Supporting Information

Synthesis and Initial Evaluation of Radioactive 5-I-α-Methyl-Tryptophan: A Trp Based Agent Targeting IDO-1

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General

Methods: Proton and carbon-13 NMR spectra were obtained on a 600 MHz or 500 MHz Bruker Avance III spectrometer. Spectra are referenced using residual protiated solvent signals or inherent carbon-13 signals ($CDCl_3$: 7.26 ppm for proton NMR spectra and 77.16 ppm for carbon-13 spectra. D_2O: 4.79 ppm for proton spectra.)[1] Mass spectra were obtained on an Advion Expression L mass spectrometer. Semi-preparative and analytical HPLC were performed on a Shimadzu LC-20AT Liquid Chromatograph.

Materials: Chemicals and solvents were used as received from the vendors (Millipore Sigma, Alfa-Aesar, or Fisher Scientific). Tetrahydrofuran was dried using an Inert Technologies solvent purification system. ^{124}I-Sodium iodide was purchased from 3D Imaging, LLC (Little Rock, Arkansas, USA).

Animal Models: All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the University of North Carolina at Chapel Hill and Experiments were approved by the by the University of North Carolina Institutional Animal Care and Use Committee. Mouse Melanoma cells line B16F10 was obtained from American Type Culture (Manassas, VA, USA)

[1] Gottlieb, H.E.; Kotlyar, V.; Nudelman, A. *J. Org. Chem.* **1997**, *62*, 7512-7515.

and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Omega Scientific, Tarzana, CA, USA). The cells were maintained at 37˚C in an atmosphere containing 5% CO2. Tumor model was established in 4- to 6- week old female C57BL6 mice (Charles River Laboratory) or NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice (NOD scid gamma (NSG™) mice). The tumor cells were suspended in PBS (2×10^6/ml) and mixed with Matrigel at 1:1 (vol: vol) ratio. Then 100 µL cell suspension was inoculated subcutaneously at the right flank. The mice were used for small animal PET imaging studies when the tumor size greater than 0.6 cm in diameter (8-10 days after cell inoculation).

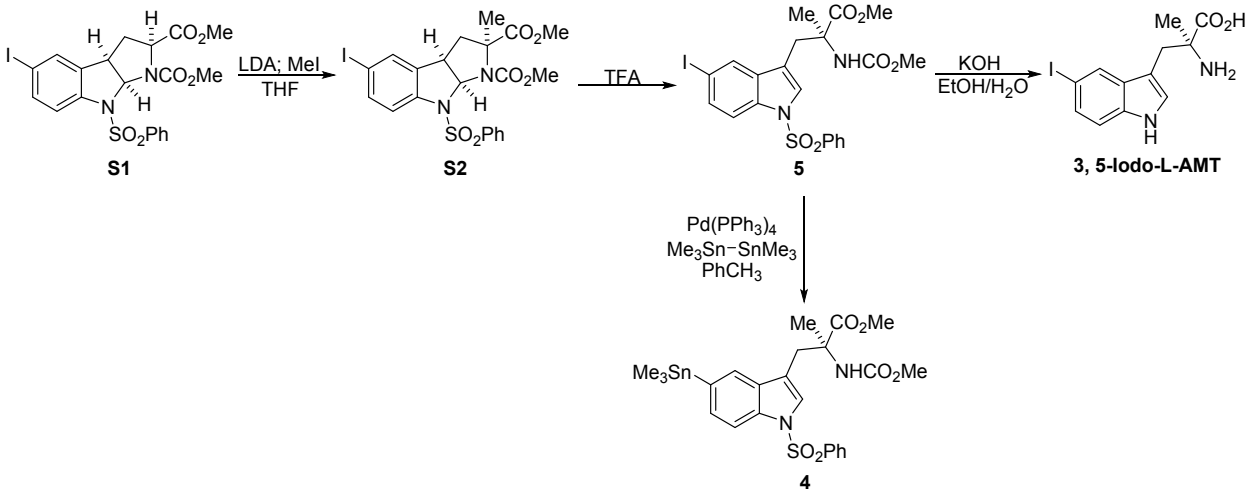
Small animal PET imaging: Mice were fasted for six hours prior to the PET studies to ensure stable plasma tryptophan and large neutral amino acid levels during the study. About 100-200 µCi dose of ^{124}I-5-I-AMT was intravenously injected into each mouse under isoflurane anesthesia (2-4% for induction and 2% for maintenance in 100% oxygen). Static scans were acquired at 0.5, 1.4 and 20.0 hours post-injection. The images were reconstructed by 2-dimensional ordered-subsets expectation maximum.

IDO1 Enzyme Assay: The IDO1 enzyme assay was performed in a 100 µL reaction system as described (PMID: 28529635). The assay buffer used in this reaction contained 50 mM K_2HPO_4 (pH6.5), 50 mM KH_2PO_4 (pH 6.5), 20 mM l (+)-ascorbic acid (pH 7.0), 10 mM methylene blue, and 100 µg/mL catalase from bovine liver. In each reaction, testing compound was incubated with five µM recombinant human IDO-1 (rhIDO; Sino Biological Inc) in the assay buffer at 37 °C for 1 h. Cl_3CCOOH (6.1 N, 100 µL) was then added. The reaction mixture was heated at 50 °C for 15 min, and centrifuged to harvest the supernatant. The equal volume of p-dimethylaminobenzaldehyde (DMAB; 2 g/ml) in 95% ethanol and concentrated HCl was added to the supernatant. The absorbance derived from kynurenine (Kyn) was measured at 490 nm with a VersaMaxSK microplate reader (Molecular Devices). L-tryptophan (20 mM, 2µL) was used as natural substrate for IDO1 as positive control. The experiment was performed in triplicate and repeated 3 times.

