Electronic Supplementary Material (ESI) for Chemical Science.

C–H Radiocyanation of Bioactive Molecules via Sequential Iodination/Copper-Mediated Cross-Coupling

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SUPPORTING INFORMATION

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1. General Information

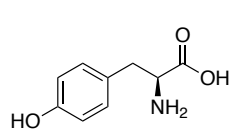
1.1 Instrument Details

NMR spectra were obtained at room temperature on Varian Vnmrs 600 (600 MHz for ^{1}H), Bruker Avance Neo 500 (500 MHz for ^{1}H, 126 MHz for ^{13}C), or Varian Vnmrs 500 (500 MHz for ^{1}H). Chemical shifts were measured in parts per million (ppm) relative to TMS ($\delta = 0.00$ ppm) and then referenced with the solvent peak. Abbreviations used in the NMR data are as follows: s (singlet); d (doublet); t (triplet); q (quartet); m (multiplet). Biotage® Isolera One was used for silica purification (column chromatography). Thermo-Nicolet IS-50 was used for the IR analyses. High resolution mass spectra (HR-MS) were recorded on Agilent 6230 TOF. HPLC-MS spectra were recorded on Agilent Q-TOF HPLC-MS. Analytical HPLC was carried out on an Agilent 1260 HPLC and mass spectrometry was performed using an Agilent 6230 LC/TOF.

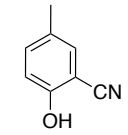
1.2 Reagents and solvents

All the reagents and solvents were purchased from commercial suppliers unless otherwise noted in Section 2.1. Unless otherwise stated, purchased reagents and synthesized materials were stored under air. Copper iodide (CuI), 1,2-dimethylethylenediamine (DMEDA), and N,N-dimethylformamide (DMF) were stored under an atmosphere of dry N_2.

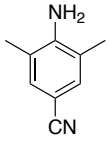
Commercial sources



2-H
Thermo Fisher Scientific



3-CN
1PlusChem



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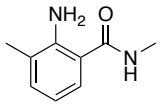
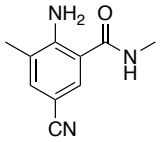
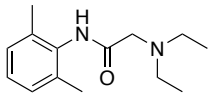
Sigma Aldrich



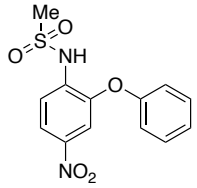
5-H
AA Block



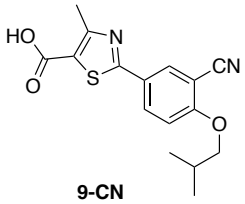
5-CN
Aaron Chemicals



6-H
TCI America



8-H
Combi-Blocks



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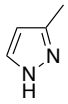
TCI America



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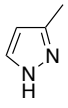
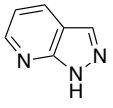
Thermo Fisher Scientific



11-CN
A2B Chem



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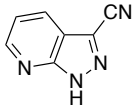
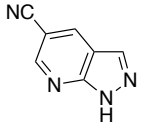
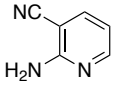
Sigma Aldrich



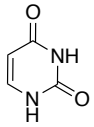
12-CN
Combi-Blocks



1H-pyrazolo[3,4-b] pyridine-5-carbonitrile
AbaChemScene



13-CN
Matrix Scientific



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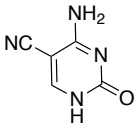
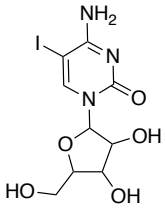
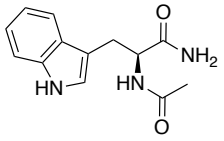
Thermo Fisher Scientific



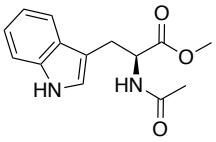
15-CN
A2B Chem



16-I
1PlusChem



19-H
Thermo Fisher Scientific



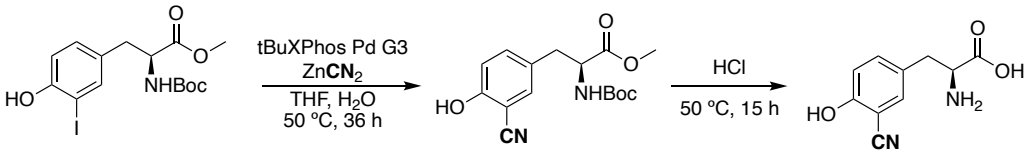
20-H
Sigma Aldrich

2. Synthesis

2.1 Preparation of Non-peptide Substrates and Authentic Standards

Unless stated otherwise, reagents were purchased from commercial sources (as indicated below) and used as received.

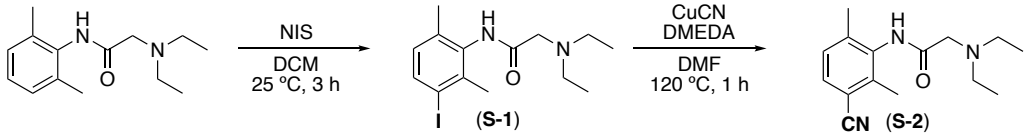
2-^{12}CN (authentic standard for 2-^{11}CN radiocyanation)



A literature procedure was followed, and **^{1}H NMR** and **^{13}C NMR** data matched those reported in from this procedure.[1]

HRMS-ESI (positive ion mode): 207.0766 (found), 207.0770 (calculated).

6-^{12}CN (the authentic standard for 6-^{11}CN radiocyanation)

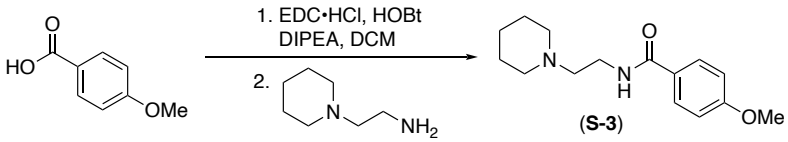


Lidocaine (99.6 mg, 0.43 mmol, 1 equiv) was dissolved in dichloromethane (DCM, 17 mL) and trifluoroacetic acid (TFA, 1 mL). Separately, N-iodosuccinimide (NIS, 96.7 mg, 0.25 mmol, 1 equiv) was dissolved in acetonitrile (1.25 mL) and then added dropwise in the dark. The mixture was stirred at room temperature for 3 h, and the volatiles were removed under vacuum. The crude product **S-1** was used for the next reaction without further purification.

Crude **S-1**, copper cyanide (76.1 mg, 0.85 mmol, 2 equiv), 1,2-dimethylethylenediamine (DMEDA, 37.5 mg, 0.43 mmol, 1 equiv), and dimethylformamide (DMF, 3.2 mL) were combined in a 4 mL vial under N_2 atmosphere. The solution was stirred at 120 ºC for 1 h. The volatiles were removed under high vacuum. The remaining solid was dissolved in DCM (~10 mL) and then extracted with 1 M aqueous sodium hydroxide (~10 mL). The dichloromethane layer was collected. Additional dichloromethane (~3 x 10 mL) was added, and extraction was repeated. The dichloromethane layers were combined and dried over sodium sulfate. The solvent was removed under reduced pressure, and the crude product was purified by chromatography on silica gel to afford **S-2 (6-^{12}CN)** as colorless oil (75 mg, 67% yield).

^{1}H NMR (500 MHz, CD_3CN) δ 9.07 (s, 1H), 7.50 (d, *J* = 7.9 Hz, 1H), 7.24 (d, *J* = 7.9 Hz, 1H), 3.16 (s, 2H), 2.67 (q, *J* = 7.1 Hz, 4H), 2.36 (s, 3H), 2.25 (s, 3H), 1.12 (t, *J* = 7.1 Hz, 6H).
^{13}C NMR (126 MHz, CD_3CN) δ 171.60, 143.02, 140.48, 137.36, 131.81, 129.59, 119.00, 111.87, 58.20, 49.48, 19.27, 17.32, 12.75.
HRMS-ESI (positive ion mode): m/z = 260.1756 (found), 260.1757 (calculated).

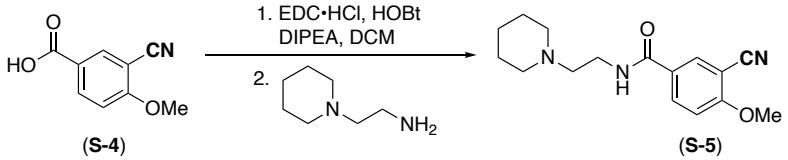
S-3 (the precursor for 7-^{11}CN radiocyanation)



4-Methoxybenzoic acid (380.5 mg, 2.5 mmol, 1 equiv), hydroxybenzotriazole (HOBt, 540 mg, 4.0 mmol, 1.6 equiv), 3-dimethylamino-propyl-ethylcarbodiimide hydrochloride (EDC•HCl 20 mg, 3.75 mmol, 1.5 equiv), and DCM (10 mL) were combined in a 20 mL vial equipped with a stir bar. Diisopropylethylamine (DIPEA, 1.4 mL) was added, and the reaction mixture was stirred for 1 h. 1-Piperidine ethanamine (357 uL, 2.5 mmol, 1 equiv) was then added, and the resulting solution was stirred for 36 h. The reaction solution was extracted with 1 M HCl (20 mL). The aqueous layer containing the protonated product was then neutralized with 1 M NaOH (20 mL) and extracted with dichloromethane (2 x 20 mL). The organic extracts were combined and dried under vacuum. The resulting oil was purified by column chromatography (100% dichloromethane → dichloromethane/MeOH = 90/10%) to afford the product **S-3** as a yellow powder (467 mg, 71% yield).

^{1}H NMR and **^{13}C NMR** spectral data matched those reported in the literature.[2]

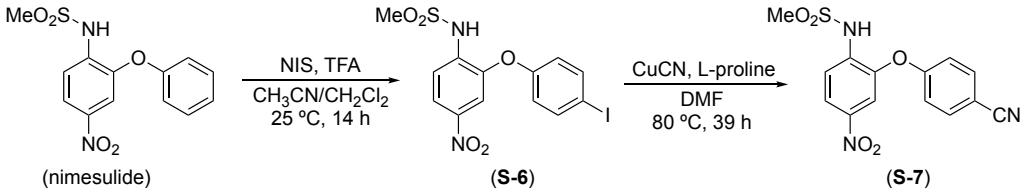
S-5 (the authentic standard for 7-^{11}CN radiocyanation)



S-4 (177.2 mg, 1 mmol, 1 equiv), hydroxybenzotriazole (HOBt, 216.2 mg, 1.6 mmol, 1.6 equiv), 3-dimethylamino-propyl-ethylcarbodiimide hydrochloride (EDC•HCl, 287.6 mg, 1.5 mmol, 1.5 equiv), and DCM (6 mL) were combined in a 20 mL vial equipped with a stir bar. Diisopropylethylamine (DIPEA, 0.56 mL) was added, and the reaction mixture was stirred for 1 h. 1-Piperidine ethanamine (143 uL, 1 mmol, 1 equiv) was then added, and the resulting solution was stirred for 2 d. The reaction solution was extracted with 1 M HCl (20 mL). The aqueous layer containing the protonated product was then neutralized with 1 M NaOH (20 mL) and extracted with dichloromethane (2 x 20 mL). The organic extracts were combined and dried under vacuum. The resulting sticky solid was purified by column chromatography (100% dichloromethane → dichloromethane/MeOH = 90/10%) to afford the product **S-5** as a yellow powder (158 mg, 56% yield).

^{1}H NMR (600 MHz, CD_3CN) δ 8.07 (s, 1H), 8.04 (d, *J* = 9.0 Hz, 1H), 7.17 (d, *J* = 9.0 Hz, 1H), 3.97 (s, 3H), 3.44 (app. q, *J* = 6.1 Hz, 2H), 2.52 (t, *J* = 6.3 Hz, 2H), 2.47 (m, 4H), 1.57 (m, 4H), 1.44 (m, 2H).
^{13}C NMR (126 MHz, CD_3CN) δ 165.53, 164.10, 134.65, 133.90, 128.59, 116.83, 112.70, 102.06, 58.34, 57.45, 55.09, 37.60, 26.53, 24.94.
HRMS-ESI (positive ion mode): 288.1778 (found), 288.1707 (calculated).

S-7 (the authentic standard for 8-^{11}CN radiocyanation)



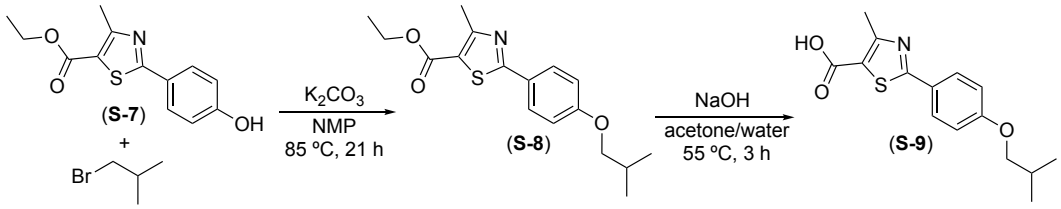
Nimesulide (77.1 mg, 0.25 mmol, 1 equiv) was dissolved in dichloromethane (10 mL) and trifluoroacetic acid (TFA, 1 mL). Separately, *N*-iodosuccinimide (NIS, 56.2 mg, 0.25 mmol, 1 equiv) was dissolved in acetonitrile (1.25 mL) and then added dropwise. The mixture was stirred at room temperature for 14 h, and the volatiles were removed under vacuum. The crude product was dissolved DCM and extracted with a saturated aqueous solution of sodium bicarbonate. The the DMC layer was collected, and this process was repeated with additional DCM (3 x 5 mL). The DCM extracts were combined and concentrated under reduced pressure. The product was purified by column chromatography (ethyl acetate/ hexane: 0% → 30%) to afford **S-6** as a white solid (93 mg, 86% yield).

^{1}H NMR and **^{13}C NMR** spectral data for **S-6** matched those reported in the literature.[3]
HRMS-ESI (negative ion mode): 432.9474 (found), 432.9367 (calculated).

S-6 (43.4 mg, 0.10 mmol, 1 equiv), copper cyanide (17.9 mg, 0.20 mmol, 2 equiv), L-proline (11.5 mg, 0.10 mmol, 1 equiv), and dimethylformamide (DMF, 0.5 mL) were combined in a 4 mL vial under N_2 atmosphere. The solution was stirred at 80 ºC for 39 h. The volatiles were removed under vacuum. The crude product was then purified by column chromatography (ethyl acetate/hexane: 0% → 40%) to afford **S-7** as a yellow solid (32 mg, 95% yield).

^{1}H NMR (500 MHz, DMSO-d_6) δ 10.31 (s, 1H), 8.16 (dd, J = 9.1, 2.6 Hz, 1H), 7.94-7.87 (multiple peaks, 3H), 7.81 (d, J = 9.1 Hz, 1H), 7.23 (d, J = 8.9 Hz, 2H), 2.51 (s, 3H).
^{13}C NMR (126 MHz, DMSO-d_6) δ 160.28, 144.81, 143.51, 137.57, 135.06, 121.78, 121.41, 119.15, 119.07, 116.57, 106.62, 41.37.
HRMS-ESI (negative ion mode): 332.0421 (found), 332.0347 (calculated).

S-9 (the precursor for 9-^{11}CN radiocyanation)



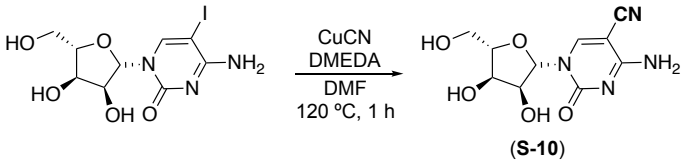
A modified literature procedure was followed.[4] **S-7** (1.5 g, 5.6 mmol, 1 equiv) and potassium carbonate (2.3 g, 16.8 mmol, 3 equiv) were weighed into a 20 mL vial and dried under high vacuum overnight. *N*-Methyl-2-pyrrolidone (NMP, 10 mL) and 1-bromo-2-methylpropane (1.2 g, 8.4 mmol, 1.5 equiv) were added, and the resulting mixture was stirred at 85 ºC for 21 h. The reaction was monitored by TLC (R_f = 0.75 in 5% methanol in dichloromethane). Water (~20 mL) was added, resulting in the precipitation of a solid. The solid was collected by filtration, recrystallized from isopropyl alcohol, and dried under high vacuum overnight to yield **S-8** as a white solid (1.4 g, 79% yield).

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.92 (d, *J* = 8.8 Hz, 2H), 7.05 (d, *J* = 8.8 Hz, 2H), 4.29 (q, *J* = 7.1 Hz, 2H), 3.83 (d, *J* = 6.5 Hz, 2H), 2.67 (s, 3H), 2.04 (hept, *J* = 6.7 Hz, 1H), 1.30 (t, *J* = 7.1 Hz, 3H), 0.99 (d, *J* = 6.7 Hz, 6H).
^{13}C NMR (126 MHz, DMSO-d_6) δ 169.01, 161.50, 160.27, 128.33, 124.73, 120.09, 115.19, 73.99, 61.08, 27.65, 18.97, 17.25, 14.17.
HRMS-ESI (positive ion mode): m/z = 320.1354 (found), 320.1320 (calculated).

A modified literature procedure was followed.[4] To a solution of **S-8** (1.0 g, 3.1 mmol, 1 equiv) in water (10 mL) and acetone (25 mL) was added sodium hydroxide (0.30 g, 7.5 mmol, 2.4 equiv). The reaction mixture was stirred at 55 ºC for 3 h. The consumption of **S-8** was monitored by TLC. After the reaction was complete, the mixture was cooled to room temperature, and an aqueous solution of trifluoroacetic acid (1 mL TFA in 20 mL water) was added, resulting in the precipitation of the product. The precipitate was collected, washed with water (10 mL), and recrystallized from ethanol to afford **S-9** as a white solid (0.74 g, 82 % yield).

^{1}H NMR (600 MHz, DMSO-d_6) δ 13.28 (s, 1H), 7.91 (d, *J* = 8.8 Hz, 2H), 7.05 (d, *J* = 8.8 Hz, 2H), 3.82 (d, *J* = 6.5 Hz, 2H), 2.65 (s, 3H), 2.04 (hept, *J* = 6.8 Hz, 1H), 0.99 (d, *J* = 6.7 Hz, 6H).
^{13}C NMR (126 MHz, DMSO-d_6) δ 168.68, 163.18, 161.35, 159.74, 128.32, 125.05, 121.76, 115.22, 74.10, 27.80, 19.09, 17.22.
HRMS-ESI (negative ion mode): m/z = 290.0850 (found), 290.0856 (calculated).

S-10 (the authentic standard for 16-^{11}CN radiocyanation)*



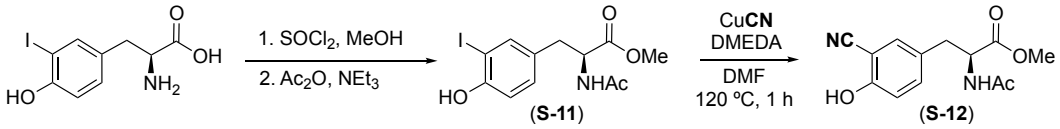
(**S-10**)

5-Iodocytidine (369.1 mg, 1.0 mmol, 1 equiv), copper cyanide (89.6 mg, 1.0 mmol, 1 equiv), 1,2-dimethylethylenediamine (DMEDA, 44.1 mg, 0.5 mmol, 0.5 equiv), and dimethylformamide (DMF, 3.0 mL) were combined in a 4 mL vial under N_2 atmosphere. The reaction was stirred at 120 ºC for 1 h. The volatiles were removed under high vacuum. The remaining solid was purified by column chromatography (methanol in dichloromethane: 0 → 10%, triethylamine was added to equilibrate the silica in the column) to afford **S-10** as a yellow solid.

^{1}H NMR (DMSO-d_6) δ 8.89 (s, 1H), 7.98 (s, 1H), 7.51 (s, 1H), 5.67 (d, J = 2.3 Hz, 1H), 5.48 (d, J = 4.6 Hz, 1H), 5.31 (t, J = 4.8 Hz, 1H), 4.99 (d, J = 6.0 Hz, 1H), 3.96 (m, 2H), 3.87 (m, 1H), 3.77 (ml, 1H), 3.59 (m, 1H).
HRMS-ESI (positive ion mode): m/z = 269.0883 (found), 269.0880 (calculated).

*The product contained residue DMEDA and DMF. Although we were unable to remove the impurities, they did not interfere with the identification of the rHPLC peak as authentic standard.

S-12 (the authentic standard for 17-^{11}CN radiocyanation)



(**S-11**) (**S-12**)

A modified literature procedure was followed.[5] To a solution of 3-iodotyrosine (750 mg, 2.4 mmol, 1 equiv) in methanol (3 mL), was added thionyl chloride (0.36 mL, 4.9 mmol, 2 equiv). The reaction mixture was stirred at room temperature for 20 h. The volatiles were removed under vacuum. The crude product was then dissolved in a mixture of 1,4-dioxane (4 mL) and water (1 mL). To this solution, was added triethylamine (0.34 mL, 2.4 mmol, 1 equiv), followed by acetic anhydride (0.23 mL, 2.4 mmol, 1 equiv). [Note: adding too much triethylamine will deprotonate the phenol and produce a side product.] The reaction was stirred for 3.5 h at room temperature. The volatiles were removed under vacuum, the crude product was dissolved in EtOAc, and extracted with H_2O. The pH of the water layer was slightly basic and was thus adjusted to pH 7 by adding HCl (1M in H_2O). The ethyl acetate layer was collected, and the water layer was extracted with additional ethyl acetate (3 x 20 mL). The combined ethyl acetate extracts were dried over sodium sulfate and concentrated under vacuum, to afford **S-11** as a white solid (835 mg, 94% yield).

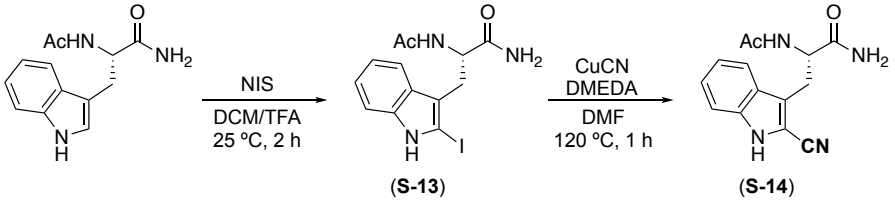
^{1}H NMR (500 MHz, CD_3CN) δ 7.54 (d, *J* = 2.1 Hz, 1H), 7.41 (s, 1H), 7.04 (dd, *J* = 8.2, 2.1 Hz, 1H), 6.81 (d, *J* = 8.3 Hz, 1H), 6.63 (s, 1H), 4.54 (td, *J* = 7.9, 5.5 Hz, 1H), 3.64 (s, 3H), 2.97 (dd, *J* = 14.0, 5.7 Hz, 1H), 2.82 (dd, *J* = 14.0, 8.1 Hz, 1H), 1.84 (s, 3H).

^{13}C NMR (126 MHz, CD_3CN) δ 172.91, 170.90, 155.97, 140.79, 131.56, 131.40, 115.77, 84.10, 54.71, 52.70, 36.72, 22.69.
HRMS-ESI (positive ion mode): m/z = 364.0037 (found), 364.0040 (calculated)

To a solution of **S-11** (72.6 mg, 0.2 mmol, 1 equiv) in dimethylformamide (0.6 mL) was added copper cyanide (26.9 mg, 0.3 mmol, 1.5 equiv) and 1,2-dimethylethylenediamine (DMEDA; 12 µL, 0.15 mmol, 0.75 equiv). The reaction mixture was stirred at 120 ºC for 2 h. Dimethylformamide and DMEDA were removed under vacuum. The crude mixture was then dissolved in ethyl acetate (~5 mL), and the ethyl acetate layer was extracted with aqueous bicarbonate buffer (pH 7; sodium bicarbonate + HCl, 10 mL). The ethyl acetate layer was collected, and the aqueous solution was extracted with additional ethyl acetate (5 x 5 mL). The combined ethyl acetate extracts were dried over sodium sulfate and concentrated under vacuum, and the resulting white solid was purified by silica chromatography, yielding **S-12** as a white solid (14.6 mg, 28% yield).

^{1}H NMR (500 MHz, CD_3CN) δ 8.38 (s, 1H), 7.36 (d, *J* = 2.3 Hz, 1H), 7.30 (dd, *J* = 8.5, 2.3 Hz, 1H), 6.92 (d, *J* = 8.5 Hz, 1H), 6.72 (d, *J* = 7.9 Hz, 1H), 4.60 (td, *J* = 8.2, 5.5 Hz, 1H), 3.65 (s, 3H), 3.03 (dd, *J* = 14.1, 5.5 Hz, 1H), 2.86 (dd, *J* = 14.1, 8.3 Hz, 1H), 1.85 (s, 3H).
^{13}C NMR (126 MHz, CD_3CN) δ 172.75, 170.93, 159.21, 136.51, 134.83, 130.12, 117.44, 117.09, 100.32, 54.38, 52.78, 36.83, 22.62.
HRMS-ESI (positive ion mode): 263.1031 (found), 263.1026 (calculated).

S-14 (the authentic standard for 19-^{11}CN radiocyanation)*



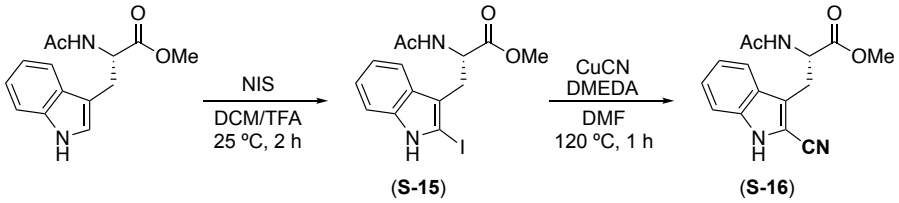
Ac-Trp-NH_2 (122.6 mg, 0.5 mmol, 1 equiv) was dissolved in a mixture of dichloromethane (DCM, 17 mL) and trifluoroacetic acid (TFA, 1.7 mL). *N*-Iodosuccinimide (NIS, 225.0 mg, 1.0 mmol, 1 equiv) was then added slowly to this solution in the dark. The mixture was stirred at room temperature for 2 h, and the volatiles were removed under vacuum. The crude product **S-13** was used for the next reaction without purification.

Crude **S-13**, copper cyanide (53.7 mg, 0.6 mmol, 1.2 equiv), 1,2-dimethylethylenediamine (DMEDA, 26.4 mg, 32 uL, 0.3 mmol, 0.6 equiv), potassium phosphate (212.3 mg, 1 mmol, 2 equiv) and dimethylformamide (DMF, 1.5 mL) were combined in a 4 mL vial under N_2 atmosphere. The solution was stirred at 120 ºC for 1 h. The volatiles were removed under vacuum. The crude product was dissolved in dichloromethane, this solution was filtered, and the filtrate was concentrated under vacuum. The resulting solid was purified by preparatory HPLC. The fractions containing pure product were frozen and lyophilized to afford **S-14** as a white solid (22 mg, 16% yield).

^{1}H NMR (500 MHz, CD_3CN) δ 9.86 (s, 1H), 7.68 (d, *J* = 8.2 Hz, 1H), 7.36 (d, *J* = 8.4 Hz, 1H), 7.28 (t, *J* = 7.6 Hz, 1H), 7.10 (t, *J* = 7.7 Hz, 1H), 6.61 (s, 1H), 6.29 (s, 1H), 5.69 (s, 1H), 4.58 (dt, *J* = 8.0, 6.3 Hz, 1H), 3.29 (dd, *J* = 14.5, 6.0 Hz, 1H), 3.15 (dd, *J* = 14.5, 6.9 Hz, 1H), 1.77 (s, 3H).
*Due to solubility in CD_3CN, ^{13}C NMR spectrum could not be obtained. The compound decomposed in DMSO-d_6.

HRMS-ESI (negative ion mode): m/z = 269.1044 (found), 269.1044 (calculated).

S-16 (the authentic standard for 20-^{11}CN radiocyanation)



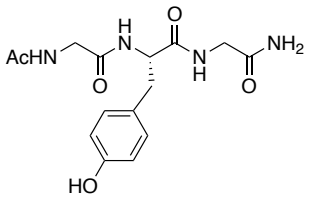
Ac-Trp-OMe (130.2 mg, 0.5 mmol, 1 equiv) was dissolved in a mixture of dichloromethane (DCM, 17 mL) and trifluoroacetic acid (TFA, 1.7 mL). *N*-Iodosuccinimide (NIS, 225.0 mg, 1.0 mmol, 1 equiv) was then added slowly to the solution in the dark. The mixture was stirred at room temperature for 2 h, and the volatiles were removed under vacuum. The crude product **S-15** was used for the next reaction without any purification.

Crude **S-15**, copper cyanide (53.7 mg, 0.6 mmol, 1.2 equiv), 1,2-dimethylethylenediamine (DMEDA, 26.4 mg, 32 uL, 0.3 mmol, 0.6 equiv), potassium phosphate (212.3 mg, 1 mmol, 2 equiv) and dimethylformamide (DMF, 1.5 mL) were combined in a 4 mL vial under N_2 atmosphere. The solution was stirred at 120 ºC for 1 h. The crude product was dissolved in dichloromethane, this solution was filtered, and the filtrate was concentrated under vacuum. The resulting solid was then purified by preparatory HPLC. The fractions containing pure product frozen and lyophilized to afford **S-16** as a white solid (30 mg, 21% yield).

^{1}H NMR (500 MHz, CD_3CN) δ 10.07 (s, 1H), 7.64 (d, *J* = 8.2 Hz, 1H), 7.45 (d, *J* = 8.4 Hz, 1H), 7.36 (t, *J* = 7.7 Hz, 1H), 7.19 (t, *J* = 7.5 Hz, 1H), 6.80 (s, 1H), 4.78 (q, *J* = 6.5 Hz, 1H), 3.64 (s, 3H), 3.35 (m, 2H), 1.87 (s, 3H).
^{1}H NMR (500 MHz, DMSO-d_6) δ 12.18 (s, 1H), 8.43 (d, *J* = 7.8 Hz, 1H), 7.66 (d, *J* = 8.1 Hz, 1H), 7.42 (d, *J* = 8.3 Hz, 1H), 7.33 (t, *J* = 7.6 Hz, 1H), 7.15 (t, *J* = 7.5 Hz, 1H), 4.57 (q, *J* = 7.2 Hz, 1H), 3.56 (s, 3H), 3.26 (m, 2H), 1.81 (s, 3H).
^{13}C NMR (126 MHz, DMSO- d_6) δ 171.44, 169.28, 136.86, 125.62, 125.53, 122.33, 120.53, 120.08, 113.95, 112.34, 105.42, 52.60, 52.00, 26.91, 22.31.
HRMS-ESI (positive ion mode): m/z = 286.1186 (found), 286.1184 (calculated).

2.3 Preparation and Characterization of Peptide Substrates and Authentic Standards

General Solid Phase Peptide Synthesis. All peptides were synthesized using standard Fmoc solid phase peptide synthesis (SPPS) or microwave-assisted SPPS on a Liberty Blue synthesizer unless otherwise noted (see details on individual peptides below). Peptides were synthesized on Rink Amide Protide resin (CEM) or 2-Chlorotritylresin (Chem Impex) and cleaved from resin using 95% trifluoroacetic acid (TFA, Sigma), 2.5% triisopropylsilane (TIPS, Sigma), and 2.5% water for 4 h. Resin was filtered, the solvent evaporated under nitrogen, and the crude peptides precipitated from cold ether and pelleted under via centrifugation. Crude peptides were dissolved in 20% acetonitrile in water, frozen, and lyophilized. Dry, crude peptides were dissolved in water and purified via preparatory HPLC on a C18 column (Phenomenex) using an Agilent 1260 HPLC with a 40 mL/min flow rate on a 0-20% acetonitrile gradient in 0.1% aqueous TFA over 20 min unless otherwise noted. Pure fractions were combined and lyophilized. Peptide purity and identity were verified by analytical HPLC on an Agilent 1260 HPLC and mass spectrometry on an Agilent 6230 LC/TOF.

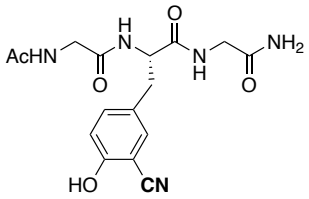


Ac-GYG-NH$_2$ was synthesized on Rink Amide Protide resin on 390 μmole scale via manual solid phase synthesis. Each Fmoc-amino acid was coupled to the resin or subsequent amino acid using Fmoc-AA (350 mg Fmoc-Gly-OH or 538 mg Fmoc-Tyr(OtBu)-OH, 1.17 mmol, 3 equiv), HBTU (445 mg, 1.17 mmol, 3 equiv), and diisopropylethylamine (DIPEA, 0.41 mL, 2.34 mmol, 6 equiv) in DMF for 2 h at RT. The resin was washed with DMF, DCM, MeOH, DMF, and DCM. Fmoc-protected resin or coupled amino acid was deprotected twice in 20% piperidine in DMF with 0.1 M HOBt for 20 min. The resin was washed again with DMF, DCM, MeOH, DMF, and DCM, and coupling was repeated until peptide was complete. The resin was capped in 10% acetic anhydride and 5% diisopropylethylamine in DMF for 30 min before evacuation and washing with DMF, DCM, MeOH, DMF, and DCM. The peptide was cleaved and purified as described above. **Ac-GYG-NH$_2$** (90 mg) was isolated in 96% purity.

^{1}H NMR (500 MHz, DMSO) δ 9.84 (d, *J* = 1.5 Hz, 1H), 8.85 (t, *J* = 5.9 Hz, 1H), 8.77 – 8.68 (m, 2H), 7.77 (d, *J* = 17.7 Hz, 2H), 7.71 – 7.65 (m, 2H), 7.34 – 7.27 (m, 2H), 5.05 (ddd, *J* = 9.3, 7.9, 4.6 Hz, 1H), 4.35 (ddd, *J* = 19.1, 16.5, 5.9 Hz, 2H), 4.24 (dt, *J* = 16.7, 5.3 Hz, 2H), 4.01 (s, 4H), 3.59 (dd, *J* = 13.9, 4.6 Hz, 1H), 3.35 (dd, *J* = 13.9, 9.4 Hz, 1H).

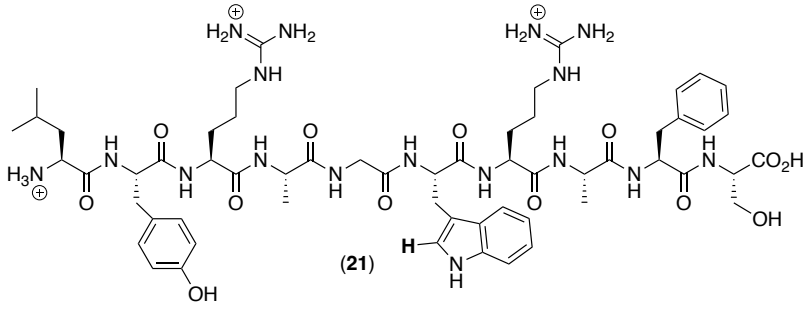
^{13}C NMR (126 MHz, DMSO) δ 171.30, 170.79, 169.71, 169.13, 155.77, 130.07, 127.83, 114.90, 54.50, 42.05, 41.93, 36.50, 22.41.

