

The first isocyanide of plant origin expands functional group diversity in cruciferous phytoalexins: synthesis, structure and bioactivity of isocyaalexin A

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General experimental

All solvents were HPLC grade and used as such, except for THF (dried over sodium). Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Flash column chromatography (FCC): silica gel, grade 60, 230-400 μm . Organic extracts were dried over Na_2SO_4 and the solvents were removed using a rotary evaporator.

NMR spectra were recorded on Bruker Avance 500 MHz spectrometers. For ^1H NMR (500 MHz) and ^{13}C NMR (125.8 MHz) spectra, the chemical shifts (δ) are reported in parts per million (ppm) relative to TMS. Fourier transform infrared (FT-IR) data were acquired on a spectrometer and spectra were measured by the diffuse reflectance method.

HPLC-DAD analysis was carried out with either Agilent 1100 series or Hewlett Packard HPLC systems equipped with quaternary pump, autosampler, diode array detector (DAD, wavelength range 190-600 nm), and degasser; Zorbax Eclipse XDB-C18 column (5 μm particle size silica, 150 \times 4.6 mm I.D.), equipped with an in-line filter, with the mobile phase $\text{H}_2\text{O}-\text{CH}_3\text{OH}$ from 50:50 to 0:100, linear gradient for 25 min, and a flow rate of 0.75 ml/min. Samples were dissolved in CH_3OH .

HPLC-DAD-MS analysis was carried out with an Agilent 1100 series HPLC system equipped with an autosampler, binary pump, degasser, and a diode array detector connected directly to a mass detector (Agilent G2440A MSD-Trap-XCT ion trap mass spectrometer) with an electrospray ionization (ESI) source. Chromatographic separations were carried out at room temperature using an Eclipse XDB-C-18 column (5 μm particle size silica, 150 \times 4.6 mm I.D.). The mobile phase consisted of a linear gradient of: Method A, in H_2O (with 0.2% HCO_2H) - CH_3CN (with 0.2% HCO_2H) from 75:25 to 25:75 in 25 min, to 0:100 in 5 min and a flow rate of 1.0 ml/min; Method B, H_2O (with 0.2% HCO_2H) - CH_3CN (with 0.2% HCO_2H) from 90:10 to 50:50 in 25 min and a flow rate of 1.0 ml/min. Data acquisition was carried out in positive and negative polarity modes in a single LC run, and data processing carried out with Agilent Chemstation Software. Samples were dissolved in CH_3CN .

Chemical structures of metabolites in Fig. 2

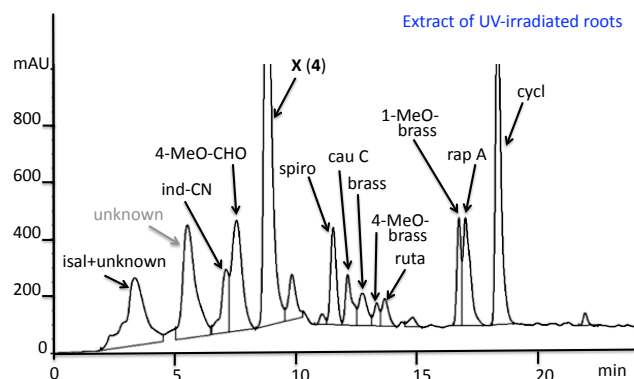
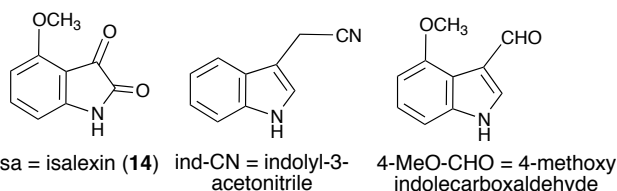
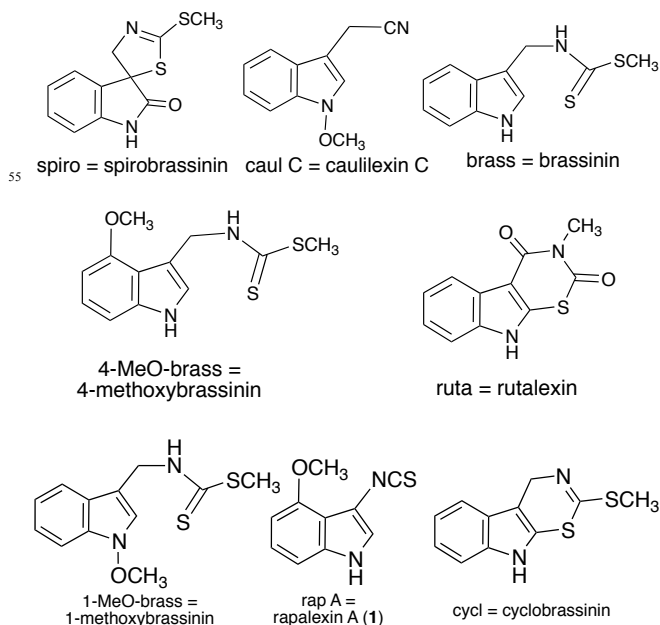


Figure 2 HPLC-DAD chromatograms (C-18 reverse phase column, $\text{H}_2\text{O}-\text{MeOH}$ elution, 50:50 to 0:100 linear gradient for 25 min, detection at 220 nm) of rutabaga root extracts: UV-irradiated for 90 min (isa = isalexin (**14**), ind-CN = indolyl-3-acetonitrile, 4-MeO-CHO = 4-methoxyindolecarboxaldehyde, spiro = spirobrassinin, cau C = caulilexin C, brass = brassinin, 4-MeO-brass = 4-methoxybrassinin, ruta = rutalexin, 1-MeO-brass = 1-methoxybrassinin, rap A = rapalexin A (**1**), cycl = cyclobrassinin) and control (no irradiation).



Antifungal bioassays

Leptosphaeria maculans, *Rhizoctonia solani* AG 2-1, *Sclerotinia sclerotiorum*, *Alternaria brassicicola* were obtained from AAFC, Saskatoon, Canada. Solid cultures were initiated with spores of *A. brassicicola* and *L. maculans*, sclerotia of *S. sclerotiorum*, and mycelia of *R. solani* were grown on potato dextrose agar (PDA) plates at 23 ± 2 °C, under constant light and mycelial plugs cut from the edge of actively growing cultures used to initiate bioassay cultures, as follows.

Solutions of each compound in DMSO were used to prepare sterile assay plates (6 wells per plate, 36 mm diameter, 2 ml per well) in PDA media (5.0×10^{-4} M, 2.0×10^{-4} M, 1.0×10^{-4} M). Control plates contained 1% DMSO in PDA. Plates containing test solutions and the control solution were inoculated with mycelia plugs (4 mm diameter) placed upside down on the centre of each plate, the plates were sealed with parafilm, and incubated at 23 ± 2 °C under constant light for 24 h for *S. sclerotiorum*, 72 h for *R. solani*, 120 h for *L. maculans* (isolates BJ-125 and Laird - 2) and *A. brassicicola*. The radial growth of mycelia was measured and compared with control plates containing only DMSO. Each experiment was conducted in triplicate and repeated three times.

Synthesis and characterization of new compounds

Synthesis of isocyalalexin (4)

Freshly distilled POCl₃ (22 µL, 0.23 mmol) was added to a stirred solution of 4-methoxyindole-3-formamide (**13**, 21.7 mg, 0.11 mmol) and Et₃N (48 µL, 0.34 mmol) in THF (1 mL) at 0 °C under inert atmosphere. The reaction mixture was stirred for 1 h at 0 °C, was neutralized (10% aq Na₂CO₃) and stirred for 30 min at rt. The reaction mixture was diluted with water (5 mL), extracted with CH₂Cl₂, the organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The crude product was subjected to column chromatography (CH₂Cl₂, 100%) to give 4-methoxyindole-3-isonitrile (**4**, 8 mg, 0.05 mmol) in 41% yield (m.p. decomposed at 160 °C).

Synthesis of indole-3-formamide (7)

Procedure A: Pd/C (50 mg) was added to a solution of 3-nitroindole (50 mg, 0.31 mmol) in EtOH (1 mL) and the reaction mixture was stirred under H₂ at balloon pressure for 5 h. The reaction mixture was cooled to -10 °C, Et₃N (129 µL, 0.93 mmol) and HCO₂H/Ac₂O (1:0.8, 2 mL) were added and the reaction mixture was further stirred for 1 h, while temperature was allowed to warm up to 0 °C. The reaction mixture was diluted with water, extracted with CH₂Cl₂, the organic phase was dried over Na₂SO₄ and concentrated. The crude product was chromatographed (CH₂Cl₂:EtOAc, 8:2) to yield indole-3-formamide (12.5 mg, 0.08 mmol) in 25% yield and indole-3-acetamide (5.2 mg, 0.03) in 9% yield.

Procedure B: Pd/C (25 mg) was added to a solution of 3-nitroindole (25 mg, 0.16 mmol) in MeOH (1 mL) and HCO₂H/Ac₂O (1.5:1) (100 µL, 1.12 mmol). The reaction mixture was stirred under H₂ balloon pressure for 3 h at -10 °C and allowed to warm up to 10 °C for an additional 1 h. The reaction mixture was diluted with water, extracted by CH₂Cl₂, the organic

phase was dried over Na₂SO₄ and concentrated to give indole-3-formamide (12.8 mg, 0.08 mmol) in 64% yield and indole-3-acetamide (4.4 mg, 0.030 mmol) in 22% yield, respectively.

Indole-3-formamide (7): HPLC t_R = 4.2 min. UV (CH₃OH-H₂O, HPLC) λ_{max} (nm): 222, 260. FTIR (KBr, cm⁻¹) ν_{max} (cm⁻¹): 3263, 3057, 2922, 1665, 1565, 1458, 1385, 1237. ¹H NMR (500 MHz, CD₃OD) δ 8.27 (1H, s), 7.69 (1H, s), 7.60 (1H, d, J = 8 Hz), 7.32 (1H, d, J = 8 Hz), 7.12 (1H, dd, J = 8, 8 Hz), 7.02 (1H, dd, J = 8, 8 Hz). ¹³C NMR (125.8 MHz, CD₃OD) δ 160.7, 135.6, 123.2, 122.0, 120.1, 118.1, 117.5, 114.7, 112.5. HRMS-EI m/z 160.0635 [M]⁺, calcd. for C₉H₈N₂O 160.0637 (100%), 131.06 (59%), 104.05 (26%). HPLC-MS-ESI m/z [M+H]⁺, 161.3 (50%), 134.3 (100%).

