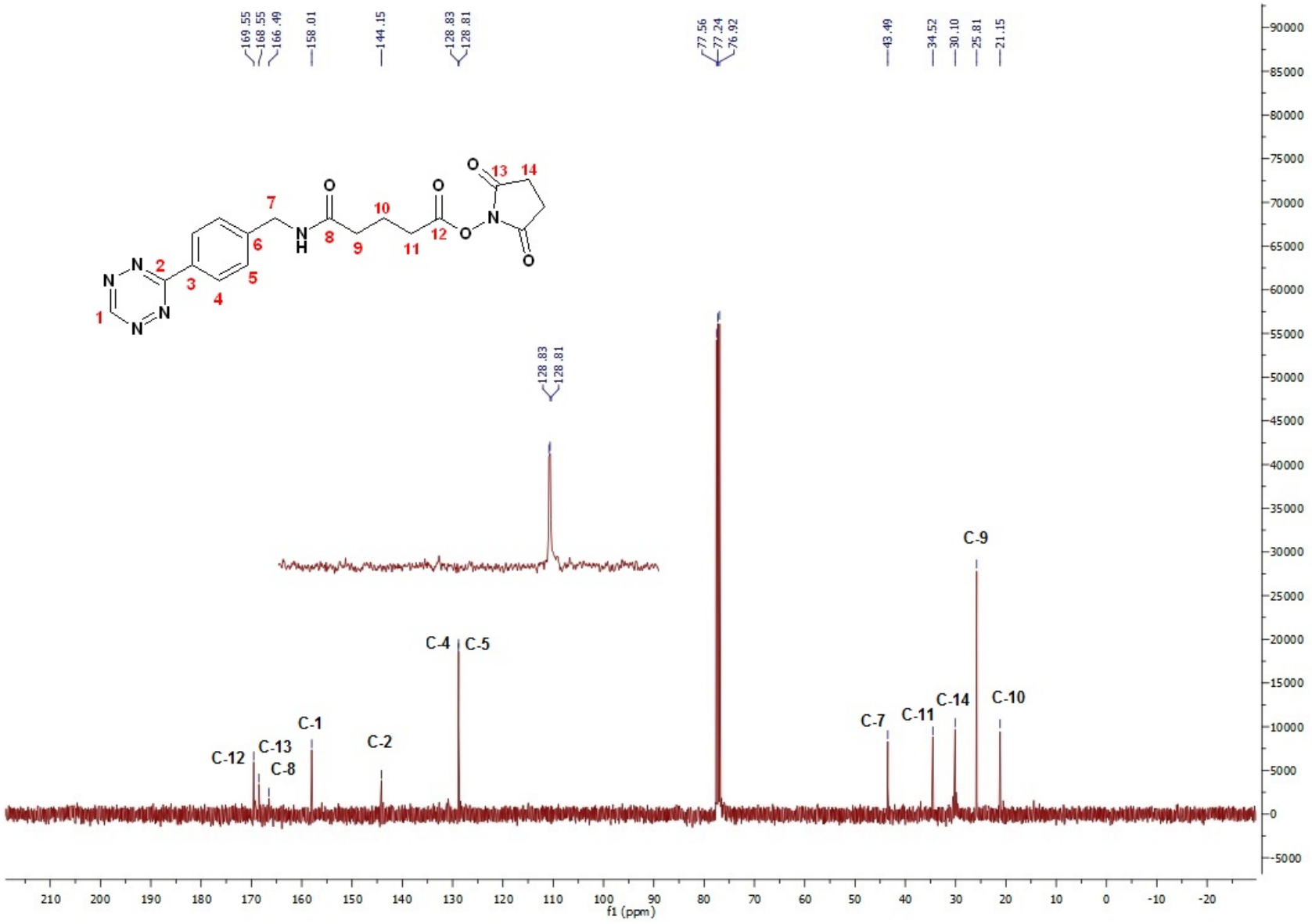
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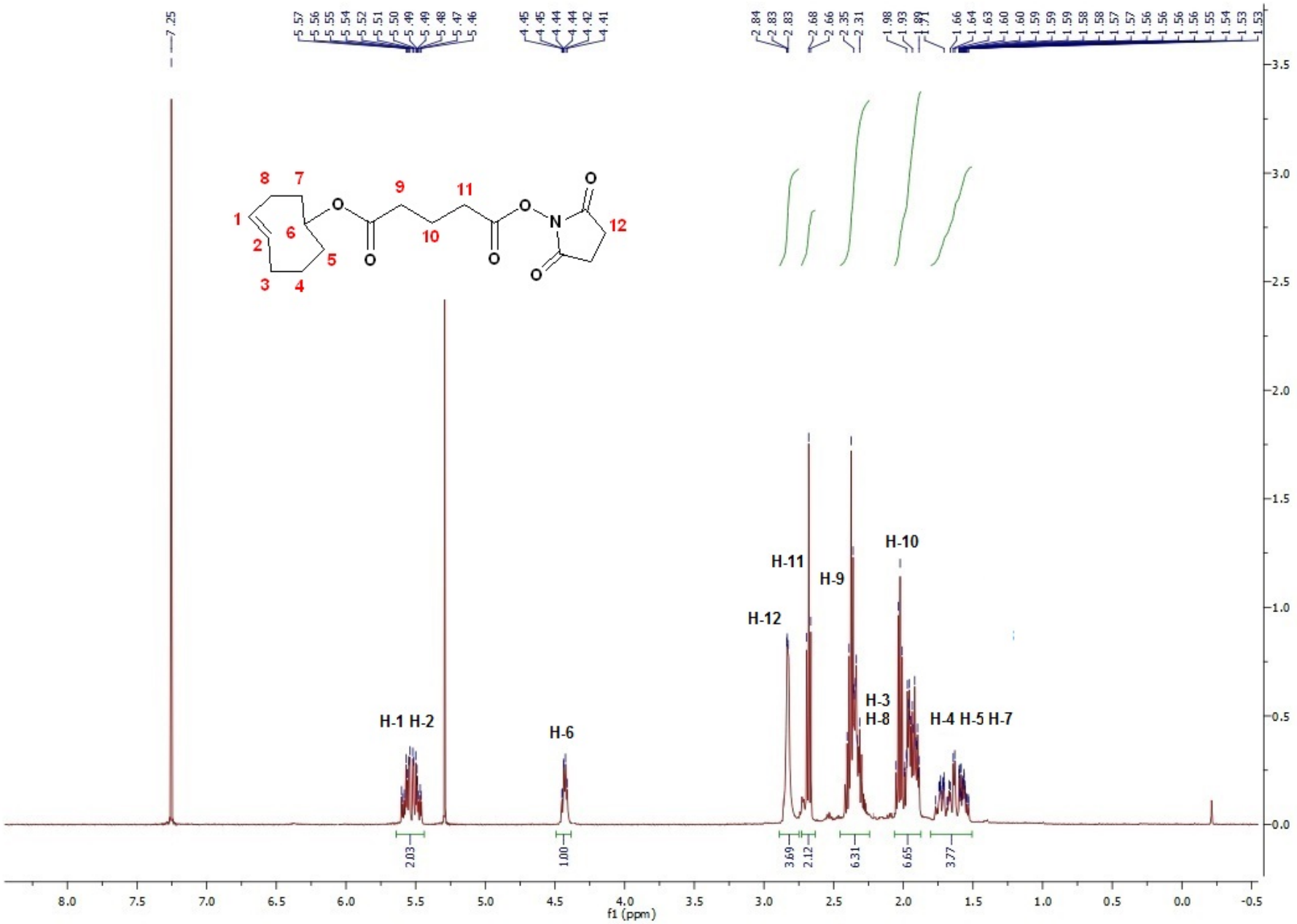