HRMS Predicted $[C_{15}H_{20}N_4O_5]H^+$: 337.1507. Found: 337.1505.



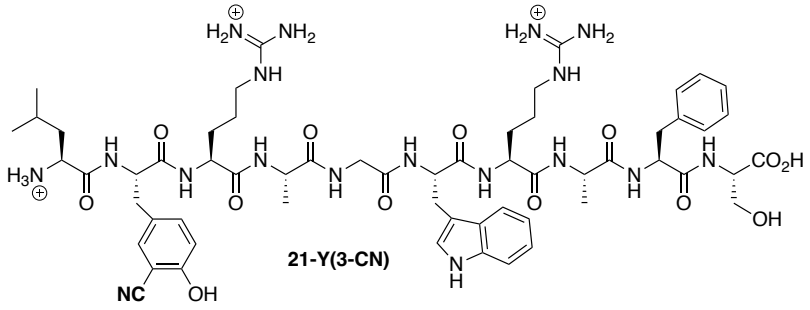
Ac-GY(3-CN)G-NH_2 was synthesized via solution phase synthesis. Boc-Tyr(3-CN)-OH (21 mg, 69 μmol) was dissolved in DMF (4 mL) and stirred with HBTU (29 mg, 77 μmol), and the resulting solution was added to a solution of glycinamide hydrochloride salt (6 mg, 81 μmol) in water (0.5 mL). To this solution was added DIPEA (31 μL, 178 μmol), and the reaction mixture was stirred overnight. The solvent was removed under vacuum, and the resulting oil suspended in 10% MeCN in water.[6] The crude suspension was filtered and purified via preparative HPLC as described in the general solid phase peptide synthesis section above. The product, Boc-Tyr(3-CN)-Gly-NH_2 (8 mg), was obtained along with glycinamide and HOBt as minor impurities, and was carried forward without further purification. This material was dissolved in 50% TFA/DCM (1 mL), the solvent was removed under vacuum, the peptide was re-dissolved in water (1 mL), and this solution was frozen and lyophilized. Acetyl glycine (Ac-Gly-OH, 26 mg, 222 μmol) was dissolved in DMF (10 mL) and stirred with N-methylmorpholine (N-MM, 22 μL, 200 μmol) and pivaloyl chloride (22 μL, 200 μmol) for 3 h at RT. The activated Ac-Gly-OH solution (2 mL) was then added to the dry, crude Boc-deprotected H-Tyr(3-CN)-Gly-NH_2 residue as well along with N-MM (11 μL, 100 μmol), and the reaction was stirred overnight. Solvent was removed under vacuum to yield a pale yellow oil that was dissolved in 10% MeCN in water. This material was then purified via HPLC. Purification was performed on a C8 column using a 0-20% acetonitrile gradient over 20 min with a flow rate of 2 mL/min. Pure fractions were combined and lyophilized to afford **Ac-GY(3-CN)G-NH_2** (5 mg) isolated with >98% purity.

HRMS Predicted $[C_{16}H_{19}N_5O_5]H^+$: 362.1459. Found: 362.1459.



Peptide **21** was synthesized on 2-chlorotrityl resin on a 200 μmol scale via automated microwave peptide synthesis as described above. The peptide was cleaved and purified as described above. Peptide **21** (79 mg) was isolated in 97% purity.

HRMS Predicted $[C_{58}H_{83}N_{17}O_{13}]H^+$: 1226.6430. Found: 1226.6428.

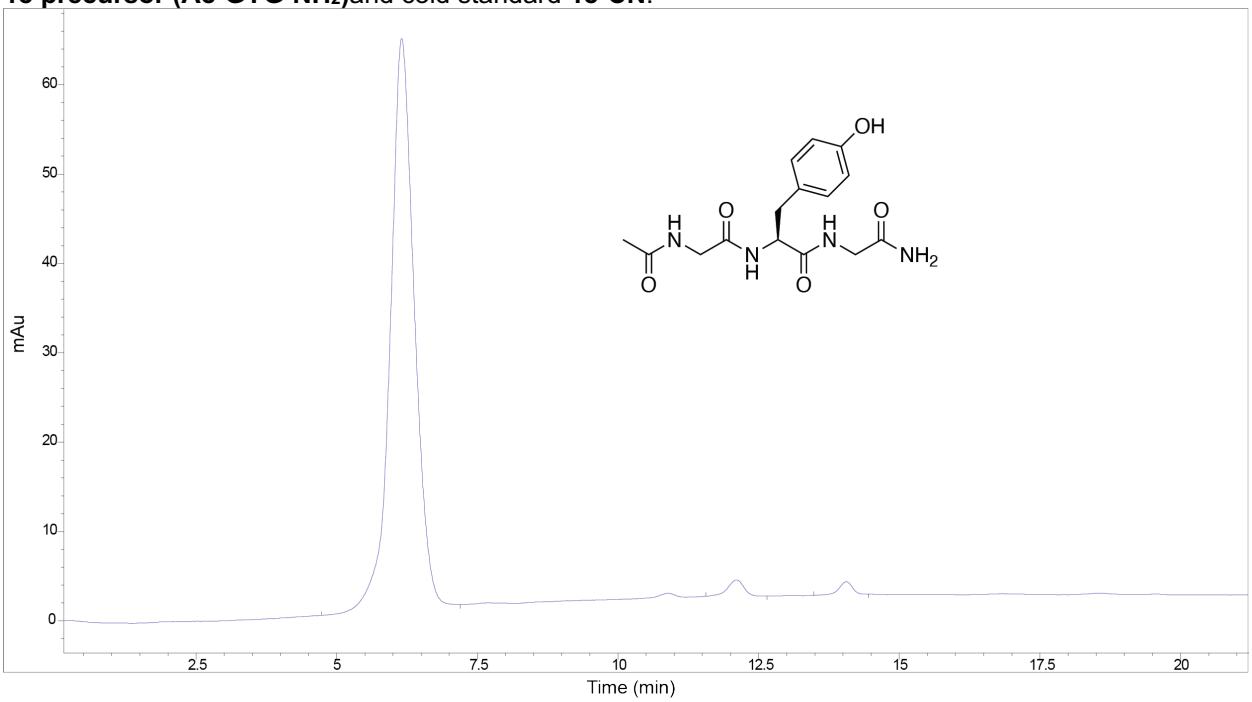


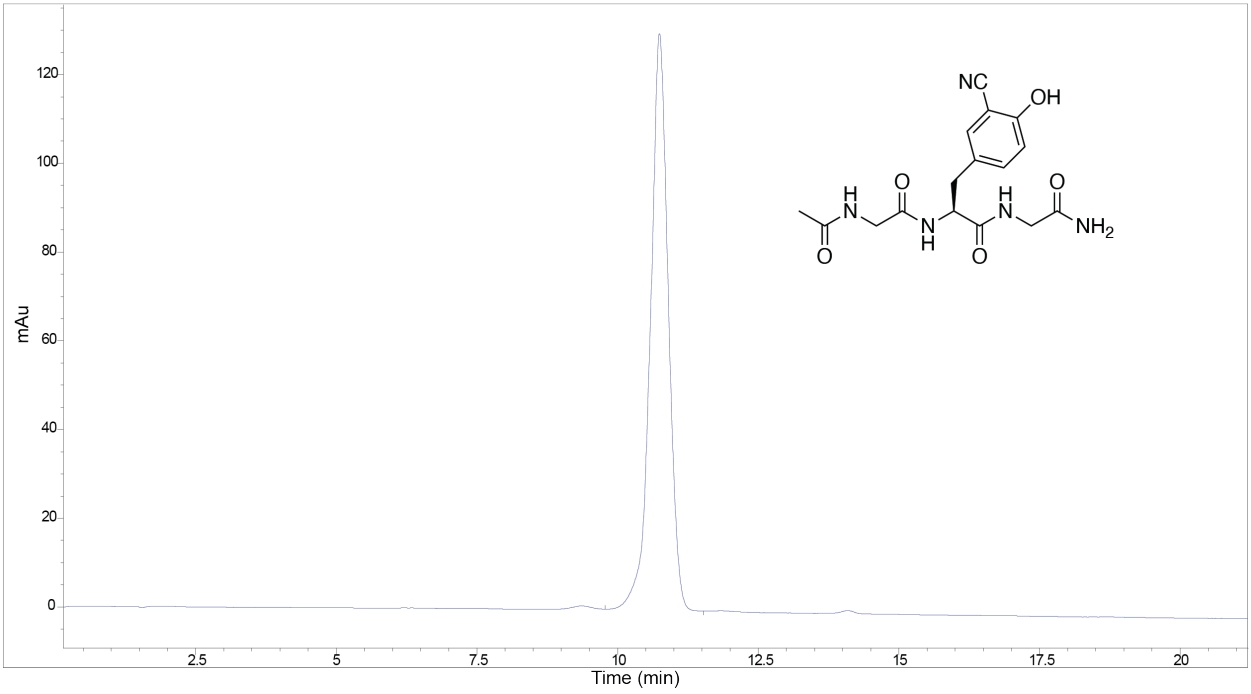
Peptide **21-Y(3-CN)** was synthesized on 2-chlorotrityl resin on a 500 μmol scale via automated microwave peptide synthesis through the second arginine residue (H-RAGWRAFS). Boc-Leu-Tyr(3-CN)-OH (10 mg, 24 μmol, see synthesis at end of section) was dissolved in DMF (1 mL) and reacted with diisopropylcarbodiimide (DIC, 4 μL, 26 μmol), and 1-hydroxy-7-azabenzotriazole (HOAt, 4 mg, 29 μmol) for 15 min in a microcentrifuge tube. To this solution was added a portion of the peptide-containing resin (73 μmol, H-RAGWRAFS), and the tube was agitated overnight. The resin was filtered, and washed with DMF (3 times) and DCM (3 times). Cleavage was performed as described above. The product was purified on a semi-preparative C18 column (10-50% MeCN gradient over 40 min in 0.1% TFA at a flow rate of 4 mL/min). Pure fractions were combined and lyophilized. Peptide **21-Y(3-CN)** (9 mg) was isolated in >98% purity.

HRMS Predicted $[C_{59}H_{82}N_{18}O_{13}]H^+$: 1251.6382. Found: 1251.6385.

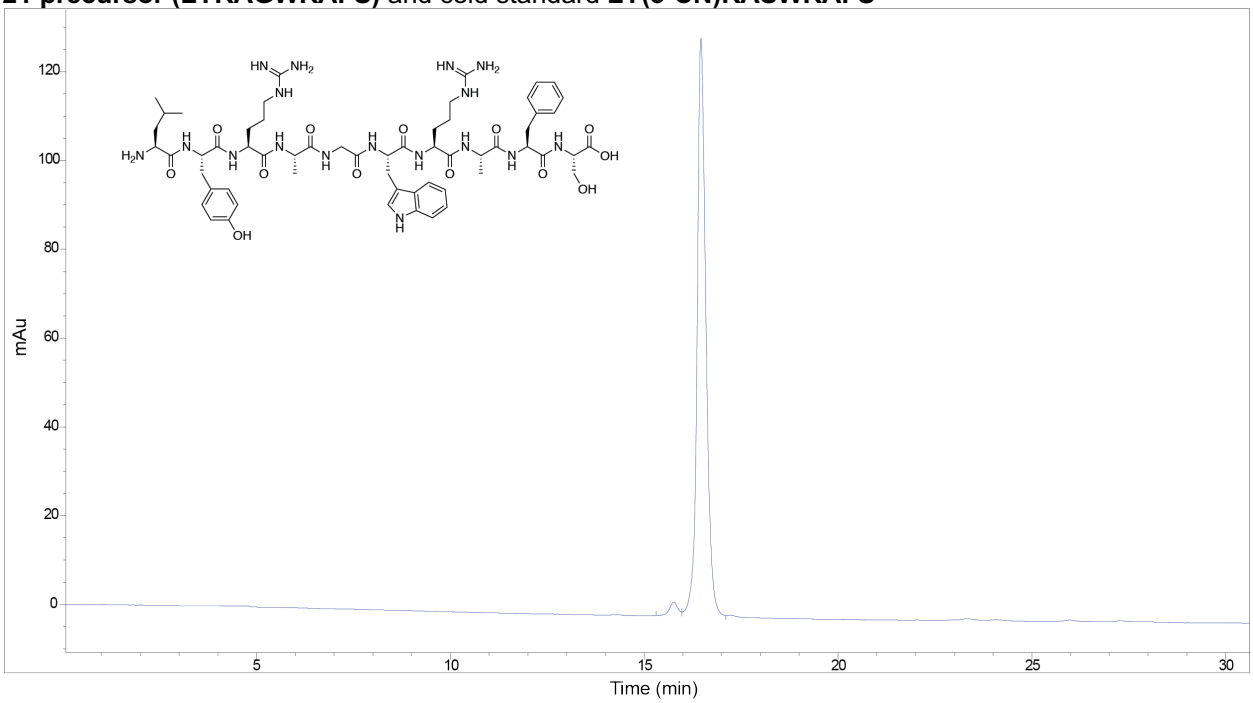
HPLC Traces of Purified Peptides.

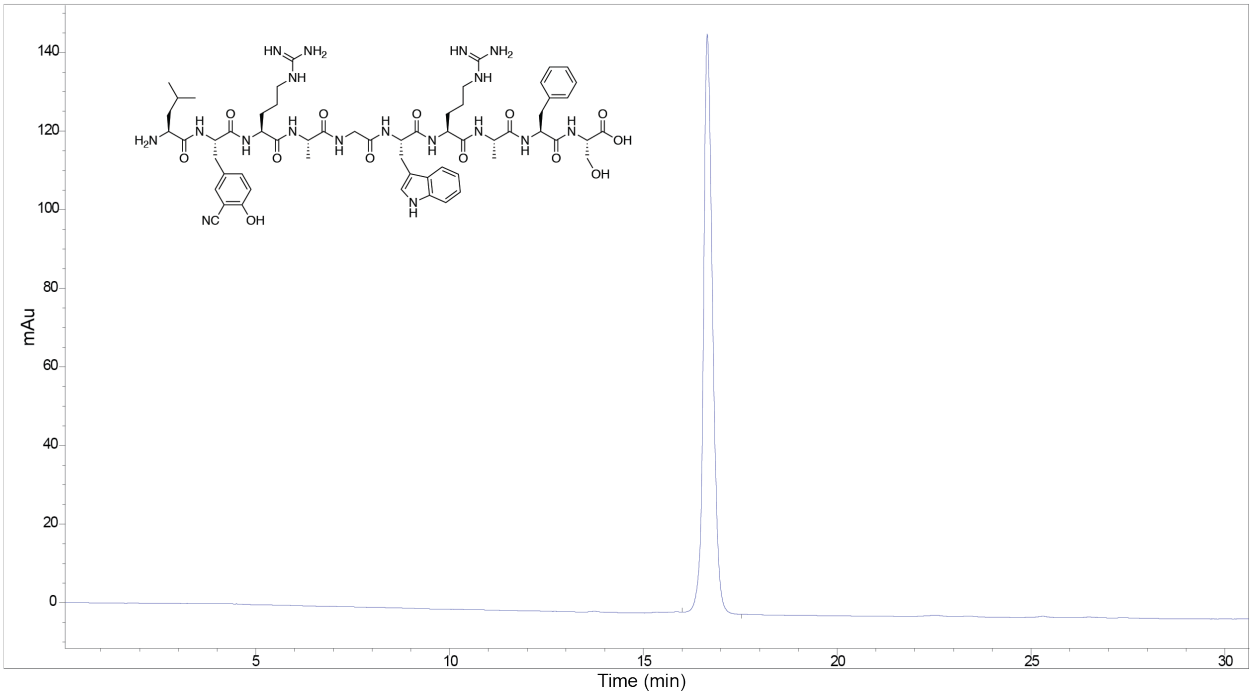
18 precursor (Ac-GYG-NH_2)and cold standard **18-CN**:





21 precursor (LYRAGWRAFS) and cold standard **LY(3-CN)RASWRAFS**





3. Iodination General Procedure

Procedure I-A: 50 mmol scale iodination. A 4 mL vial was charged with a stir bar and the substrate (5 mmol, 1 equiv). Dichloromethane (2.0 mL) and trifluoroacetic acid (TFA; 0.2 mL) were added. In the dark, a solution of the iodinating reagent (0.25 mL of a 0.2 M solution in acetonitrile, 1 equiv) was slowly added to the vial. The reaction mixture was stirred at room temperature for 2-24 h in the dark. The volatiles were removed under a stream of dry N_2, then the resulting crude material was further dried under vacuum for 2 h. The yield of the C(sp^2)–H iodination product was then obtained via ^{1}H NMR spectroscopic analysis of this crude reaction mixture.

Procedure I-B: 5 mmol scale iodination. A 4 mL vial was charged with a stir bar and the substrate (50 mmol, 1 equiv). Dichloromethane (0.4 mL) and trifluoroacetic acid (TFA; 40 µL) were added. In the dark, a solution of iodinating reagent (25 µL of a 0.2 M solution in acetonitrile, 1 equiv) was slowly added to the vial. The reaction mixture was stirred at room temperature for 2 h in the dark. The crude reaction solution was transferred to a reactor vial, and the volatiles were removed under a stream of dry N_2, then the resulting crude material was further dried under vacuum for 2 h.

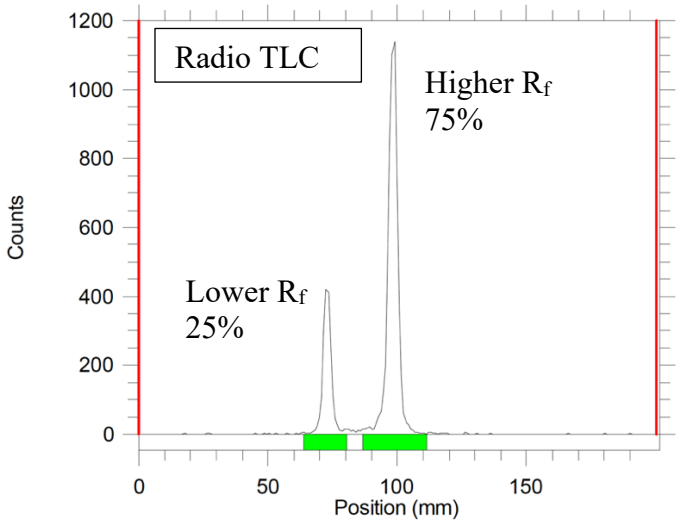
4. Radiochemistry General Details

4.1 Radiochemistry Terms[1,7,8]

Activity = the amount of radioactivity. In our research article and Supporting Information, Ci and mCi are used as units.

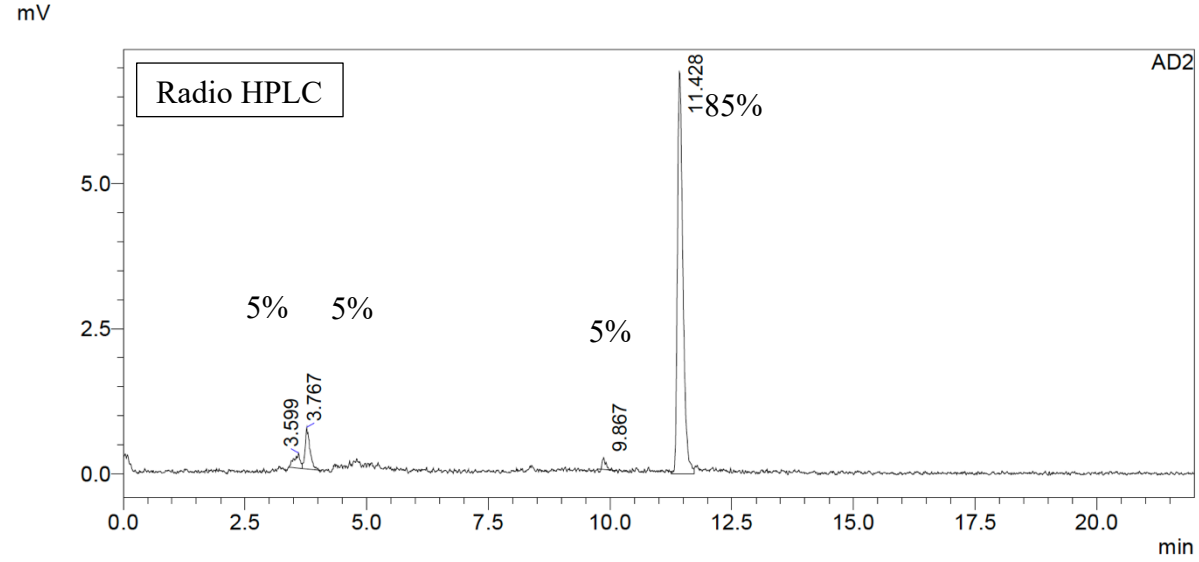
Radiochemical conversion (RCC) = % conversion of [^{11}C]KCN to [^{11}C]CN-labeled organic product as determined generally either by radio-TLC or radio-HPLC (as specified)

RCC (by rTLC) = RCC measured by radio-TLC (rTLC). It is defined as the amount of radioactivity in the higher R_f spot (organic product) relative to the radioactivity of the crude reaction.
An example with an RCC of 75% is shown below.

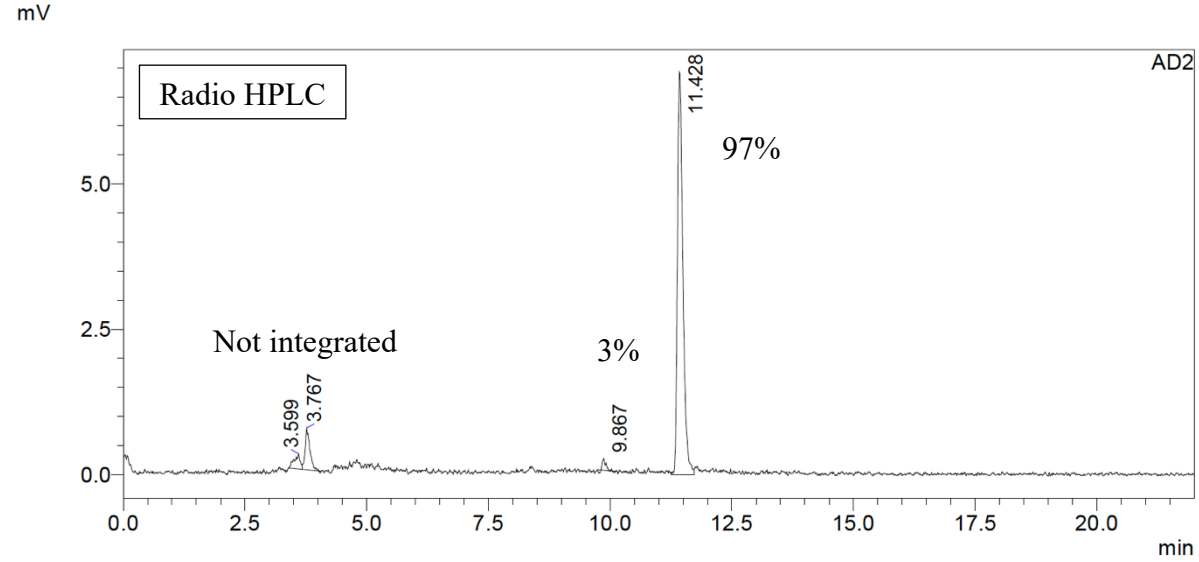


RCC (by rHPLC) = RCC determined by radio-HPLC (rHPLC). It is defined as as the amount of radioactivity in the target product relative to the total radioactivity in the reaction. However, in this study, for the reactions for which rTLC analysis was used, we did not integrate HPLC peaks estimated to be ionic ($^{11}CN^-$) (gamma peaks eluting before the solvent front with high % organic mobile phase).

When rTLC is NOT performed RCC (by rHPLC) = 85%



When rTLC is performed, RCC (by rHPLC) = 97%



Radiochemical yield (RCY) = the yield of [^{11}C]CN-labeled organic product. There are two types of RCY (non-isolated and isolated).

Non-isolated RCY = Radiochemical yield for manual reactions. If rTLC is used, non-isolated RCY is defined as RCC (by rTLC) multiplied by RCC (by rHPLC). If rTLC is not used, non-isolated RCY is equal to RCC (by rHPLC)

Isolated RCY is calculated after the final product is isolated by preparatory HPLC in a synthesis module. Decay corrected.

$$RCY = \frac{Isolated\ activity\ (mCi)}{Starting\ activity\ (mCi)} \text{ x } 100$$

Starting activity = the maximum activity of [^{11}C]KCN used in a radiosynthesis. The maximum activity reading was taken from the radiation detector in the hot cell.

Isolated activity = the activity of purified radiolabeled product.

Decay correction = the calculation used to compare activities of multiple compounds/ aliquots that are measured at different time points.

$$A_T = A_0 \times \left(\frac{1}{2}\right)^{T/20.38} \leftrightarrow A_0 = \frac{A_T}{\left(\frac{1}{2}\right)^{T/20.38}}$$

A_T = The radioactivity before decay correction (mCi)
A_0 = The radioactivity after decay correction (mCi)
T = difference (min) between two time points

Molar activity (A_m) = the Activity (Ci) of the radiolabeled product (μmol) of compound.

Beam length = Duration of cyclotron irradiation to generate [^{11}C]CO_2.

4.2 Generation of [^{11}C]HCN

GE Medical Systems PETtrace cyclotron irradiated the $^{14}N_2$ + 0.5%$^{16}O_2$ target and produced [^{11}C]CO_2 via the $^{14}N(\rho,\alpha)^{11}C$ nuclear reaction. A 16.4 MeV proton irradiation at 60 μA for 10 min generates ~1690 mCi of [^{11}C]CO_2. The [^{11}C]CO_2 was then transferred to and trapped on molecular sieves in a GE PETtrace Carbon-11 process panel. The molecular sieves were heated at 350 ºC to release [^{11}C]CO_2, which was then reduced to [^{11}C]CH_4 over a pre-heated nickel oven at 400 ºC under a mixture of H_2 and He gases. This gas was then passed through Ascarite and Sicapent columns to remove residue [^{11}C]CO_2 and water, and was then was passed through a pre-heated platinum oven at 950 ºC with ammonia gas to form ~1100 mCi of [^{11}C]HCN. The [^{11}C]HCN was then transferred to a synthesis module for trapping processes.

5. Manual Radiocyanation Reactions

5.1 Generation of [^{11}C]KCN

In a 4 mL vial, K_3PO_4 (115 mg) was dissolved in milli-Q water (1.0 mL). The vial was placed in a GE TRACERlab FX_M synthesis module. [^{11}C]HCN was generated with a 50 s to 1.5 min beam (see Section 4.2), and then the [^{11}C]HCN gas was bubbled through the K_3PO_4 solution, resulting in trapping as [^{11}C]KCN (up to 50 mCi).

5.2 General Manual Radiocyanation Setup

See Section 3 (Iodination General Procedure) for the first step of the C–H radiocyanation.

CuI/dmeda stock solution. In a N_2 atmosphere glove box, a 4 mL vial was charged with CuI (61.9 mg), dmeda (70 μL), and DMF (566 μL). The vial was shaken until the CuI completely dissolved.

Radiocyanation Procedure R-A. After the iodination and drying processes, the crude iodinated precursor was dissolved in DMF (88 μL) under a nitrogen atmosphere. On the bench top, 25 μL of the [^{11}C]KCN solution and 50 μL of the CuI/dmeda stock solution were added, being careful to maintain a N_2 atmosphere in the reaction. The reaction was heated at 130 ºC for 5 min, and then allowed to cool to room temperature. Appropriate solvents were added to dissolve any insoluble compounds before analysis by radio-TLC and radio-HPLC.

Radiocyanation Procedure R-B. After the iodination and drying process, the crude iodinated precursor was dissolved in DMF (50 μL) under a N_2 atmosphere. On the bench top, 100 μL of the [^{11}C]KCN solution and 50 μL of the CuI/dmeda stock solution were added being careful to maintain a N_2 atmosphere in the reaction. The reaction was heated at 130 ºC for 5 min, and then allowed to cool to room temperature. Appropriate solvents were added to dissolve any insoluble compounds before analysis by radio-TLC and radio-HPLC.

Radiocyanation Procedure R-C. After the iodination and drying process, the crude iodinated precursor was dissolved in DMF (50 μL), to which sodium ascorbate (9.9 mg, 50 μmol) was added under a N_2 atmosphere. On the bench top, 100 μL of the [^{11}C]KCN solution and 50 μL of the CuI/dmeda stock solution were added being careful to maintain a N_2 atmosphere in the reaction. The reaction was heated at 130 ºC for 5 min, and then allowed to cool to room temperature. Appropriate solvents were added to dissolve any insoluble compounds before analysis by radio-TLC and radio-HPLC.

5.3 Analysis of Manual Radiocyanation Reactions

5.3.1 Radio TLC

AR-2000 TLC Scanner (Eckert & Ziegler) was used to scan TLC plates for RCC analyses.

5.3.2 Analytical HPLC

Column A: Ultremex 5µ C18 250 x 4.6 mm 5 micron
P/N = 00G-0048-E0

Column B: Synergi 10µ Hydropro-RP 80Å 250 x 4.6 mm
P/N = 00G-4376-E0

Column C: Luna 5µ C8(2) 100 Å 250 x 4.6 mm
P/N = 00G-4249-E0

Column D: Luna 5µ C8 100 Å 100 x 2.00 mm
P/N = 00D-4040-B0

Column E: Luna 5µ C5 100 Å 150 x 4.6 mm 5micron
P/N = 00F-4043-E0

HPLC Method 1
Flow rate: 1.0 mL/min
UV detection = 220 nm
Solvent A = H_2O + 0.5% TFA; Solvent B = MeCN + 0.5% TFA
0-2 min: 5% B (hold)
2-15 min: 5 → 95% B
15-16 min: 95% B (hold)
16-16.5 min: 95 → 5% B
16.5-22 min: 5% B (hold)

HPLC Method 2
Flow rate: 1.0 mL/min
UV detection = 254 nm
Solvent A = H_2O + 0.5% TFA; Solvent B = MeCN + 0.5% TFA
0-2 min: 5% B (hold)
2-15 min: 5 → 95% B
15-16 min: 95% B (hold)
16-16.5 min: 95 → 5% B
16.5-22 min: 5% B (hold)

HPLC Method 3
Flow rate: 1.0 mL/min
UV detection = 254 nm
Solvent A = H_2O + 0.5% TFA; Solvent B = MeCN + 0.5% TFA
0-4 min: 5% B (hold)

4-5 min: 5 → 25% B
5-15 min: 25 → 90% B
15-20 min: 90% B (hold)
20-21 min: 90 → 5% B
21-25 min: 5% B (hold)

HPLC Method 4
Flow rate: 1.0 mL/min
UV detection = 220 nm
Solvent A = H_2O + 0.5% TFA; Solvent B = MeCN + 0.5% TFA
0-5 min: 0% B (hold)
5-6 min: 0 → 15% B
6-10 min: 15% B (hold)
10-15 min: 15 → 90% B
15-16 min: 90% B (hold)
16-17 min: 90 → 5% B
17-22 min: 5% B (hold)

HPLC Method 5
Flow rate: 1.0 mL/min
UV detection = 254 nm
Solvent A = H_2O + 0.5% TFA; Solvent B = MeCN + 0.5% TFA
0-7 min: 0% B (hold)
7-12 min: 0 → 65% B
12-14 min: 65% B (hold)
14-15 min: 65 → 0% B
15-20 min: 0% B (hold)

HPLC Method 6
Flow rate: 1.0 mL/min
UV detection = 254 nm
Solvent A = H_2O + 0.5% TFA; Solvent B = MeCN + 0.5% TFA
0-4 min: 5% B (hold)
4-5 min: 5 → 20% B
5-15 min: 20 → 90% B
15-20 min: 90% B (hold)
20-21 min: 90 → 5% B
21-25 min: 5% B (hold)

HPLC Method 7
Flow rate: 1.0 mL/min
UV detection = 280 nm
Solvent A = H_2O + 0.5% TFA; Solvent B = MeCN + 0.5% TFA
0-4 min: 5% B (hold)
4-5 min: 5 → 20% B
5-8 min: 20% B (hold)

8-12 min: 20 → 90% B
12-15 min: 90% B (hold)
15-16 min: 90 → 5% B
16-20 min: 5% B (hold)

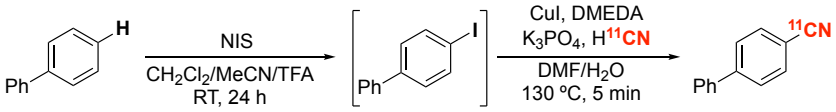
HPLC Method 8
Flow rate: 1.0 mL/min
UV detection = 280 nm
Solvent A = H_2O + 0.5% TFA; Solvent B = MeCN + 0.5% TFA
0-5 min: 5% B (hold)
5-6 min: 5 → 15% B
6-10 min: 15% B (hold)
10-15 min: 15 → 90% B
15-16 min: 90% B (hold)
16-17 min: 90 → 5% B
17-22 min: 5% B (hold)

HPLC Method 9
Flow rate: 2.0 mL/min
UV detection = 254 nm
Solvent A = H_2O + 0.5% TFA; Solvent B = MeCN + 0.5% TFA
0-3 min: 5% B (hold)
3-10 min: 5 → 60% B
10-12 min: 60 → 95% B
12-15 min: 95% B (hold)
At 15min, 95 → 5% B
15-18 min: 5% B (hold)

5.3.3 Manual Radiocyanation Yields and Analysis Spectra

The presence of the desired products was confirmed by co-injection (the authentic standard and the crude solution were injected into a radio-HPLC, and both the authentic standard and the desired product eluted at the same retention time). Due to different injection volumes, different solvents to dissolve HPLC samples, and different radio-HPLCs used, the retention times of the desired product in the UV and radio traces may be slightly different. Also, the eluent is detected by the UV detector first and then by the radio detector (the gamma counter), which affected the retention times of the UV and radio spectra.