Synthesis of ethyl indole-3-carbamate (8)

Pd/C (20 mg) was added to a solution of 3-nitroindole (20 mg, 0.12 mmol) in EtOH (1 mL) under H₂ at balloon pressure. The reaction mixture was stirred for 5 h, was cooled to -15 °C and then Et₃N (166 µL, 1.2 mmol) and Cl₂CO (325 µL, 0.62 mmol) were added and the reaction mixture was stirred for further 5 min. The reaction mixture was diluted with water (5 mL), extracted with CH₂Cl₂, and the organic layer was dried over Na₂SO₄ and concentrated. The crude product was purified by FCC (CH₂Cl₂-MeOH, 95:5) to yield ethyl indole-3-carbamate (**8**, 7.7 mg, 0.04 mmol) in 32% yield.

Synthesis of indole-3-isonitrile (9)

Freshly distilled POCl₃ (35 µL, 0.38 mmol) was added to a stirred solution of indole-3-formamide (**7**, 30 mg, 0.19 mmol) and Et₃N (78 µL, 0.56 mmol) in THF (1 mL) at 0 °C under inert atmosphere. The reaction mixture was stirred for 1 h at 0 °C, was neutralized (10% aq Na₂CO₃) and further stirred for 30 min at rt. The reaction mixture was diluted with water (5 mL), extracted with CH₂Cl₂, the organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The crude product was chromatographed (CH₂Cl₂, 100 %) to yield indole-3-isonitrile (**9**, 14 mg, 0.10 mmol) in 54% yield (m.p. decomposed at 153 °C).

Indole-3-isonitrile (9): HPLC t_R = 10.3 min. UV (CH₃OH-H₂O, HPLC) λ_{max} (nm): 240, 279. FTIR (KBr, cm⁻¹) ν_{max} : 3217, 2144, 1436, 1339, 1241. ¹H NMR (500 MHz, CD₃OD) δ 7.55 (1H, s, H-2), 7.54 (1H, d, J = 8 Hz), 7.39 (1H, d, J = 8 Hz), 7.20 (1H, ddd, J = 8, 8, 1 Hz), 7.15 (1H, ddd, J = 8, 8, 1 Hz). ¹³C NMR (125.8 MHz, CD₃OD) δ 165.3, 135.7, 124.8, 124.6, 124.2, 122.1, 118.2, 113.4, 104.3 (t, 16 Hz, C-3). HRMS-EI m/z 142.0531 [M]⁺, calcd. for C₉H₆N₂ 142.0531 (100%), 115.04 (44%). HPLC-MS-ESI m/z [M-H]⁻ 141.0 (100%).

Synthesis of 4-methoxyindole-3-formamide (13)

TFA (503 mg, 0.93 mmol) was added to a solution of 3-nitroindole (**5**, 100 mg, 0.62 mmol) in TFA (3 mL) and the reaction mixture was stirred for 4 h at 30 °C. The solvent was evaporated under reduced pressure, I₂ (472 mg, 1.86 mmol) and CuI (472 mg, 2.48 mmol) in DMF (4 mL) were added to the reaction mixture and the mixture was stirred for 1 h at 25 °C. NaOCH₃ (prepared from Na, 800 mg, 34.8 mmol, in anhydrous MeOH, 5 mL) was added to the reaction mixture and the mixture was refluxed at 110 °C for 1 h. The reaction mixture was

neutralized (pH 7, HCl, 1 M), was filtered through celite and the solvent was evaporated. The residue was taken in water (20 mL), extracted with EtOAc, dried over Na₂SO₄ and concentrated. The crude product was subjected to column chromatography (EtOAc-hex, 1:1) to yield 4-methoxy-3-nitroindole (**10**, 77 mg, 0.40 mmol) in 64% yield.

Procedure A: Pd/C (52 mg) was added to a solution of 4-methoxy-3-nitroindole (**10**, 52 mg, 0.27 mmol) in EtOH (1 mL) and reaction mixture was stirred under H₂ at balloon pressure for 5 h. The reaction mixture was cooled to -10 °C, Et₃N (112 μL, 0.81 mmol) and a solution of HCO₂H/Ac₂O (1:0.8, 2 mL) were added and the mixture was further stirred for 1 h, while allowing the temperature to rise to 0 °C. The reaction mixture was diluted with water, extracted with CH₂Cl₂, the organic phase was dried over Na₂SO₄ and concentrated. The crude product was subjected to column chromatography (CH₂Cl₂:MeOH, 98:2) to yield 4-methoxyindole-3-formamide (**13**, 24 mg, 0.13 mmol) in 47% yield.

Procedure B: Pd/C (20 mg) was added to a solution of 4-methoxy-3-nitroindole (20 mg, 0.10 mmol) in MeOH (1 mL) and solution of HCO₂H/Ac₂O (1.5:1) (88 μL, 0.62 mmol). The reaction mixture was stirred under H₂ at balloon pressure for 3 h at -10 °C and allowed to warm up to 10 °C for an additional 1 h. The reaction mixture was diluted with water, extracted with CH₂Cl₂, the organic phase was dried over Na₂SO₄ and concentrated to give 4-methoxyindole-3-formamide (**13**, 12.6 mg, 0.07 mmol) in 66 % yield and 4-methoxy indole-3-acetamide (1.4 mg, 0.007 mmol) in 7% yield.

4-Methoxyindole-3-formamide (13): HPLC *t*_R = 5.6 min. UV (CH₃OH-H₂O, HPLC) λ_{max} (nm): 223, 250, 285. FTIR (KBr, cm⁻¹) ν_{max}: 3399, 3302, 1661, 1502, 1263, 1085. ¹H NMR (500 MHz, CD₃OD) δ 8.25 (1H, s), 7.68 (1H, s), 7.00 (1H, dd, *J* = 8, 8 Hz), 6.91 (1H, d, *J* = 8 Hz), 6.46 (1H, d, *J* = 8 Hz), 3.93 (3H, s). ¹³C NMR (125.8 MHz, CD₃OD) δ 160.5, 155.0, 136.6, 124.1, 115.5, 115.1, 111.3, 106.0, 99.9, 55.7. HRMS-EI *m/z* 190.0736 [M]⁺, calcd. for C₁₀H₁₀N₂O₂ 190.0742 (100%), 162.08 (20%), 147.0557 (74%). HPLC-MS-ESI *m/z* [M+H]⁺ 191.2 (40%), 163.2 (100%).

Synthesis of [²H₃]rapalexin A (**12**)

TFA (252 mg, 0.46 mmol) was added to a solution of 3-nitroindole (**5**, 50 mg, 0.31 mmol) in TFA (3 mL) and the reaction mixture was stirred for 4 h at 30 °C. The solvent was evaporated under reduced pressure, I₂ (236 mg, 0.93 mmol) and CuI (236 mg, 1.24 mmol) in DMF (4 mL) were added to the reaction mixture and the mixture was stirred for 1 h at 25 °C. NaOC²H₃ (prepared from Na, 400 mg, 56 mmol, in ²H₃CO²H, 5 mL) was added to the reaction mixture and the mixture was refluxed at 110 °C for 1 h. The reaction mixture was neutralized (pH 7, HCl, 1 M), was filtered through celite and the solvent was evaporated. The residue was taken in water (10 mL), extracted with EtOAc, dried over Na₂SO₄ and concentrated. The crude product was subjected to column chromatography (EtOAc-hex, 1:1) to yield 4-methoxy-3-nitroindole (**10**, 65.3 mg, 0.33 mmol) in 54% yield.

Pd/C (25 mg) was added to a solution of 4-methoxy-3-nitroindole (**10**, 25 mg, 0.13 mmol) in THF (1 mL) and EtOH (200 μL) and the reaction mixture was stirred under H₂ balloon at pressure for

5 h. The reaction mixture was cooled to -10 °C, Et₃N (54 μL, 0.39 mmol) and CSeCl₂ (12 μL, 0.16 mmol) were added and further stirred for 10 min. the reaction mixture was diluted with water, extracted with CH₂Cl₂, the organic phase was dried over Na₂SO₄ and concentrated. The crude product was flash chromatographed (CH₂Cl₂, 100%) to yield [²H₃]rapalexin A (**12**, 13.8 mg, 0.67 mmol) in 51%.

HPLC *t*_R = 16.9 min. UV (CH₃OH-H₂O, HPLC) λ_{max} (nm): 222, 292. FTIR (KBr, cm⁻¹) ν_{max}: 3399, 2072, 1592, 1508, 1280, 1100, 1075, 774, 729. ¹H NMR (500 MHz, CDCl₃) δ 7.94 (1H, s), 7.27 (1H, s), 7.16 (1H, dd, *J* = 8, 8 Hz), 7.07 (1H, d, *J* = 3 Hz), 6.94 (1H, d, *J* = 8 Hz), 6.56 (1H, d, *J* = 8). HRMS-EI *m/z* 207.0544 [M]⁺, calcd. for C₁₀H₅²H₃N₂S 207.0546 (100%), 189.01 (52%), 161.01 (27%). HPLC-MS-ESI *m/z* [M-H]⁻, 206.1 (100%), 188.1 (44%).