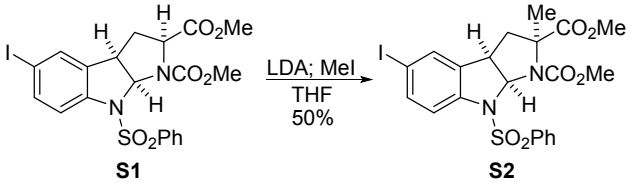
Immunohistochemistry analysis: Immunohistochemistry analysis was performed in in formalin-fixed, paraffin-embedded B16F10 tumor and spleen harvested from NSG and C57BL6 mice using an INDO polyclonal antibody (Product # PA5-24598; Thermofishers; 1:100 dilution), followed by HRP-conjugated secondary antibody and DAB staining. The images were viewed and exported using Aperio eSlide Manager.

Compound Synthesis and Characterization Data

Synthesis of 5-Iodo-L-α-Methyl-Tryptophan and Stannane Precursor 4



Synthesis of S2

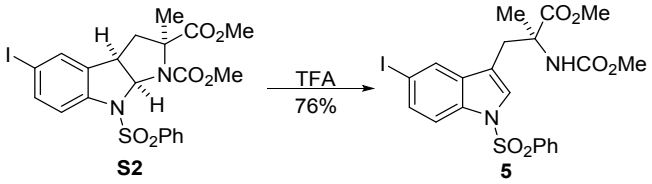


Compounds **S1 and S2** were prepared as previously described.[2] **S2** was synthesized using a minor modification to the reported procedure. Diisopropylamine (0.12 mL, 0.88 mmol, 1.6 eq) was dissolved in 5.5 mL of THF, placed under an atmosphere of nitrogen, and cooled to approximately -55 °C using a 50% aqueous methanol dry ice slush. n-Butyllithium (0.52 mL of a 1.6 M solution in hexanes, 0.83 mmol, 1.5

[2] Giglio, B.C.; Fei, H.; Wang, M.; Wang, H.; He, L.; Feng, H.; Wu, Z.; Lu, H.; Li, Z. *Theranostics* **2017**, *7*, 1524-1530.

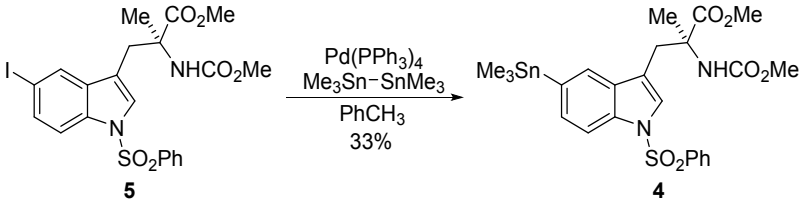
eq) was added dropwise, and the solution was allowed to stir for 10 minutes. **S1** (0.3 g, 0.55 mmol, 1 eq) was dissolved in THF (18 mL) and added slowly to the LDA solution. After 5 minutes, iodomethane (0.1 mL, 1.65 mmol, 3 eq) was added slowly. The 50% aqueous methanol/dry ice bath was then removed and replaced with an ice water bath. After 1 hour, TLC analysis indicated that the reaction was mostly complete. The reaction was quenched with 80 mL of saturated NH_4Cl, and further diluted with 50 mL of water. The THF layer which separated from the aqueous phase was collected, and the aqueous phase was extracted twice with EtOAc. The combined organic phases were washed once with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The crude produce was purified using silica gel chromatography (1:1 hexanes/EtOAc) to afford 0.1546g of **S2** in 50% yield. Physical and spectral characteristics matched those previously reported.[2]

Synthesis of S3



Compound **S2** (98 mg, 0.176 mmol) was dissolved in 1.8 mL of trifluoroacetic acid (TFA), and stirred at room temperature until TLC analysis indicated that the reaction was complete (approximately 1 hour). The TFA was removed via rotary evaporation. Residual TFA was removed from the crude product by codistillation with 5 mL of dichloromethane. Two codistillations were performed. The crude residue was purified by silica gel chromatography (2:1 hexanes/EtOAc) to yield 74.9 mg of compound **5** in 76% yield. Analytical data for **5**: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.79 (m, 2H), 7.76 (s, 1H), 7.71 (d, J = 9.2 Hz, 2H), 7.55 (m, 2H), 7.45 (m, 2H), 7.22 (s, 1H), 5.48 (br. s, 1H), 3.75 (s, 3H), 3.72 (s, 3H), 3.60 (d, J = 14.6 Hz), 3.22 (d, J = 14.6 Hz, 1H), 1.66 (s, 3H) . ^{13}C NMR (151 MHz, $CDCl_3$): δ = 173.98, 155.40, 137.84, 134.21, 133.77, 133.33, 129.50, 128.77, 126.77, 125.85, 117.08, 115.60, 87.65, 60.66, 53.12, 52.43, 30.92, 23.88. LRMS (ESI) Calc. for $[C_{21}H_{21}IN_2O_6S + H]^+$ = 557.02, Found 557.07.