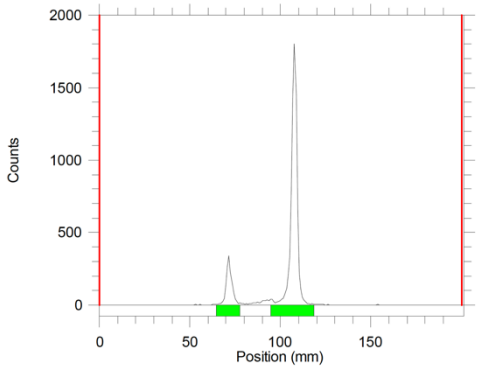
Radiocyanation and analysis of 1-^{11}CN



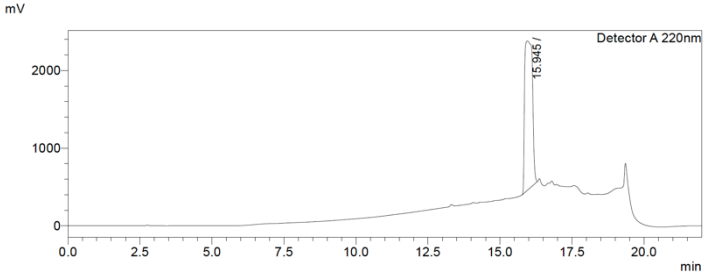
Iodination conditions: **Procedure I-A (24 h)**. Radiocyanation conditions: **Procedure R-A**. HPLC column: **Column A**. HPLC condition: **Method 1**. Non-isolated RCY = 68 ± 14 %

n	RCC [%]	RCP [%]	RCY [%]
1	62	84	52
2	66	84	55
3	73	82	60
4	82	95	78
5	83	97	80
6	85	100	85

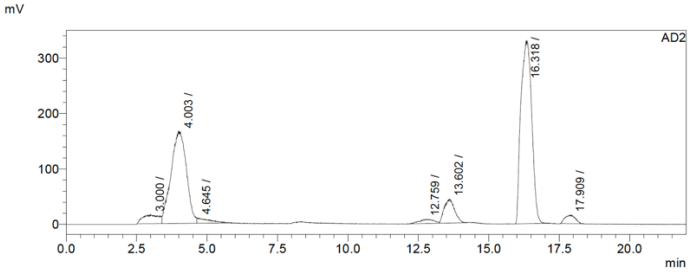
Radio-TLC (Hex:EtOAc = 1:1)



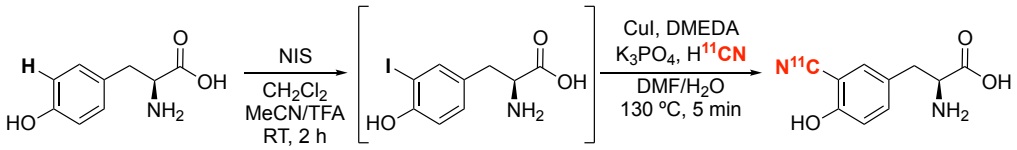
HPLC (UV trace): authentic standard **1-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



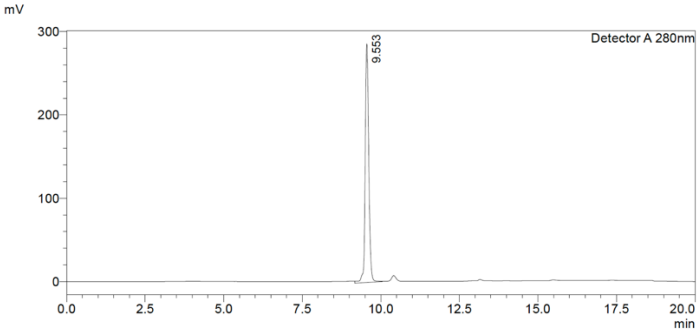
Radiocyanation and analysis of 2-^{11}CN



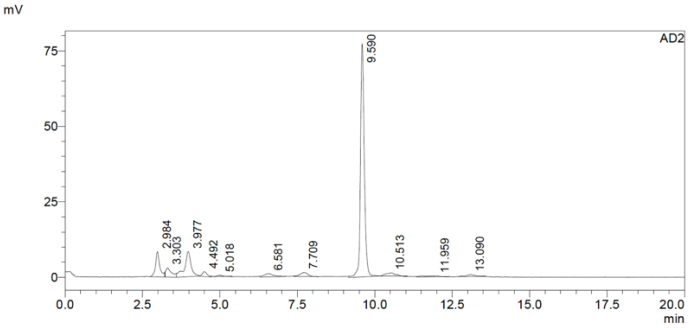
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-B**. HPLC column: **Column B**. HPLC condition: **Method 7**. Non-isolated RCY = 68 ± 4 %

n	RCY [%]
1	65
2	72
3	63
4	70

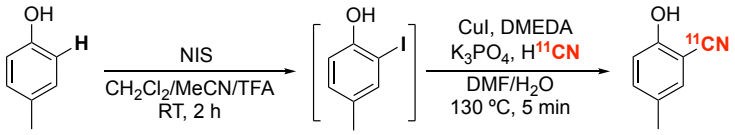
HPLC (UV trace): authentic standard **2-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



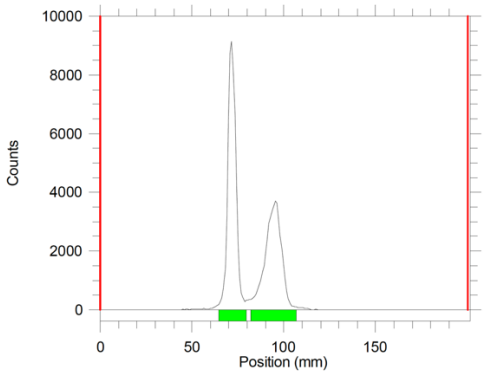
Radiocyanation and analysis of 3-^{11}CN



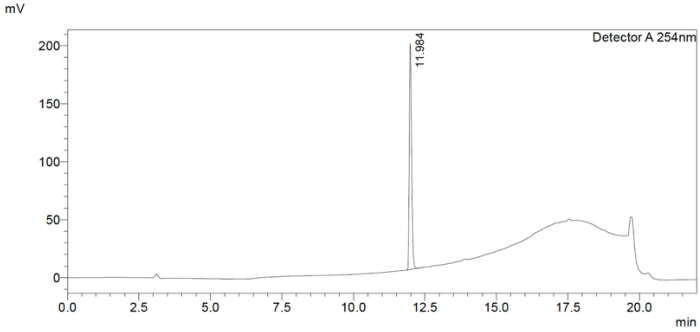
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-A**. HPLC column: **Column A**. HPLC condition: **Method 1**. Non-isolated RCY = 36 ± 2 %

n	RCC [%]	RCP [%]	RCY [%]
1	37	94	35
2	45	88	39
3	36	98	35

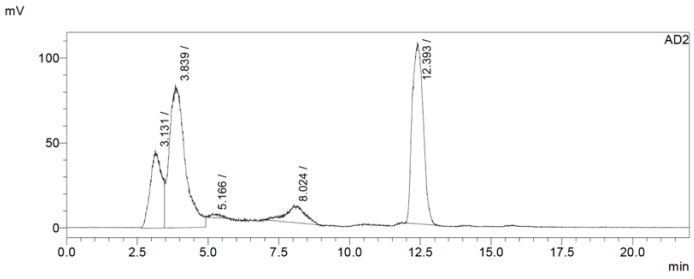
Radio-TLC (Hex : EtOAc = 1:1)



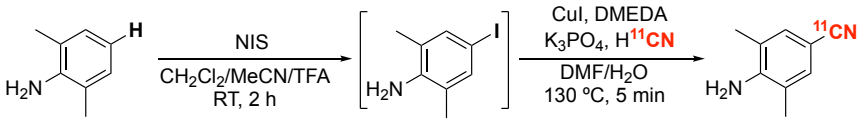
HPLC (UV trace): authentic standard **3-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



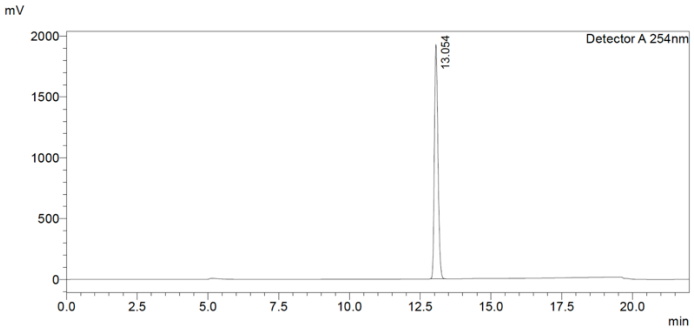
Radiocyanation and analysis of 4-^{11}CN



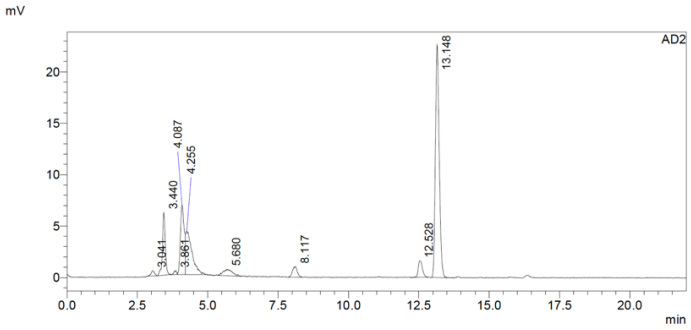
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-A**. HPLC column: **Column B**. HPLC condition: **Method 2.** Non-isolated RCY = 51 ± 8 %

n	RCY [%]
1	56
2	43
3	59

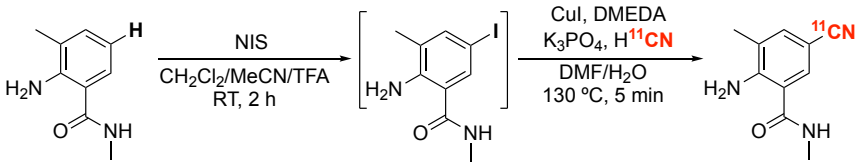
HPLC (UV trace): authentic standard **4-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



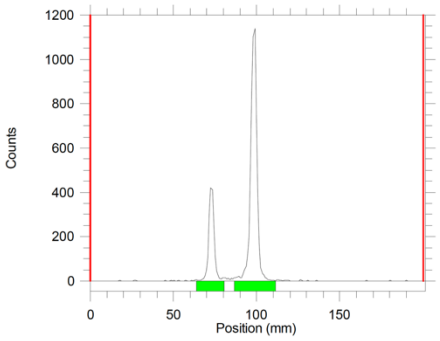
Radiocyanation and analysis of 5-^{11}CN



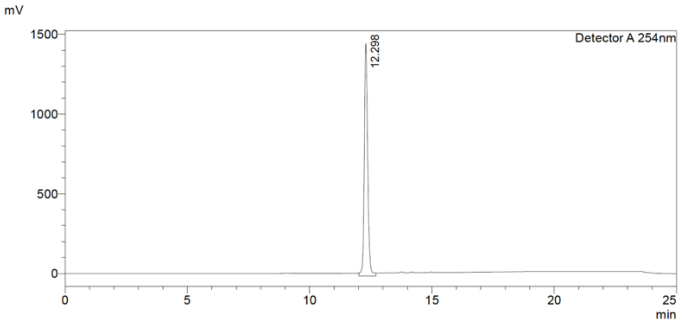
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-A**. HPLC column: **Column B**. HPLC condition: **Method 3**. Non-isolated RCY = 68 ± 5%

n	RCC [%]	RCP [%]	RCY [%]
1	69	93	64
2	73	100	73
3	76	90	68

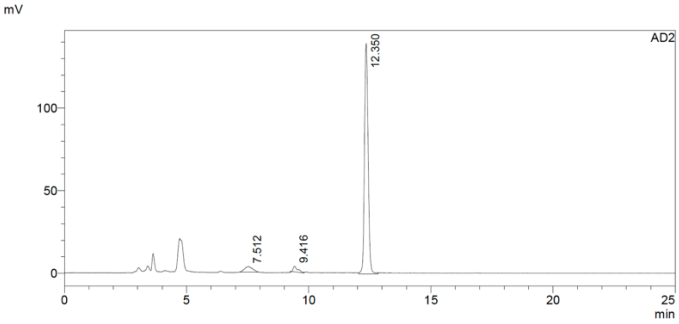
Radio-TLC (DCM:MeOH = 9:1)



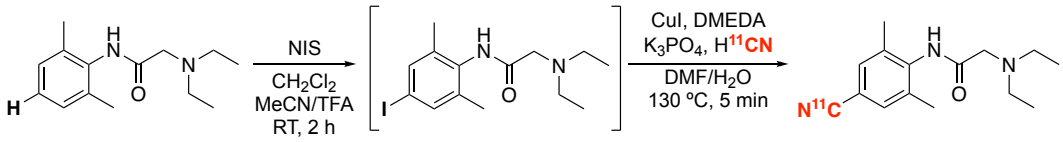
HPLC (UV trace): authentic standard **5-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



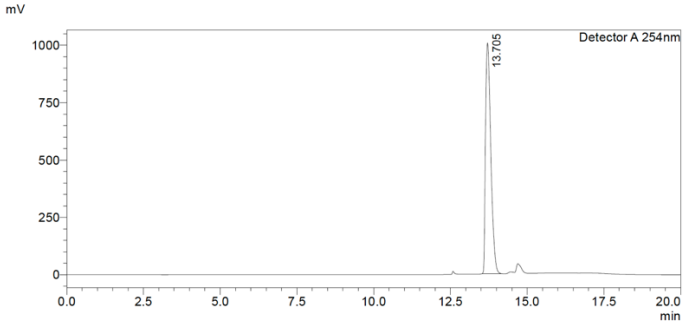
Radiocyanation and analysis of 6-^{11}CN



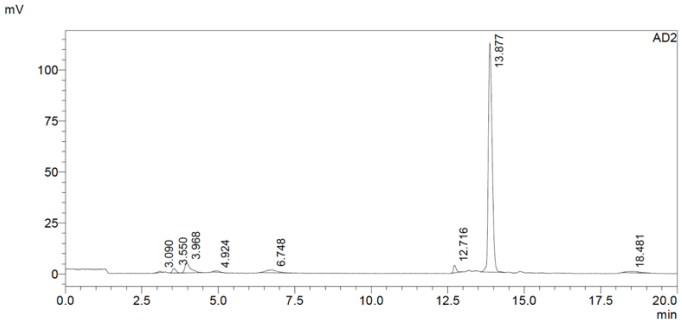
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-B**. HPLC column: **Column B**. HPLC condition: **Method 5.** Non-isolated RCY = 85 ± 10%

n	RCY [%]
1	88
2	95
3	72
4	84

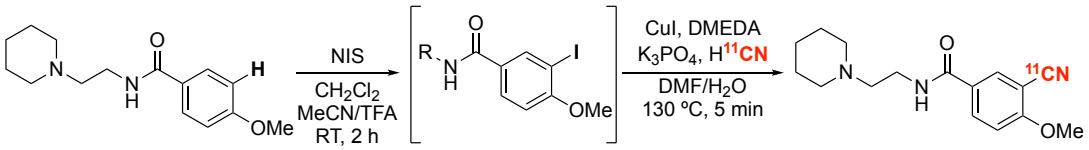
HPLC (UV trace): authentic standard **6-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



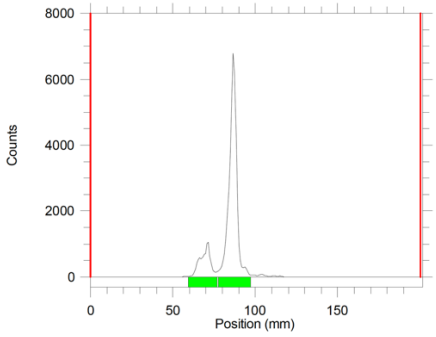
Radiocyanation and analysis of 7-^{11}CN



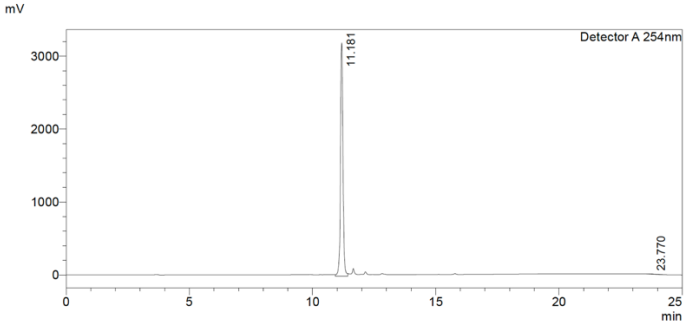
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-B**. HPLC column: **Column B**. HPLC condition: **Method 6.** Non-isolated RCY = 67 ± 17%

n	RCC [%]	RCP [%]	RCY [%]
1	83	93	77
2	50	93	46
3	80	96	77

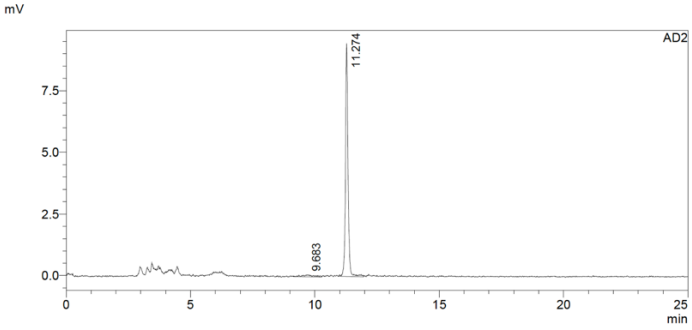
Radio-TLC (Hex:EtOAc = 1:1)



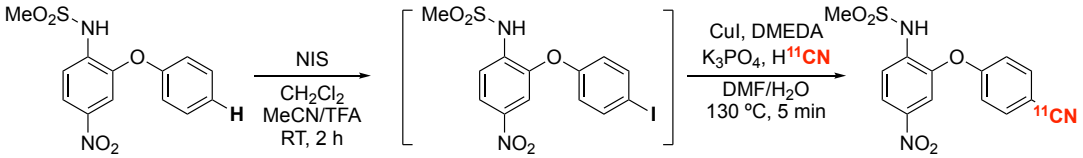
HPLC (UV trace): authentic standard **7-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



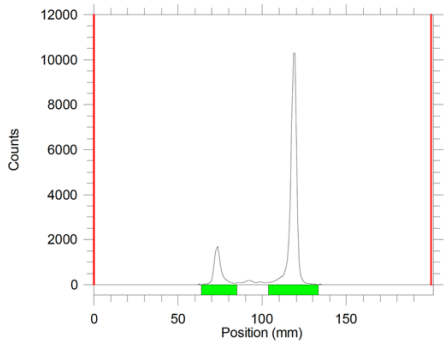
Radiocyanation and analysis of 8-^{11}CN



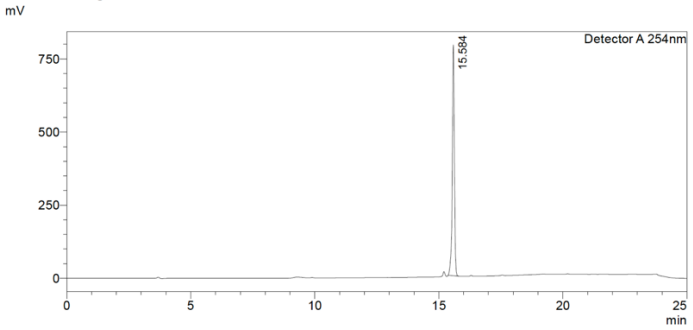
Iodination conditions: **Procedure I-A (24 h)**. Radiocyanation conditions: **Procedure R-B**. HPLC column: **Column B**. HPLC condition: **Method 6.** Non-isolated RCY = 46 ± 6%

n	RCC [%]	RCP [%]	RCY [%]
1	66	65	43
2	77	55	42
3	66	78	52

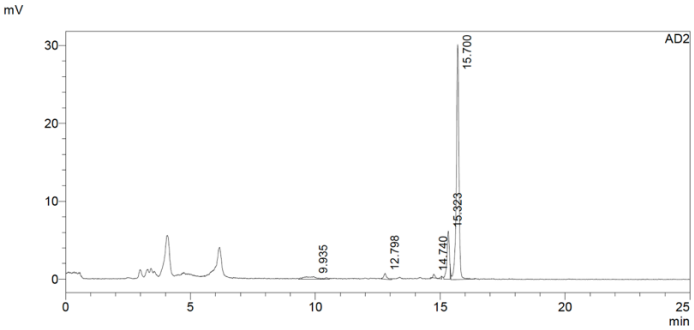
Radio-TLC (DCM:MeOH = 9:1)



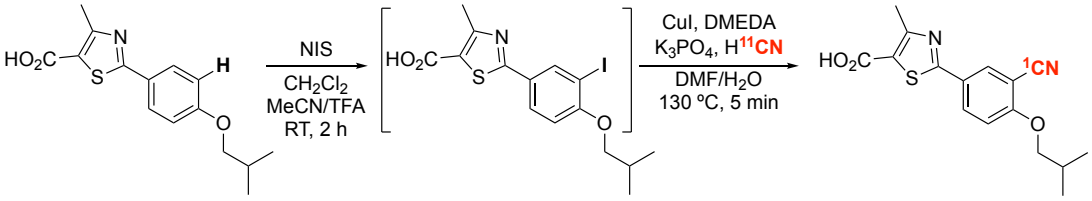
HPLC (UV trace): authentic standard **8-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



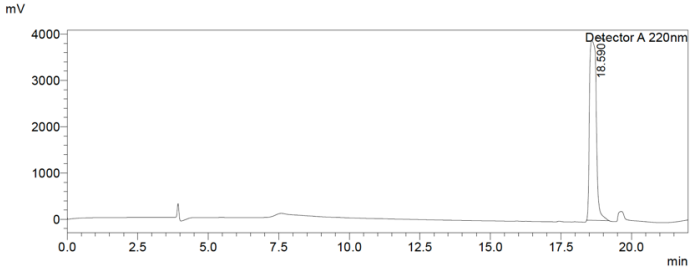
Radiocyanation and analysis of 9-^{11}CN



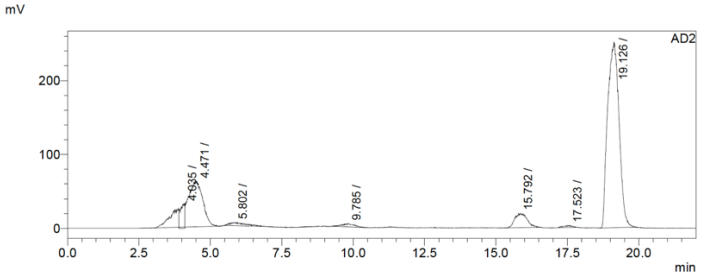
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-B**. HPLC column: **Column C**. HPLC condition: **Method 1.** Non-isolated RCY = 53 ± 15%

n	RCY [%]
1	55
2	39
3	73
4	43

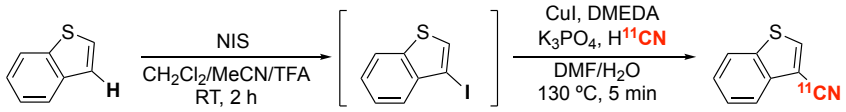
HPLC (UV trace): authentic standard **9-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



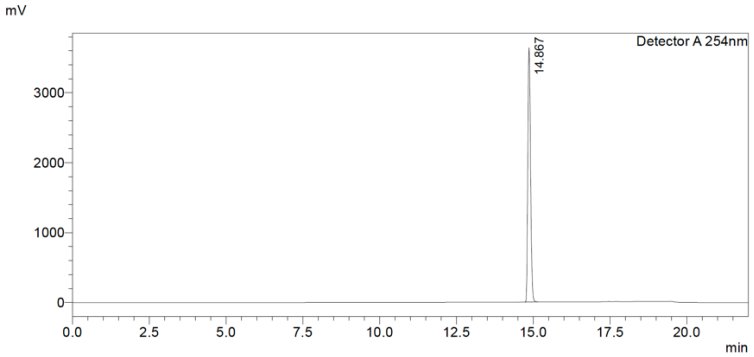
Radiocyanation and analysis of **10-^{11}CN**



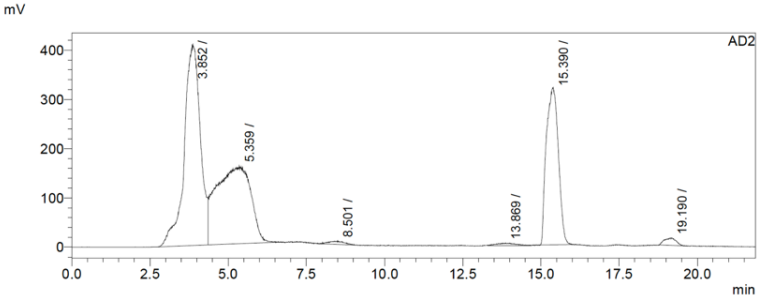
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-A (but at 100 °C)**. HPLC column: **Column A**. HPLC condition: **Method 2.** Non-isolated RCY = 51 ± 2%

n	RCY [%]
1	52
2	52
3	48

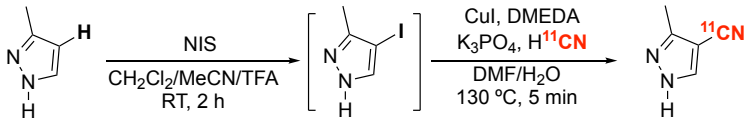
HPLC (UV trace): authentic standard **10-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



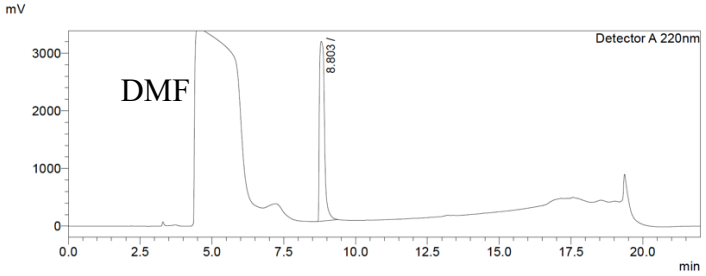
Radiocyanation and analysis of 11-^{11}CN



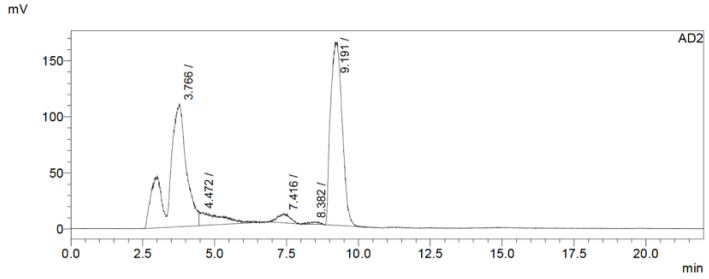
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-A**. HPLC column: **Column A**. HPLC condition: **Method 1.** Non-isolated RCY = 43 ± 7 %

n	RCY [%]
1	35
2	44
3	48

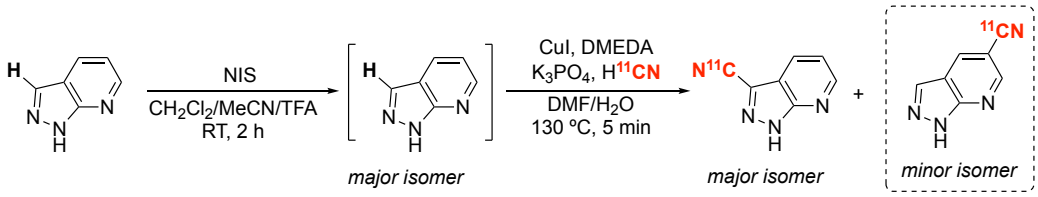
HPLC (UV trace): authentic standard **11-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



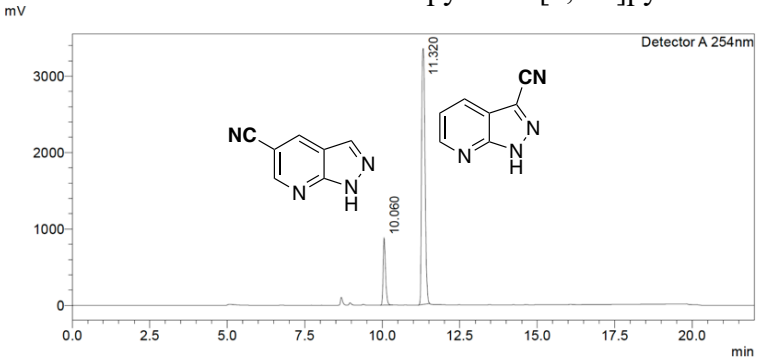
Radiocyanation and analysis of 12-^{11}CN



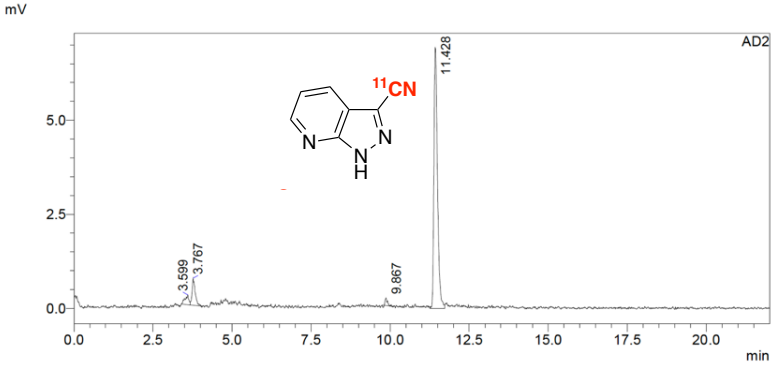
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-A**. HPLC column: **Column C**. HPLC condition: **Method 2.** Non-isolated RCY = 79 ± 14 %

n	Major product RCY [%]	Minor product RCY [%]
1	84	4
2	90	1
3	64	0

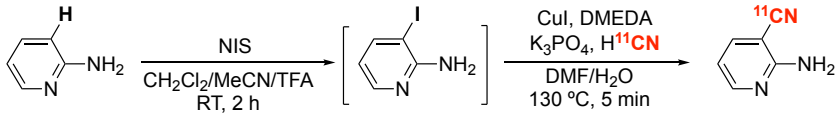
HPLC (UV trace): authentic standard **12-^{12}CN** and pyrazolo[3,4-b]pyridine-5-carbonitrile



HPLC (radio trace): radiocyanation reaction (crude)



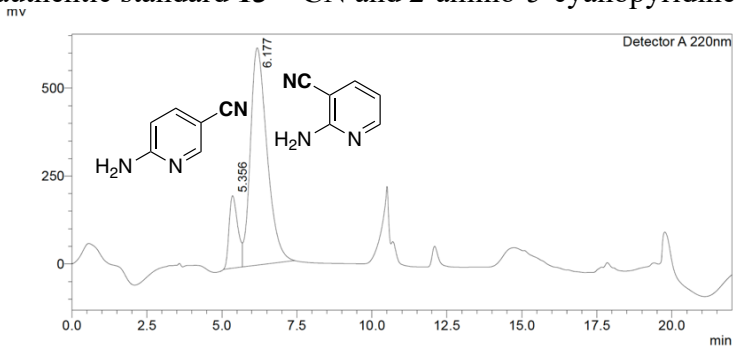
Radiocyanation and analysis of 13-^{11}CN



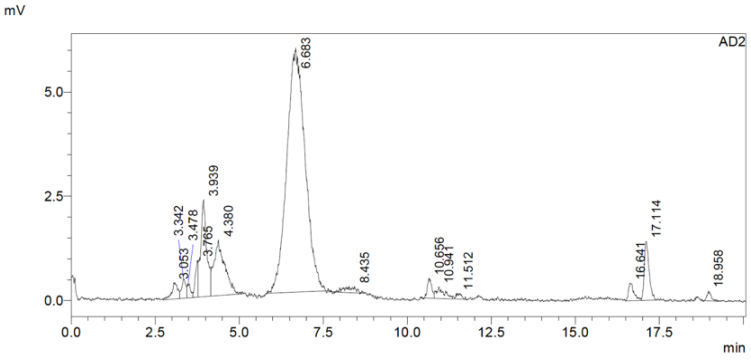
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-A**. HPLC column: **Column B**. HPLC condition: **Method 4.** Non-isolated RCY = 57 ± 16 %

n	RCY [%]
1	38
2	66
3	66

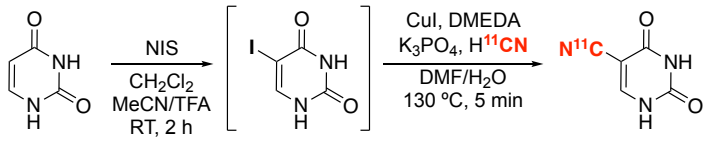
HPLC (UV trace): authentic standard **13-^{12}CN** and 2-amino-5-cyanopyridine



HPLC (radio trace): radiocyanation reaction (crude)



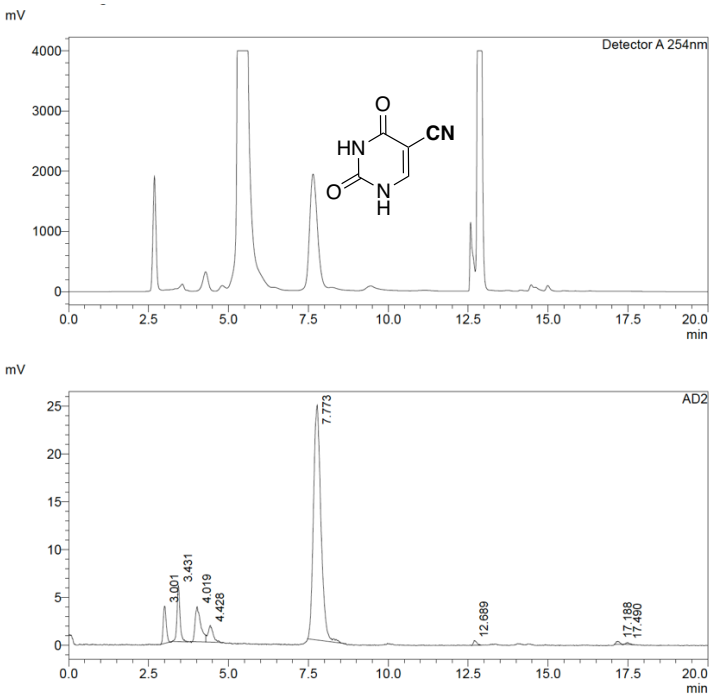
Radiocyanation and analysis of 14-^{11}CN



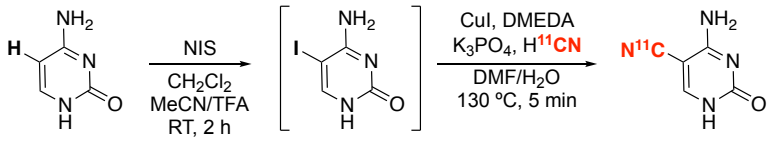
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-B**. HPLC column: **Column B**. HPLC condition: **Method 5.** Non-isolated RCY = 69 ± 11%

n	RCY [%]
1	77
2	57
3	73

HPLC (UV trace, top): reaction crude co-injected with authentic standard **14-^{12}CN**
HPLC (radio trace, bottom): radiocyanation reaction (crude)



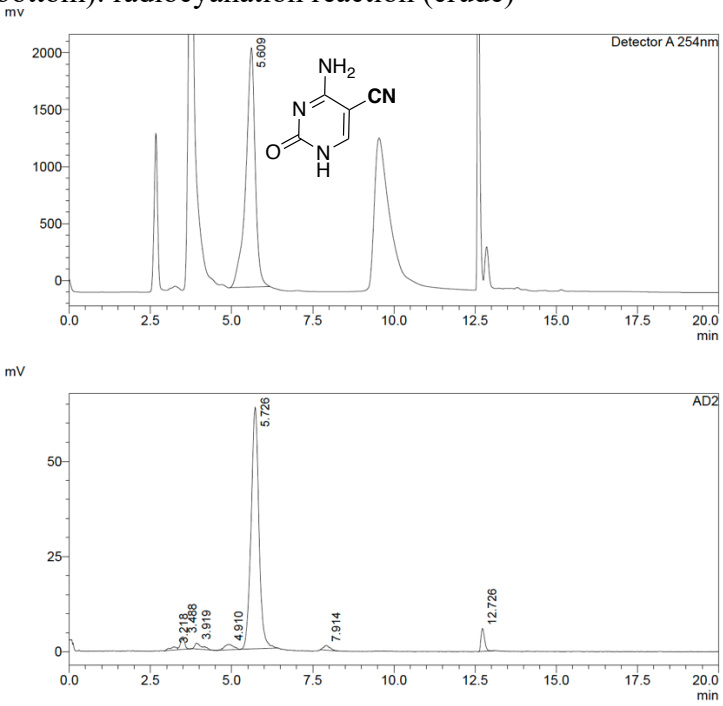
Radiocyanation and analysis of 15-^{11}CN



