

## Supplementary Material

# The synthesis and anti-inflammatory evaluation of 1,2,3-triazole linked isoflavone benzodiazepine hybrids

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## 1. Synthesis of Previously Reported Compounds

### 7-Hydroxy-3-(4-nitrophenyl)-4H-chromen-4-one (**8**)<sup>29,30</sup>

A solution of resorcinol (20 mmol, 2.2 g) and 4-nitrophenylacetic acid (20 mmol, 3.62 g) in  $\text{BF}_3 \cdot \text{Et}_2\text{O}$  (102 mmol, 12.6 mL) and under nitrogen, was stirred and heated at 100 °C for 3 h. The reaction mixture was allowed to cool down to room temperature and dry DMF (30 mL) was gradually added. The mixture was heated to 50 °C and a solution of methanesulfonyl chloride (60 mmol, 4.64 mL) in dry DMF (75 mmol, 5.8 mL) was added dropwise. After reaction at 100 °C for 3 h, the resulting mixture was cooled to room temperature and quenched with aq. sodium acetate (10%, 50 mL). The precipitate was filtered, washed with water (3 × 75 mL) and dried in air. The crude was purified by column chromatography ( $\text{CHCl}_3/\text{CH}_3\text{OH}$ , 100:1) to give isoflavone **8** (2 g, 35%) as a pale brown solid; mp = 295–296 °C, lit. mp > 270 °C.<sup>29</sup>  $R_f$  = 0.2 ( $\text{CHCl}_3/\text{CH}_3\text{OH}$ , 97:3).

<sup>1</sup>H-NMR (400 MHz, DMSO):  $\delta$  = 10.94 (s, 1 H, OH-7), 8.62 (s, 1 H), 8.30 (d,  $J$  = 8.6 Hz, 2 H), 8.01 (d,  $J$  = 8.7 Hz, 1 H), 7.91 (d,  $J$  = 8.6 Hz, 2 H), 6.99 (dd,  $J$  = 1.8, 8.7 Hz, 1 H), 6.93 (d,  $J$  = 1.8 Hz, 1 H).

<sup>13</sup>C-NMR (100 MHz, DMSO):  $\delta$  = 174.3 (C=O), 163.4 (qC), 157.9 (qC), 155.9 (CH), 147.2 (qC), 139.8 (qC), 130.4 (2 CH), 127.8 (CH), 123.7 (2 CH), 122.1 (qC), 116.9 (qC), 116.0 (CH), 102.8 (CH).

HRMS (Dual ESI): calc  $m/z$  for  $\text{C}_{15}\text{H}_9\text{NO}_5$ : 283.0481 [M], 284.0553 [M+H]<sup>+</sup>; found: 283.0480 [M], 284.0552 [M+H]<sup>+</sup>.

FT-IR ( $\text{cm}^{-1}$ ):  $\nu$  = 3361, 3078, 2999, 2933, 1624, 1597, 1513, 1451, 1340, 1269, 1229, 1188, 1098, 1042, 797.

### 3-(4-Aminophenyl)-7-hydroxy-4H-chromen-4-one (**9**)<sup>29,30</sup>

To a solution of the nitroisoflavone **8** (4.94 mmol, 1.4 g) in ethanol (20 mL), iron powder (49.43 mmol, 2.76 g) and  $\text{NH}_4\text{Cl}$  (9.89 mmol, 0.53 g in 2 mL water) were added and the mixture was stirred and heated to 90 °C for 4 h. The resulting solution was filtered hot, and the filtrate concentrated under vacuum. The crude material was purified by flash chromatography ( $\text{CHCl}_3/\text{CH}_3\text{OH}$ , 100:5) to give the aminoisoflavone **9** (0.83 g, 66%) as a white solid; mp = 253 °C (decomp.), lit. mp = 250 °C (decomp.).<sup>29</sup>  $R_f$  = 0.16 ( $\text{CHCl}_3/\text{CH}_3\text{OH}$ , 95:5).

<sup>1</sup>H-NMR (400 MHz, DMSO):  $\delta$  = 10.76 (s, 1 H, OH-7), 8.24 (s, 1H), 7.96 (d,  $J$  = 8.7 Hz, 1 H), 7.24 (d,  $J$  = 8.5 Hz, 2 H), 6.92 (dd,  $J$  = 2.2, 8.7 Hz, 1 H), 6.85 (d,  $J$  = 2.2 Hz, 1 H), 6.59 (d,  $J$  = 8.5 Hz, 2 H), 5.21 (br s, 2 H,  $\text{NH}_2$ ).

<sup>13</sup>C-NMR (100 MHz, DMSO):  $\delta$  = 175.3 (C=O), 162.8 (qC), 157.8 (qC), 152.5 (CH), 148.9 (qC), 129.9 (2 CH), 127.7 (CH), 124.3 (qC), 119.4 (qC), 117.1 (qC), 115.4 (CH), 113.8 (2 CH), 102.5 (CH).