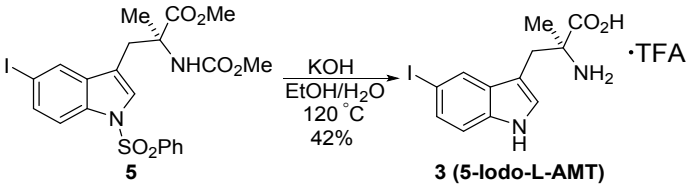
Synthesis of 4



Stannane **4** was synthesized using the palladium catalyzed stannylation procedure described by Neumaier.[3] Compound **5** (79.7 mg, 0.143 mmol, 1 eq), $Pd(PPh_3)_4$ (33.0 mg, 0.0286 mmol, 0.2 eq), and hexamethylditin (107.9 mg, 0.329 mmol, 2.3 eq) were dissolved in toluene (1.4 mL). After degassing with a gentle stream of nitrogen gas for 20 minutes, the reaction mixture was heated at 110 °C until TLC analysis indicated that all starting material was consumed (approximately 4 hours). The reaction mixture was diluted with ethyl acetate, filtered through Celite, and concentrated by rotary evaporation. Purification by silica gel chromatography (2:1 hexanes/EtOAc) afforded stannane **4** (28.5 mg, 33.5% yield).

Analytical data for **4**: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.94 (m, 1H), 7.84 (m, 2H), 7.55 (m, 2H), 7.44 (m, 2H), 7.39 (d, J = 7.6 Hz, 1H), 7.26 (s, 1H), 5.48 (br. s, 1H), 3.70 (s, 3H), 3.64 (s, 3H), 3.59 (d, J = 14.2 Hz, 1H), 3.31 (d, J = 14.7 Hz, 1H), 1.66 (s, 3H), 0.30 (m, 9H) . ^{13}C NMR (151 MHz, $CDCl_3$): δ = 174.16, 155.44, 138.17, 136.00, 135.23, 133.90, 131.87, 131.37, 129.36, 126.94, 126.84, 124.64, 117.45, 113.37, 60.46, 52.97, 52.18, 31.28, 29.84, 23.92, -9.20. LRMS (ESI) Calc. for $[C_{24}H_{30}N_2O_6SSn + H]^+$ = 595.09, Found 595.17.

Synthesis of 5-Iodo-L-α-Methyl-Tryptophan

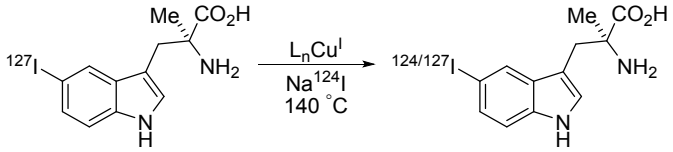


Hydrolysis of compound **4** was achieved using a modified version of Chakraborty's procedure.[4] Compound **5** (8.1 mg, 0.018 mmol) was placed into a 1 dram vial and dissolved in 0.25 mL of ethanol.

[3] Zlatopolskiy, B.D.; Zischler, J.; Krapf, P.; Zarrad, F.; Urusova, E.A.; Kordys, E.; Endepols, H.; Neumaier, B. *Chem. Eur. J.* **2015**, *21*, 5972.

Aqueous KOH (5N, 0.25 mL, 1.25 mmol, 69 eq) was added. The vial was sealed with a PTFE lined cap, and stirred at 130 °C for 30 minutes. After cooling to room temperature, the reaction was acidified with 0.25 mL of 50% (v/v) aqueous acetic acid, and then diluted to a total volume of 2 mL with 5% (v/v) aqueous acetic acid. The product was purified via semi-prep HPLC with UV detection (212 nm and 254 nm) using the following conditions: Column: ACE 5 AQ (250 x 10 mm); Solvent system: MeCN (0.1% TFA) and Water (0.1% TFA); Gradient: 5% MeCN held for 2 minutes, then ramped to 95% MeCN over 20 minutes; Flow rate: 3 mL/min. The product eluted from the column with a retention time of 15 minutes. Removal of the solvents by rotary evaporation and lyophilization afforded 2.8 mg of the trifluoroacetate salt of 5-Iodo-L-α-Methyl-Tryptophan (42% yield). Analytical data for **3**: ^{1}H NMR (500 MHz, D_2O): δ = 8.06 (d, *J* = 1.6 Hz, 1H), 7.53 (dd, *J* = 8.5 Hz, 1.7 Hz, 1H), 7.34 (d, *J* = 8.5 Hz, 1H), 7.27 (s, 1H), 3.45 (d, *J* = 15.5 Hz, 1H), 3.23 (d, *J* = 15.4 Hz, 1H), 1.63 (s, 3H). ^{13}C NMR (126 MHz, D_2O): δ = 175.28, 135.14, 130.10, 129.86, 127.29, 126.40, 117.48, 113.95, 105.83, 82.20, 61.37, 32.31, 27.75. ^{19}F{^{1}H} NMR (471 MHz, D_2O): δ = -75.59. LRMS (ESI) Calc. for $[C_{12}H_{13}IN_2O_2 + H]^+$ = 345.01, Found 345.08.