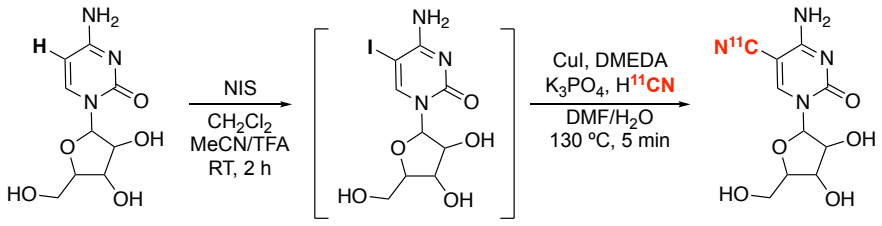
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-B**. HPLC column: **Column B**. HPLC condition: **Method 5.** Non-isolated RCY = 84 ± 4%

n	RCY [%]
1	87
2	86
3	79

HPLC (UV trace, top): reaction crude co-injected with authentic standard 15-^{12}CN
HPLC (radio trace, bottom): radiocyanation reaction (crude)



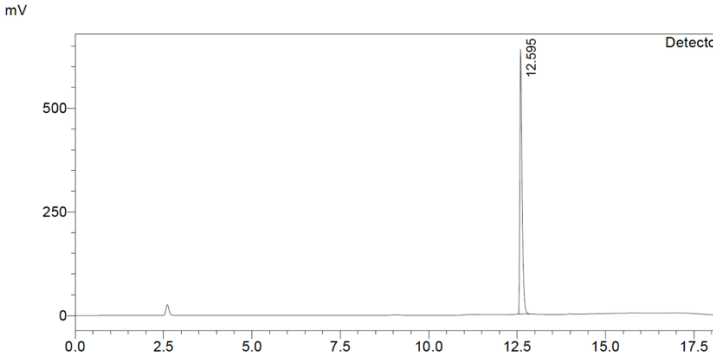
Radiocyanation and analysis of 16-^{11}CN



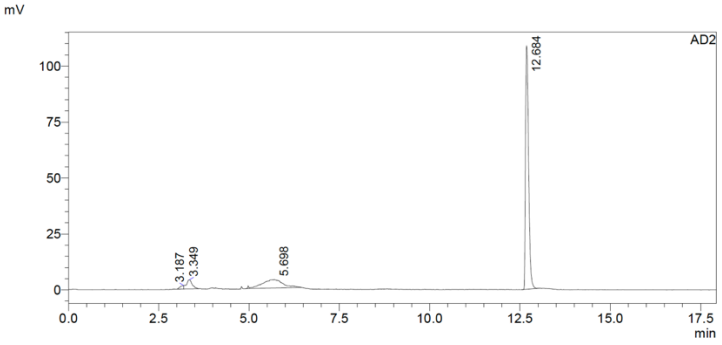
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-B**. HPLC column: **Column B**. HPLC condition: **Method 5.** Non-isolated RCY = 75 ± 11%

n	RCY [%]
1	63
2	80
3	81

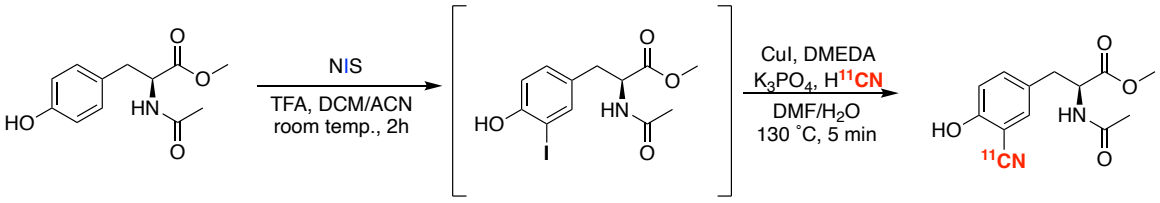
HPLC (UV trace): authentic standard **16-^{12}CN**



HPLC (radio trace): radiochemistry reaction (crude)



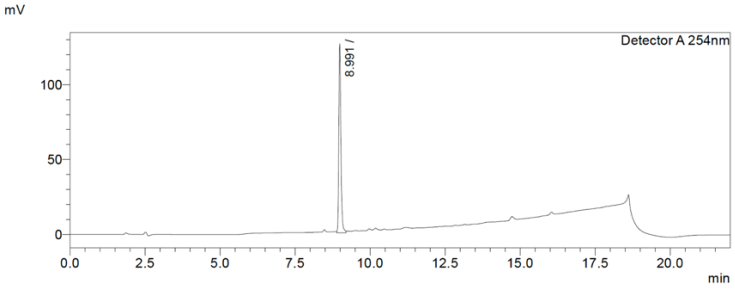
Radiocyanation and analysis of 17-^{11}CN



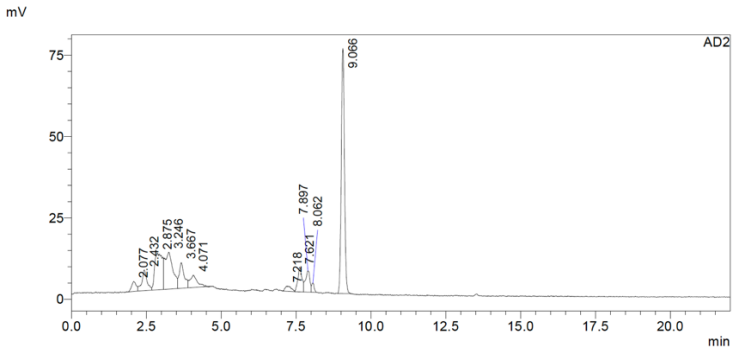
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-C**. HPLC column: **Column D**. HPLC condition: **Method 2.** Non-isolated RCY = 75 ± 5%

n	RCY [%]
1	50
2	41
3	43

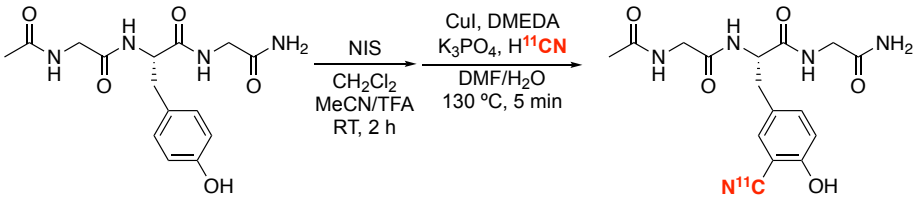
HPLC (UV trace): authentic standard **17-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



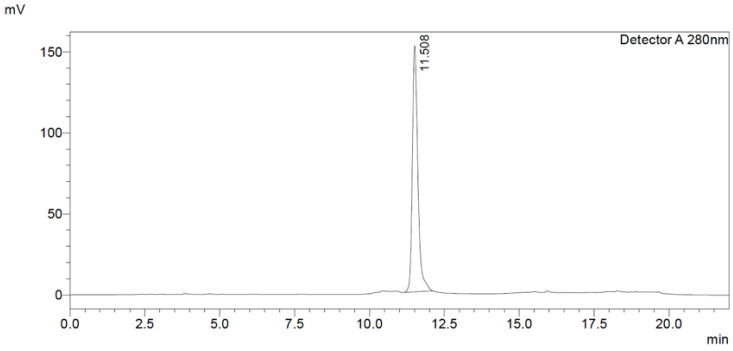
Radiocyanation and analysis of 18-^{11}CN



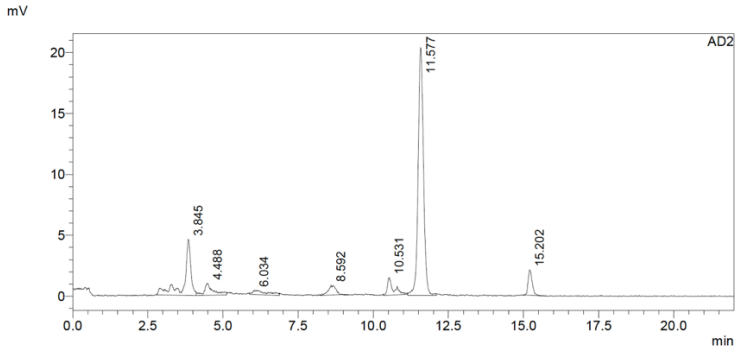
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-B**. HPLC column: **Column B**. HPLC condition: **Method 8.** Non-isolated RCY = 67 ± 7%

n	RCY [%]
1	66
2	74
3	61

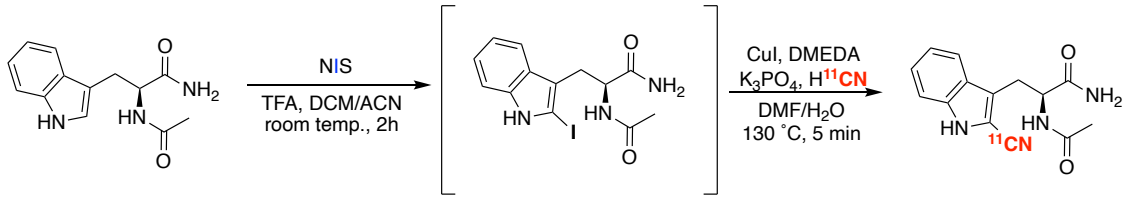
HPLC (UV trace): authentic standard **18-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



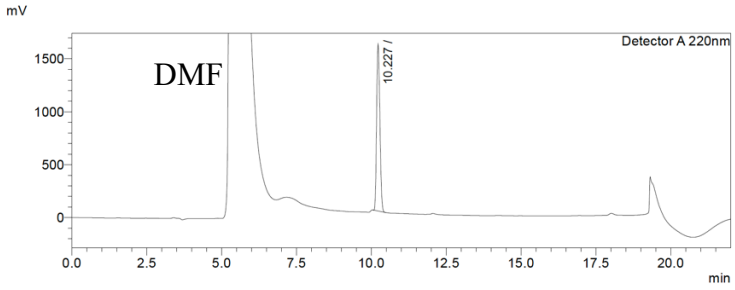
Radiocyanation and analysis of 19-^{11}CN



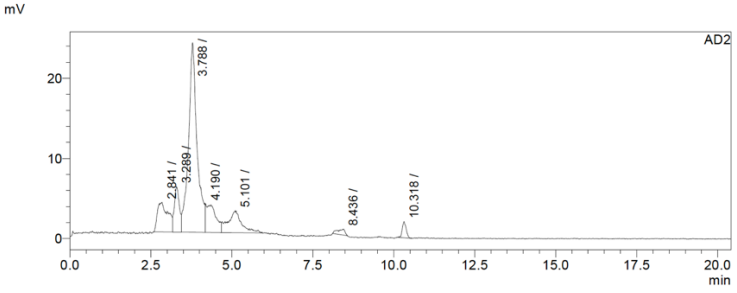
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-C**. HPLC column: **Column B**. HPLC condition: **Method 1.** Non-isolated RCY = 2 ± 1%

n	RCY [%]
1	2
2	3
3	1
4	3
5	3

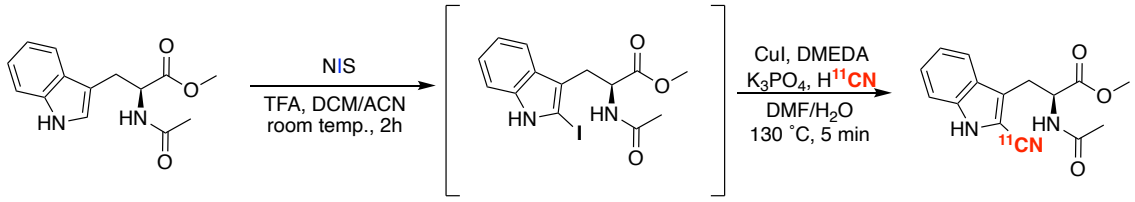
HPLC (UV trace): authentic standard **19-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



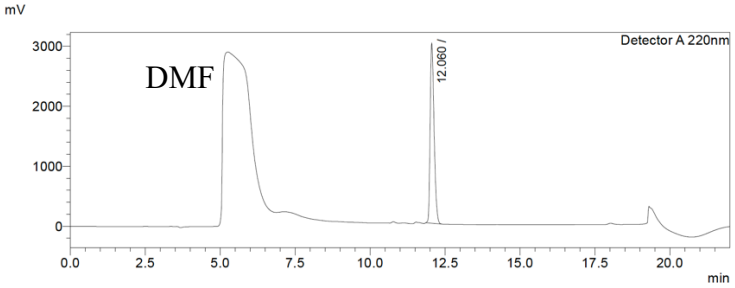
Radiocyanation and analysis of **20-^{11}CN**



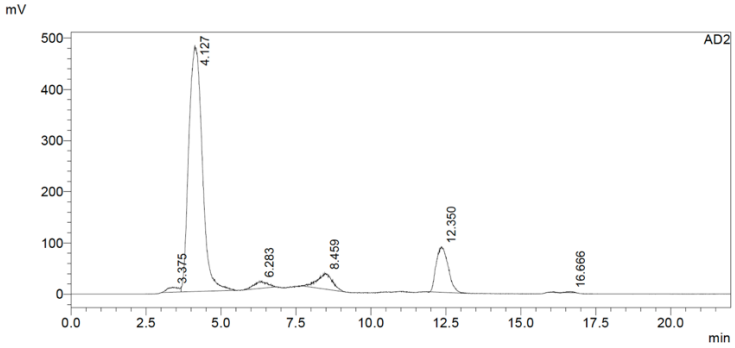
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-C**. HPLC column: **Column B**. HPLC condition: **Method 1.** Non-isolated RCY = 10 ± 3%

n	RCY [%]
1	8
2	9
3	9
4	16
5	11

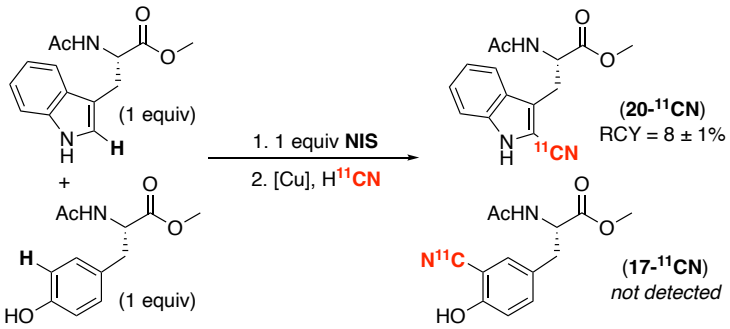
HPLC (UV trace): authentic standard **20-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



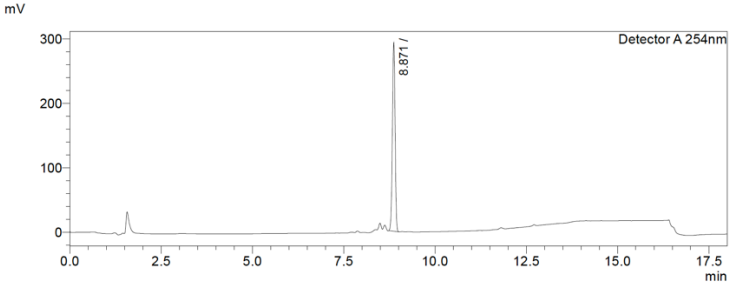
Radiocyanation and analysis of competition study (20-^{11}CN vs 17-^{11}CN)



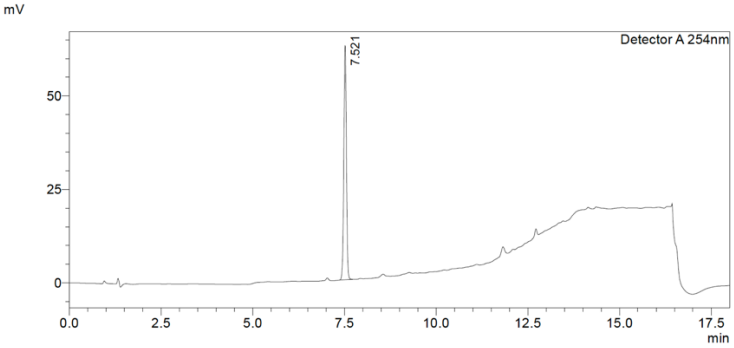
Iodination conditions: **Procedure I-A (2 h)**. Radiocyanation conditions: **Procedure R-C**. HPLC column: **Column E**. HPLC condition: **Method 9**. Non-isolated RCY of **20-^{11}CN** = 8 ± 1%

n	RCY [%]
1	9
2	8
3	6
4	7

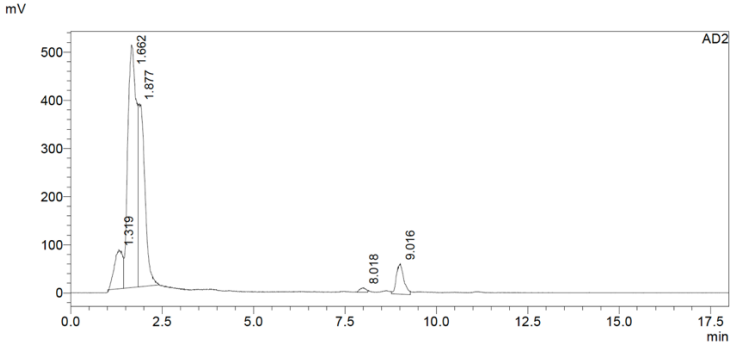
HPLC (UV trace): authentic standard **20-^{12}CN**



HPLC (UV trace): authentic standard **17-^{12}CN**



HPLC (radio trace): radiocyanation reaction (crude)



6. Automated Radiocyanation

6.1 Reaction Setup

6.1.1 Synthesis Module Configuration

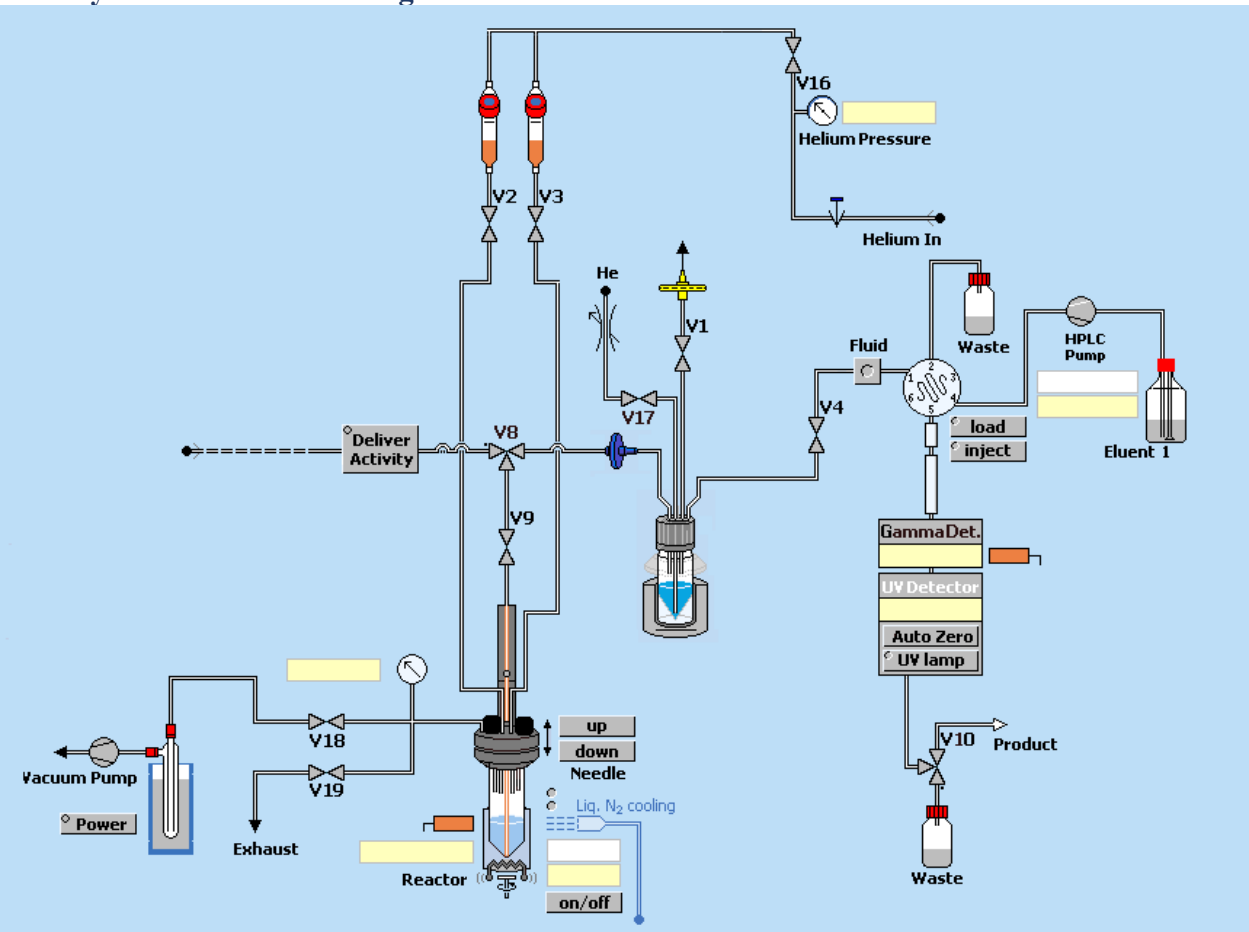


Figure 1. GE TRACERlab FX_M Synthesis Module Configuration for Automated Radiocyanation

6.1.2 General Reaction Setup

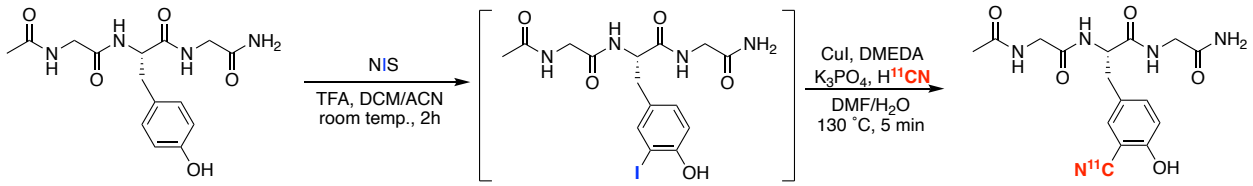
See Section 3 (Iodination General Procedure) for the first step of the C–H Radiocyanation (Procedure I-B). C—H iodination was carried out on a 5 μmol scale. The reaction mixture was immediately transferred to the synthesis module reactor vial and the solvent was removed under a stream of nitrogen gas. Subsequently, the reactor vial was placed in a vacuum chamber under high vacuum for 1-2 hrs to remove solvent. The reactor vial with a dry precursor inside was charged with a solution of milli-Q water (200 μL) and potassium phosphate (2.7 mg, 20 μmol, 4 equiv) under air. The reactor vial was then attached to the synthesis module.

In a N_2 atmosphere glove box, a 4 mL vial was charged with CuI (3.8 mg, 20 μmol), DMEDA (4.4 μL), and DMF (396 μL) to make a CuI stock solution. The vial was shaken until the CuI completely dissolved. 100 μL of CuI stock solution was transferred to Vial 2 (N_2 balloon was used to minimize air exposure)

In Vial 3, 1 mL of HPLC solution (H_2O/MeCN = 50/50 with 0.1% of trifluoroacetic acid; TFA).

[^{11}C]HCN was produced in a cyclotron (10 min beam) and bubbled through the potassium phosphate solution in the reactor vial for 5 minutes, which trapped as [^{11}C]KCN. The CuI stock solution in Vial 2 was dispensed into the reactor vial. And, the reaction solution was stirred at 130 ˚C for 5 minutes under Ar atmosphere. The reaction was then cooled down to 70 ˚C, and the HPLC buffer was dispensed from Vial 3. The crude reaction mixture was then either sent to the preparatory-HPLC for purification or removed from the hot cell to be analyzed by an analytical HPLC.

6.2 Ac-GYG-NH_2*



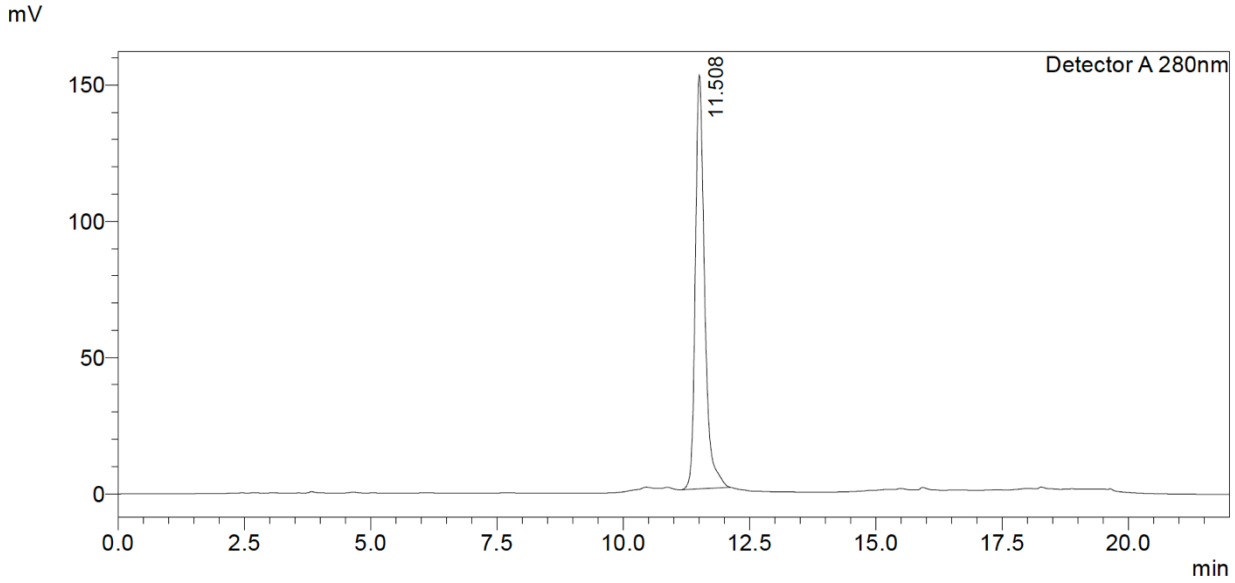
*The second step was automated in a synthesis module in a hot cell.

n	RCC [%]	RCY [%]	Molar Acitivity [Ci/µmol]
1	46	29	1.1
2	27	16	1.2
3	47	NA	NA
4	32	NA	NA
5	27	NA	NA

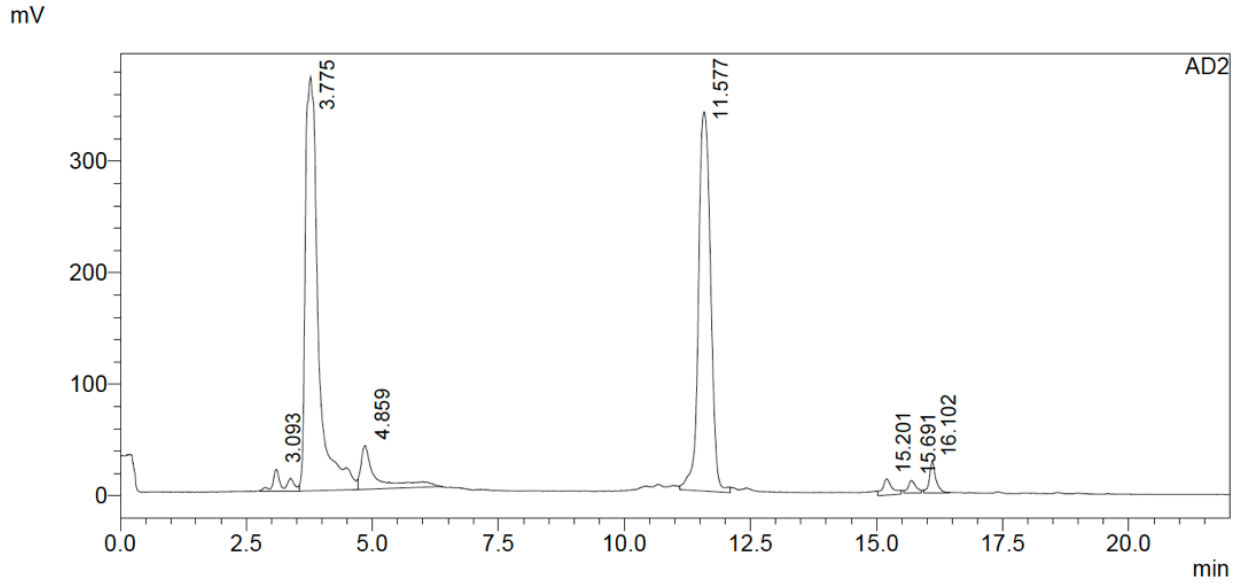
RCC = 36 ± 9%
NA = not isolated

	Column	Eluent
Analytical	Synergi 10µ Hydropro-RP 80Å 250 x 4.6 mm P/N = 00G-4376-E0	Method 8 (Section 5.4.2)
Preparatory	Synergi 10µ Hydro-RP 80Å LC Column 250 x 10 mm P/N = 00G-4376-N0	90% H_2O + 0.1% TFA 10% MeCN + 0.1% TFA Isocratic method

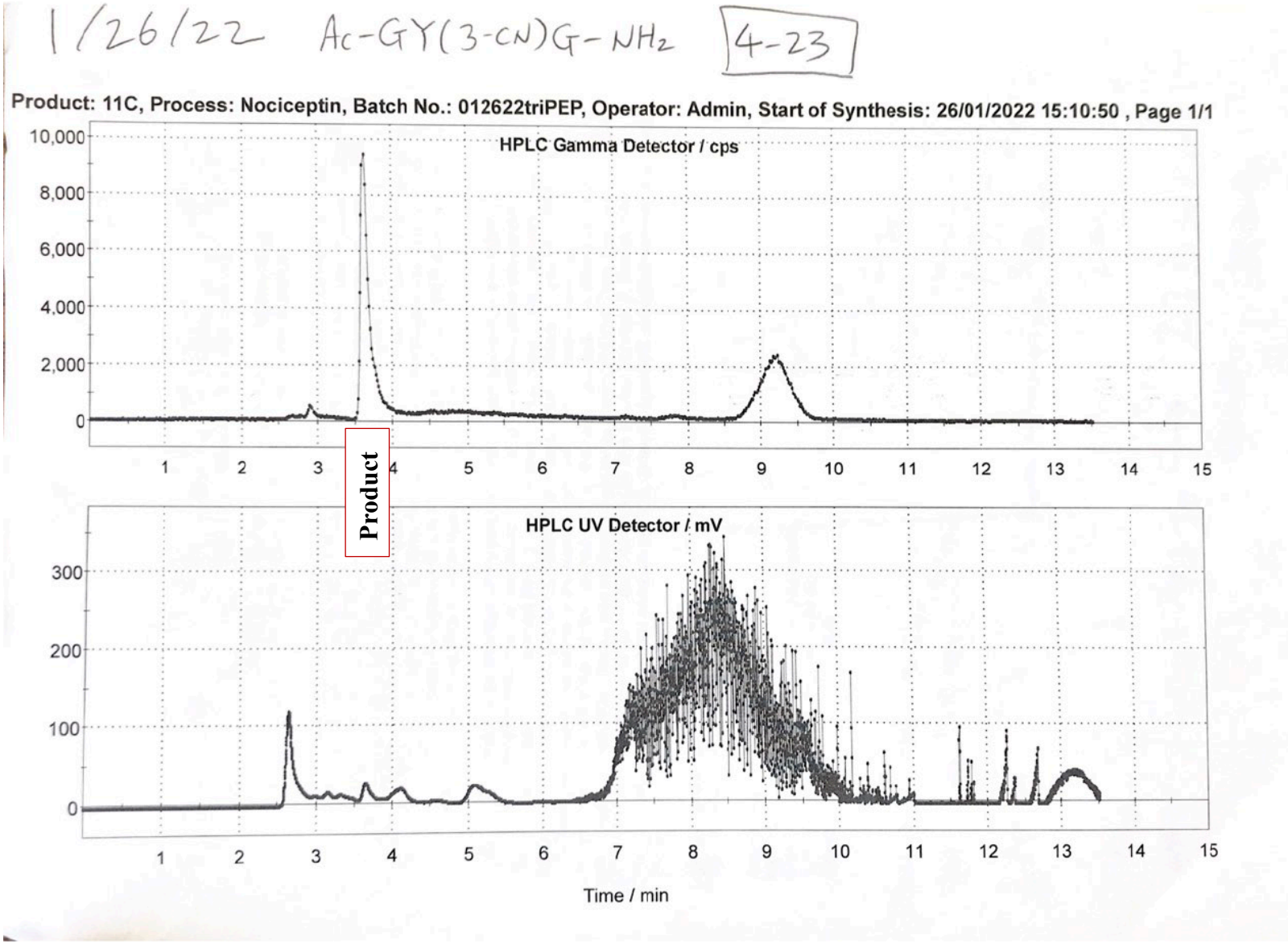
Analytical HPLC (UV trace): authentic standard



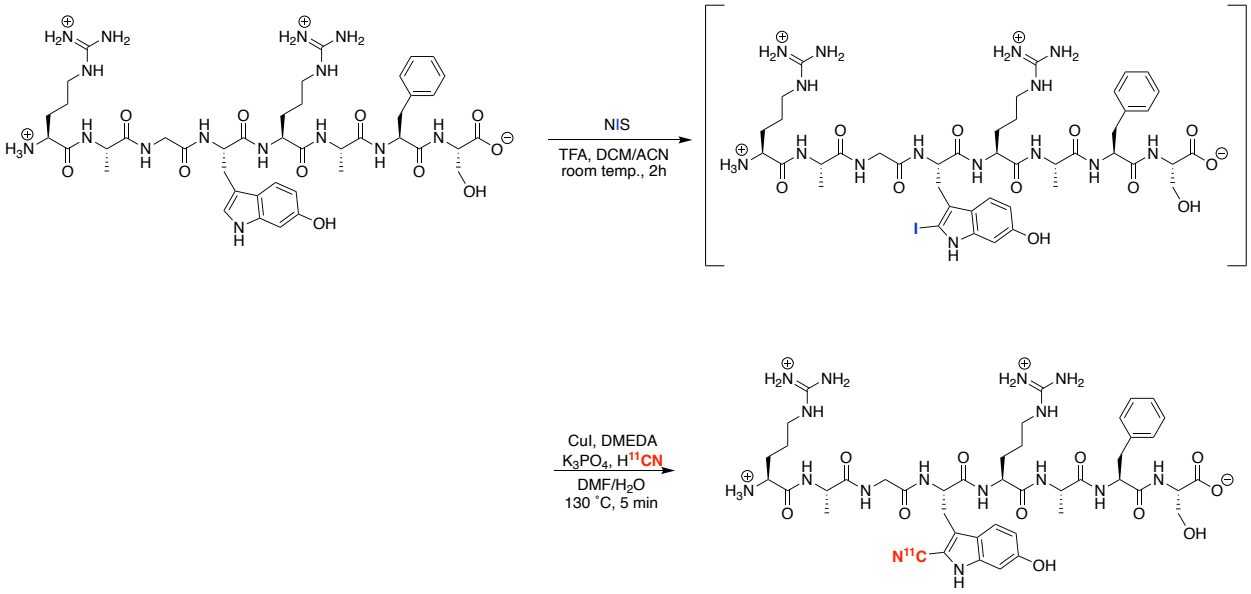
Analytical HPLC (radio trace): authentic standard



Prep-HPLC


1/26/22 Ac-GY(3-CN)G-NH2 4-23
Product: 11C, Process: Nociceptin, Batch No.: 012622triPEP, Operator: Admin, Start of Synthesis: 26/01/2022 15:10:50 , Page 1/1
HPLC Gamma Detector / cps
10,000
8,000
6,000
4,000
2,000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Product
HPLC UV Detector / mV
300
200
100
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time / min

6.3 LYRAGWRAFS*



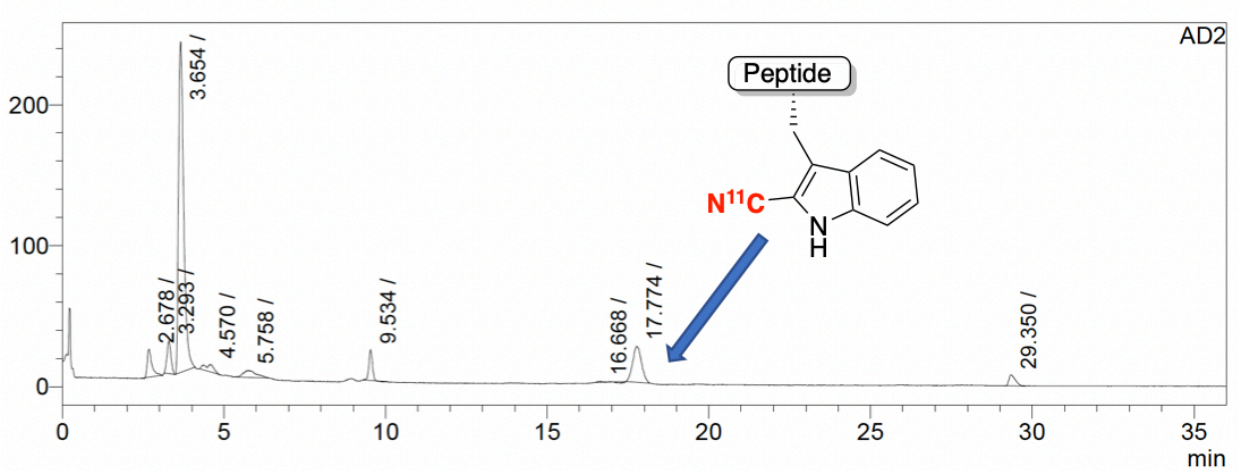
*The second step was automated in a synthesis module in a hot cell.