HRMS (Dual ESI): calc  $m/z$  for  $\text{C}_{15}\text{H}_{11}\text{NO}_3$ : 253.0739 [M], 254.0812 [M+H]<sup>+</sup>; found: 253.0741 [M], 254.0814 [M+H]<sup>+</sup>.

FT-IR ( $\text{cm}^{-1}$ ):  $\nu$  = 3337, 3247, 3156, 3056, 2918, 1626, 1608, 1590, 1514, 1462, 1376, 1246, 1191, 1097, 840.

### 1,2,3,11a-Tetrahydro-5H-benzo[e]pyrrolo[1,2-*a*][1,4]diazepine-5,11(10H)-dione (**16**)<sup>44</sup>

A solution of isatoic anhydride **12** (10 mmol, 1.63 g) and L-proline **14** (11 mmol, 1.26 g) in DMSO (10 mL) was stirred and heated at 120 °C for 4 h. The resulting mixture was allowed to cool to RT and water (20 mL) was added. The precipitate that appeared was allowed to fully form in the freezer overnight, was filtered off, washed with cold water, and dried under vacuum suction to give **16** (2.15 g, 99%) as a pale brown solid; mp = 219–220 °C, lit. mp = 220–222 °C,<sup>44</sup>  $R_f$  = 0.61 ( $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ , 96:4).

<sup>1</sup>H-NMR (400 MHz, DMSO):  $\delta$  = 10.48 (s, 1 H, NH), 7.76 (dd,  $J$  = 1.6, 7.8 Hz, 1 H), 7.54 – 7.45 (m, 1 H), 7.24 – 7.17 (m, 1 H), 7.14 – 7.08 (m, 1 H), 4.14 – 4.04 (m, 1 H), 3.63 – 3.51 (m, 1 H), 3.48 – 3.38 (m, 1 H), 2.00 – 1.70 (m, 3 H).

<sup>13</sup>C-NMR (100 MHz, DMSO):  $\delta$  = 171.2 (qC), 165.0 (qC), 136.8 (qC), 132.5 (CH), 130.7 (CH), 127.0 (qC), 124.3 (CH), 121.7 (CH), 56.6 (CH), 47.3 ( $\text{CH}_2$ ), 26.2 ( $\text{CH}_2$ ), 23.5 ( $\text{CH}_2$ ).

FT-IR ( $\text{cm}^{-1}$ ):  $\nu$  = 3418, 3202, 3050, 2940, 2869, 1693, 1673, 1601, 1575, 1443, 1395, 1289, 755.

**7-Bromo-1,2,3,11a-tetrahydro-5H-benzo[e]pyrrolo[1,2-*a*][1,4]diazepine-5,11(10H)-dione (18)<sup>34</sup>**

To a solution of **16** (10 mmol, 2.16 g) in glacial acetic acid (20 mL), sodium acetate (10 mmol, 0.82 g) was added, followed by a dropwise addition of a solution of bromine (12 mmol, 1.92 g, 0.62 mL) in 20 mL of glacial acetic acid. After stirring the resulting solution at room temperature for 16 h, water (50 mL) was added.<sup>201</sup> The aqueous phase was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 50 mL) and the organic phase was washed with water (2 × 100 mL), dried (MgSO<sub>4</sub>), filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (CH<sub>2</sub>Cl<sub>2</sub>/Acetone, 20:1) to give **18** (2.06 g, 70%) as a white solid; mp = 213-215 °C, lit. mp = 218-220 °C;<sup>34</sup> *R*<sub>f</sub> = 0.43 (ethyl acetate/petroleum ether, 4:1).

<sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ = 8.87 (s, 1 H, NH), 8.11 (d, *J* = 2.3 Hz, 1 H), 7.56 (dd, *J* = 2.3, 8.5 Hz, 1 H), 6.92 (d, *J* = 8.5 Hz, 1 H), 4.06 (d, *J* = 7.3 Hz, 1 H), 3.86 – 3.75 (m, 1 H), 3.65 – 3.54 (m, 1 H), 2.82 – 2.69 (m, 1 H), 2.12 – 1.94 (m, 3 H).

<sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ = 171.0 (qC), 164.0 (qC), 135.4 (CH), 134.3 (qC), 133.8 (CH), 128.6 (qC), 122.7 (CH), 118.2 (qC), 56.7 (CH), 47.5 (CH<sub>2</sub>), 26.3 (CH<sub>2</sub>), 23.5 (CH<sub>2</sub>).

FT-IR (cm<sup>-1</sup>): ν = 3219, 3154, 3050, 2938, 2872, 1698, 1613, 1475, 1445, 1366, 1253, 838, 752.