Radiochemistry



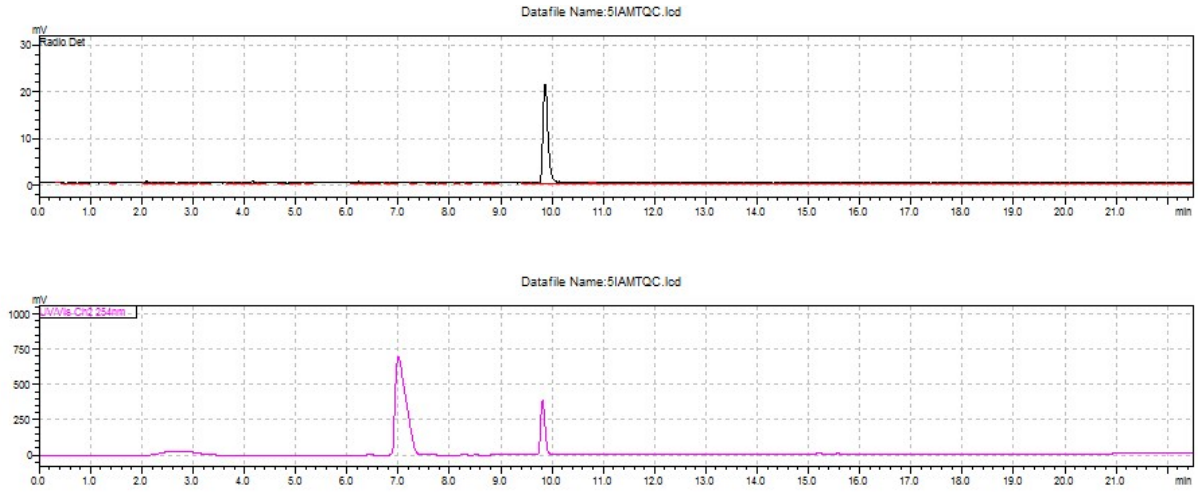
^{124}I-5-Iodo-L-α-Methyl-Tryptophan was prepared using the copper mediated halogen exchange reaction described by Guilloteau.[5] A stock of acidic reducing solution was prepared by dissolving 25 mg of 2,5-dihydroxybenzoic acid (gentisic acid), 35 mg citric acid, 35 µL of glacial acetic acid, and 1 mg of $SnSO_4$ in 2.5 mL of degassed (vacuum-sonication method) 18 megaOhm water. A stock solution of $CuSO_4$ was prepared by dissolving 32.5 mg of $CuSO_4 \cdot 5H_2O$ in 10 mL of degassed 18 megaOhm water. A 5 mL V-vial

[4] Chakraborty, P.K.; Mangner, T.J.; Chugani, D.C.; Muzik, O.; Chugani, H.T. *Nucl. Med. Biol.* **1996**, *23*, 1005-1008.

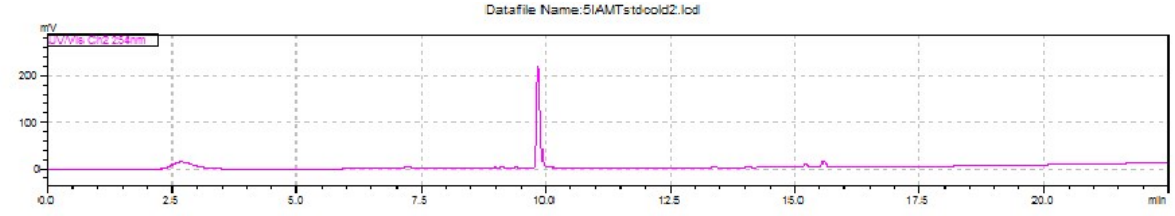
[5] Lena, I.; Ombetta, J.E.; Chalon, S.; Dognon, A.M.; Baulieu, J.L.; Frangin, Y.; Garreau, L.; Besnard, J.C.; Guilloteau, D. *Nucl. Med. Biol.* **1995**, *22*, 727-736.

was charged with 0.5 mg of non-radioactive 5-Iodo-L-α-Methyl-Tryptophan (dissolved in 50 uL of water), 455 uL of the stock reducing solution, 30 uL of the stock solution of $CuSO_4$, and 1.28 mCi of ^{124}I-NaI. The vial was placed under an atmosphere of nitrogen by gently blowing nitrogen into the headspace (**Caution**: Acidic solutions of radioiodide are volatile. This must be done in a fume hood.), sealed with a PTFE lined crimp cap, and heated at 140 °C for 30 minutes in an aluminum heating block. After cooling to room temperature, the reaction mixture was diluted to approximately 900 µL with 5% acetic acid and purified using the semi-preparitve HPLC method described above for the purification of the non-radioactive precursor. Following purification, 1.22 mCi of ^{124}I-5-Iodo-L-α-Methyl-Tryptophan was obtained (95% isolated yield, 97% decay-corrected yield). An 11.3 µCi sample was analyzed by analytical HPLC using the following conditions: Column: Phenomenex Kinetix 5µm EVO C18 100Å (150 x 4.6 mm); Solvent system: MeCN (0.1% TFA) and Water (0.1% TFA); Gradient: 5% MeCN held for 2 minutes, then ramped to 95% MeCN over 20 minutes; Flow rate: 1 mL/min. The product eluted from the column with a retention time of 10 minutes. Gentisic acid (retention time of 7 minutes) was present as at a concentration of approximately 2 µg per uCi of the dose. The specific activity of the product was estimated to be 0.74 mCi/umol based upon comparison of the area of the UV signal (254 nm) of the cold mass with the UV response produced by a known quantity of 5-Iodo-L-AMT standard. A dose of the product was prepared for injection into mice by neutralization of the TFA with 1N NaOH and by buffering to pH 6-7 with phosphate buffered saline. Acetonitrile was removed via rotary evaporation.