HPLC column: Column B (Section 5.4.2).
HPLC condition: Flow rate: 1.0 mL/min
UV detection = 280 nm
Solvent A = H_2O + 0.5% TFA; Solvent B = MeCN + 0.5% TFA
0-4 min: 5% B (hold)
4-5 min: 5 → 20% B
5-25 min: 20 → 27% B
25-27 min: 27 → 90% B
27-31 min: 90% B (hold)
31-32 min: 90 → 5% B
32-36 min: 5% B (hold)

n	RCC [%]
1	17
2	20
3	13

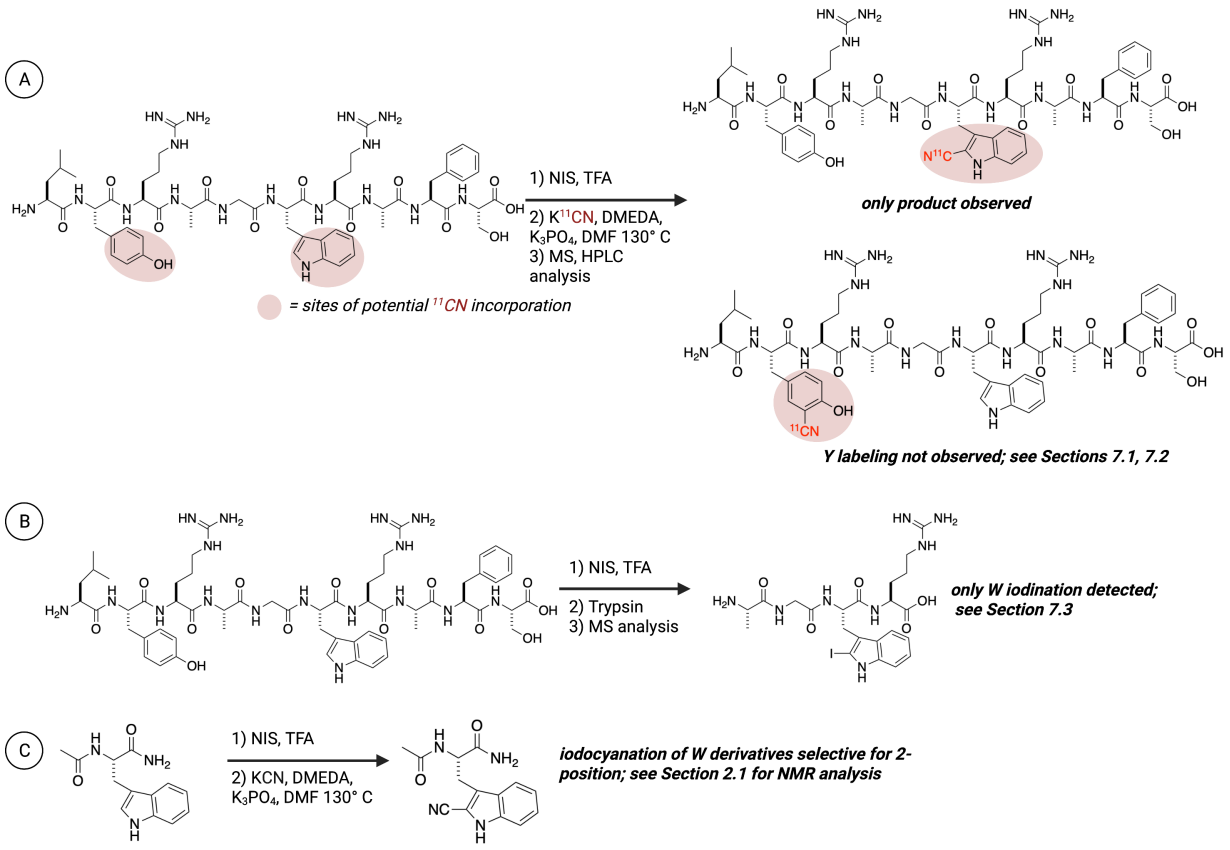
RCC = 17 ± 3%

Analytical HPLC (radio trace):



7. LYRAGWRAFS Analysis

The LYRAGW(3-^{12}CN)RAFS standard proved challenging to synthesize, so we established the structure based on the following experiments.



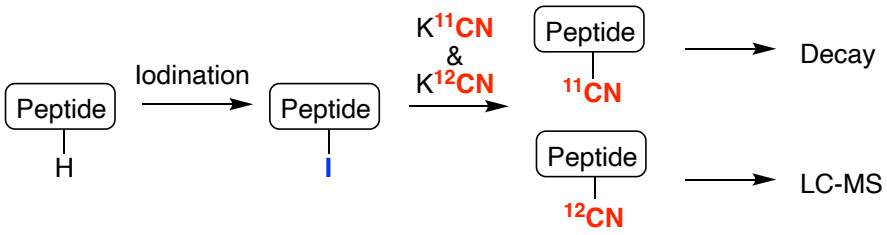
Scheme 1. A. C—H radiocyanation of LYRAGWRAFS followed by LC-MS analysis. B. Trypsin digestion experiment to determine the labeling residue. C. Cyanation of tryptophan derivative to determine the selectivity at C-2

7.1 Confirmation of Radiocyanation of LYRAGWRAFS

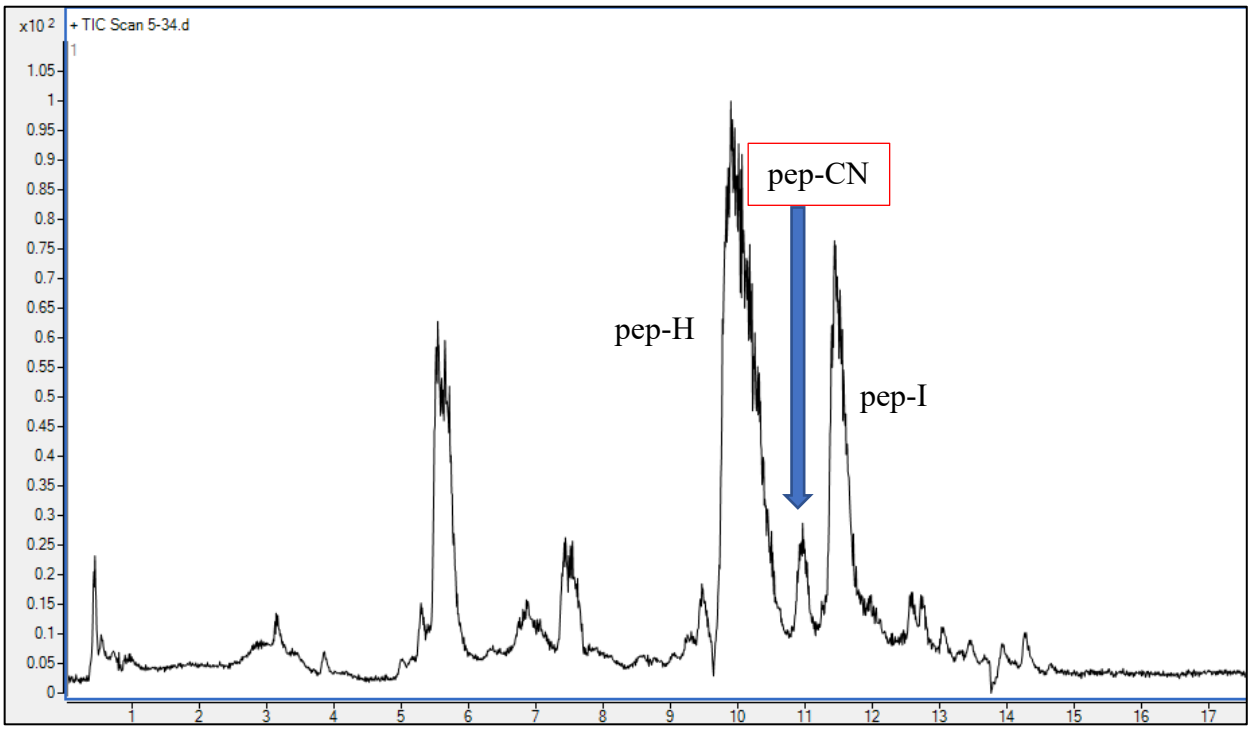
The occurrence of the C—H iodination followed by radiocyanation was confirmed by the following experiment:

Procedure I-B was used for the iodination of the peptide LYRAGWRAFS (See Section 3). The reaction mixture was transferred to the synthesis module reactor vial and the solvent was removed under a stream of nitrogen gas. Subsequently, the reactor vial was placed in a vacuum chamber under high vacuum for 1-2 hrs to remove solvent. The reactor vial with a dry precursor inside was charged with a solution of milli-Q water (200 µL), potassium phosphate (2.7 mg, 20 µmol, 4 equiv), and **KCN (10 µg)** under air. The reactor vial was then attached to the synthesis module. The following steps were carried out as described in Section 6.2.2.

By intentionally adding [^{12}C]KCN, the reaction was able to be analyzed by Agilent Q-TOF HPLC-MS.



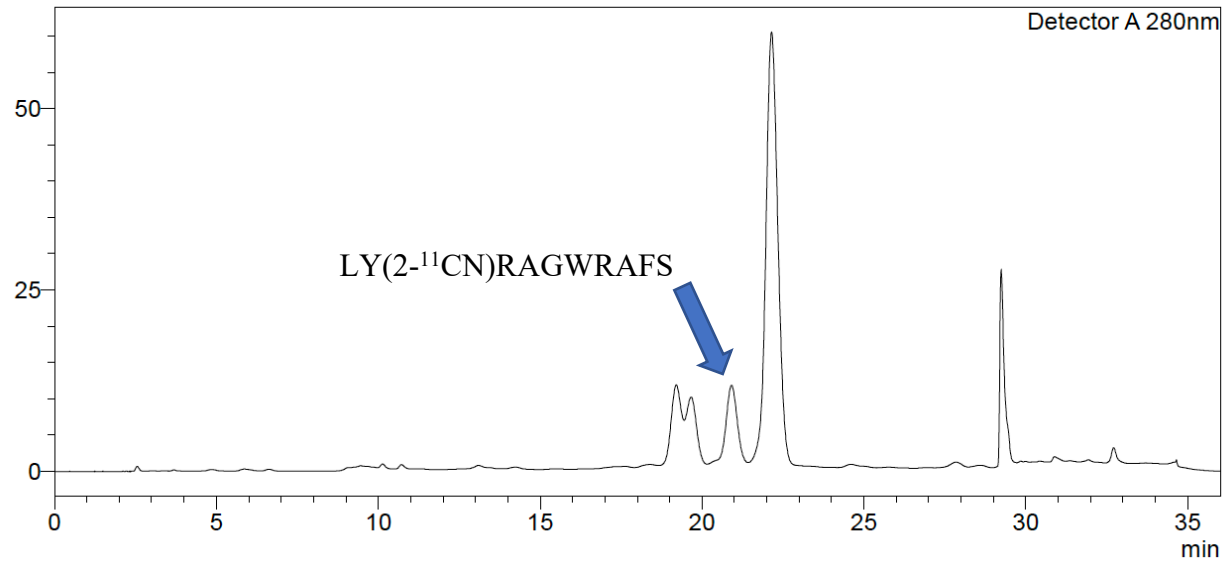
LC-MS Spectrum of the reaction crude



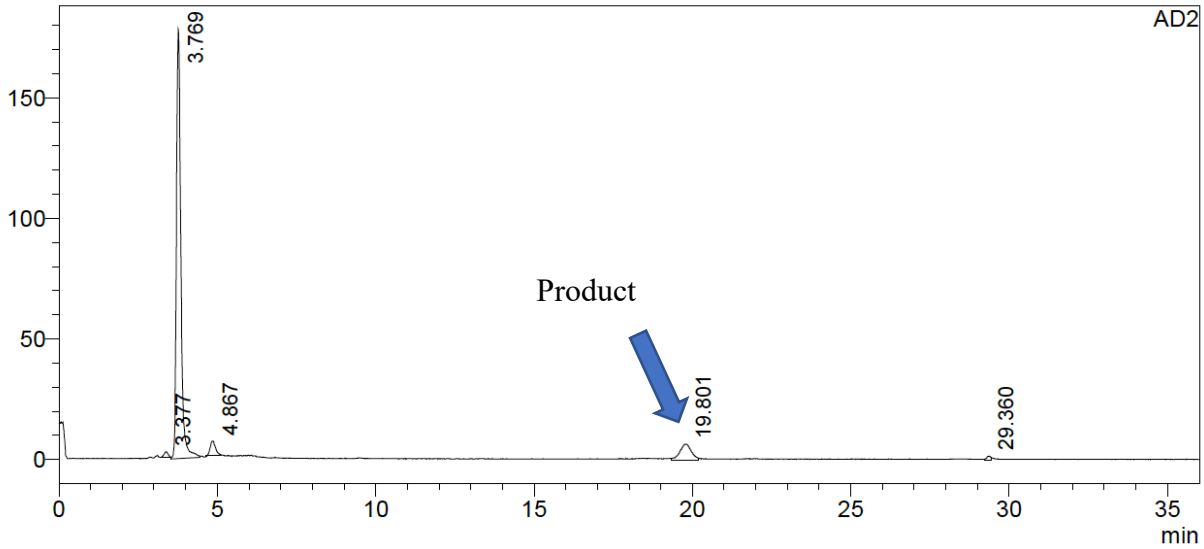
7.2 Comparison with LY(2-CN)RAGWRAFS authentic standard

The LY(2-^{12}CN)RAGWRAFS authentic standard did not match with the main radio peak. Based on this co-inject analysis, our product was not LY(2-^{11}CN)RAGWRAFS.

HPLC (UV trace): co-inject with authentic standard LY(2-^{11}CN)RAGWRAFS



HPLC (radio trace): radiocyanation reaction (crude)



7.3 Identifying the labeled residue

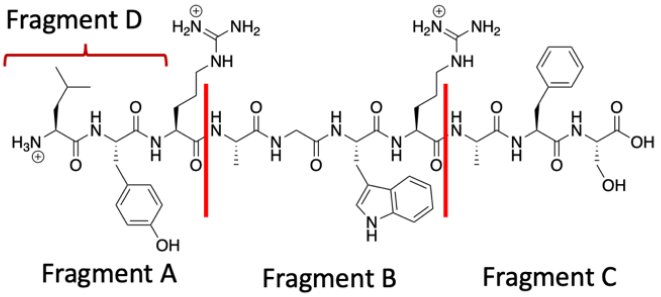
The tyrosine, tryptophan, and phenylalanine residue could be iodinated under the electrophilic aromatic iodination condition. To identify which amino acid residue underwent the reaction, the following experiment was performed.

Iodination of the peptide → cutting the peptide into three fragments → LC-MS analysis

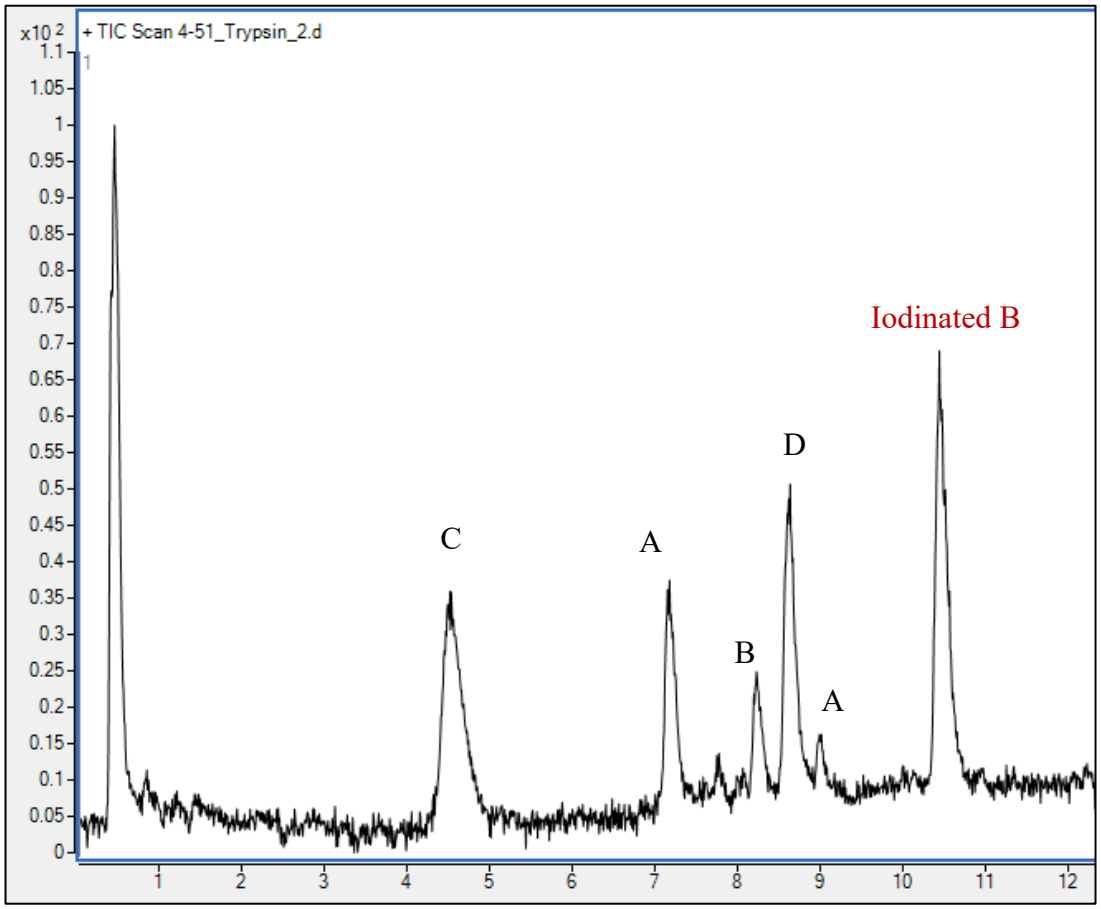
Procedure I-B was used for the iodination of the peptide LYRAGWRAFS on a 5 μmol scale. After the reaction completed, 186 μL of the crude solution was transferred to a clean 4-mL vial. The voltiles were removed under a stream of nitrogen and then under high vacuum. To this vial, 50 mM Tris-HCl was added until the crude product (solid) fully dissolved (~ 0.9mL). Then, 80 μL of Gibco™ Trypsin-EDTA (0.25%) was added to this vial and stirred at 37 ˚C overnight. The proteolysis reaction was quenched by addition of trifluoroacetic acid 10 μL. This crude reaction mixture was injected to Agilent Q-TOF HPLC-MS for analysis without further purification.

LC-MS condition:
Flow rate: 0.4 mL/min
Solvent A = H_2O + 0.5% TFA; Solvent B = MeCN + 0.5% TFA
0-1 min: 0% B (hold)
1-5 min: 0 → 10% B
5-10 min: 10 → 15% B
10-20 min: 15 → 50% B
20-25 min: 50% B (hold)
25-30 min: 50 → 0% B

Result:



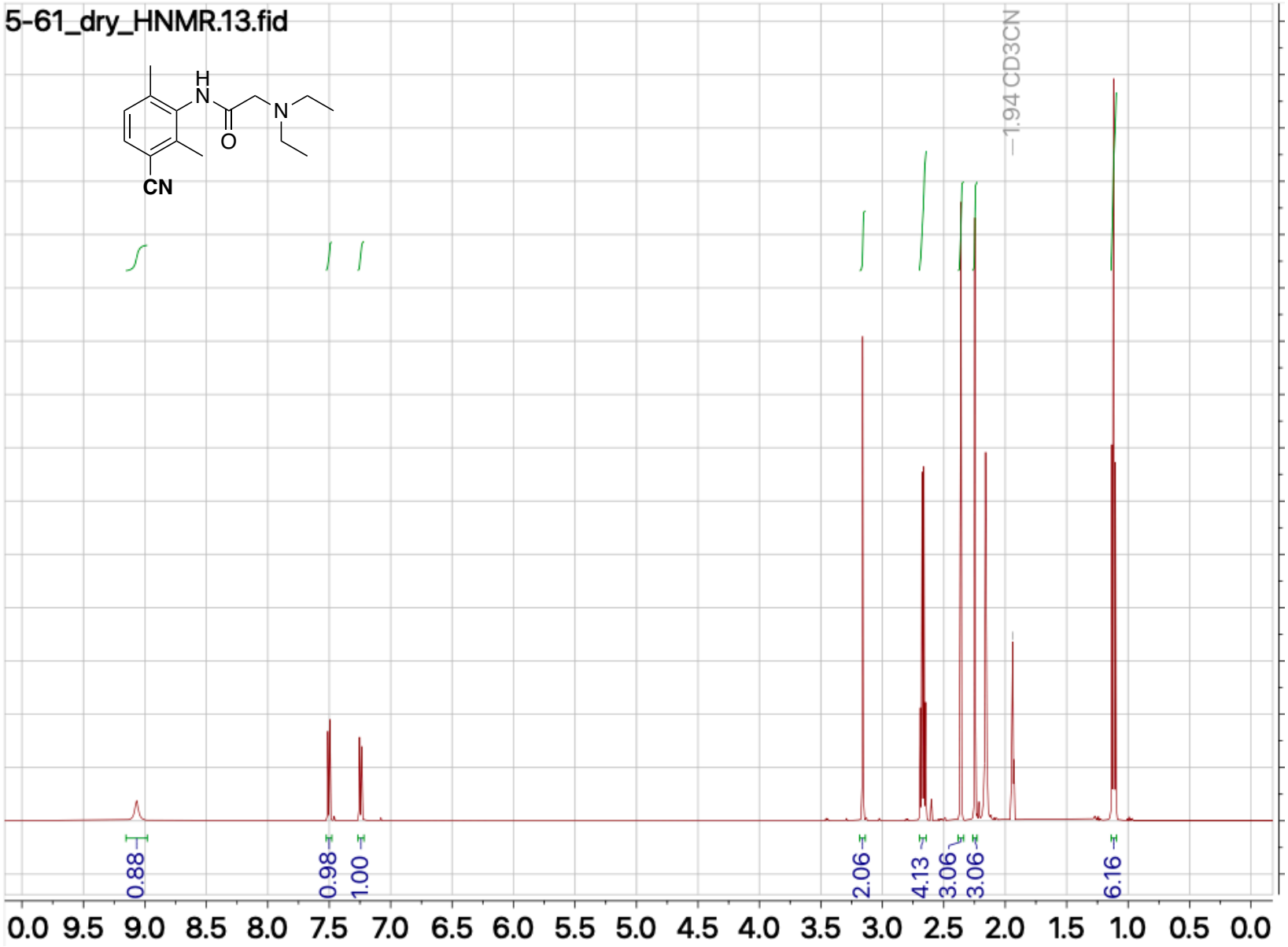
LC-MS spectrum



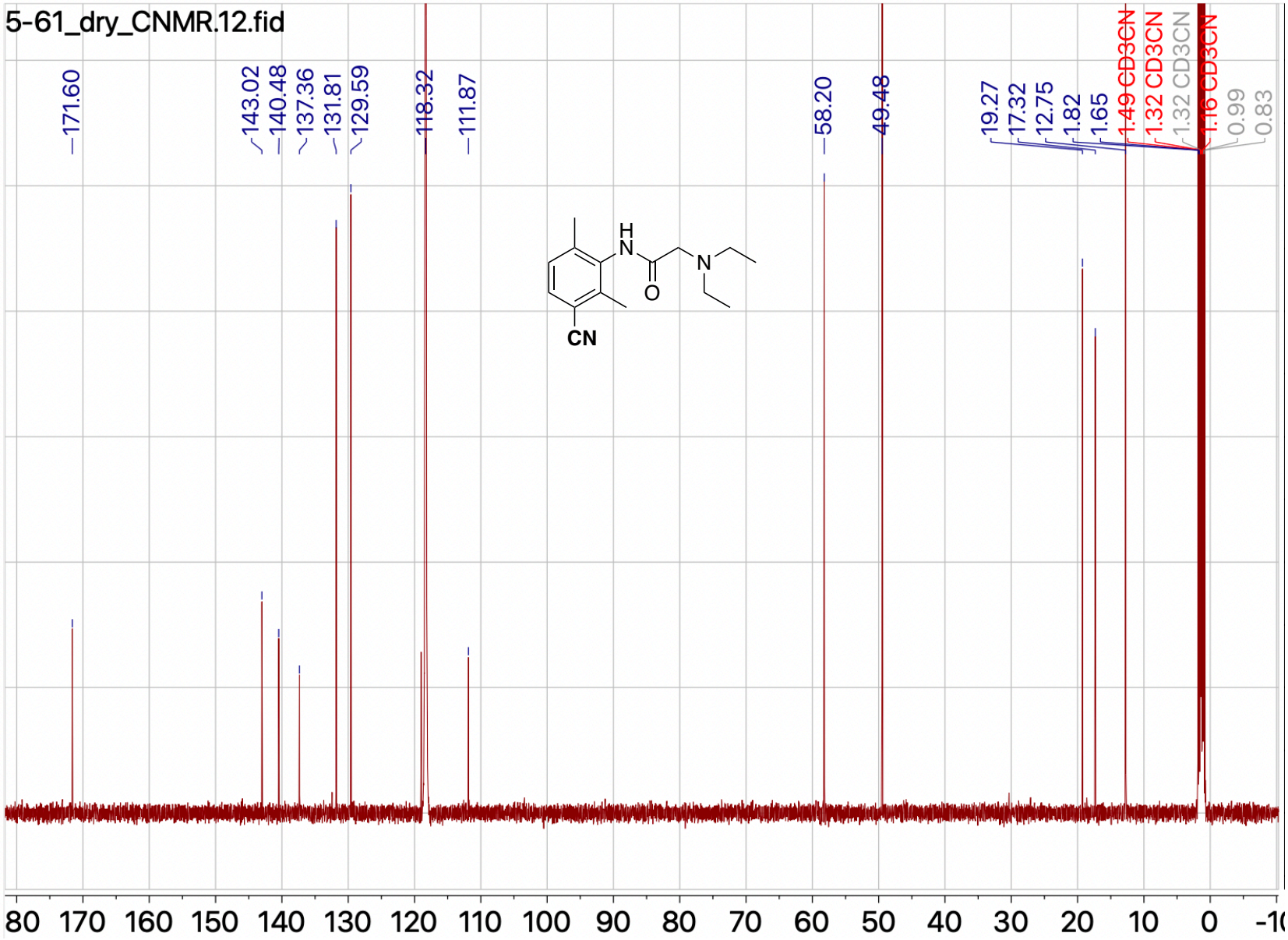
Counts(%) vs Acquisition Time (min)

Because the tryptophan residue is iodinated, this residue gets radiocyanated as well.

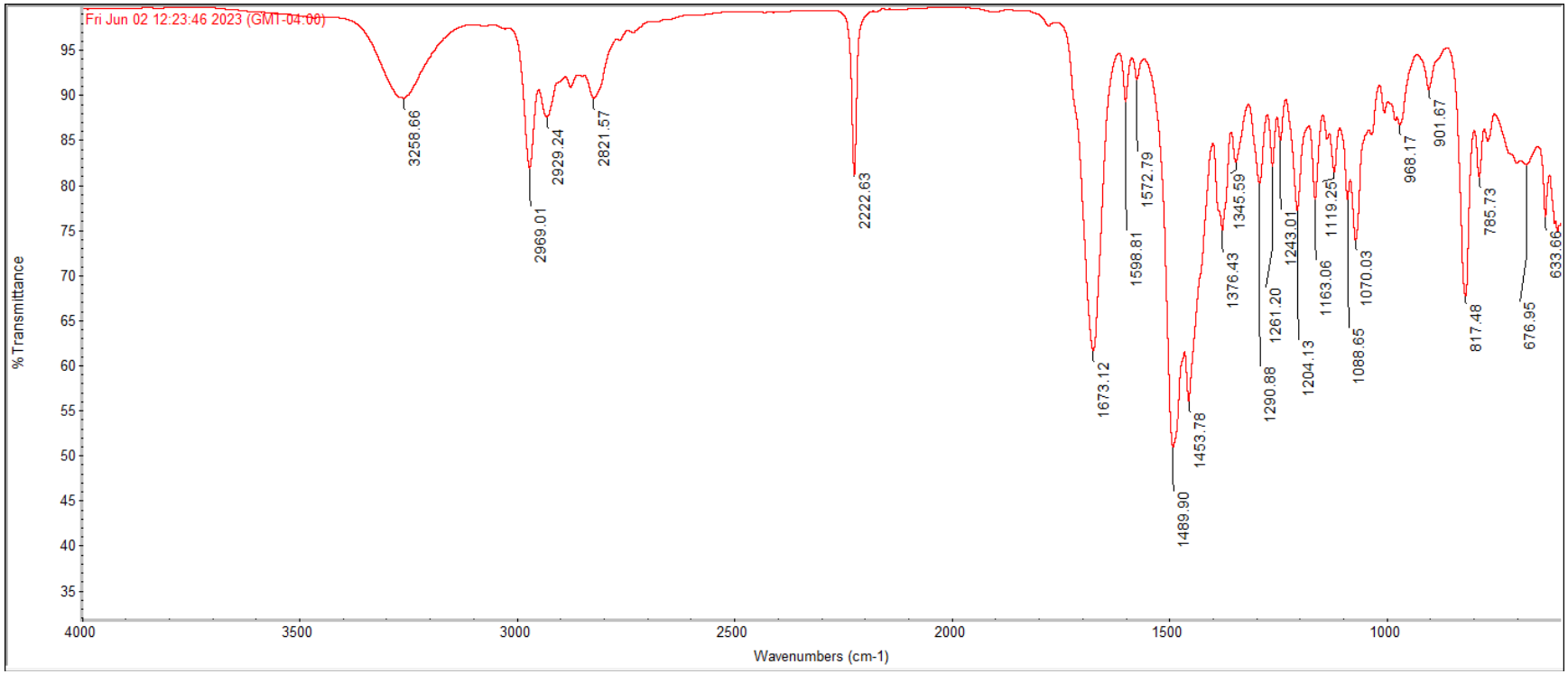
^{1}H NMR of S-2 in CD_3CN



^{13}C NMR of S-2 in CD_3CN



IR of S-2


Fri Jun 02 12:23:46 2023 (GMT-04:00)
% Transmittance
Wavenumbers (cm-1)
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3000
2500
2000
1500
1000
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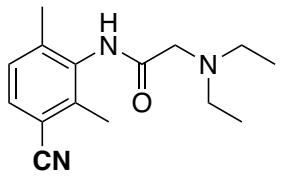
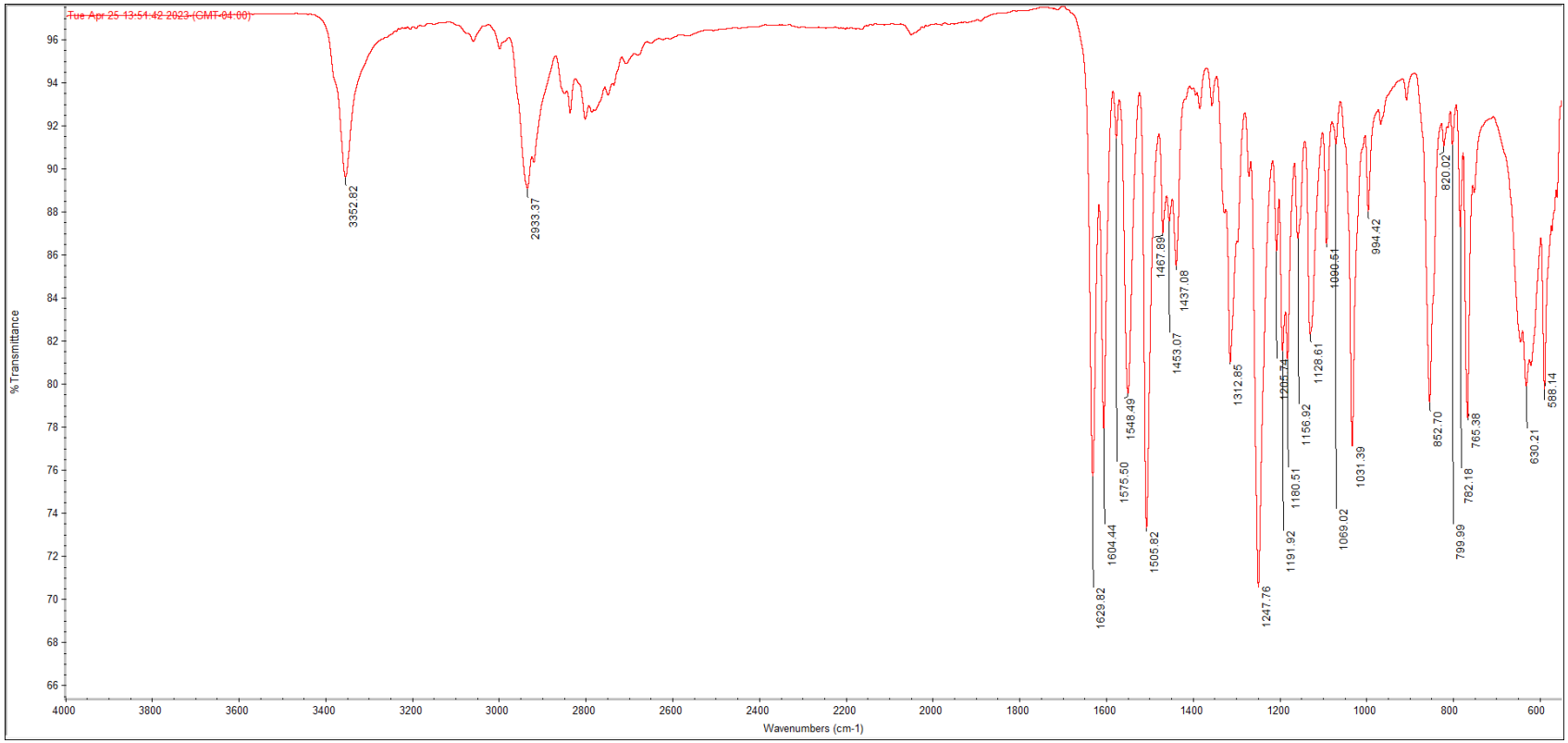
2969.01
2929.24
2821.57
2222.63
1673.12
1598.81
1572.79
1489.90
1453.78
1376.43
1345.59
1290.88
1261.20
1243.01
1204.13
1163.06
1119.26
1088.65
1070.03
968.17
901.67
817.48
785.73
676.95
633.66