**Ethyl 7-bromo-9-oxo-11,12,13,13a-tetrahydro-9H-benzo[e]imidazo[5,1-*c*]pyrrolo[1,2-*a*][1,4]diazepine-1-carboxylate (22)<sup>35</sup>**

To a solution of **18** (1.69 mmol, 210 mg) in anhydrous THF (30 mL), cooled to 0 °C and under N<sub>2</sub>, *t*-BuOK (1.86 mmol, 210 mg) was added and the mixture was stirred at 0 °C for 20 min. After cooling the reaction mixture to -35 °C, diethyl chlorophosphate **19** (2.2 mmol, 0.32 mL) was added dropwise. The resulting mixture was brought to 0 °C and stirred for 30 min. The mixture was again cooled to -35 °C and ethyl isocynoacetate **20** (1.86 mmol, 0.2 mL), followed by *t*-BuOK (1.86 mmol, 210 mg) were added. The reaction was allowed to warm to RT and stirred for 4 h. Saturated aqueous NaHCO<sub>3</sub> (50 mL) was added and the aqueous layer was extracted with ethyl acetate (3 × 50 mL). The organic phase was dried (MgSO<sub>4</sub>), filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (EtOAc/PE, 1:1) to give **22** (0.35 g, 53%) as a white solid; mp = 237-238 °C, lit. mp = 248.5-249 °C;<sup>35</sup> *R*<sub>f</sub> = 0.1 (EtOAc/PE, 3:1). 70 mg of starting **18** were recovered.

<sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ = 8.26 (d, *J* = 2.3 Hz, 1 H), 7.83 (s, 1 H), 7.78 (dd, *J* = 2.3, 8.5 Hz, 1 H), 7.28 (d, *J* = 8.5 Hz, 1 H), 4.75 (d, *J* = 6.8 Hz, 1 H), 4.41 (q, *J* = 7.1 Hz, 2 H), 3.82 – 3.74 (m, 1 H), 3.61 – 3.48 (m, 2 H), 2.39 – 2.13 (m, 3 H), 1.44 (t, *J* = 7.1 Hz, 3 H).

<sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ = 162.4 (qC), 162.3 (qC), 137.5 (qC), 135.9 (CH), 135.7 (CH), 134.6 (CH), 131.6 (qC), 130.9 (qC), 127.7 (qC), 124.8 (CH), 122.9 (qC), 61.5 (CH<sub>2</sub>), 53.3 (CH), 46.7 (CH<sub>2</sub>), 28.4 (CH<sub>2</sub>), 24.4 (CH<sub>2</sub>), 14.3 (CH<sub>3</sub>).

FT-IR (cm<sup>-1</sup>): ν = 3059, 2978, 2877, 1711, 1626, 1592, 1546, 1441, 1251, 1119, 1061, 958, 836.

**Ethyl 9-oxo-7-((trimethylsilyl)ethynyl)-11,12,13,13a-tetrahydro-9H-benzo[e]imidazo[5,1-*c*]pyrrolo[1,2-*a*][1,4]diazepine-1-carboxylate (31)<sup>18</sup>**

Prepared as described for compound **29** using bromide **22** (1.7 mmol, 663 mg), Et<sub>3</sub>N (25 mL), CH<sub>3</sub>CN (20 mL), PdCl<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub> (0.17 mmol, 120 mg, 10 mol %) and trimethylsilyl alkyne **23** (3.4 mmol, 334 mg, 0.47 mL); purified by flash chromatography (CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH, 100:1) to give **31** (538 mg, 78%) as a pale brown oily solid; mp = 95-97 °C, lit.<sup>18</sup> mp not reported; *R*<sub>f</sub> = 0.11 (CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH, 100:1).

<sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ = 8.13 (d, *J* = 1.9 Hz, 1 H), 7.80 (s, 1 H), 7.62 (dd, *J* = 1.9, 8.3 Hz, 1 H), 7.30 (d, *J* = 8.3 Hz, 1 H), 4.69 (d, *J* = 7.0 Hz, 1 H), 4.35 (q, *J* = 7.1 Hz, 2 H), 3.78 – 3.66 (m, 1 H), 3.57 – 3.38 (m, 2 H), 2.33 – 2.04 (m, 3 H), 1.37 (t, *J* = 7.1 Hz, 3 H), 0.21 (s, 9 H).

$^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 163.1 (qC, C=O), 162.7 (qC, C=O), 137.5 (qC), 135.9 (CH), 135.4 (CH), 135.2 (CH), 132.1 (qC), 129.3 (qC), 128.1 (qC), 124.1 (qC), 123.2 (CH), 102.4 (qC), 97.9 (qC), 61.2 ( $\text{CH}_2$ ), 53.3 (CH), 46.6 ( $\text{CH}_2$ ), 28.4 ( $\text{CH}_2$ ), 24.3 ( $\text{CH}_2$ ), 14.3 ( $\text{CH}_3$ ), -0.2 (3  $\text{CH}_3$ ).