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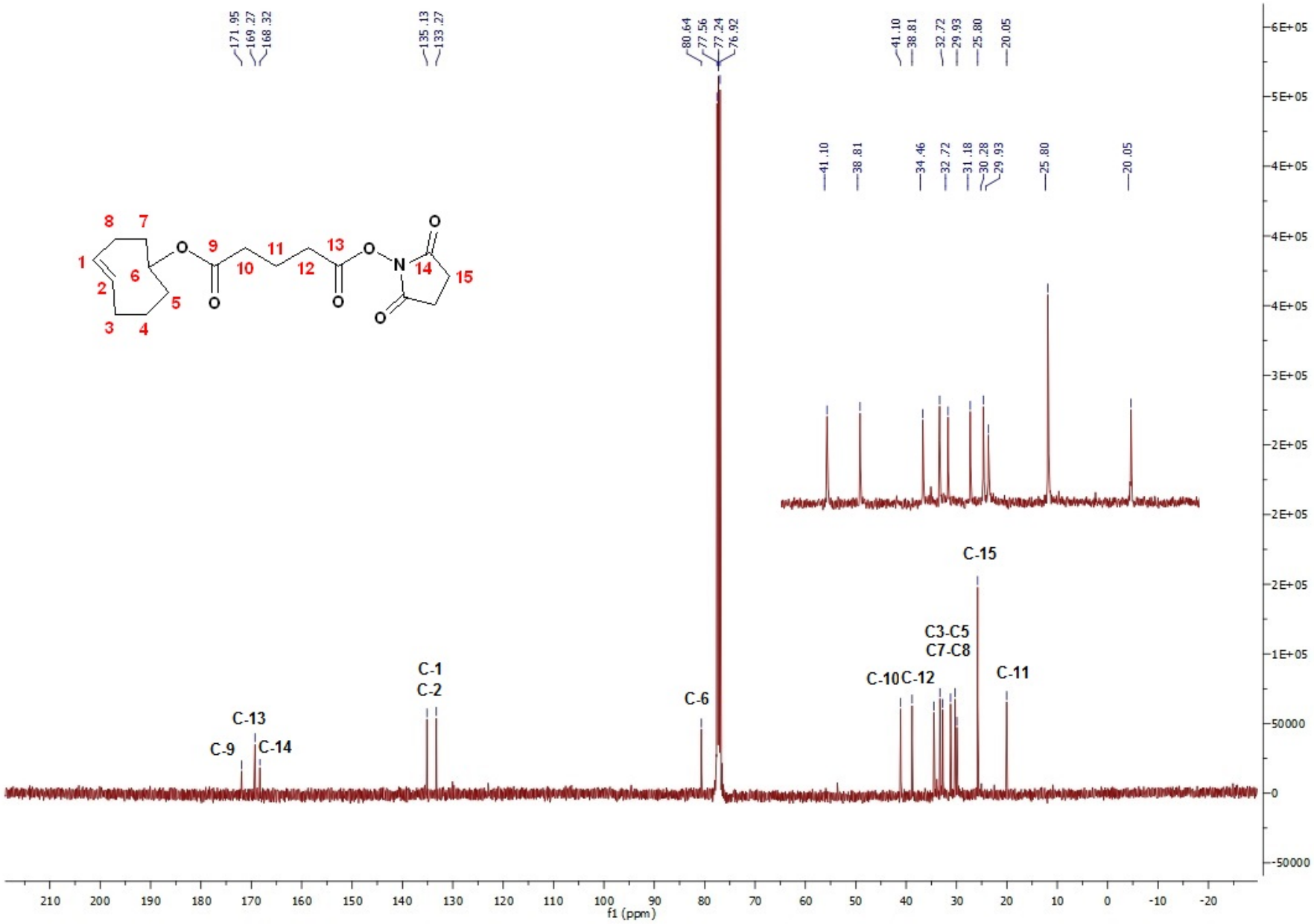
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References

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