H
N
O
N
CN

IR of S-3


Tue Apr 25 13:51:42 2023 (GMT-04:00)
% Transmittance
Wavenumbers (cm-1)
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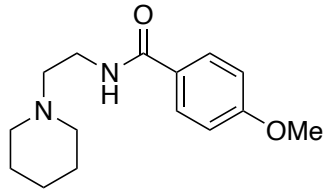
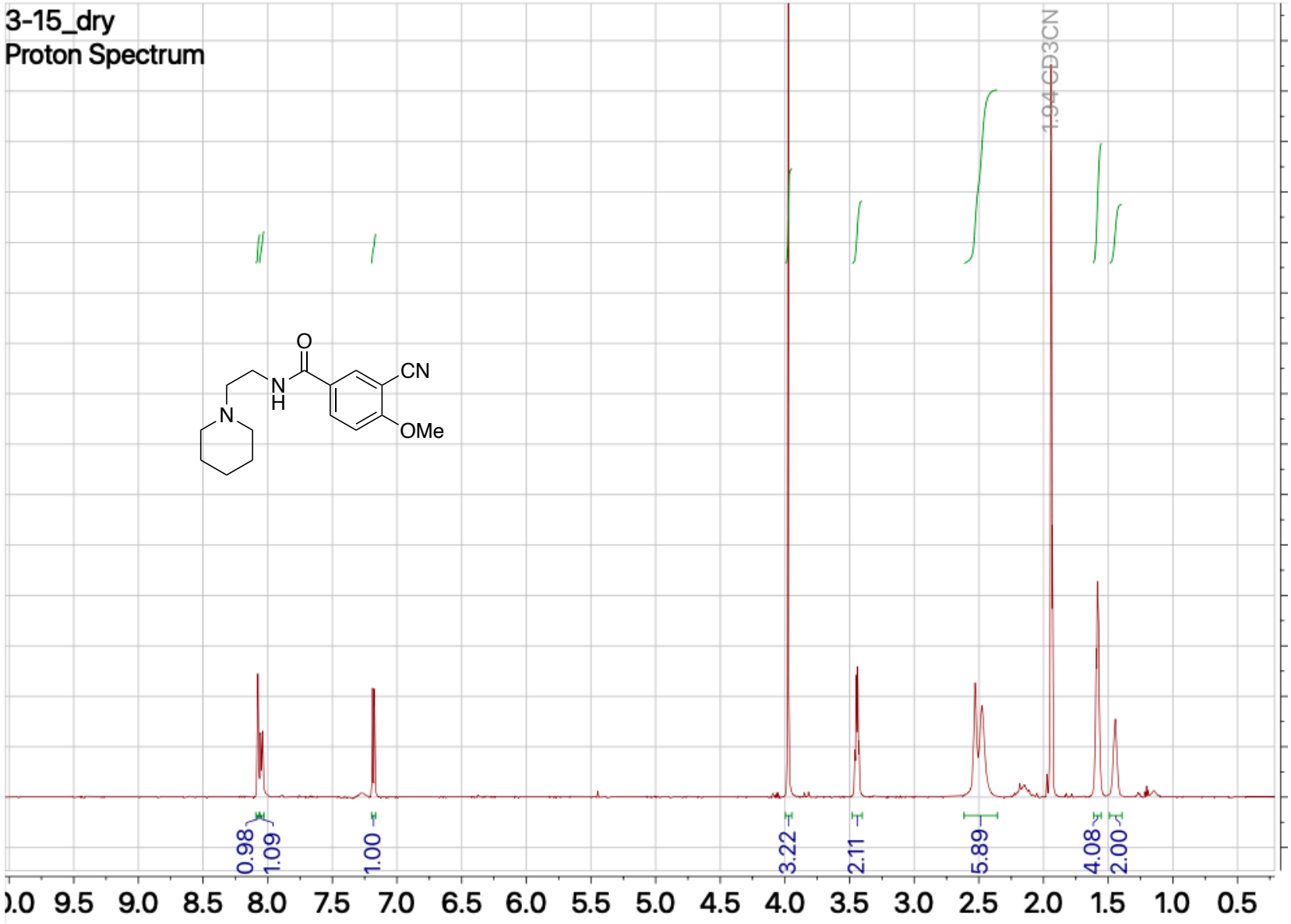
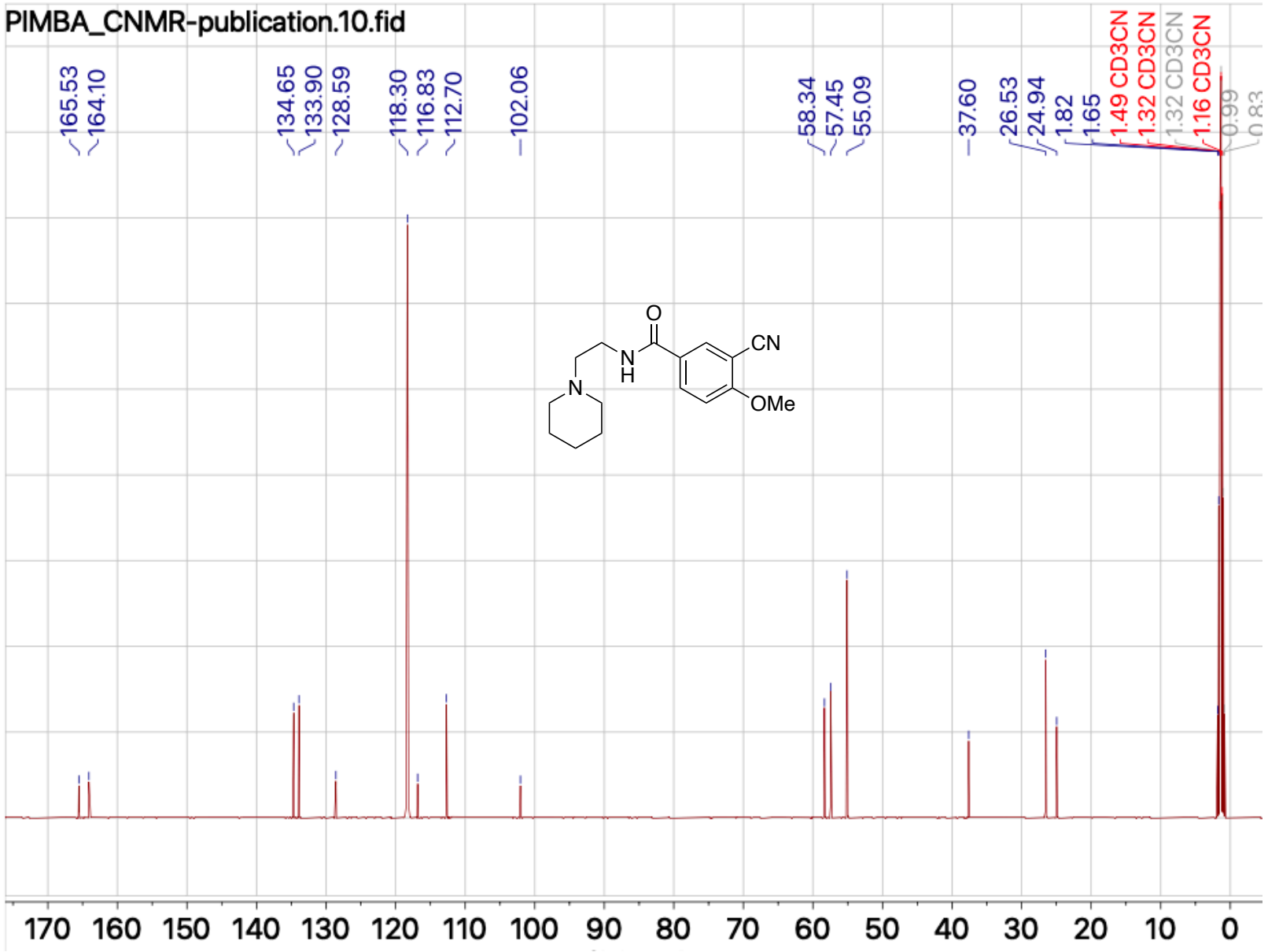
2933.37
1629.82
1604.44
1575.50
1548.49
1505.82
1467.89
1437.08
1453.07
1312.85
1247.76
1205.74
1191.92
1180.51
1156.92
1128.61
1069.02
1060.61
1031.39
994.42
852.70
820.02
799.99
782.18
765.38
630.21
588.14


O
N
H
N
OMe

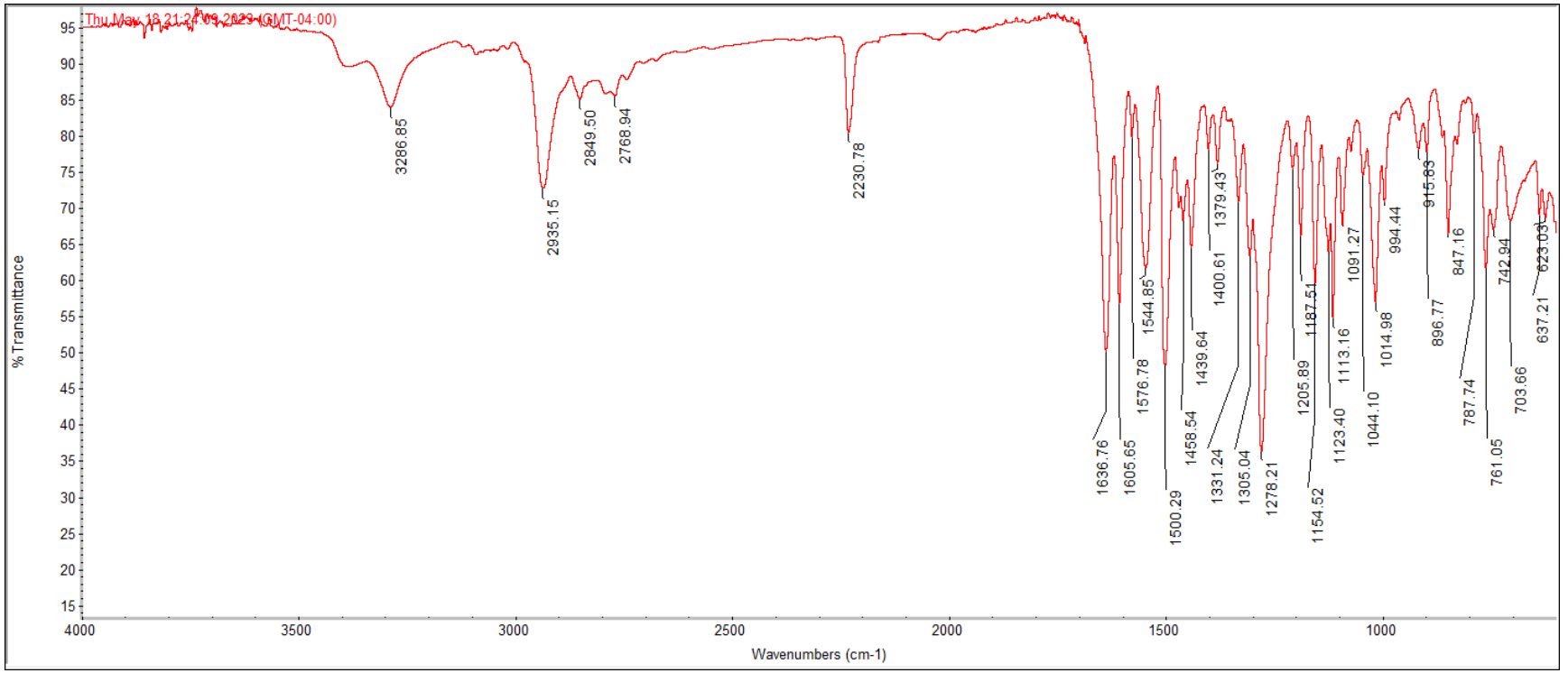
^{1}H NMR of S-5 in CD_3CN



^{13}C NMR of S-5 in CD_3CN



IR of S-5


Thu May 18 21:24:09 2023 (GMT-04:00)
% Transmittance
Wavenumbers (cm-1)
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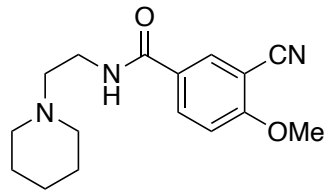
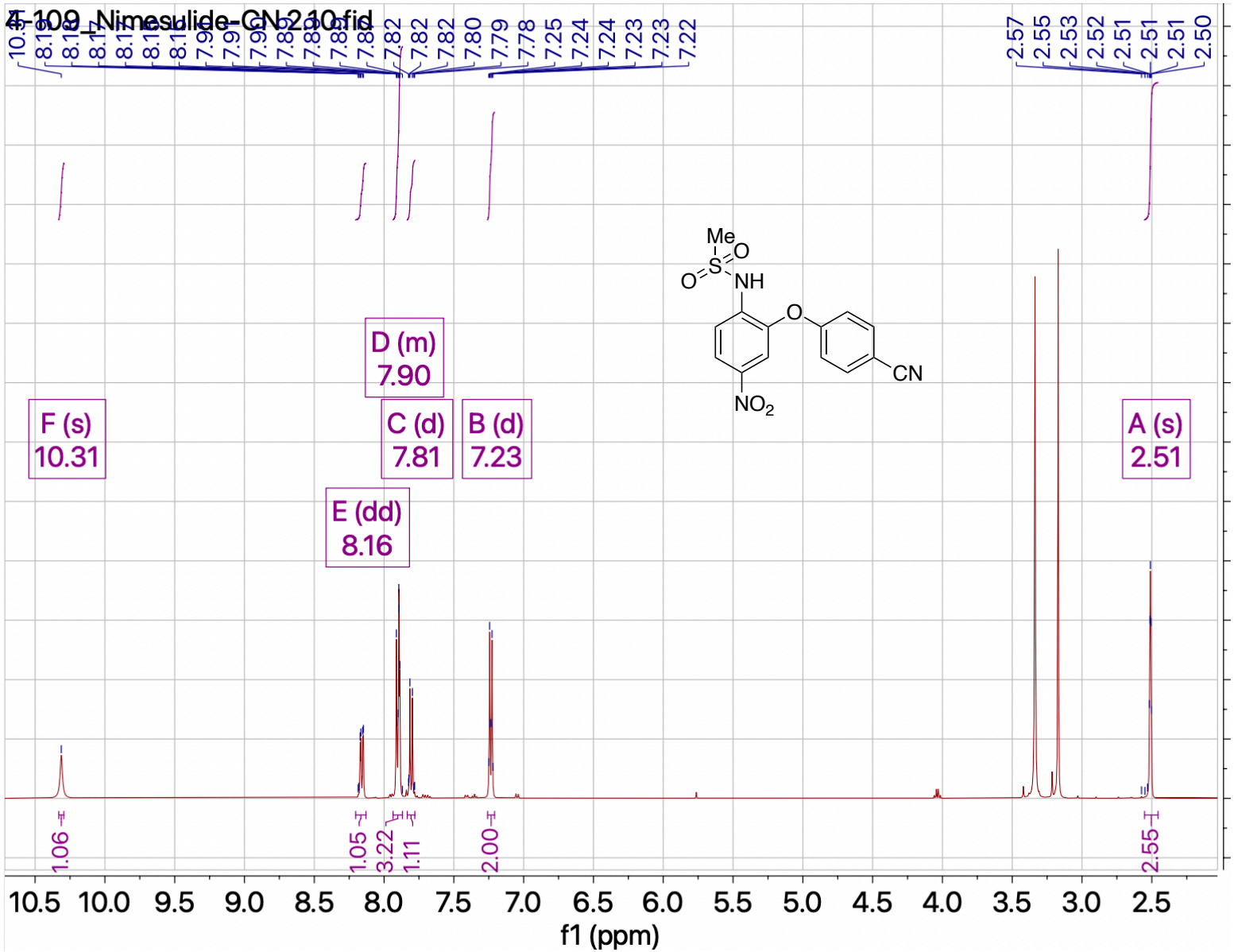
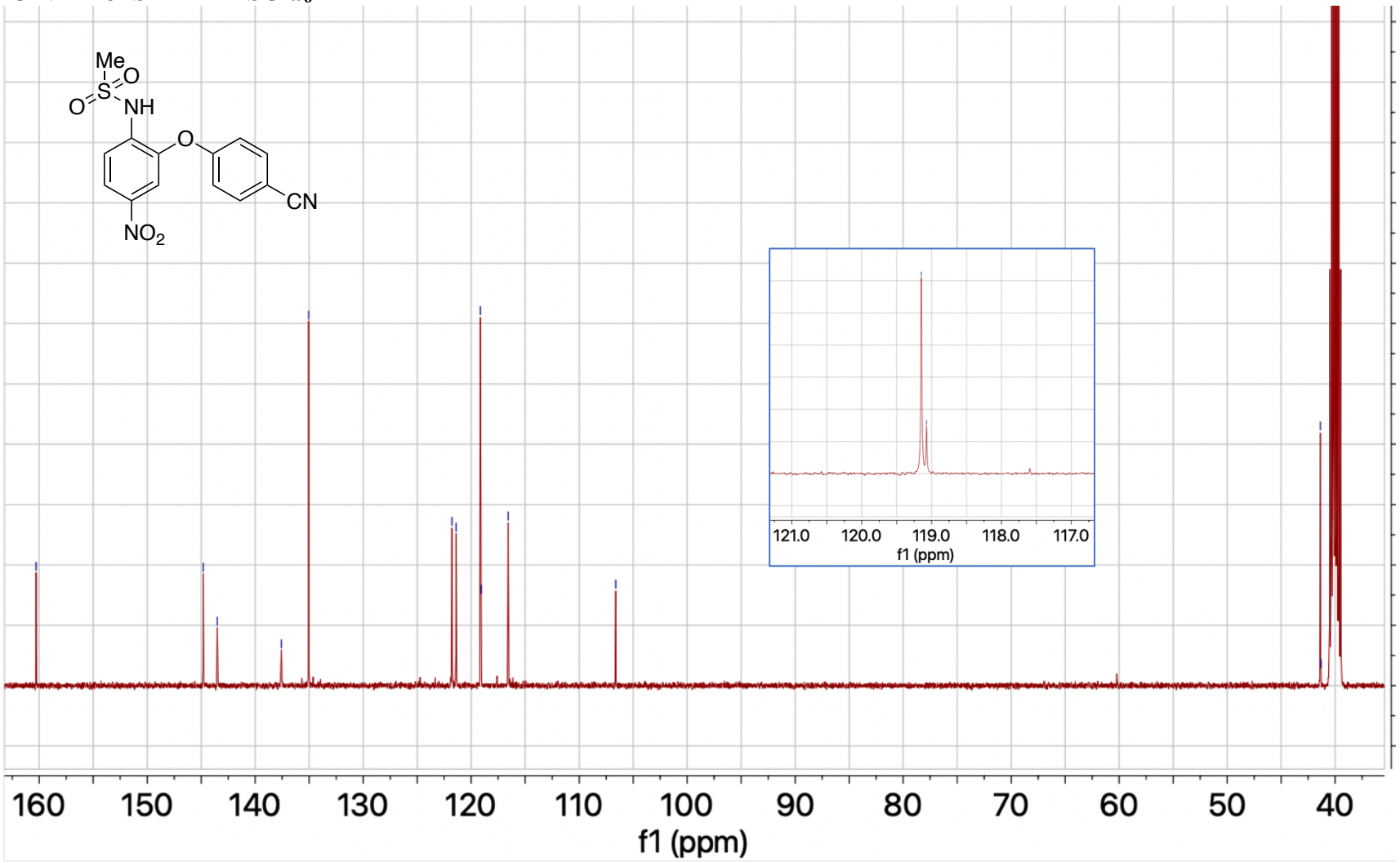
2849.50
2768.94
2230.78
1636.76
1605.65
1576.78
1544.85
1500.29
1458.54
1439.64
1400.61
1379.43
1331.24
1305.04
1278.21
1205.89
1187.51
1154.52
1123.40
1113.16
1091.27
1044.10
1014.98
994.44
915.83
896.77
847.16
787.74
761.05
742.94
703.66
637.21
623.03


O
N
H
N
CN
OMe

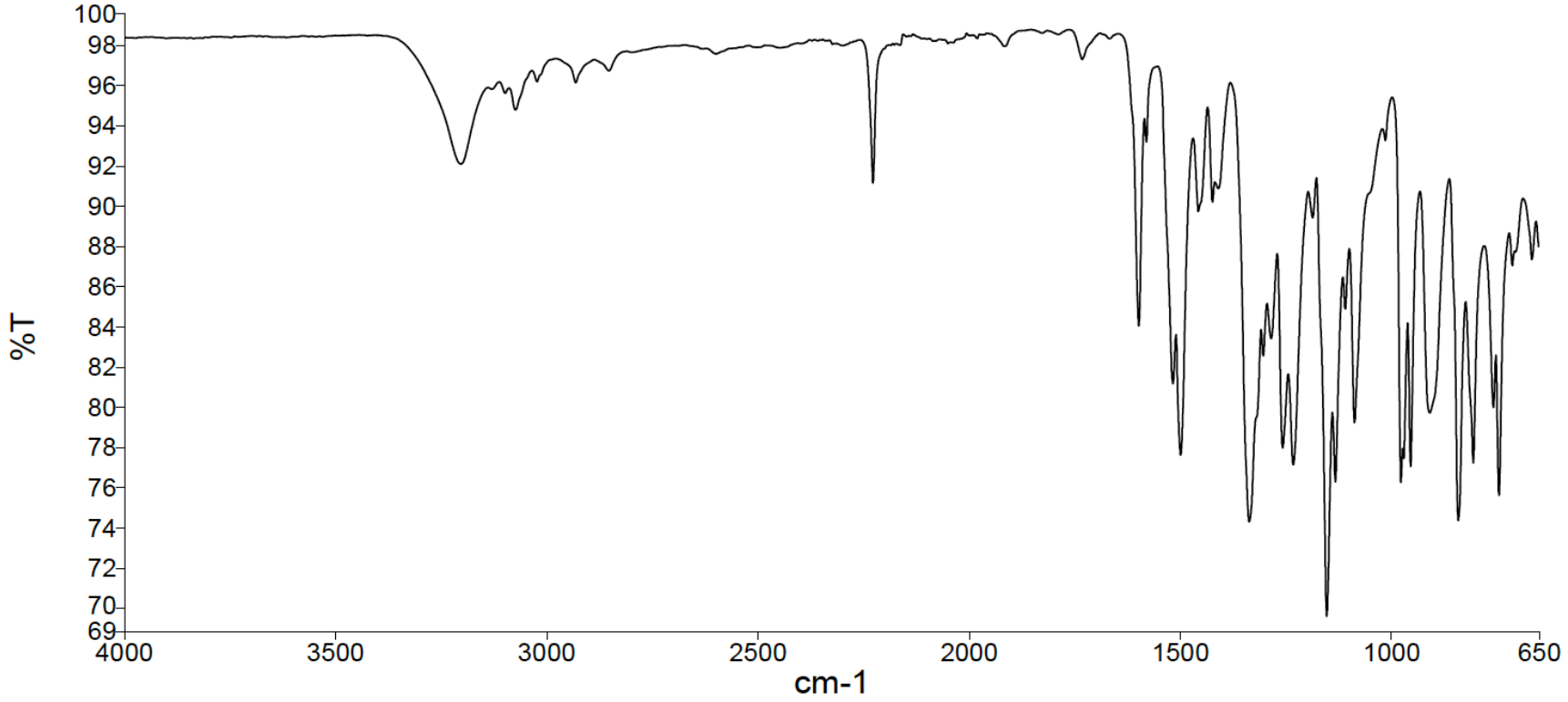
^{1}H NMR of S-7 in DMSO-d_6

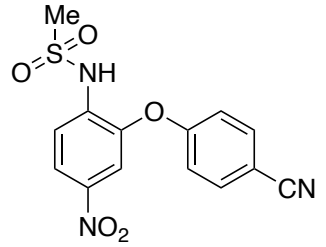


^{13}C NMR of S-7 in DMSO-d_6

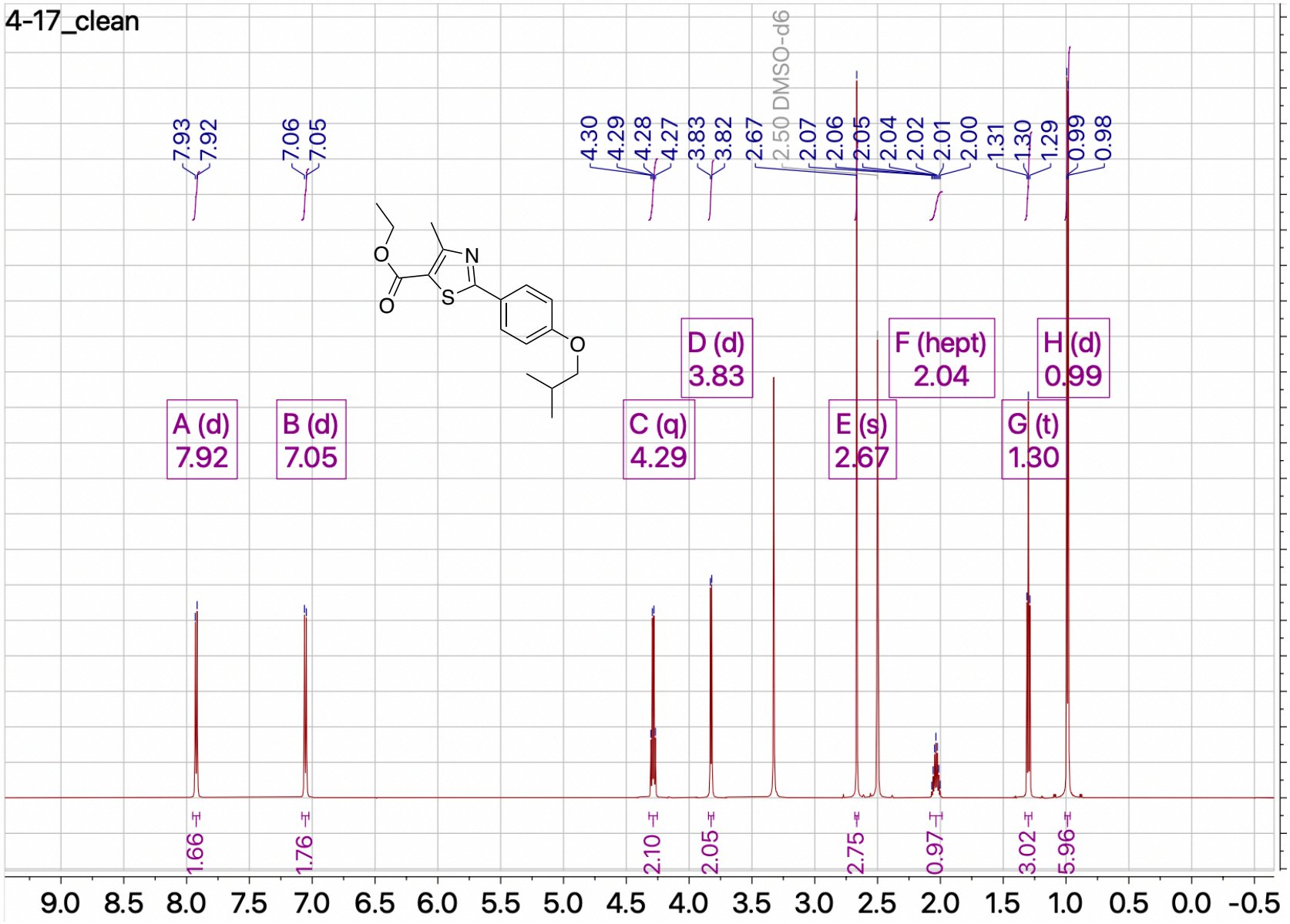


IR of S-7

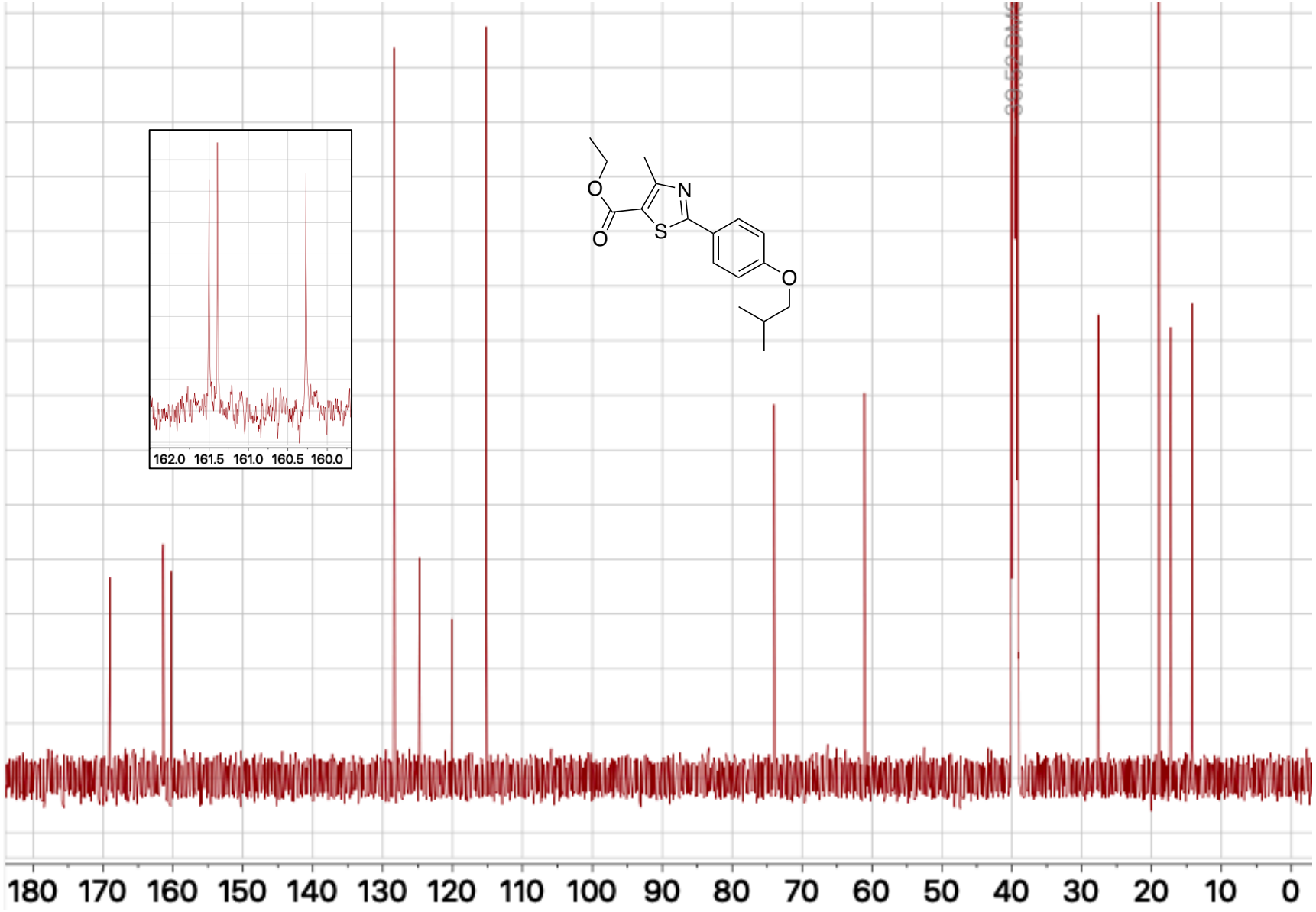

100
98
96
94
92
90
88
86
84
82
80
78
76
74
72
70
69
%T
4000
3500
3000
2500
2000
1500
1000
650
cm-1