HPLC Traces of 11.3 µCi of ^{124}I-5-Iodo-α-Methyl Tryptophan (Radio detection (top) and 254 nm UV absorption (bottom))

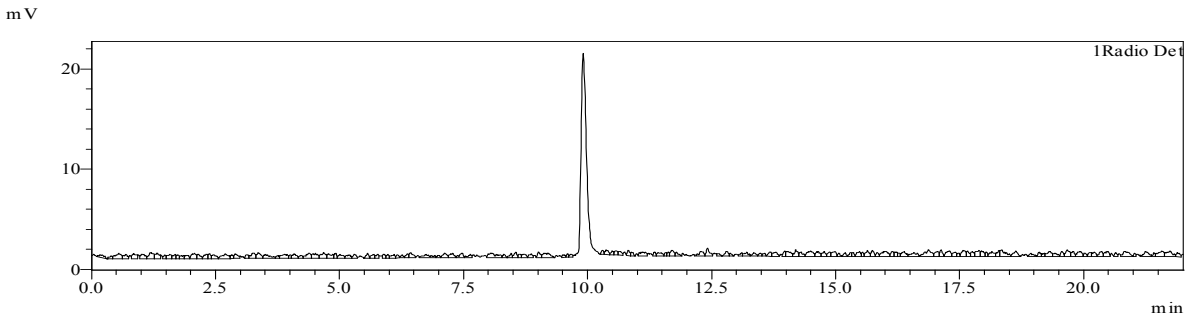


HPLC Trace of 5-Iodo-α-Methyl Tryptophan Standard (254 nm UV absorption)



Radiochemical Stability Study Radio-HPLC Traces of ^{124}I-5-Iodo-α-Methyl Tryptophan

Approximately 6 Hours after End of Synthesis:



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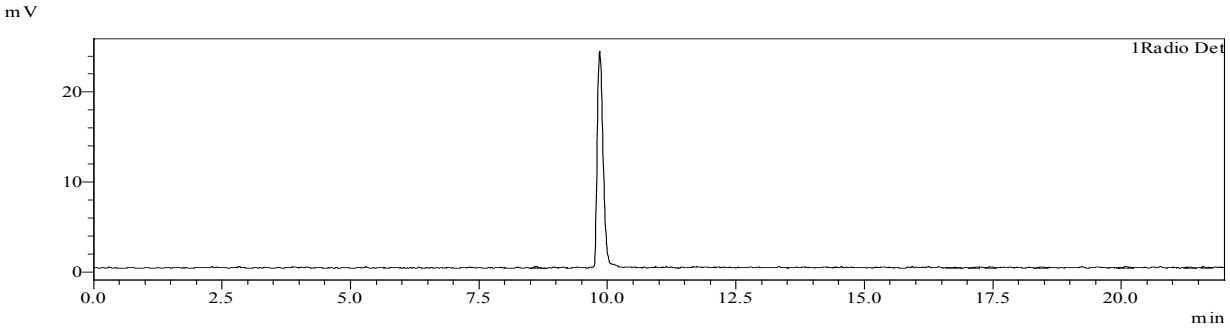
mV
1Radio Det
20
10
0
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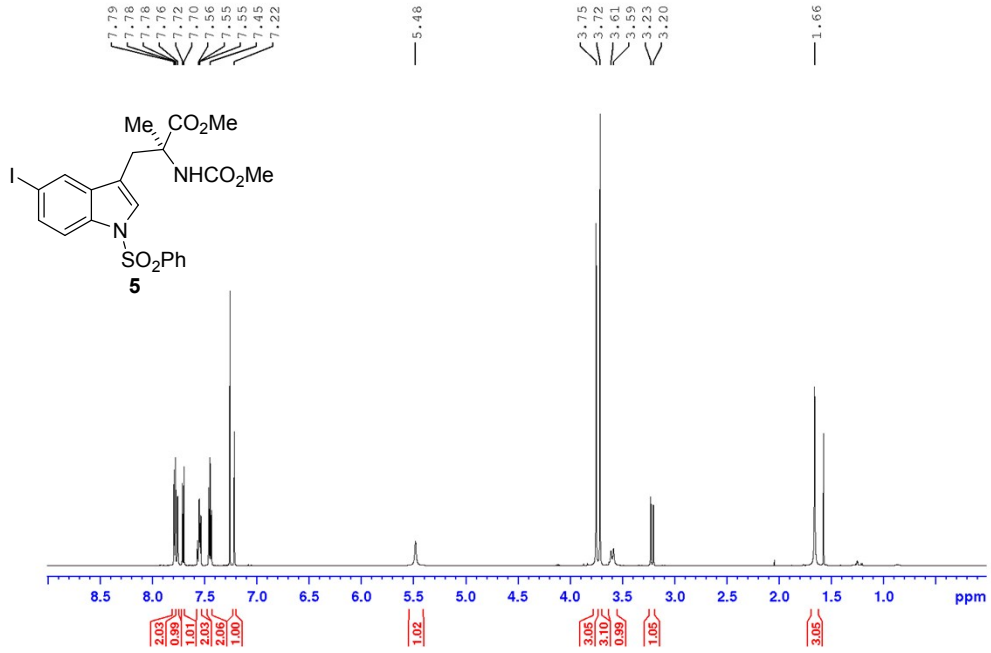
5.0
7.5
10.0
12.5
15.0
17.5
20.0
min