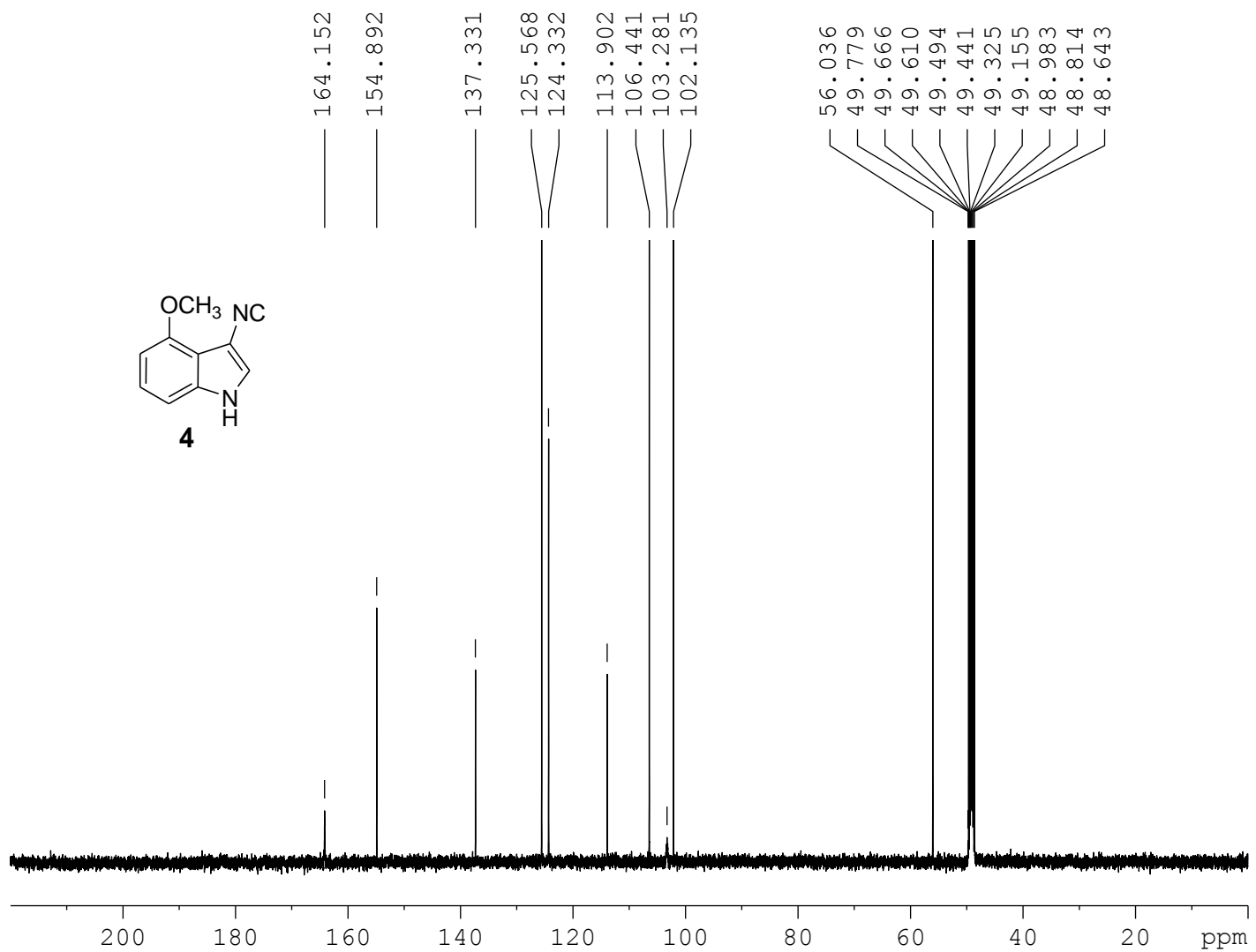
Synthesis of 4-methoxyindole-3-carbonitrile

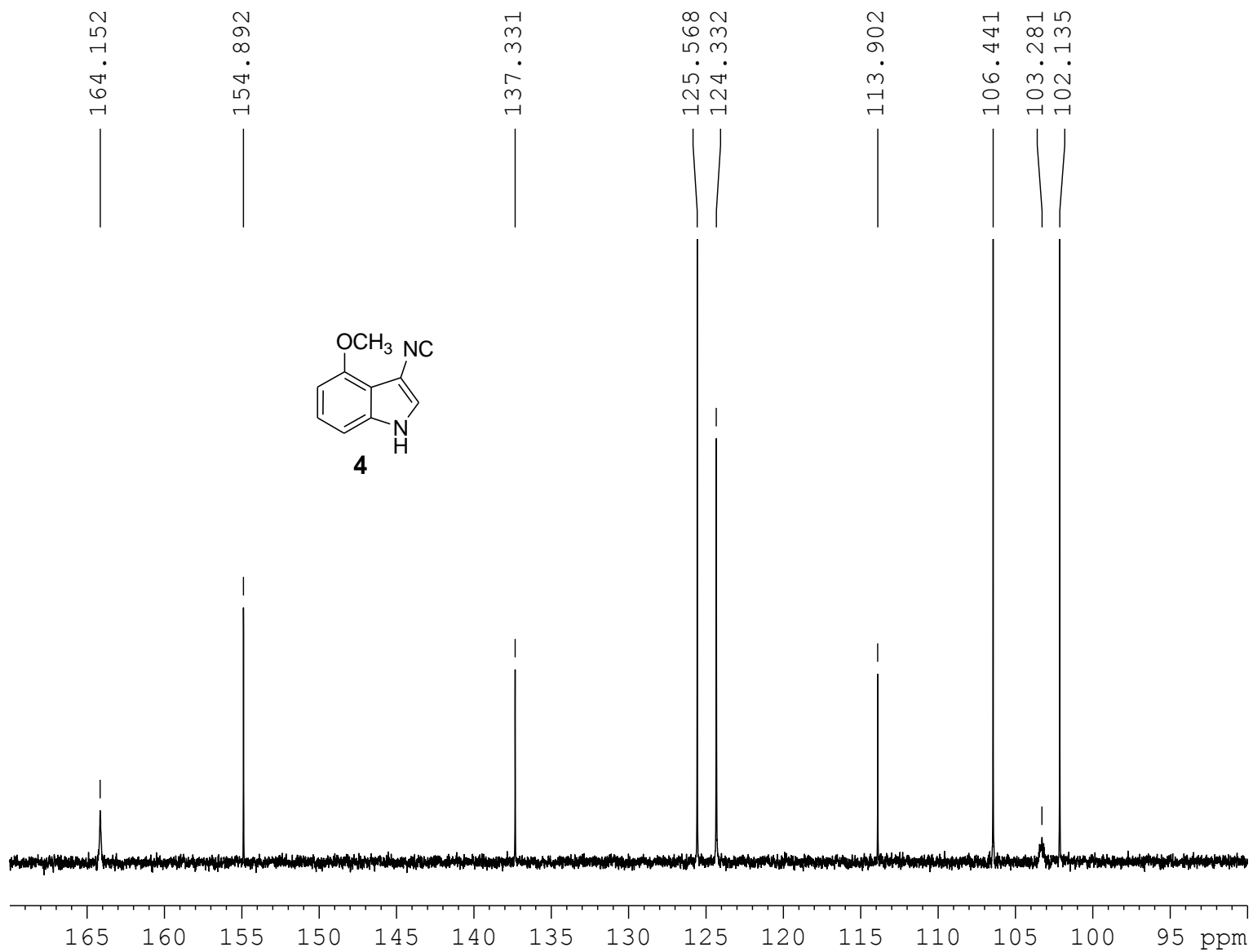
4-Methoxyindole-3-carboxaldehyde oxime [prepared from 4-methoxyindole-3-carboxaldehyde¹ using a standard procedure: NH₂OH.HCl (28 mg, 0.40 mmol) and Na₂CO₃ (43 mg, 0.40 mmol) in 1 mL H₂O, added to aldehyde (35 mg, 0.20 mmol) in 3 mL EtOH and refluxed at 55 °C for 1 h](20 mg, 0.10 mmol) was acetylated using Ac₂O-pyridine (4:1, 100 μL). After standing at rt for 30 min, the reaction mixture was diluted with toluene and concentrated to dryness in a rotary evaporator. The reaction mixture was subjected to column chromatography (CH₂Cl₂, 100%) to yield 4-methoxyindole-3-carbonitrile in 76%.

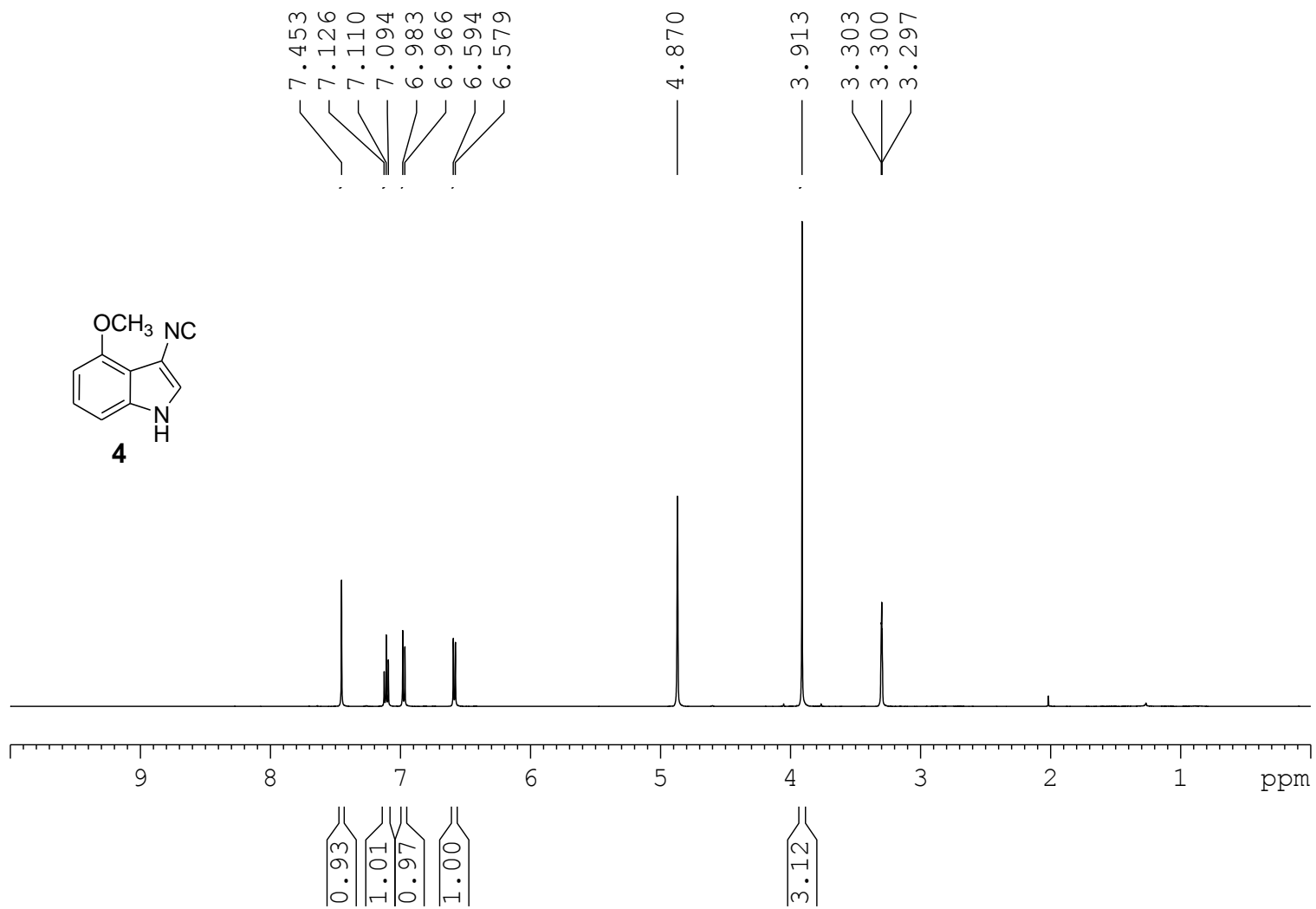
4-Methoxyindole-3-carbonitrile: HPLC *t*_R = 7.2 min. UV (CH₃OH-H₂O, HPLC) λ_{max} (nm): 222, 275. FTIR (KBr, cm⁻¹) ν_{max}: 3330, 2219, 1591, 1519, 1417, 1270, 1091. ¹H NMR (500 MHz, CD₃OD) δ 7.83 (1H, s), 7.22 (1H, dd, *J* = 8, 8 Hz), 7.10 (1H, d, *J* = 8 Hz), 6.68 (1H, d, *J* = 8 Hz), 3.96 (3H, s). ¹³C NMR (125.8 MHz, CD₃OD) δ 154.8, 138.6, 134.2, 126.0, 118.5, 118.2, 111.3, 106.6, 102.7, 84.8, 56.0. HRMS-EI *m/z* 172.0637 [M]⁺, calcd. for C₁₀H₈N₂O 172.0637 (100%), 157.04 (60%), 129.04 (43%). HPLC-MS-ESI *m/z* [M-H]⁻, 171.2 (100%), 157.1 (70%).