Me
O
S
O
NH
O
CN
NO2

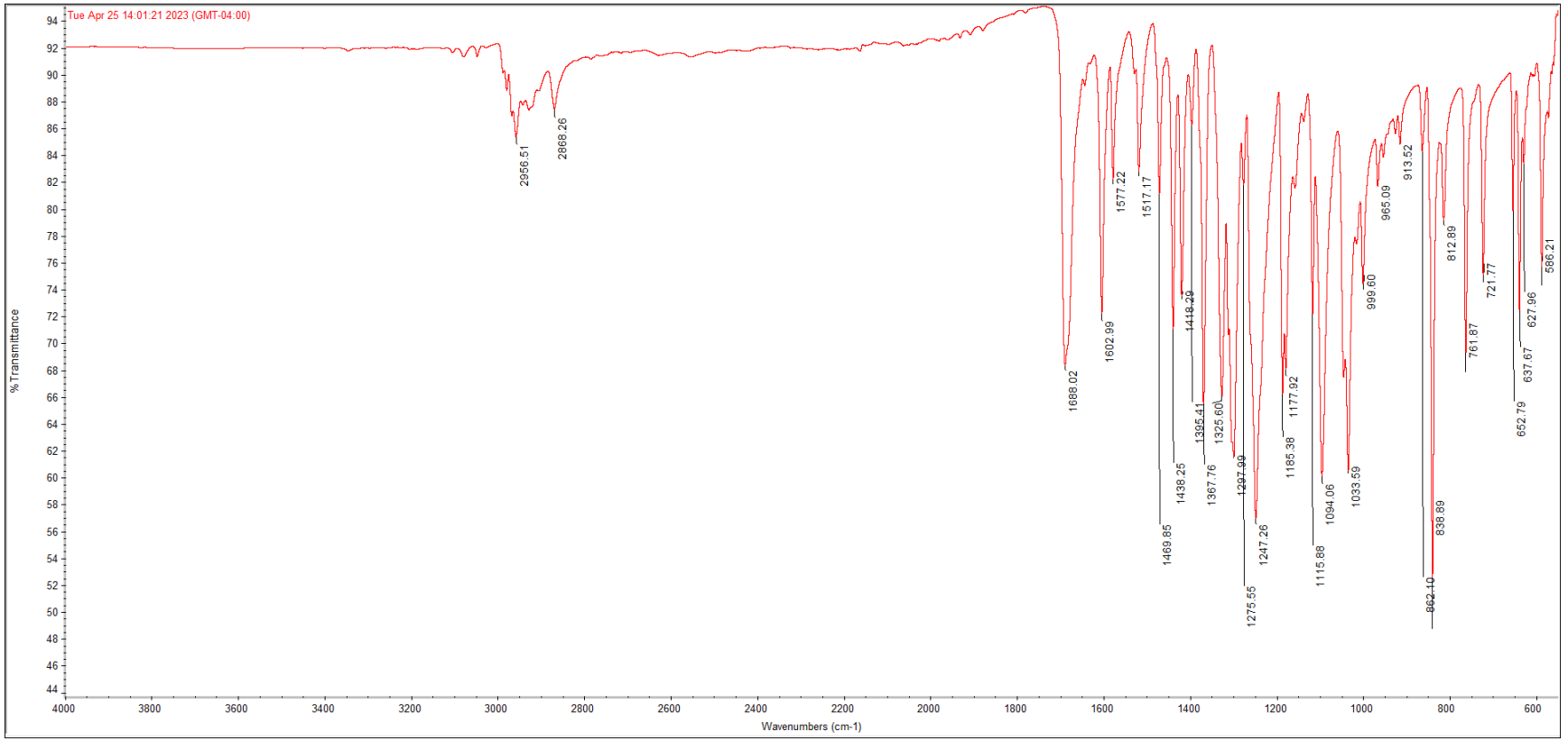
^{1}H NMR of S-8 in DMSO-d_6



^{13}C NMR of S-8 in DMSO-d_6



IR of S-8


Tue Apr 25 14:01:21 2023 (GMT-04:00)
%Transmittance
Wavenumbers (cm-1)
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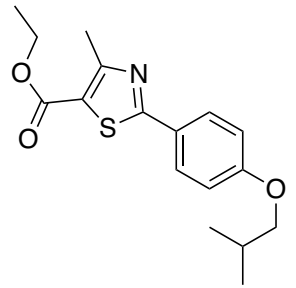
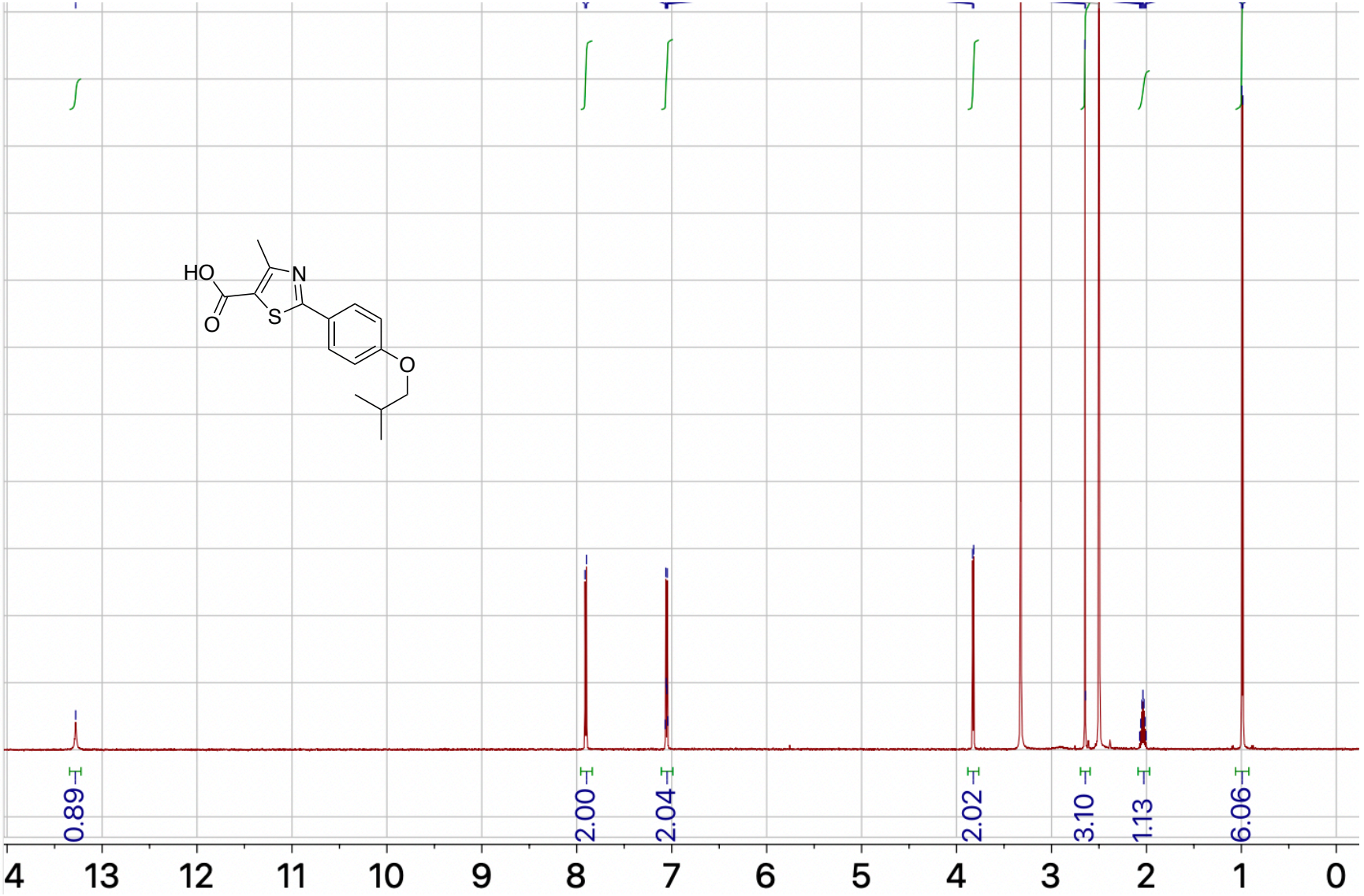
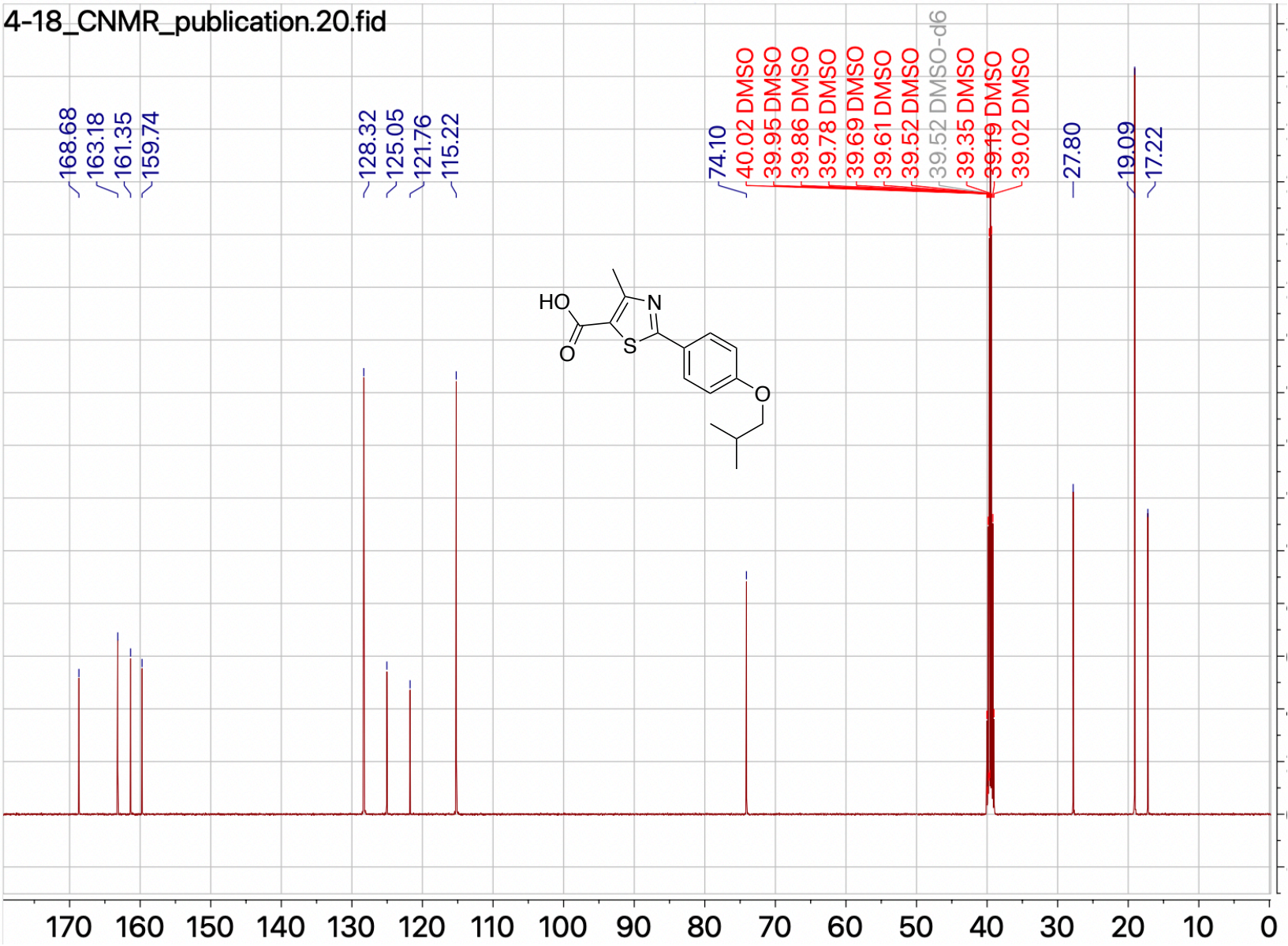
2868.26
1688.02
1602.99
1577.22
1517.17
1469.85
1438.25
1418.29
1395.41
1367.76
1325.60
1297.90
1275.55
1247.26
1185.38
1177.92
1115.88
1094.06
1033.59
999.60
965.09
913.52
862.10
838.89
812.89
761.87
721.77
652.79
637.67
627.96
586.21


O
O
N
S
O

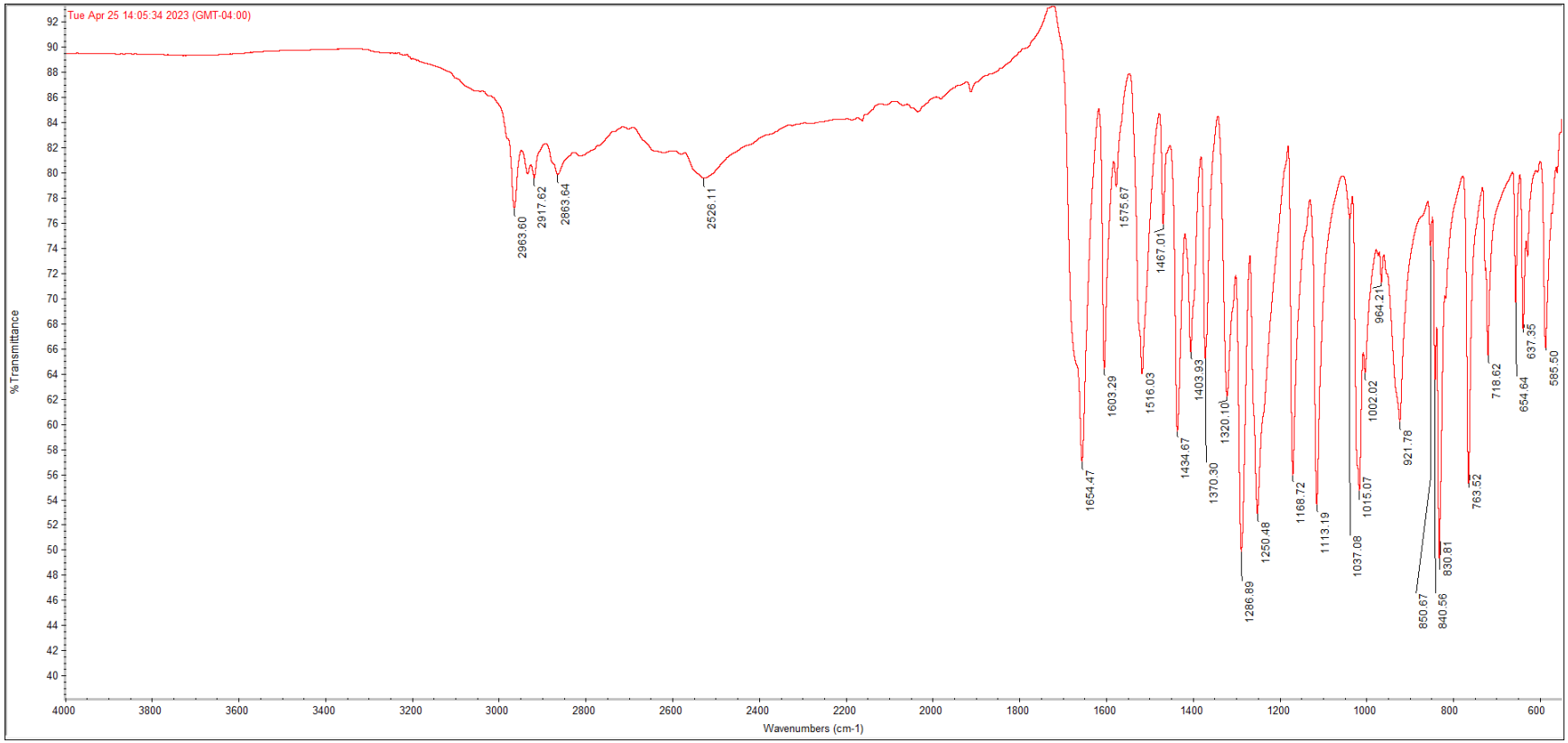
^{1}H NMR of S-9 in DMSO-d_6



^{13}C NMR of S-9 in DMSO-d_6



IR of S-9


Tue Apr 25 14:05:34 2023 (GMT-04:00)
%Transmittance
Wavenumbers (cm-1)
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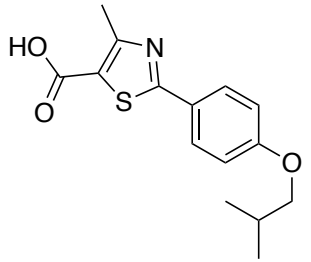
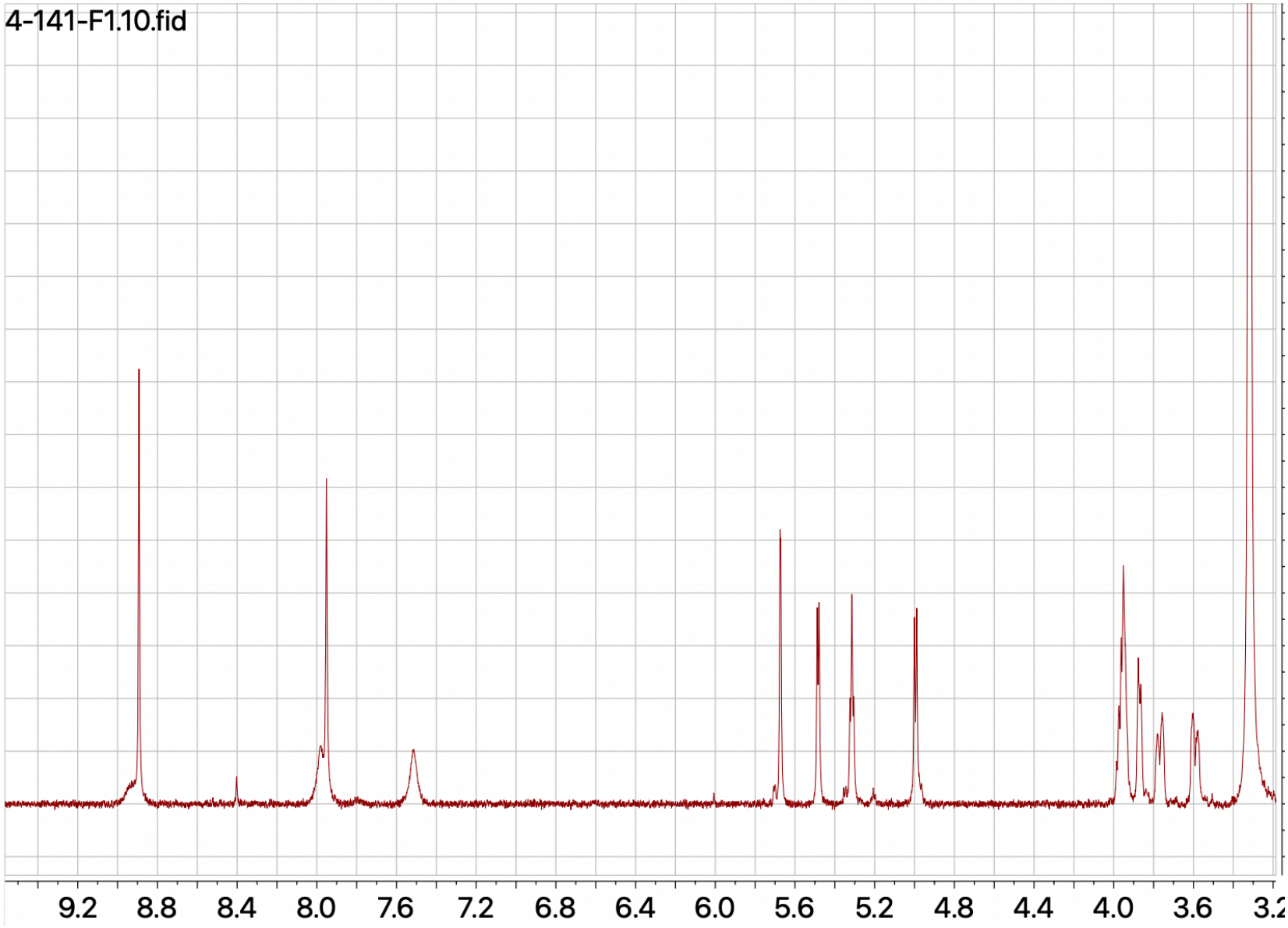
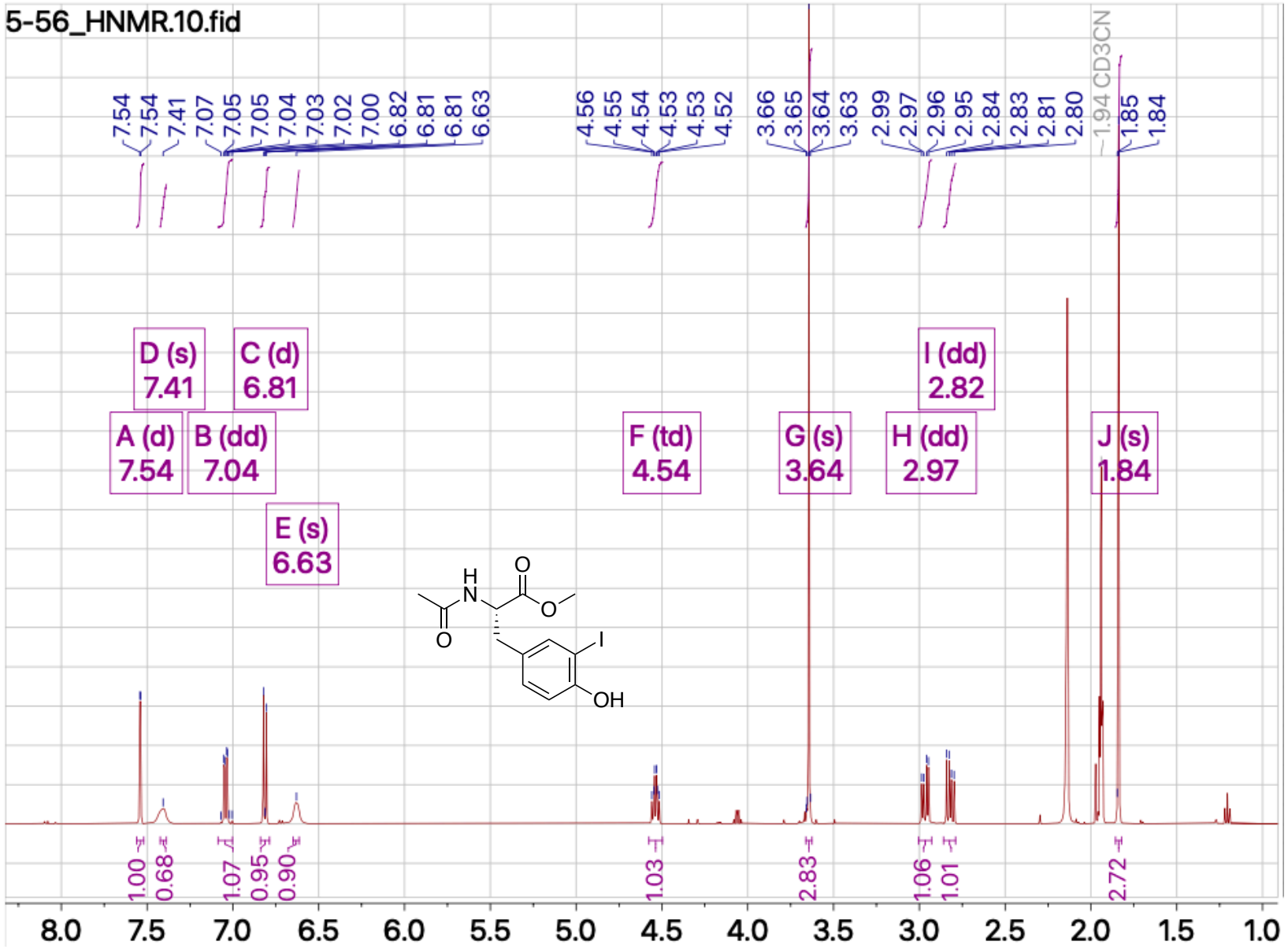
2917.62
2863.64
2526.11
1654.47
1603.29
1575.67
1516.03
1467.01
1434.67
1403.93
1370.30
1320.10
1286.89
1250.48
1168.72
1113.19
1037.08
1015.07
1002.02
964.21
921.78
850.67
840.56
830.81
763.52
718.62
654.64
637.35
585.50


HO
O
N
S
O

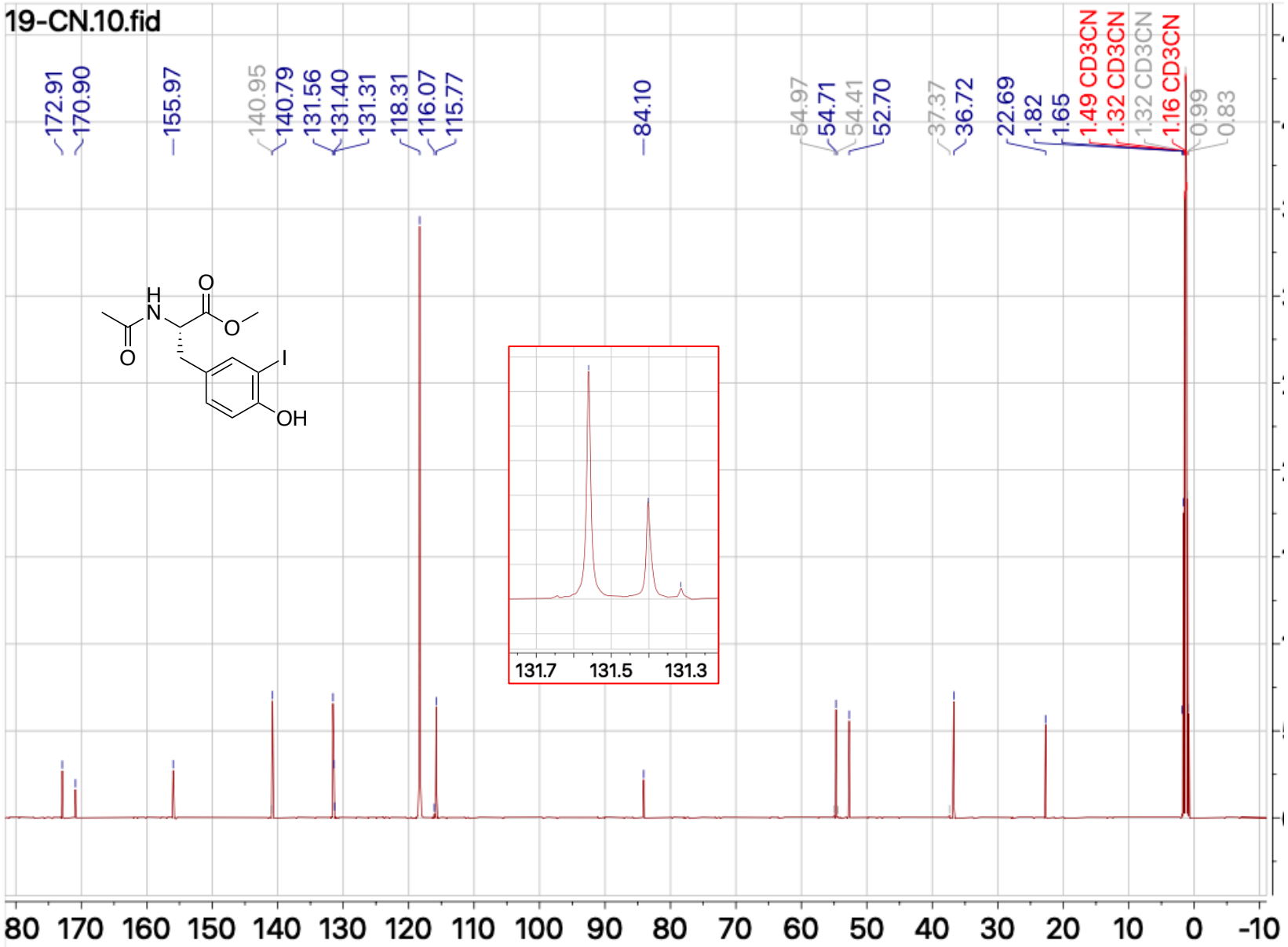
^{1}H NMR of S-10 in DMSO-d_6



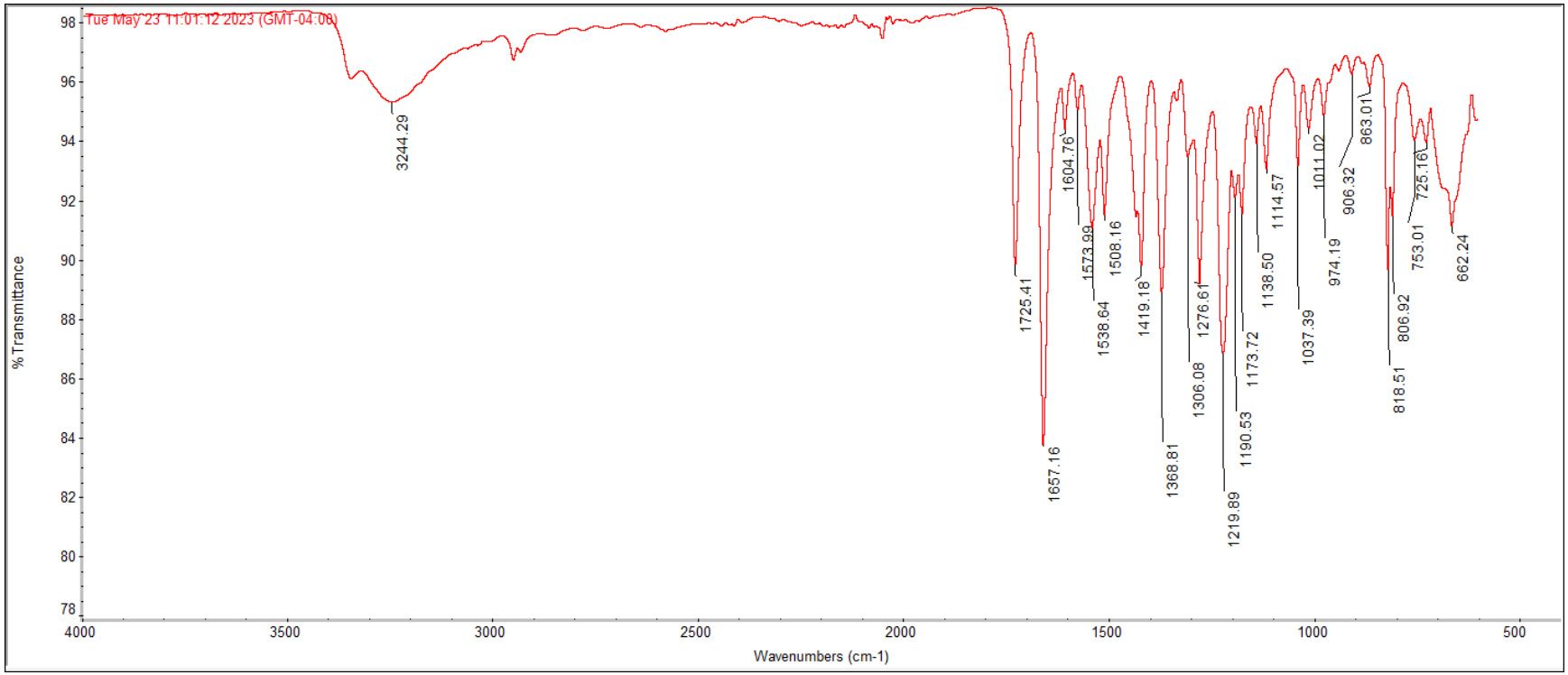
^{1}H NMR of S-11 in CD_3CN



^{13}C NMR of S-11 in CD_3CN



IR of S-11


Tue May 23 11:01:12 2023 (GMT-04:00)
% Transmittance
Wavenumbers (cm-1)
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96
94
92
90
88
86
84
82
80
78
4000
3500
3000
2500
2000
1500
1000
500
3244.29
1725.41
1657.16
1604.76
1573.99
1538.64
1508.16
1419.18
1368.81
1306.08
1276.61
1219.89
1190.53
1173.72
1138.50
1114.57
1037.39
1011.02
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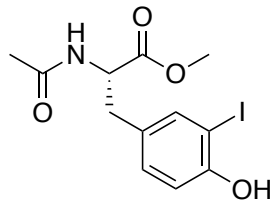
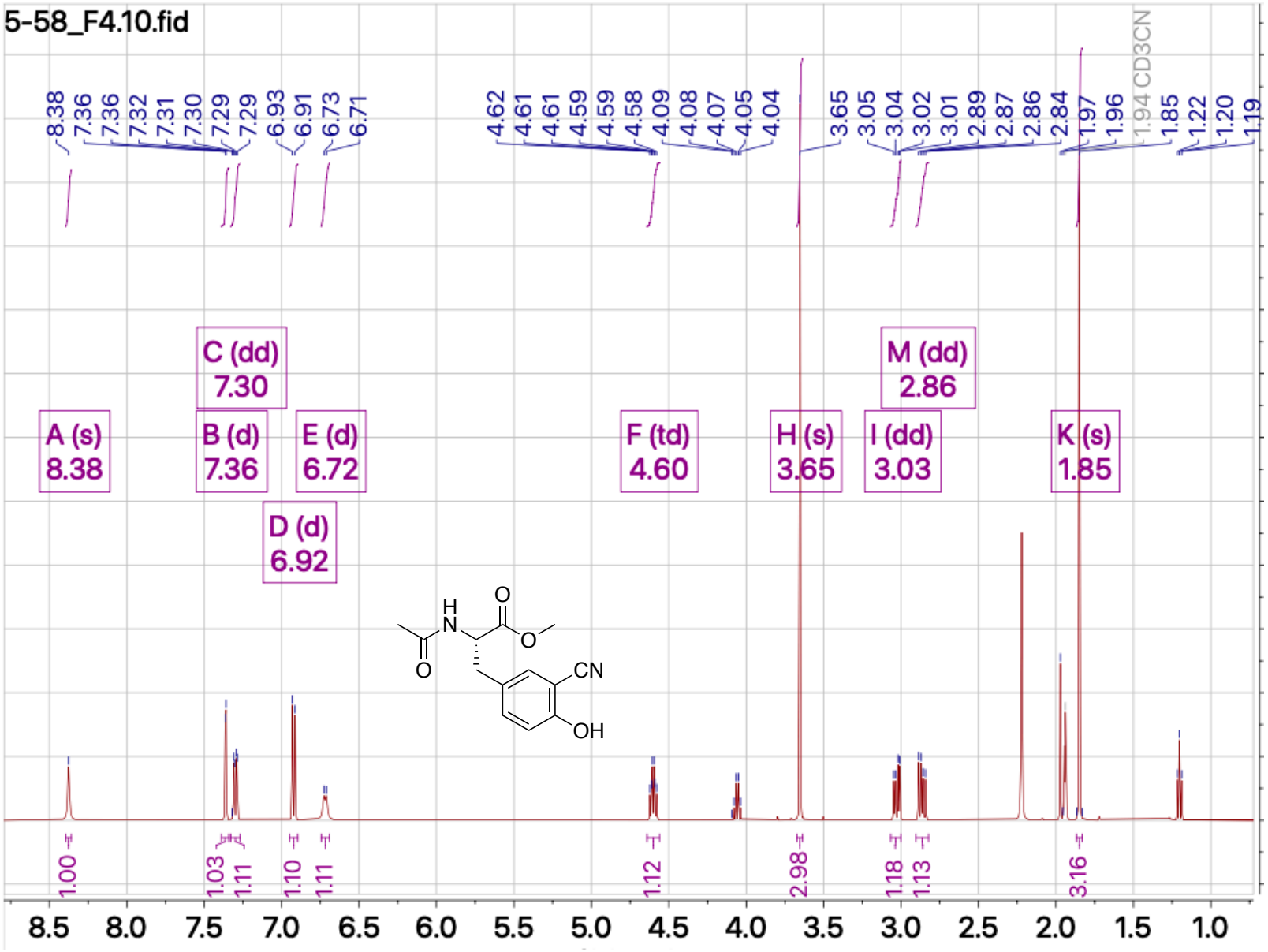
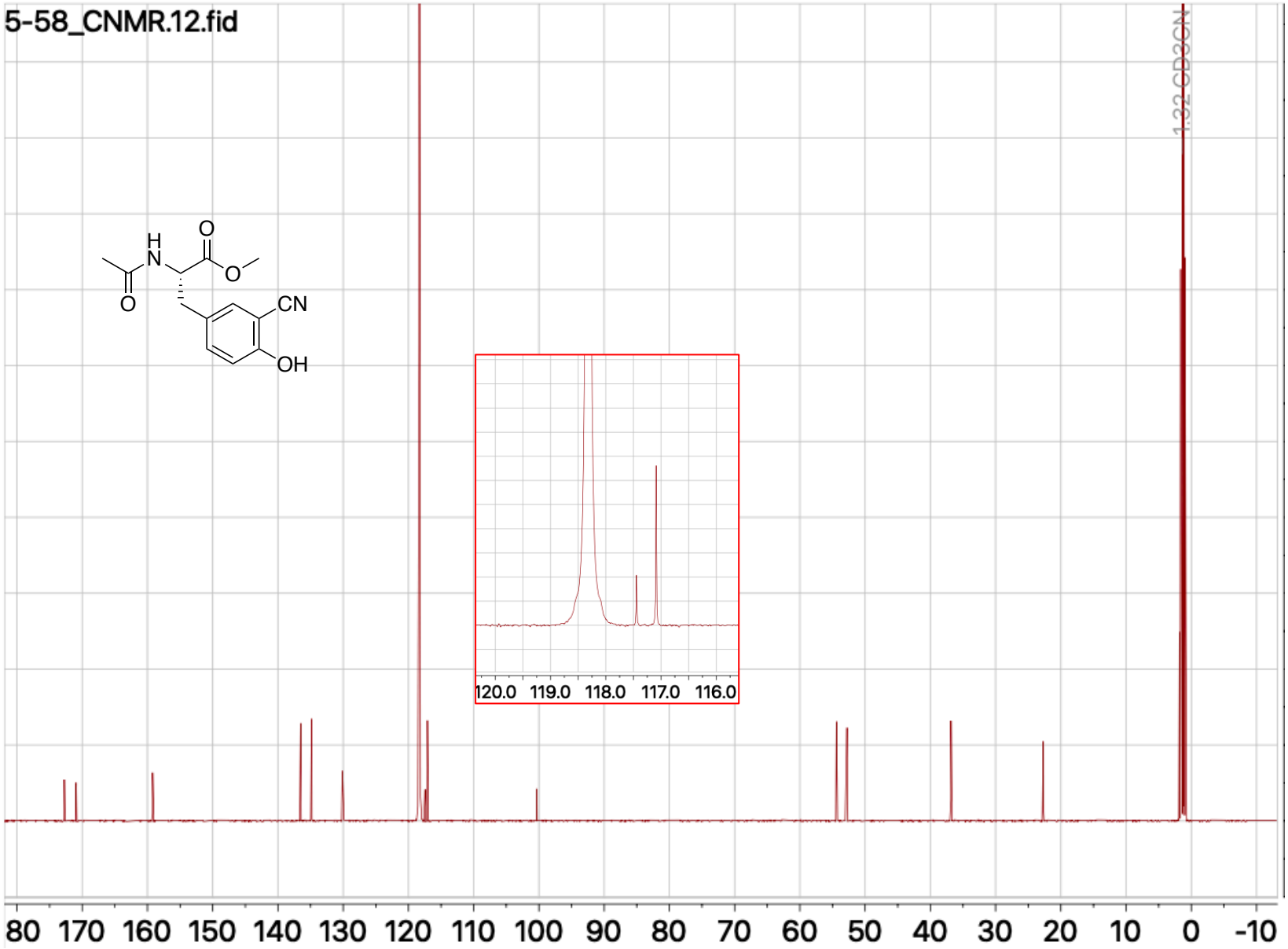
906.32
863.01
818.51
806.92
753.01
725.16
662.24