NMR Spectra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7.70
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7.45
7.22
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3.75
3.72
3.61
3.59
3.23
3.20
1.66
Me
CO2Me
NHCO2Me
I
N
SO2Ph
5
8.5
8.0
7.5
7.0
6.5
6.0
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5.0
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4.0
3.5
3.0
2.5
2.0
1.5
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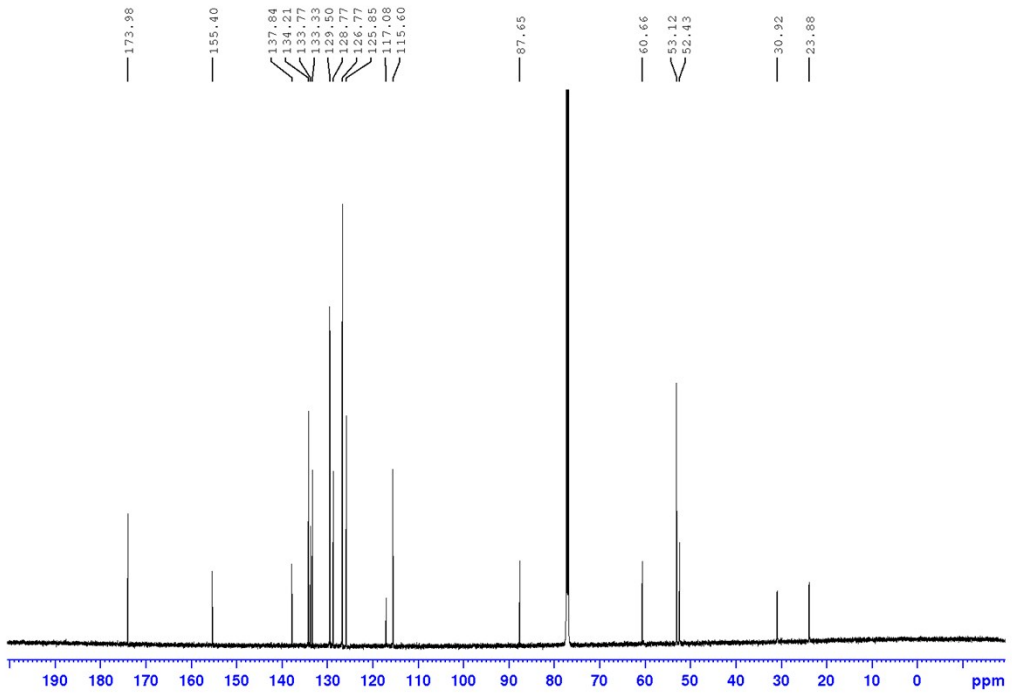
3.05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53.12
52.43
30.92
23.88
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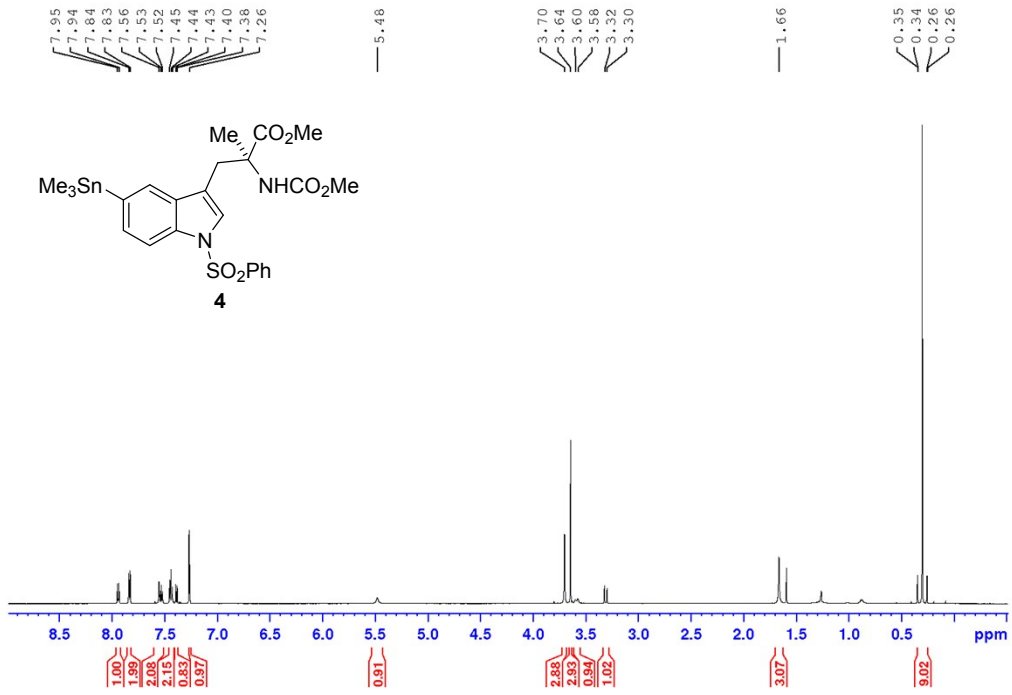
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7.26
5.48
3.70
3.64
3.60
3.58
3.32
3.30
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0.34
0.26
0.26
Me
CO2Me
NHCO2Me