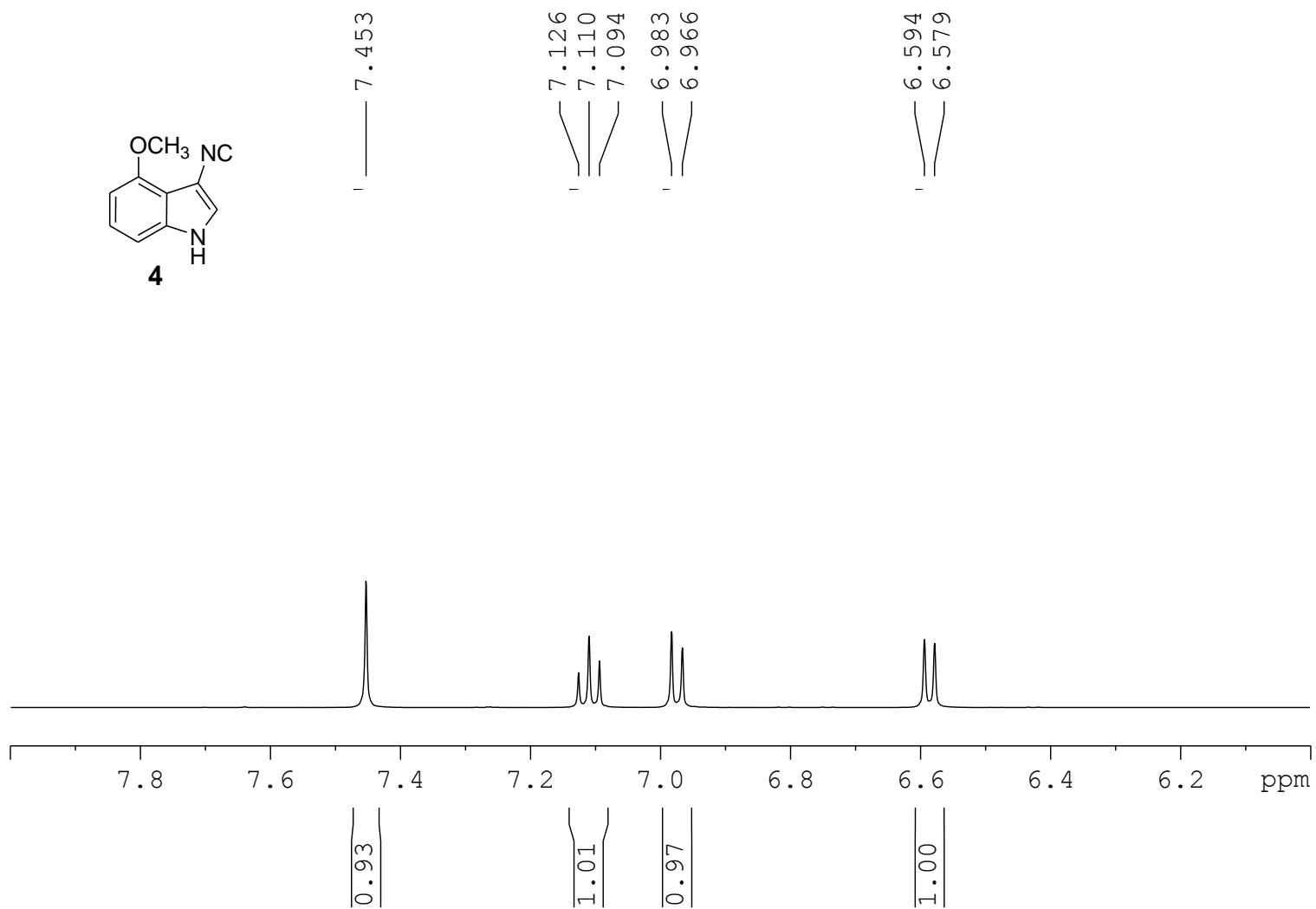
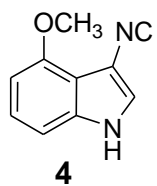
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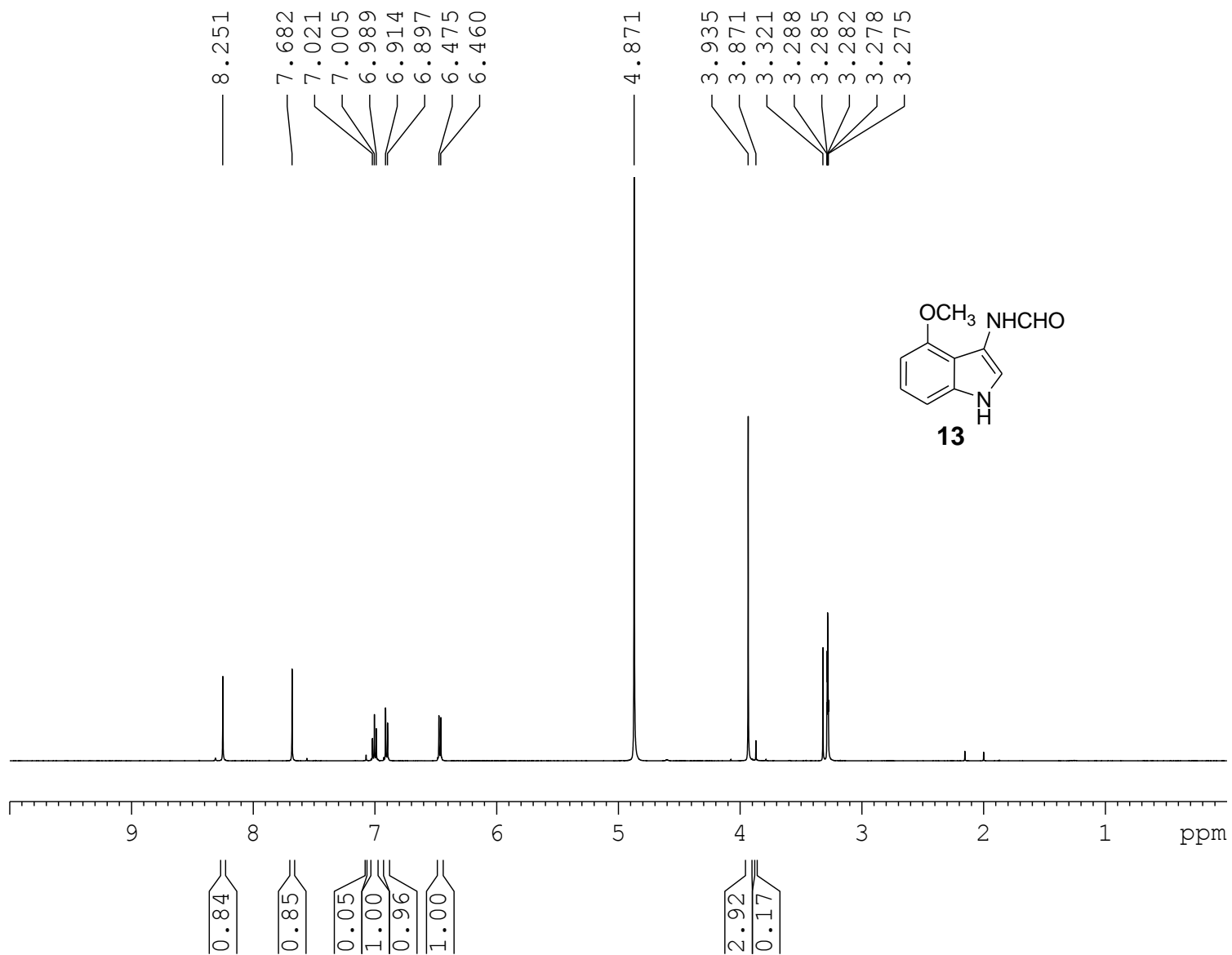
- 1 M. Somei, F. Yamada, M. Kunitomo, C. Kaneko, *Heterocycles*, 1984, **22**, 797-801.