HRMS (Dual ESI): calc  $m/z$  for  $\text{C}_{22}\text{H}_{25}\text{N}_3\text{O}_3\text{Si}$ : 407.1665 [M], 408.1738 [M+H] $^+$ ; found: 407.1674 [M], 408.1745 [M+H] $^+$ .

FT-IR ( $\text{cm}^{-1}$ ):  $\nu$  = 3100, 2958, 2898, 2158, 1715, 1636, 1600, 1543, 1494, 1368, 1249, 1178, 1159, 1112, 1042.

#### **Ethyl 7-ethynyl-9-oxo-11,12,13,13a-tetrahydro-9H-benzo[e]imidazo [5,1-c]pyrrolo[1,2-a][1,4]diazepine-1-carboxylate (34)<sup>18</sup>**

Prepared as described for compound **35** using TBAF (1.48 mmol, 386 mg, 1.48 mL), compound **31** (1.06 mmol, 430 mg) and THF (10 mL) at RT for 15 min; purified by flash chromatography ( $\text{CH}_2\text{Cl}_2$ /acetone, 9:1) to give **34** (348 mg, 98%) as a white solid; mp = 159-160  $^\circ\text{C}$ , lit.<sup>18</sup> mp not reported;  $R_f$  = 0.16 ( $\text{CH}_2\text{Cl}_2$ /acetone, 9:1).

$^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 8.26 (d,  $J$  = 1.9 Hz, 1 H), 7.88 (s, 1 H), 7.74 (dd,  $J$  = 1.9, 8.3 Hz, 1 H), 7.39 (d,  $J$  = 8.3 Hz, 1 H), 4.77 (d,  $J$  = 6.9 Hz, 1 H), 4.44 (q,  $J$  = 7.1 Hz, 2 H), 3.86 – 3.77 (m, 1 H), 3.64 – 3.50 (m, 2 H), 3.25 (s, 1 H), 2.39 – 2.16 (m, 3 H), 1.46 (t,  $J$  = 7.1 Hz, 3 H).

$^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 163.0 (C=O), 162.6 (C=O), 137.5 (qC), 135.9 (CH), 135.8 (CH), 135.4 (CH), 132.6 (qC), 129.6 (qC), 128.1 (qC), 123.3 (CH), 123.1 (qC), 81.2 (qC,  $\text{C}\equiv\text{CH}$ ), 80.2 ( $\text{C}\equiv\text{CH}$ ), 61.3 ( $\text{CH}_2$ ), 53.3 (CH), 46.7 ( $\text{CH}_2$ ), 28.5 ( $\text{CH}_2$ ), 24.4 ( $\text{CH}_2$ ), 14.4 ( $\text{CH}_3$ ).

HRMS (Dual ESI): calc  $m/z$  for  $\text{C}_{19}\text{H}_{17}\text{N}_3\text{O}_3$ : 335.1270 [M], 336.1343 [M+H] $^+$ ; found: 335.1271 [M], 336.1345 [M+H] $^+$ .

FT-IR ( $\text{cm}^{-1}$ ):  $\nu$  = 3292, 3212, 3077, 2923, 2852, 1709, 1637, 1601, 1541, 1438, 1365, 1249, 1179, 1112, 1044.

#### **4-Methyl-3,4-dihydro-1H-benzo[e][1,4]diazepine-2,5-dione (15)<sup>31</sup>**

Prepared as described for compound **16** using isatoic anhydride **12** (10 mmol, 1.63 g), sarcosine **13** (11 mmol, 0.98 g) and DMSO (10 mL), to furnish compound **15** (1.43 g, 75%) as a pale brown solid; mp = 244-245  $^\circ\text{C}$ , lit. mp = 243-246  $^\circ\text{C}$ ;<sup>31</sup>  $R_f$  = 0.13 (EtOAc/PE, 2/1).

$^1\text{H}$ -NMR (400 MHz, DMSO):  $\delta$  = 10.47 (s, 1 H, NH), 7.74 (dd,  $J$  = 1.4, 7.8 Hz, 1 H), 7.54 – 7.47 (m, 1 H), 7.25 – 7.19 (m, 1 H), 7.12 – 7.08 (m, 1 H), 3.84 (s, 2 H), 3.12 (s, 3 H).

$^{13}\text{C}$ -NMR (100 MHz, DMSO):  $\delta$  = 170.2 (qC), 167.0 (qC), 137.4 (qC), 132.4 (CH), 131.3 (CH), 126.6 (qC), 124.3 (CH), 121.1 (CH), 52.6 ( $\text{CH}_2$ ), 36.3 ( $\text{CH}_3$ ).

FT-IR ( $\text{cm}^{-1}$ ):  $\nu$  = 3204, 3150, 3053, 2980, 2889, 1693, 1630, 1579, 1477, 1373, 1243, 1148, 989, 697, 500.