Me3Sn
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SO2Ph
4
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
1.00
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2.93
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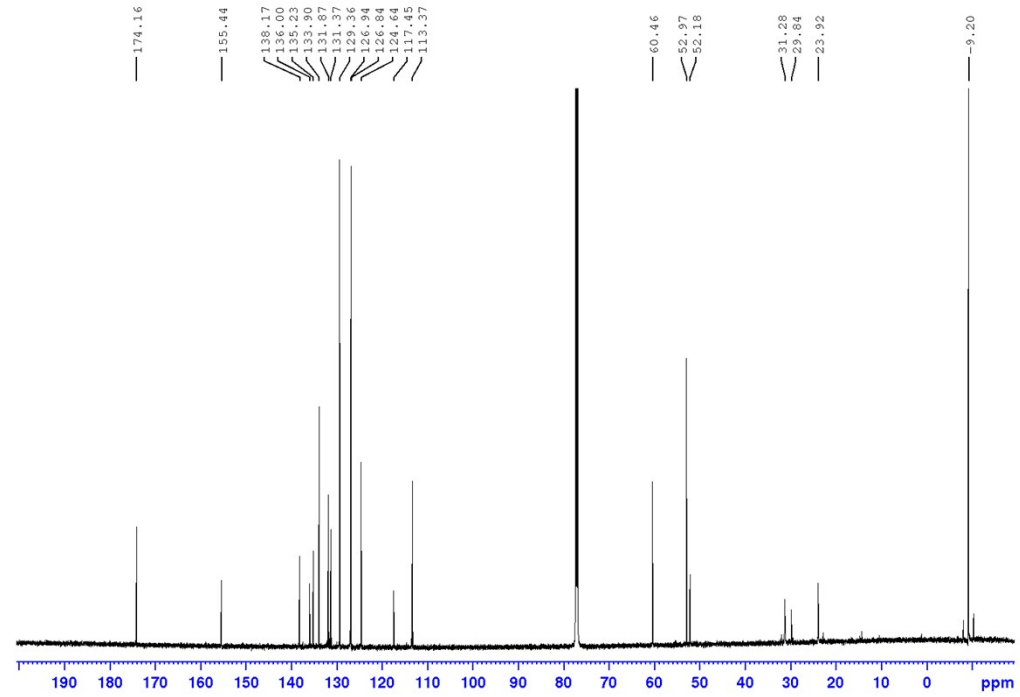
1.02
3.07
9.02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113.37
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31.28
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23.92
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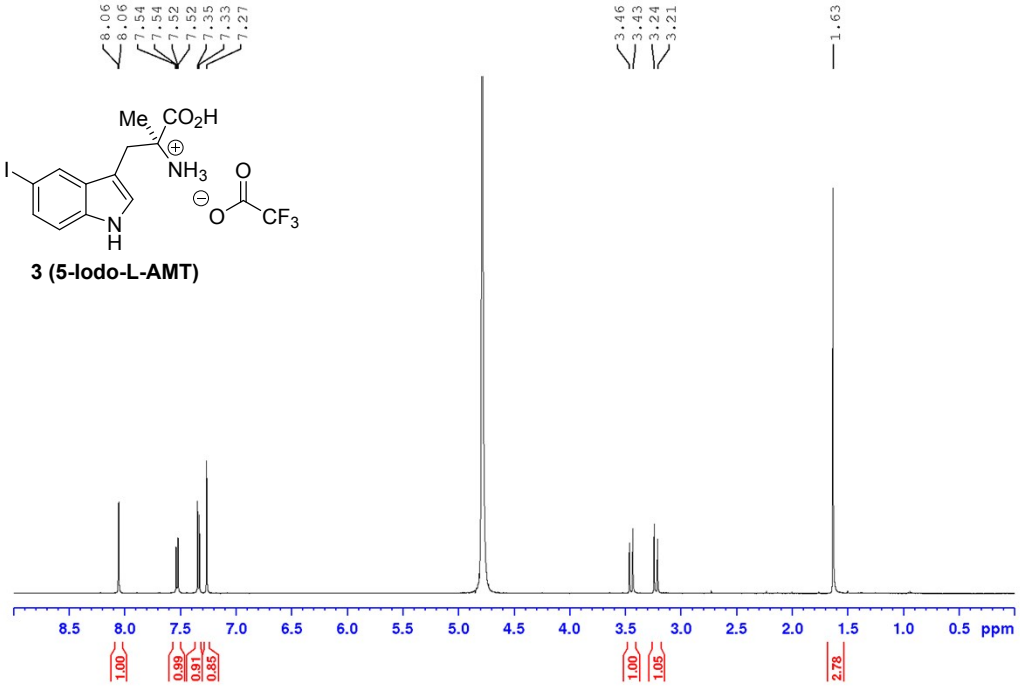
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7.33
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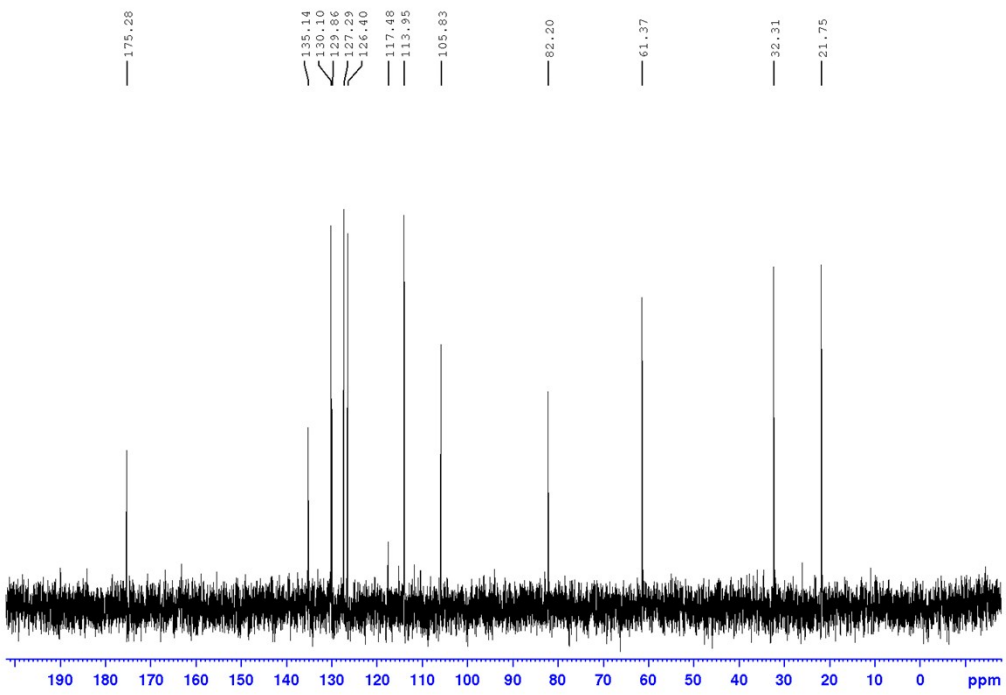
3.46
3.43
3.24
3.21
1.63
Me
CO2H
NH3
I
N
H
O
O
CF3
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
1.00
0.99
0.91
0.85
1.00
1.05
2.78