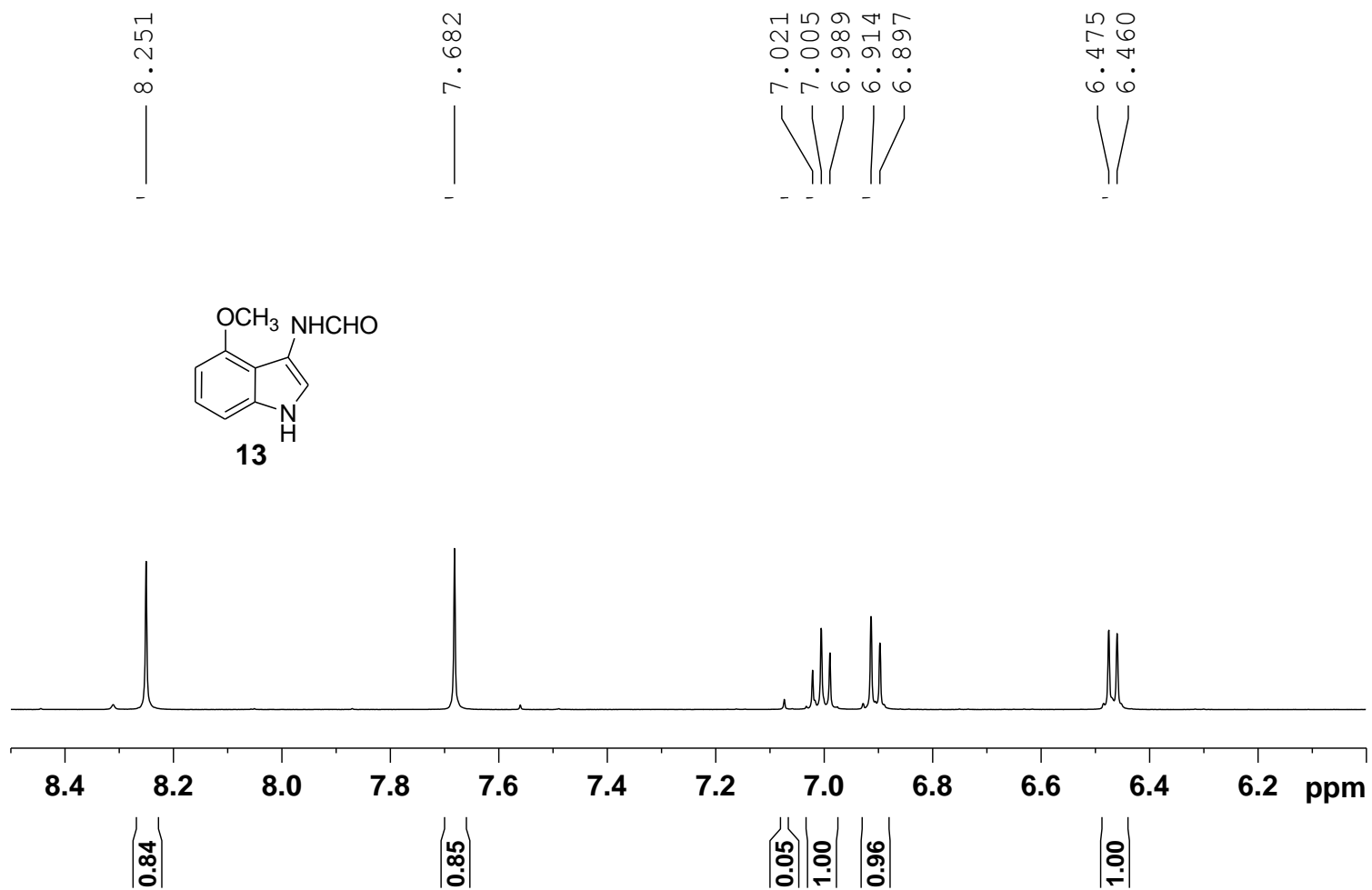


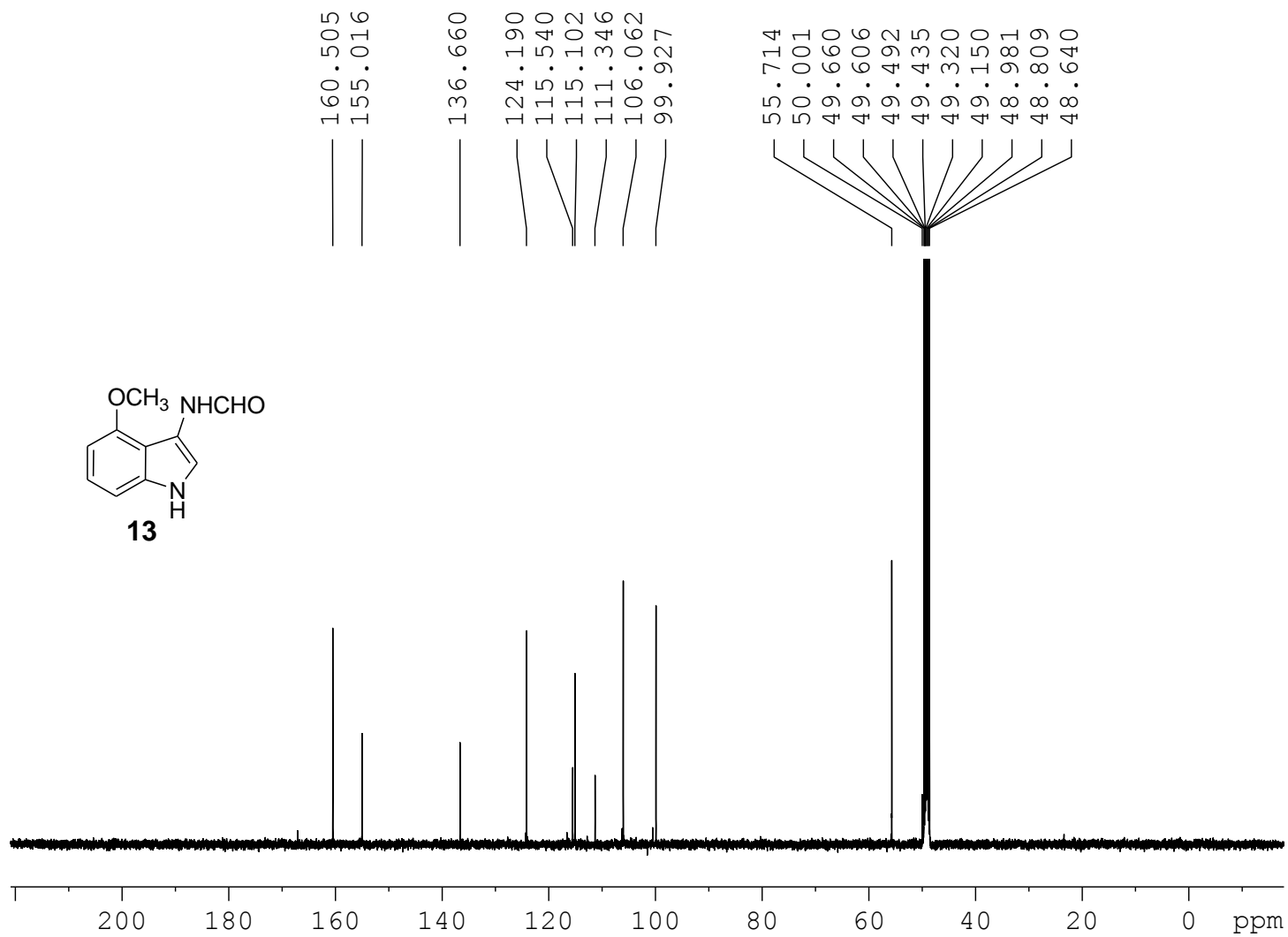
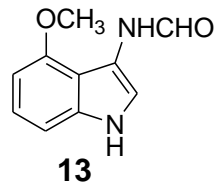


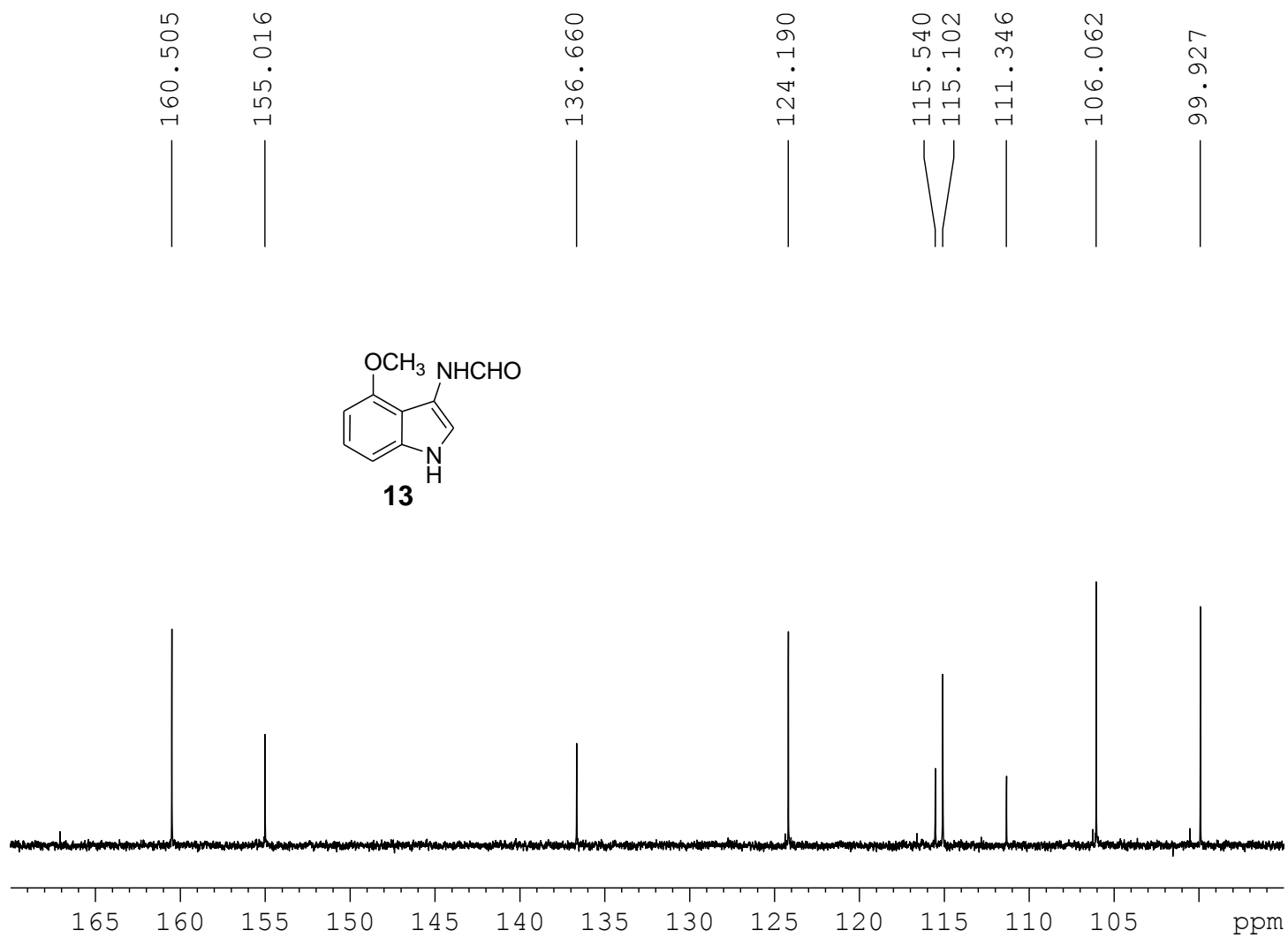
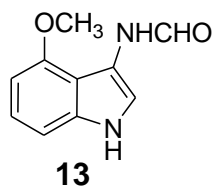