#### **7-Bromo-4-methyl-3,4-dihydro-1H-benzo[e][1,4]diazepine-2,5-dione (17)<sup>33</sup>**

Prepared as described for compound **18**, but using compound **15** (7.36 mmol, 1.4 g), glacial acetic acid (15 mL), NaOAc (7.36 mmol, 0.6 g), bromine (8.83 mmol, 1.41 g, 0.45 mL) in 15 mL of glacial acetic acid, RT for 16 h; purified by flash chromatography (EtOAc/PE, 2:1) to give **17** (1.2 g, 60%) as a white solid; mp = 255-257  $^\circ\text{C}$ , lit. mp = 260-261  $^\circ\text{C}$ ;<sup>33</sup>  $R_f$  = 0.23 (EtOAc/PE, 2:1).

$^1\text{H}$ -NMR (400 MHz, DMSO):  $\delta$  = 10.57 (s, 1 H, NH), 7.82 (d,  $J$  = 2.4 Hz, 1 H), 7.69 (dd,  $J$  = 2.4, 8.6 Hz, 1 H), 7.05 (d,  $J$  = 8.6 Hz, 1 H), 3.88 (s, 2 H), 3.11 (s, 3 H).

$^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 170.0 (qC), 165.7 (qC), 136.8 (qC), 135.1 (CH), 133.4 (CH), 128.4 (qC), 123.4 (CH), 116.1 (qC), 52.4 ( $\text{CH}_2$ ), 36.4 ( $\text{CH}_3$ ).

FT-IR ( $\text{cm}^{-1}$ ):  $\nu$  = 3202, 3143, 3054, 2950, 1693, 1615, 1594, 1474, 1422, 1361, 1251, 1150, 988, 821, 769.

#### **7-Ethynyl-4-methyl-3,4-dihydro-1H-benzo[e][1,4]diazepine-2,5-dione (32)<sup>37</sup>**

Method A: Prepared as described for compound **35** using TBAF (1.86 mmol, 485 mg, 1.86 mL), trimethylsilyl precursor **27** (1.33 mmol, 380 mg) and THF (10 mL), RT for 15 min; purified by flash chromatography ( $\text{CH}_2\text{Cl}_2$ /MeOH, 100:1 to 100:2) to give the desired compound **32** (240 mg, 84%) as a white solid; mp = 263-264  $^\circ\text{C}$ , lit.<sup>37</sup> mp not available;  $R_f$  = 0.15 ( $\text{CH}_2\text{Cl}_2$ /acetone, 9:1).

Method B: Prepared as described for compound **35** using TBAF (0.86 mmol, 223 mg, 0.86 mL), triethylsilyl precursor **28** (0.61 mmol, 201 mg) and THF (7 mL), RT for 10 min; purified by flash chromatography (CH<sub>2</sub>Cl<sub>2</sub>/acetone, 9:1 to 7:3) to give the desired compound **32** (123 mg, 94%) as above.

<sup>1</sup>H-NMR (400 MHz, DMSO): δ = 10.63 (s, 1 H, NH), 7.78 (d, *J* = 2.0 Hz, 1 H), 7.59 (dd, *J* = 2.0, 8.4 Hz, 1 H), 7.10 (d, *J* = 8.4 Hz, 1 H), 4.23 (s, 1 H, C≡CH), 3.88 (s, 2 H), 3.11 (s, 3 H).

<sup>13</sup>C-NMR (100 MHz, DMSO): δ = 170.0 (qC, C=O), 166.1 (qC, C=O), 137.7 (qC), 135.2 (CH), 134.8 (CH), 136.7 (qC), 121.6 (CH), 117.4 (qC), 82.8 (qC, C≡CH), 81.4 (C≡CH), 52.5 (CH<sub>2</sub>), 36.3 (CH<sub>3</sub>).

HRMS (Dual ESI): calc *m/z* for C<sub>12</sub>H<sub>10</sub>N<sub>2</sub>O<sub>2</sub>: 214.0742 [M], 215.0815 [M+H]<sup>+</sup>; found: 214.0742 [M], 215.0814 [M+H]<sup>+</sup>.

FT-IR (cm<sup>-1</sup>): ν = 3249, 3192, 3127, 3010, 2929, 1669, 1613, 1603, 1488, 1375, 1196, 995, 842, 778, 696.