H
N
O
O
O
O
I
OH

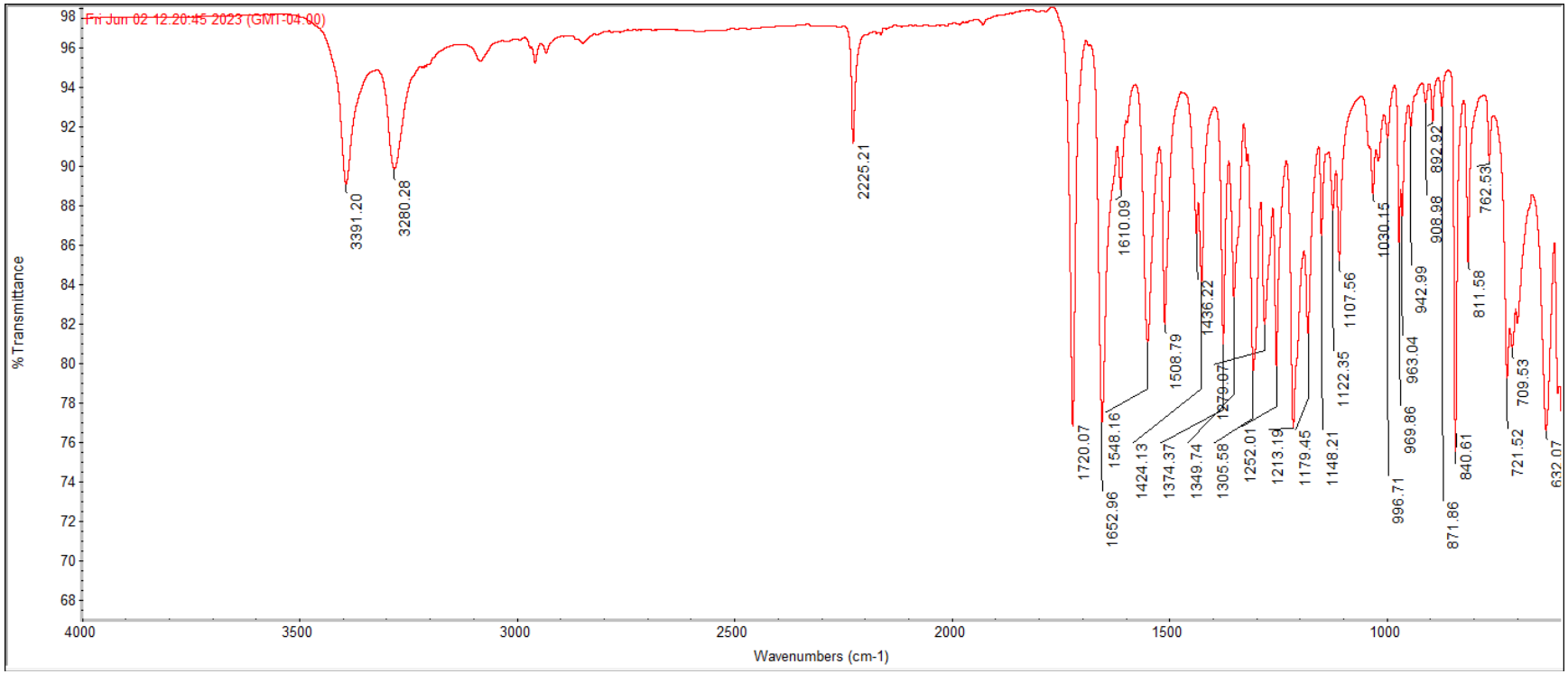
^{1}H NMR of S-12 in CD_3CN



^{13}C NMR of S-12 in CD_3CN



IR of S-12


Fri Jun 02 12:20:45 2023 (GMT-04:00)
% Transmittance
Wavenumbers (cm-1)
3391.20
3280.28
2225.21
1720.07
1652.96
1610.09
1548.16
1508.79
1436.22
1424.13
1374.37
1349.74
1305.58
1279.07
1252.01
1213.19
1179.45
1148.21
1122.35
1107.56
1030.15
996.71
969.86
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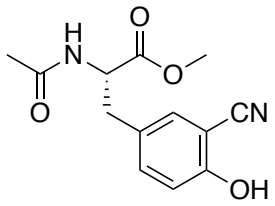
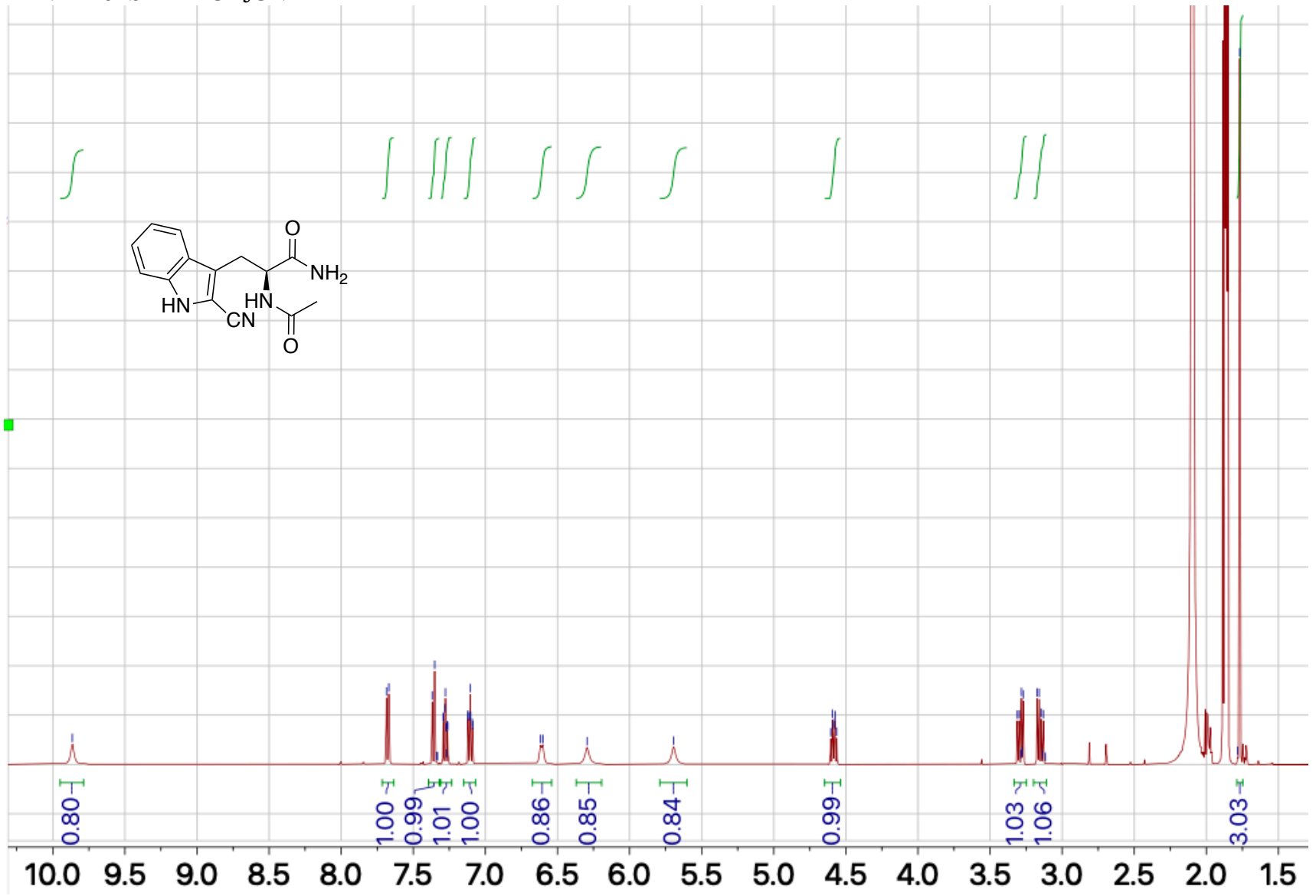
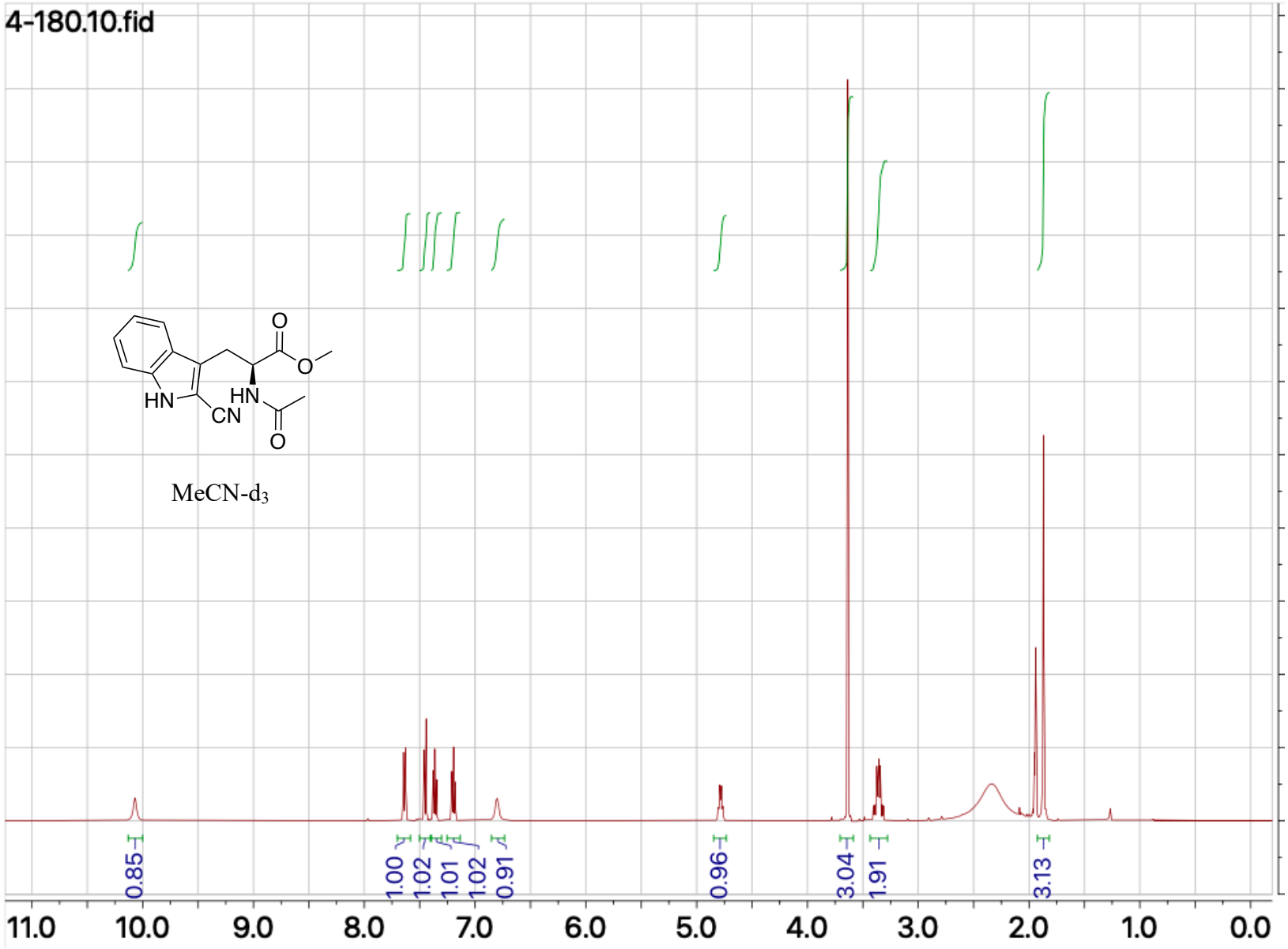
811.58
762.53
721.52
709.53
632.07


H
N
O
O
O
CN
OH

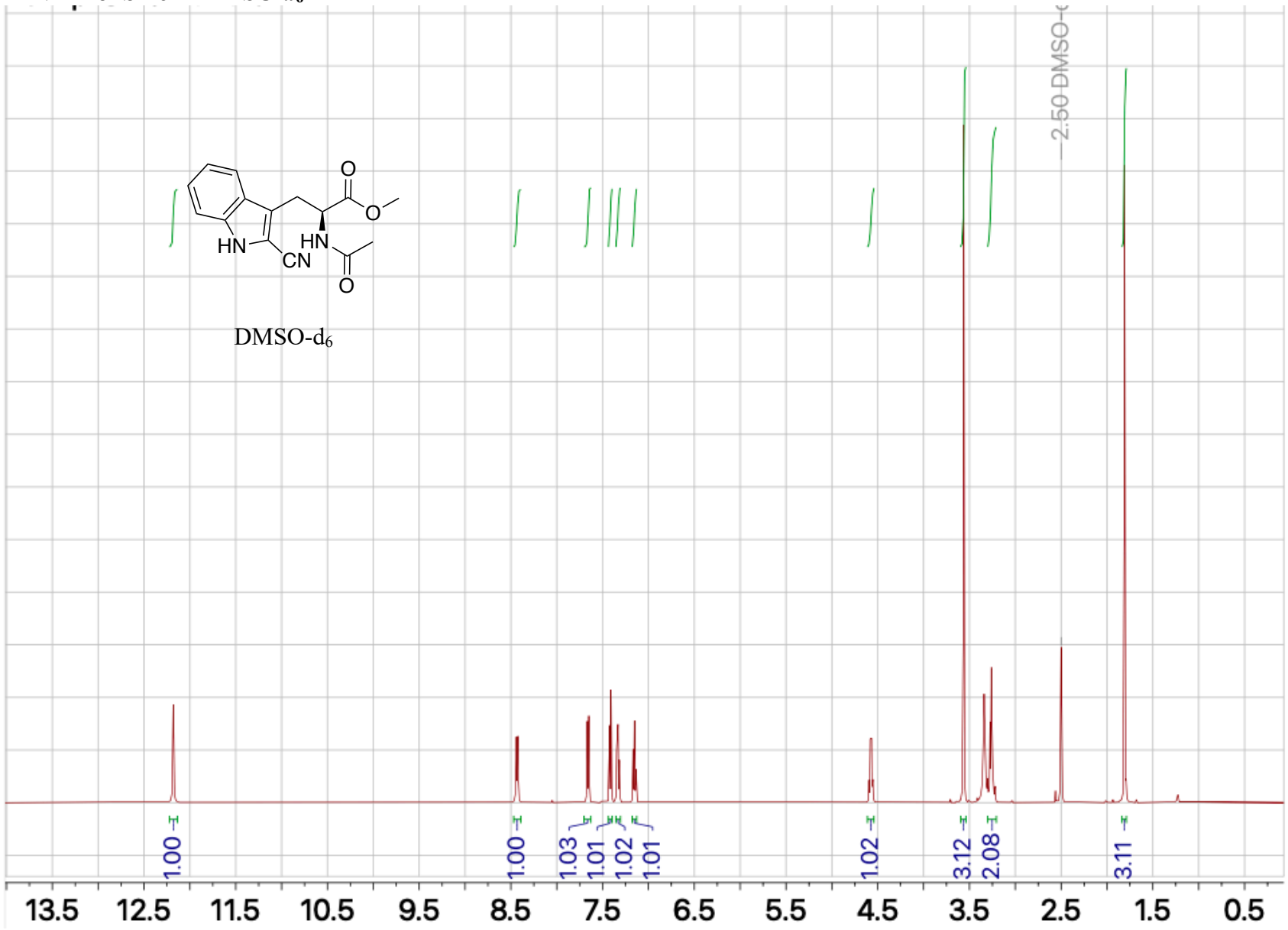
^{1}H NMR of S-14 in CD_3CN



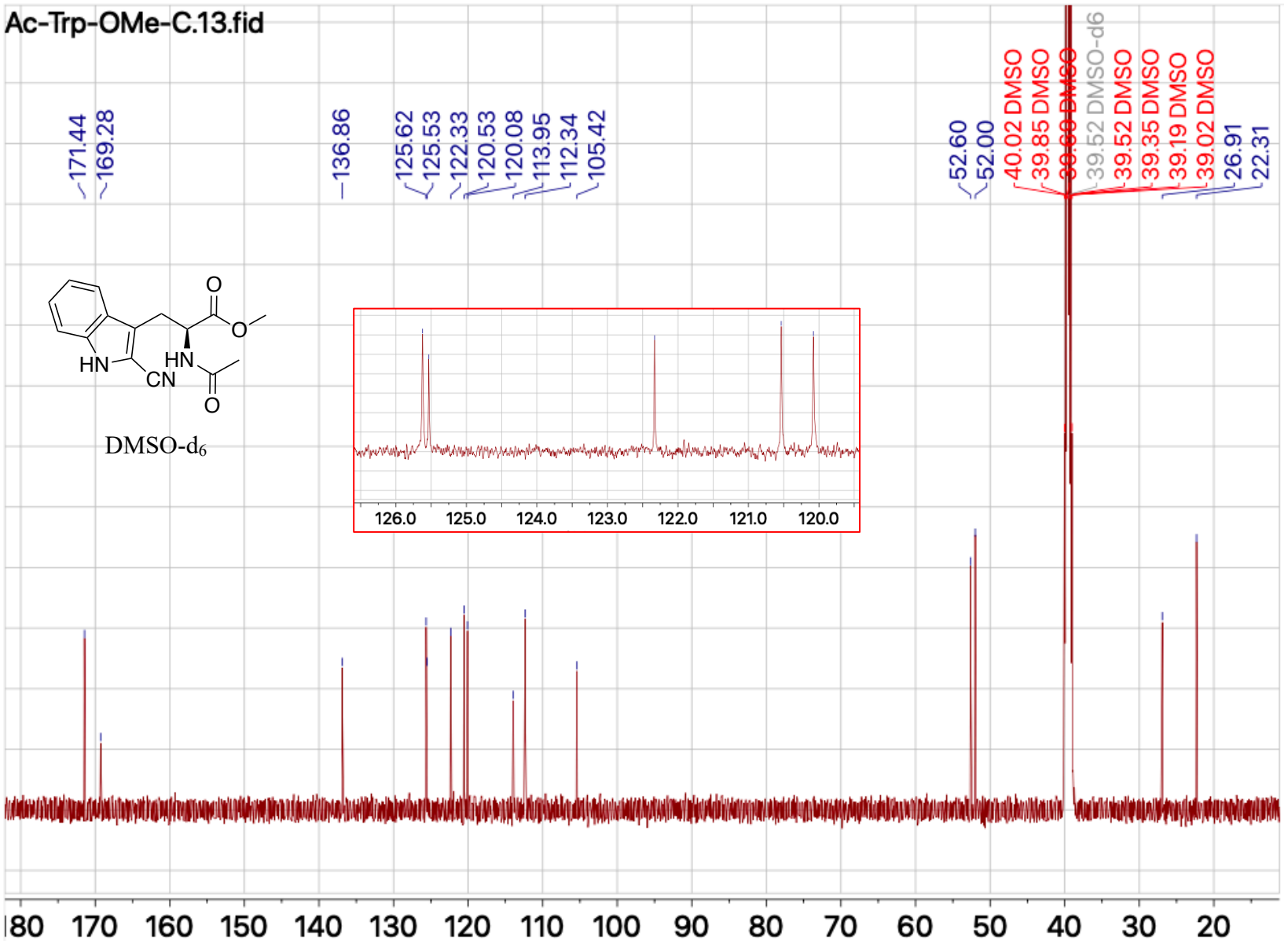
^{1}H NMR of S-16 in CD_3CN



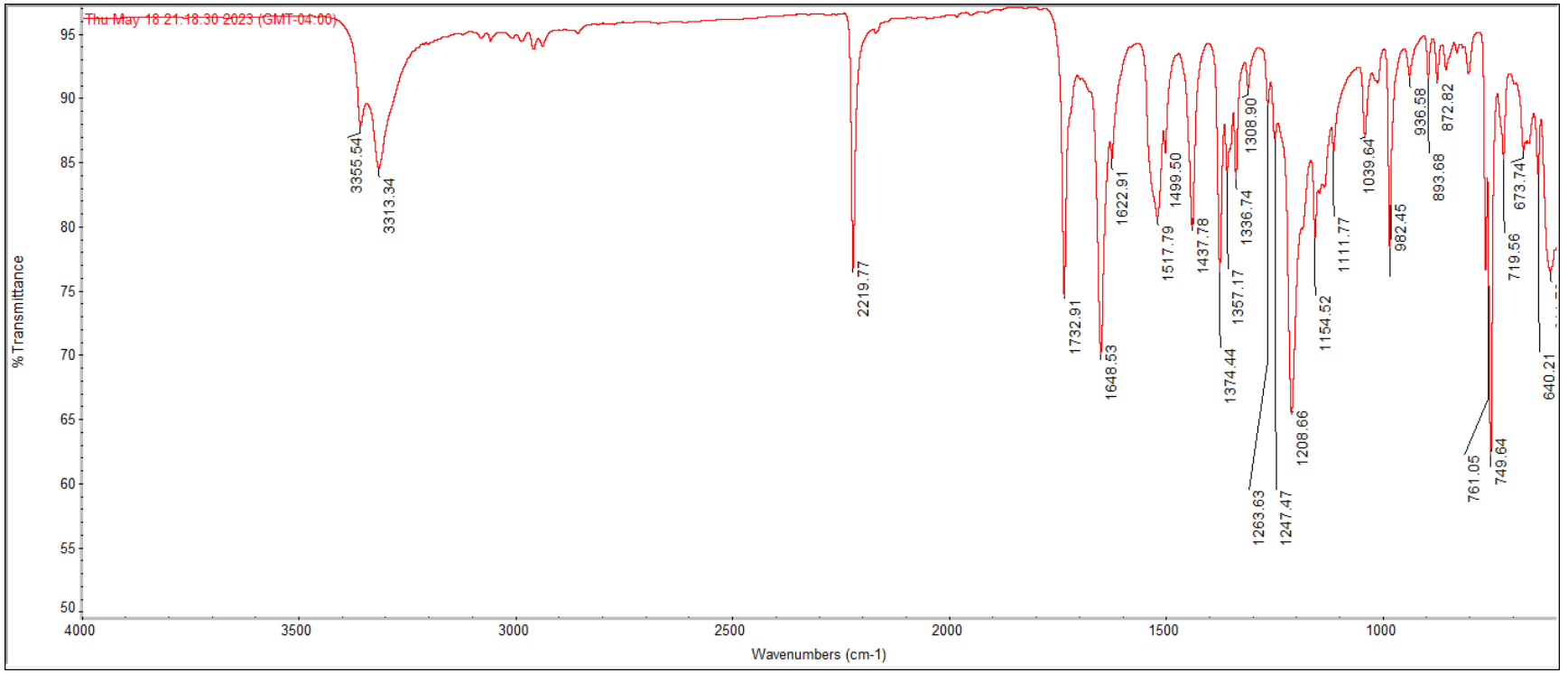
^{1}H NMR of S-16 in DMSO-d_6

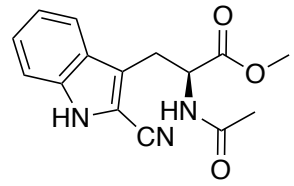


^{13}C NMR of S-16 in DMSO-d_6

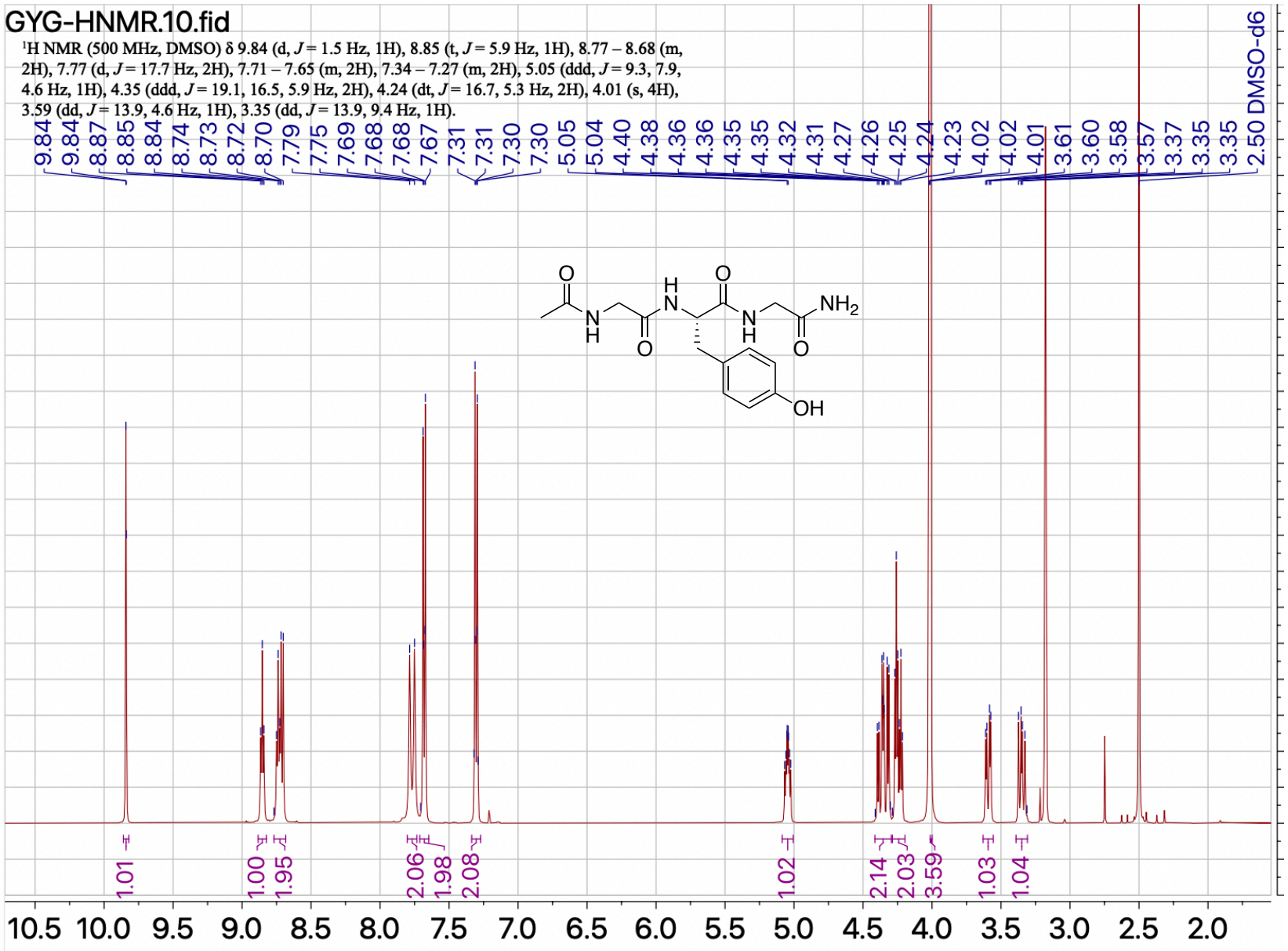


IR of S-16

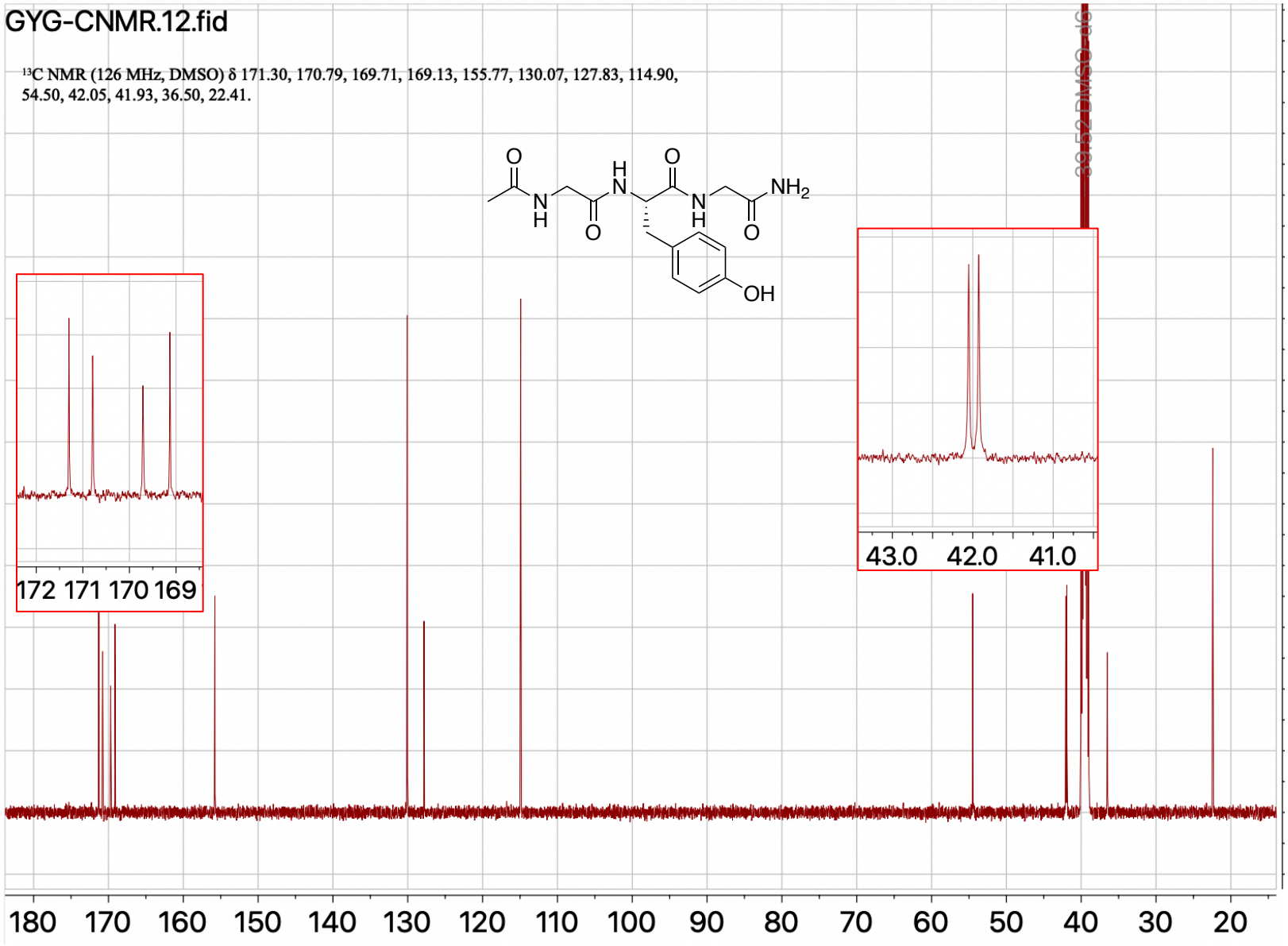

Thu May 18 21:18:30 2023 (GMT-04:00)
% Transmittance
Wavenumbers (cm-1)
95
90
85
80
75
70
65
60
55
50
4000
3500
3000
2500
2000
1500
1000
3355.54
3313.34
2219.77
1732.91
1648.53
1622.91
1517.79
1499.50
1437.78
1374.44
1357.17
1336.74
1308.90
1263.63
1247.47
1208.66
1154.52
1111.77
1039.64
982.45
936.58
893.68
872.82
761.05
749.64
719.56
673.74
640.21


O
O
HN
HN
CN
O

^{1}H NMR of Ac-GYG-NH$_2$ in DMSO-d_6



^{13}C NMR of Ac-GYG-NH$_2$ in DMSO-d_6



8. References

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