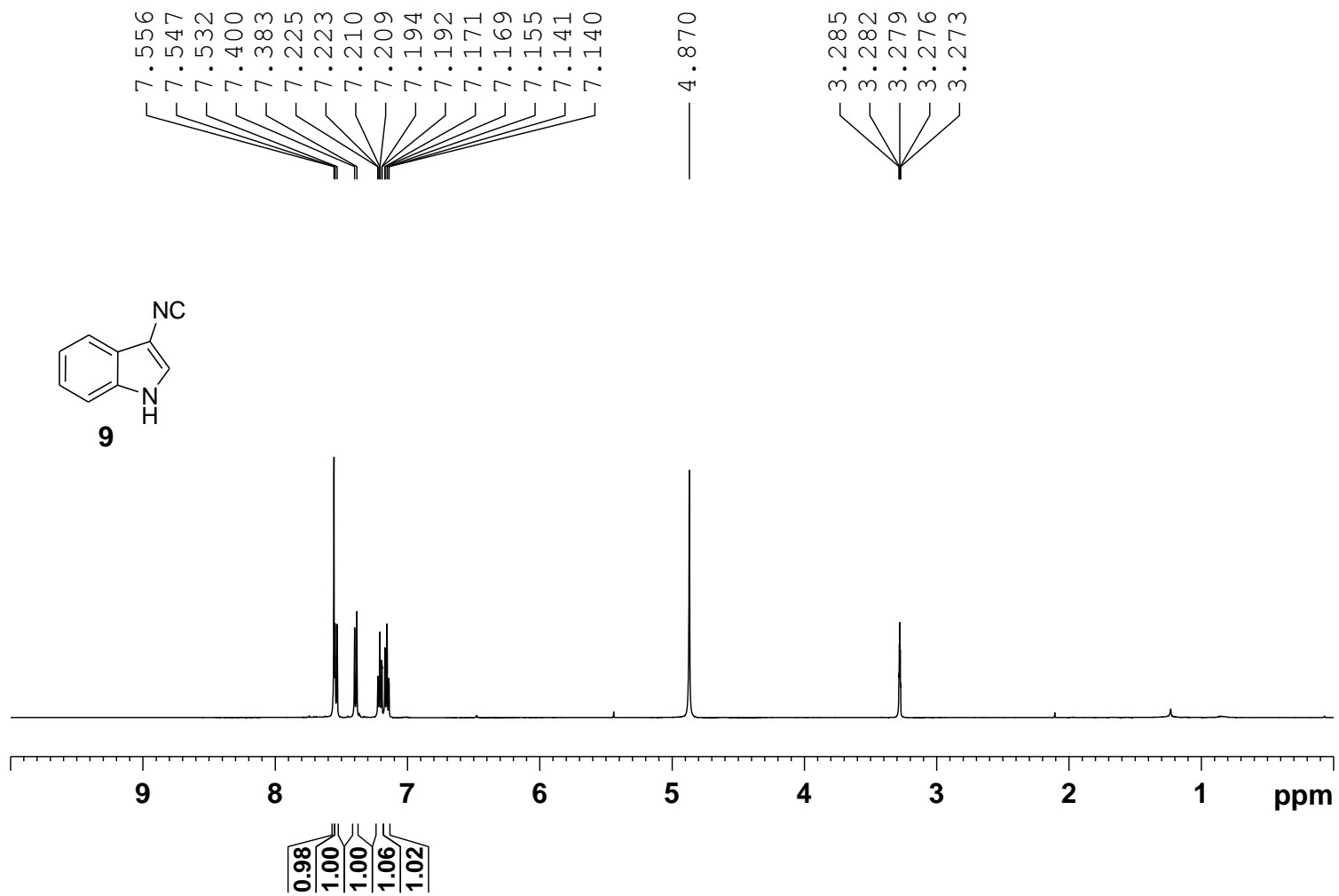


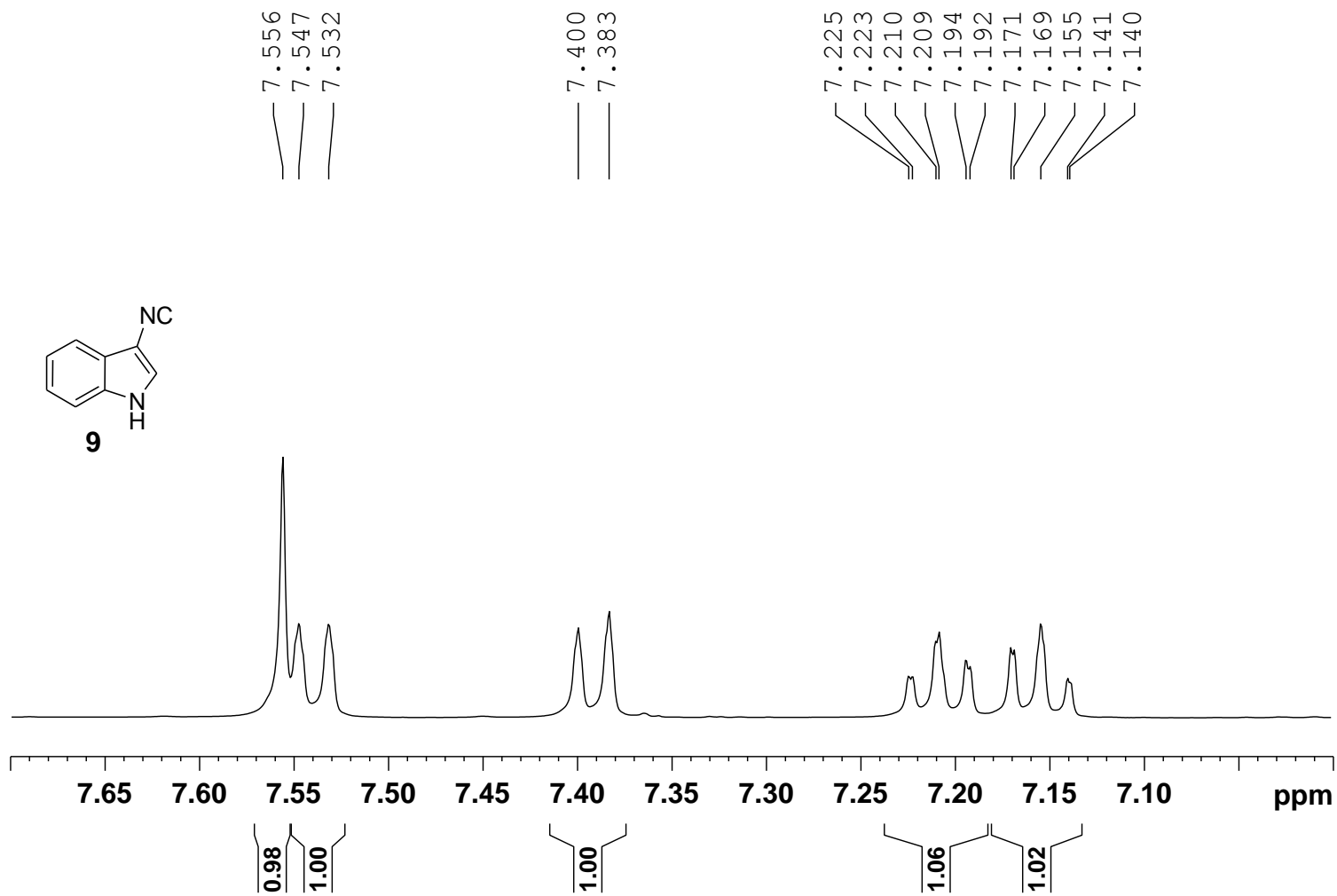


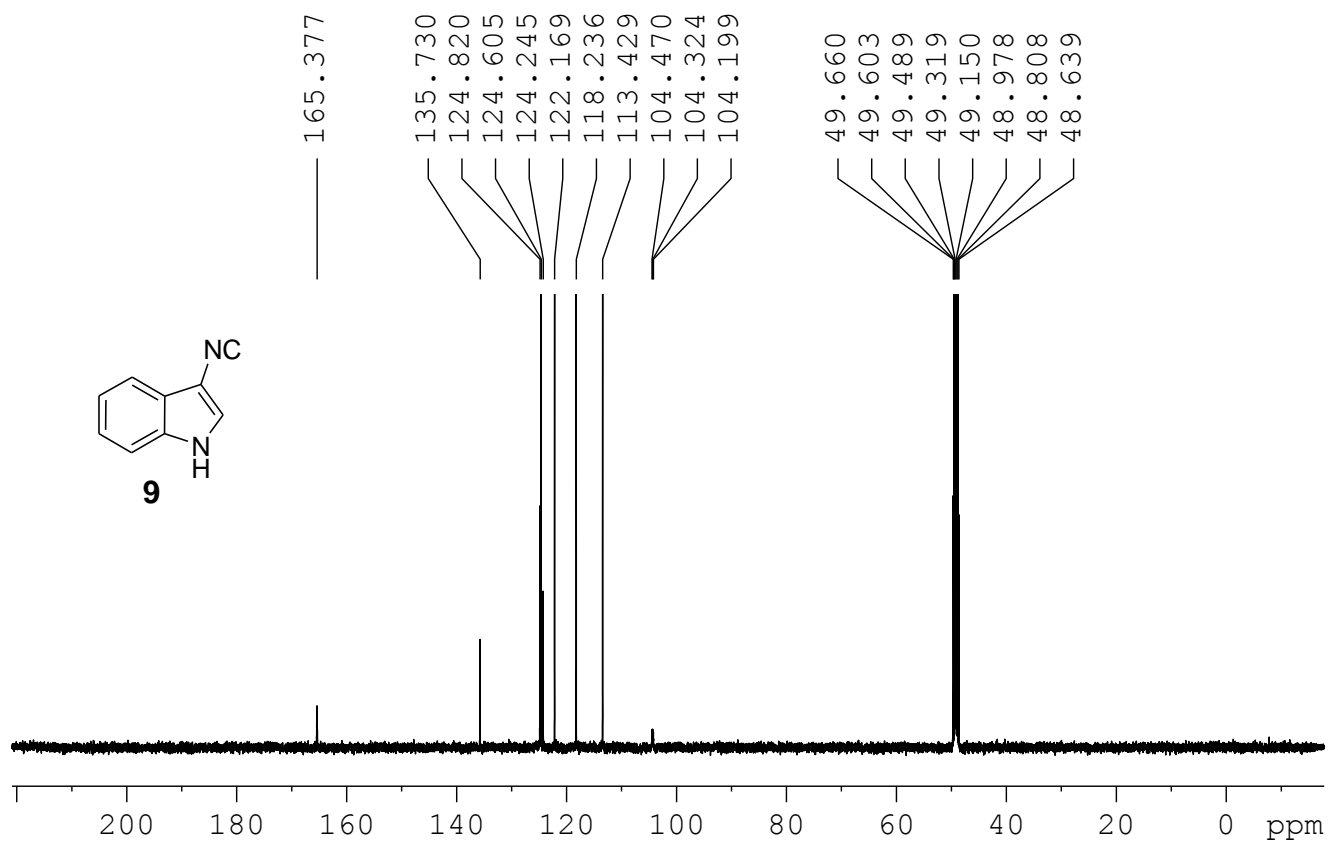