#### **Ethyl 8-bromo-5-methyl-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate (21)<sup>35</sup>**

To a solution of **17** (2.86 mmol, 770 mg) in anhydrous THF (50 mL), cooled to 0 °C and under N<sub>2</sub>, *t*-BuOK (3.15 mmol, 353 mg) was added and the mixture was stirred at 0 °C for 20 min. After cooling the reaction mixture to -35 °C, diethyl chlorophosphate **19** (3.72 mmol, 0.54 mL) was added dropwise and the resulting mixture was brought to 0 °C and stirred for 30 min. The mixture was cooled to -78 °C and ethyl isocynoacetate **20** (3.15 mmol, 0.35 mL), followed by *t*-BuOK (3.15 mmol, 353 mg) were added. The reaction was allowed to warm to room temperature and stirred for 4 h. After this time, saturated aqueous NaHCO<sub>3</sub> (100 mL) was added and the aqueous layer was extracted with ethyl acetate (3 × 75 mL). The organic phase was dried over MgSO<sub>4</sub>, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (EtOAc/PE, 2:1) to give **21** (0.42 g, 40%) as a white solid; mp = 187-188 °C, lit. mp = 192-193 °C;<sup>35</sup> *R*<sub>f</sub> = 0.18 (EtOAc/PE, 3:1). 150 mg of starting material **17** were recovered.

<sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ = 8.23 (d, *J* = 2.1 Hz, 1 H), 7.90 (s, 1 H), 7.77 (dd, *J* = 2.1, 8.5 Hz, 1 H), 7.33 (d, *J* = 8.5 Hz, 1 H), 5.23 (app s, 1 H), 4.53 – 4.30 (m, 3 H), 3.26 (s, 3 H), 1.47 (t, *J* = 7.1 Hz, 3 H).

<sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ = 165.1 (qC), 162.8 (qC), 135.7 (CH), 135.5 (CH), 135.3 (qC), 134.8 (CH), 130.9 (qC), 130.6 (qC), 128.9 (qC), 123.4 (CH), 122.5 (qC), 61.1 (CH<sub>2</sub>), 42.2 (CH<sub>2</sub>), 36.0 (CH<sub>3</sub>), 14.3 (CH<sub>3</sub>).

FT-IR (cm<sup>-1</sup>): ν = 3101, 2979, 2900, 1721, 1628, 1593, 1494, 1343, 1251, 1154, 1111, 938, 829, 658, 517.

#### **Ethyl 5-methyl-6-oxo-8-((trimethylsilyl)ethynyl)-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate (25)<sup>36</sup>**

Prepared as described for compound **29** using bromide **21** (1.7 mmol, 620 mg), Et<sub>3</sub>N (25 mL), CH<sub>3</sub>CN (20 mL), PdCl<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub> (0.17 mmol, 120 mg, 10 mol %) and trimethylsilyl alkyne **23** (3.4 mmol, 334 mg, 0.47 mL); purified by flash chromatography (CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH, 100:1) to give **25** (583 mg, 90%) as a pale brown oily solid; mp = 166-168 °C, lit.<sup>36</sup> mp not reported; *R*<sub>f</sub> = 0.1 (CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH, 100:1).

<sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ = 8.06 (d, *J* = 1.9 Hz, 1 H), 7.84 (s, 1 H), 7.60 (dd, *J* = 1.9, 8.3 Hz, 1 H), 7.33 (d, *J* = 8.3 Hz, 1 H), 5.11 (app s, 1 H), 4.50 – 4.20 (m, 3 H), 3.16 (s, 3 H), 1.36 (t, *J* = 7.1 Hz, 3 H), 0.19 (s, 9 H).

<sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ = 165.6 (qC, C=O), 162.8 (qC, C=O), 136.1 (CH), 135.4 (CH), 135.2 (qC), 134.8 (CH), 131.2 (qC), 129.0 (qC), 128.7 (qC), 123.9 (qC), 121.8 (CH), 102.3 (qC), 97.7 (qC), 60.9 (CH<sub>2</sub>), 42.2 (CH<sub>2</sub>), 35.8 (CH<sub>3</sub>), 14.3 (CH<sub>3</sub>), -0.2 (3 CH<sub>3</sub>).

HRMS (Dual ESI): calc *m/z* for C<sub>20</sub>H<sub>23</sub>N<sub>3</sub>O<sub>3</sub>Si: 381.1509 [M], 382.1581 [M+H]<sup>+</sup>; found: 381.1517 [M], 382.1586 [M+H]<sup>+</sup>.

FT-IR (cm<sup>-1</sup>): ν = 3128, 2980, 2955, 2893, 2163, 1719, 1650, 1604, 1560, 1504, 1251, 1166, 1118, 1077, 965.

#### **Ethyl 8-ethynyl-5-methyl-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate (33)<sup>36</sup>**

Method A: Prepared as described for compound **35** using TBAF (1.79 mmol, 467 mg, 1.79 mL), trimethylsilyl precursor **25** (1.28 mmol, 487 mg) and THF (10 mL), RT for 15 min; purified by flash chromatography

(CH<sub>2</sub>Cl<sub>2</sub>/acetone, 9:1) to give **33** (364 mg, 92%) as a white solid; mp = 200-202 °C, lit. mp = 206-207 °C;<sup>36</sup> *R*<sub>f</sub> = 0.2 (CH<sub>2</sub>Cl<sub>2</sub>/acetone, 9:1).