3 (5-Iodo-L-AMT)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127.29
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32.31
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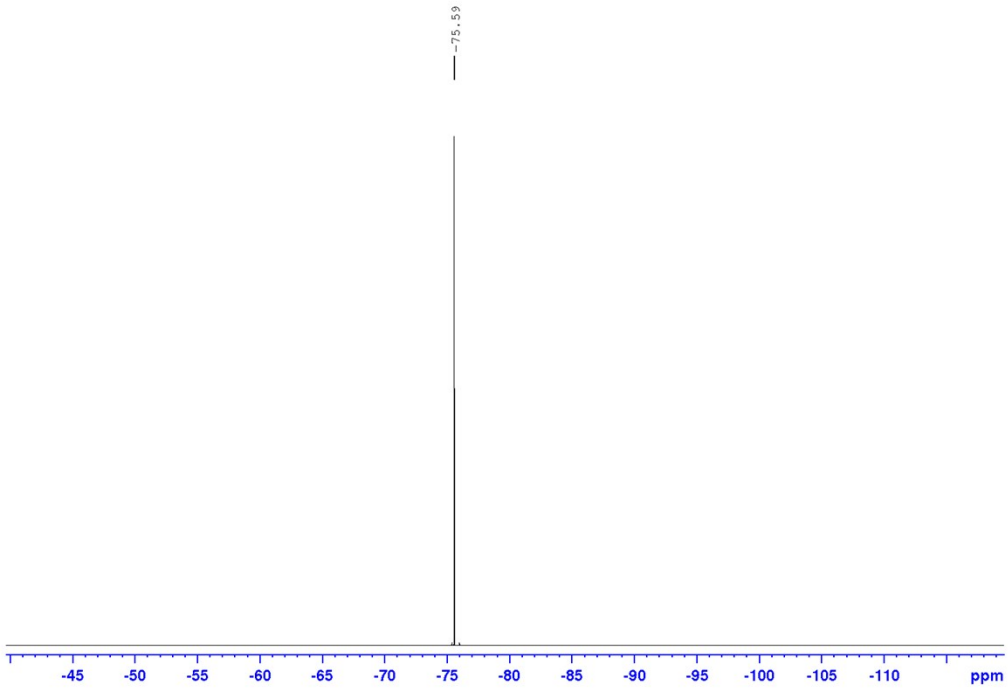
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-45 -50 -55 -60 -65 -70 -75 -80 -85 -90 -95 -100 -105 -110 ppm