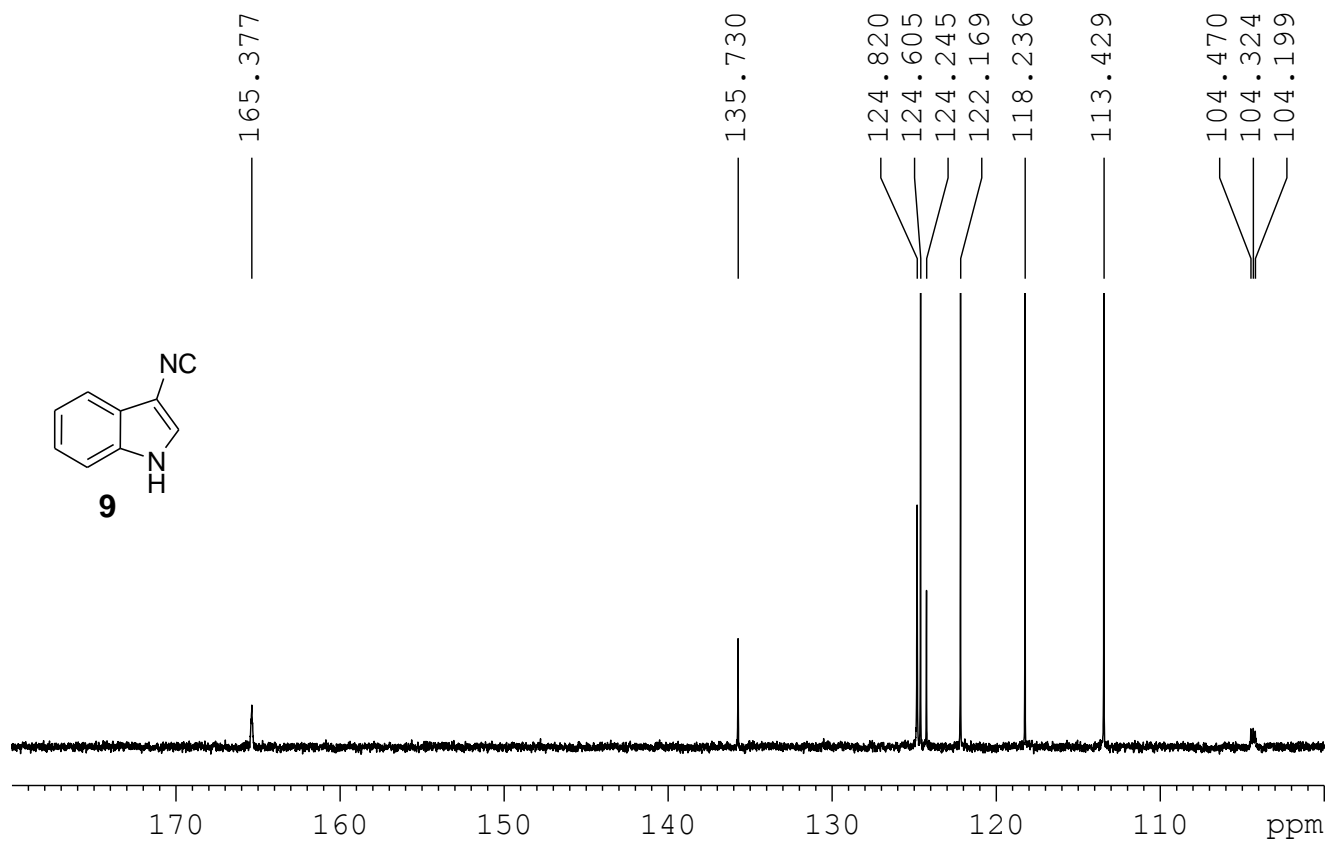


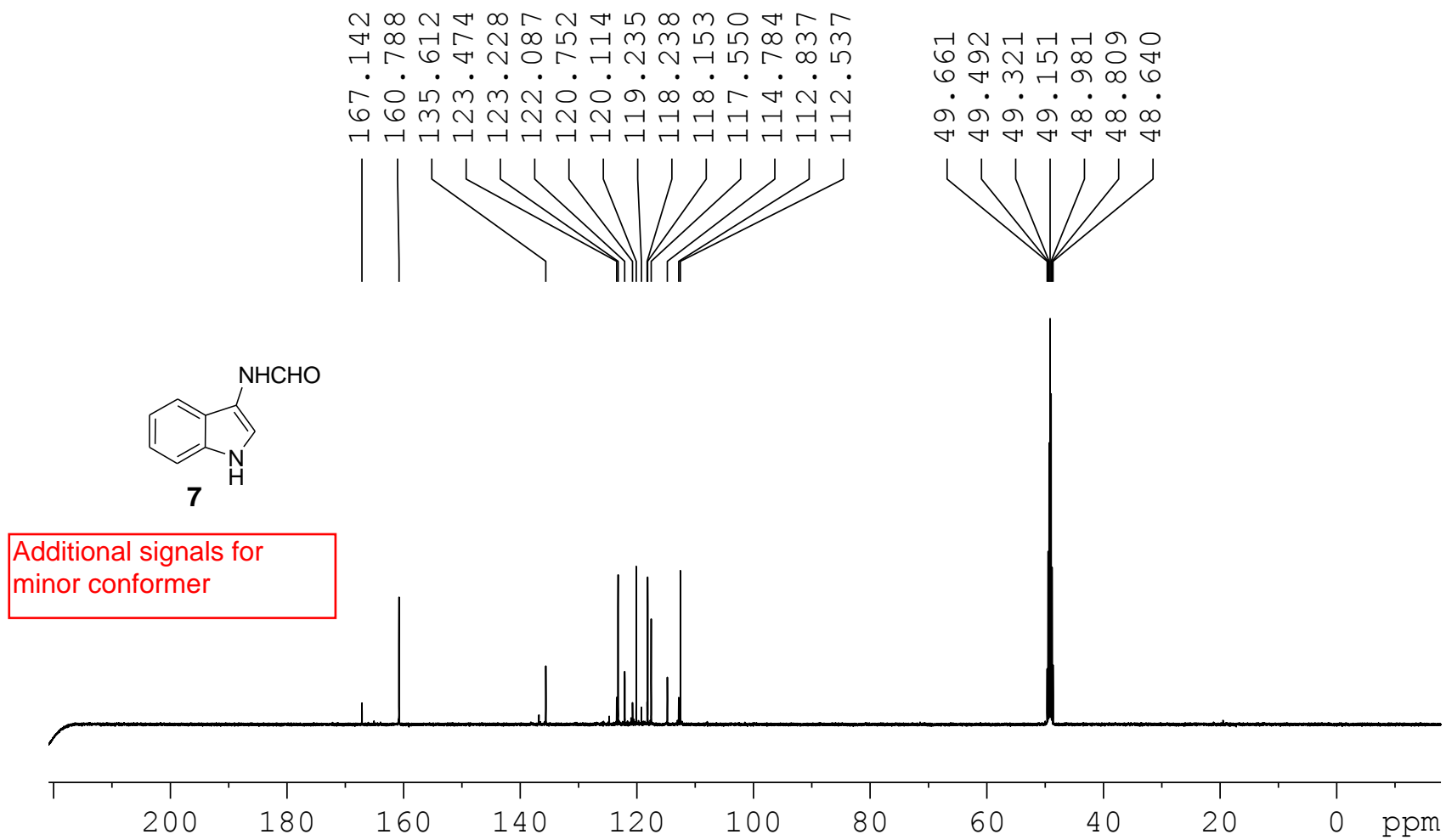


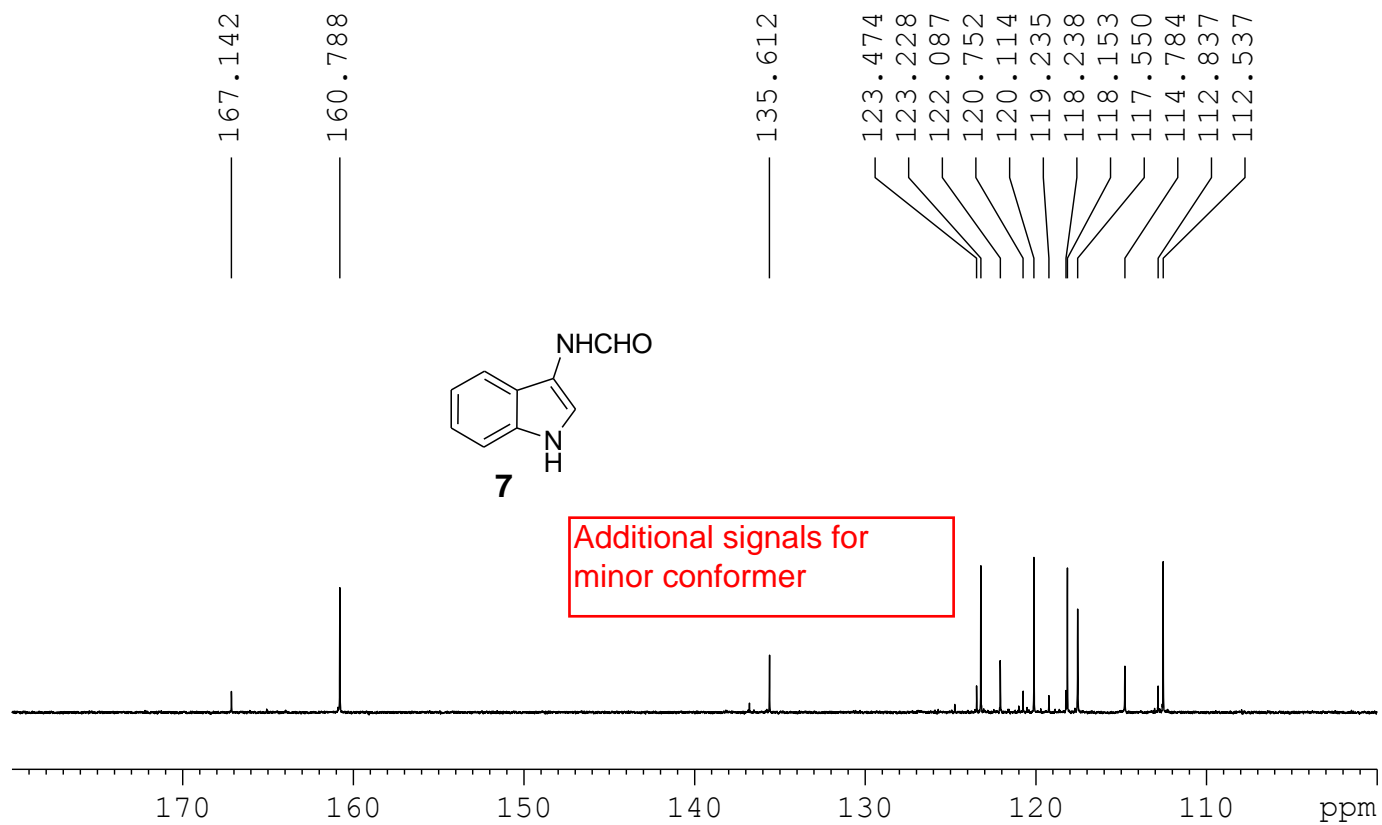