Method B: Prepared as described for compound **35** using TBAF (0.35 mmol, 91 mg, 0.35 mL), triethylsilyl precursor **26** (0.25 mmol, 105 mg) and THF (4 mL), RT for 10 min; purified by flash chromatography (CH<sub>2</sub>Cl<sub>2</sub>/acetone, 9:1) to give **33** (62 mg, 81%) as described above.

<sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ = 8.20 (d, *J* = 1.6 Hz, 1 H), 7.95 (s, 1 H), 7.74 (app d, *J* = 6.8 Hz, 1 H), 7.43 (d, *J* = 8.1 Hz, 1 H), 5.22 (app s, 1 H), 4.54 – 4.29 (m, 3 H), 3.27 (s, 3 H), 3.25 (s, 1 H), 1.47 (t, *J* = 7.1 Hz, 3 H).

<sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ = 165.6 (qC), 162.8 (qC), 136.5 (CH), 135.8 (CH), 135.4 (qC), 134.9 (CH), 131.7 (qC), 129.3 (qC), 128.8 (qC), 123.1 (qC), 122.0 (CH), 81.2 (qC), 80.1 (CH), 61.1 (CH<sub>2</sub>), 42.3 (CH<sub>2</sub>), 35.9 (CH<sub>3</sub>), 14.3 (CH<sub>3</sub>).

HRMS (Dual ESI): calc *m/z* for C<sub>17</sub>H<sub>15</sub>N<sub>3</sub>O<sub>3</sub>: 309.1113 [M], 310.1186 [M+H]<sup>+</sup>; found: 309.1114 [M], 310.1187 [M+H]<sup>+</sup>.

FT-IR (cm<sup>-1</sup>): ν = 3235, 3112, 2979, 2913, 2850, 1698, 1636, 1602, 1495, 1393, 1251, 1189, 1111, 1059, 924.

## 2. Biological Methodology

### Materials

The BV2 mouse brain microglial cells, cell line ICLCATL03001, was purchased from Interlab Cell Line Collection - Banca Biologica e Cell Factory, Italy. The cells were cultured in Gibco™ Roswell Park Memorial Institute (RPMI) 1640 Medium (Life Technologies). The RPMI medium was supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Sigma), 1 mM sodium pyruvate (Sigma), and streptomycin (100 units/mL) – penicillin G (100 mg/mL) (Sigma) to obtain the complete RPMI medium. The cells were stimulated with LPS from *Salmonella typhimurium*, S-form TLRpure™ Sterile Solution (Innaxon Biosciences). The cells were maintained at 37 °C in 5% CO<sub>2</sub> humidified atmosphere (autoclaved ddH<sub>2</sub>O with 1% AQUAGUARD-1) in NuAire CO<sub>2</sub> incubator (TripleRed). The cell culture procedures were carried out in a NuAire Class II Biological Safety Cabinet (TripleRed). Cell confluency was assessed using an EVOS phase contrast microscope (Life Technologies). Cells were washed with DPBS (Life Technologies) and detached from the T75 flask using TrypLE™ Express Enzyme (TrypleX, Life Technologies) or a 0.05% trypsin/ 0.02% EDTA solution. Cells were counted using a haemocytometer with a Neubauer chamber. For the 96-well plates, to add the LPS solution into the well, an Eppendorf Repeater Xstream Pipette with a 0.2 mL Eppendorf Combitip was used. The T75 tissue culture flask with vent (75 cm<sup>2</sup>), cell culture plates (24-, 48- and 96-well plates), serological pipettes (2, 5, 10 and 25 mL), centrifuge tubes (5, 15 and 50 mL), Eppendorf tubes (0.5 and 1.5 mL), pipette tips (10, 200 and 1000 μL), and pipetting reservoirs were purchased from Sarstedt. For pipetting, Eppendorf Research® Plus adjustable-volume pipettes (0.1-2.5 μL, 0.5-10 μL, 2-20 μL, 10-100 μL, 20-200 μL, 100-1000 μL), Gilson PIPETMAN L multi 8 channel (20-200 μL) and HTL Discovery Comfort pipettes (0.5-10 μL, 2-20 μL, 20-200 μL, 100-1000 μL, multi 8 channel pipette 20-200 μL) were used. The equipment was sanitized using 70% (v/v) ethanol in ddH<sub>2</sub>O. Before disposal, cells were treated with 1% Virkon solution. The cell viability assay was performed using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT, Invitrogen) and *N*-methyl dibenzopyrazine methyl sulfate (PMS, Sigma), and the absorbance was read using a microplate reader (Infinite® F50, Tecan) at 450 nm. The NO production was determined using the Griess Reagent System purchased from Promega or prepared (0.1 g of sulfanilamide in 10 mL of 5% H<sub>3</sub>PO<sub>4</sub>, and 10 mg of *N*-1-naphthylethylenediamine dihydrochloride in 10 mL of water). The absorbance for Griess Assay was read at 540 nm in the microplate reader.