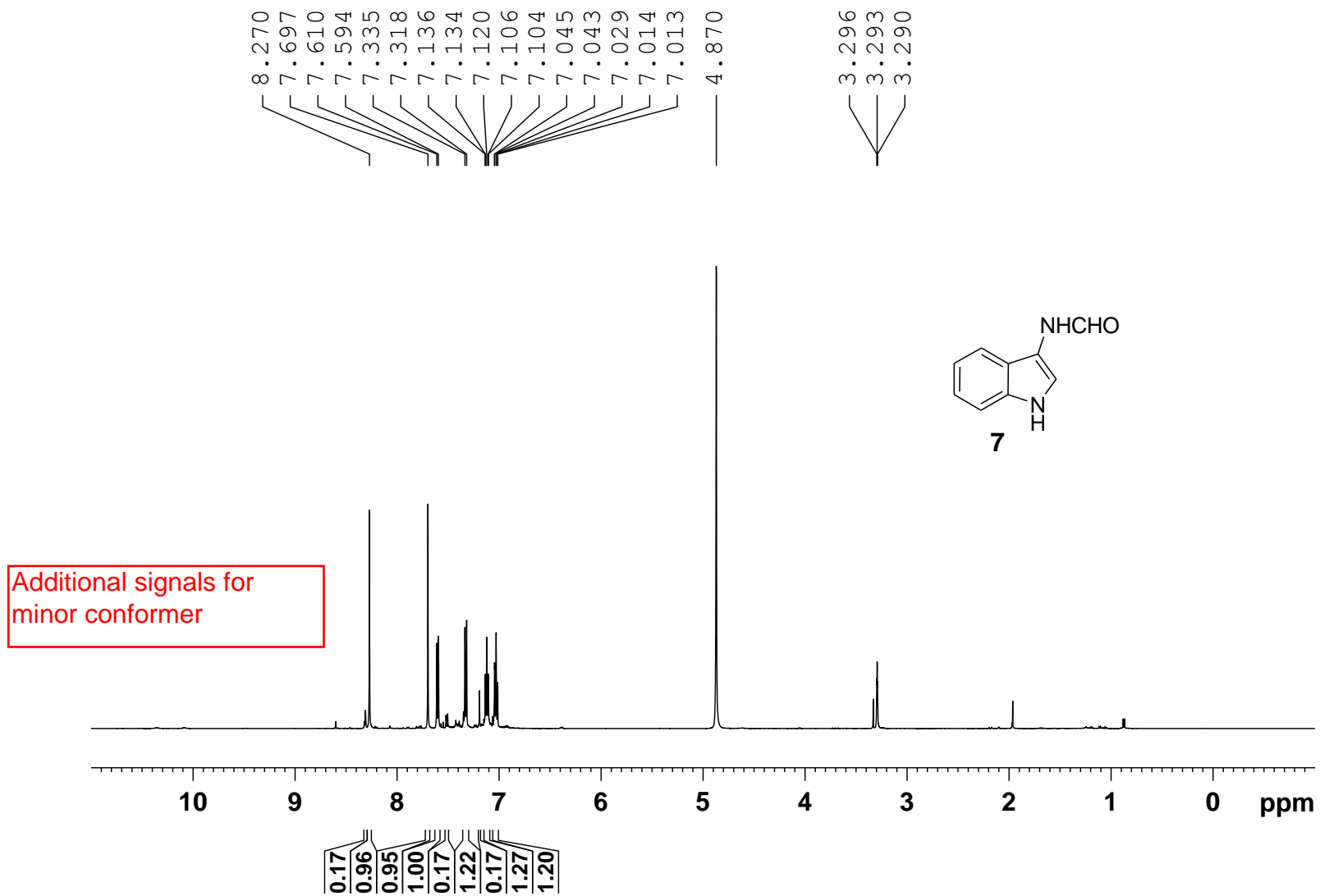


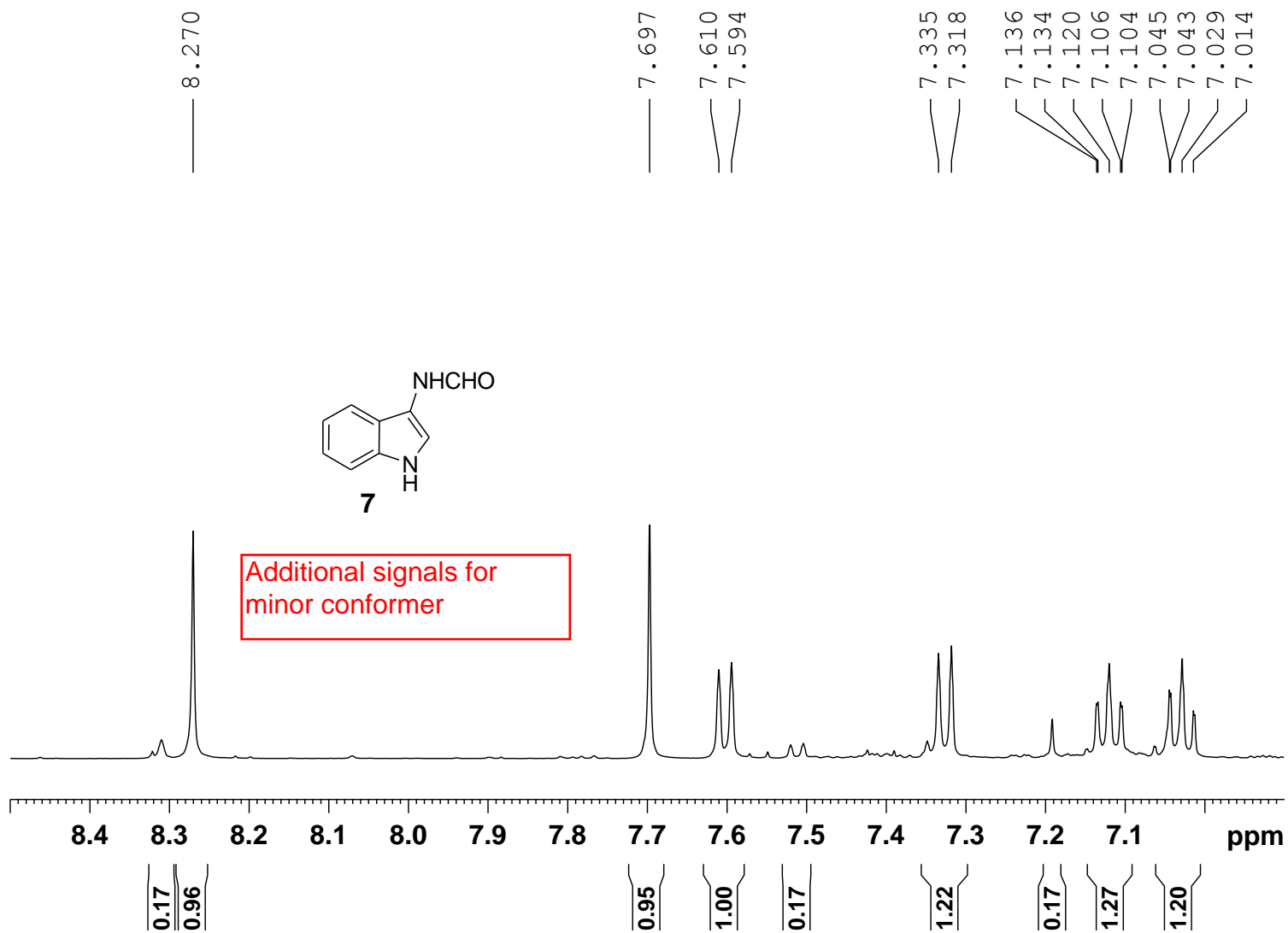












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