Each assay was performed at least three times and with each sample in duplicate.

### **Cell culture**

The appropriate vial with BV2 cells (in DMSO) was removed from the liquid N<sub>2</sub> tank and was quickly thawed in a 37 °C water bath until sides were melted and the centre still frozen. The BV2 cells were quickly transferred into pre-warmed complete RPMI medium (37 °C, 10 mL) in a centrifuge tube (50 mL). The tube was centrifuged at 1200 rpm for 5 min. The supernatant was aspirated, and the cell pellet was dissolved in another 10 mL of complete RPMI medium and transferred in a T75 flask. The flask was incubated at 37 °C in 5% CO<sub>2</sub> humidified atmosphere. After 48 h the supernatant from the flask was aspirated, 10 mL of pre-warmed complete RPMI medium was added, and the cells incubated again. When the cells reached 80% confluency (~24 h), they were subcultured. Since BV2 microglia are semi-adherent cells, both attached and suspended cells must be subcultured.

The medium from the T75 flask (~10 mL) was transferred into a 50 mL centrifuge tube and the attached cells were washed with PBS (5 mL). The PBS was aspirated, and 2.5 mL of 0.05% trypsin/ 0.02% EDTA solution or TrypleX was added to detach the cells. After incubating the mixture for 1-2 min at 37 °C in 5% CO<sub>2</sub>, the flask was gently tapped, the cells were checked under a microscope, and 8 mL of complete RPMI medium were added to inactivate trypsin or TrypleX. The mixture was transferred into the same centrifuge tube as the suspended cells, and the tube was centrifuged at 1200 rpm for 5 min. The supernatant was aspirated, the tube was flicked to break the cell pellet, and 10 mL of complete RPMI was added to dissolve the cell pellet. To create a new passage, 1 mL of cell solution was transferred into a new T75 flask, and 9 mL of complete RPMI medium was added. The cells were grown and maintained at 37 °C in 5% CO<sub>2</sub> humidified atmosphere until they reached 80% confluency, then, as needed, the medium was changed or the cells subcultured again.

To seed out the cells in the required well plates, cells were first counted using the haemocytometer, then diluted to the required concentration of  $2 \times 10^5$  cells/mL with complete RPMI medium and seeded. The cells were grown at 37 °C in 5% CO<sub>2</sub> humidified atmosphere until 80% confluency, and then used in different experiments.

### **Determination of cell viability by XTT Assay**

The BV2 cells were seeded out in a 96-well plate (200 µL in each well) at a concentration of  $2 \times 10^5$  cells/mL and were grown at 37 °C in 5% CO<sub>2</sub> humidified atmosphere until 80% confluency (~20 h). The medium was changed to serum free RPMI, and the cells were incubated for at least 2 hours. Subsequently the BV2 cells were treated with the synthesized compounds (20 µM final concentration, 0.4 µL of a 10 mM solution in 100% DMSO) in duplicates and maintained for 30 min at 37 °C in 5% CO<sub>2</sub>. Cells were stimulated with LPS (100 ng/mL final concentration into the well, 2 µL of a 10 µg/mL or 0.2 µL of a 100 µg/mL solution in sterile PBS) and incubated for 24 h. Each 96-well plate had a negative control well (NC), a negative control well with 0.4 µL DMSO, and an LPS-stimulated control well. 100 µL of cell culture medium was carefully removed from each well, centrifuged at 2500 rpm for 5 min at 4 °C and used for determination of NO production.

XTT Assay was conducted according to XTT Cell Viability Assay Protocol by Thermo Fisher Scientific. 5 mg of XTT were dissolved in 5 mL of warm serum free RPMI. 3 mg of PMS were dissolved in 1 mL of PBS to prepare a 10 mM PMS solution. Right before labelling the cells, to the 5 mL XTT solution, 12.5 µL of PMS solution were added to obtain an XTT/PMS solution. 25 µL of XTT/PMS solution were added to each well (96-well plate, 100 µL cell culture medium), and the mixture was incubated for 2 h at 37 °C in 5% CO<sub>2</sub>. After, the absorbance was read using the microplate reader at 450 nm.

XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, is a tetrazolium salt that is reduced to an orange coloured formazan derivative under the action of cellular enzymes of a living cell.<sup>38</sup> The reaction takes place at cell surface, and the sensitivity is enhanced when an electron carrier such as PMS (5-methylphenazin-5-ium methyl sulfate) is used. Formation of the bright orange soluble formazan product allows the absorbance reading and direct determination of cell viability.

#### ***Determination of NO production by Griess Assay***

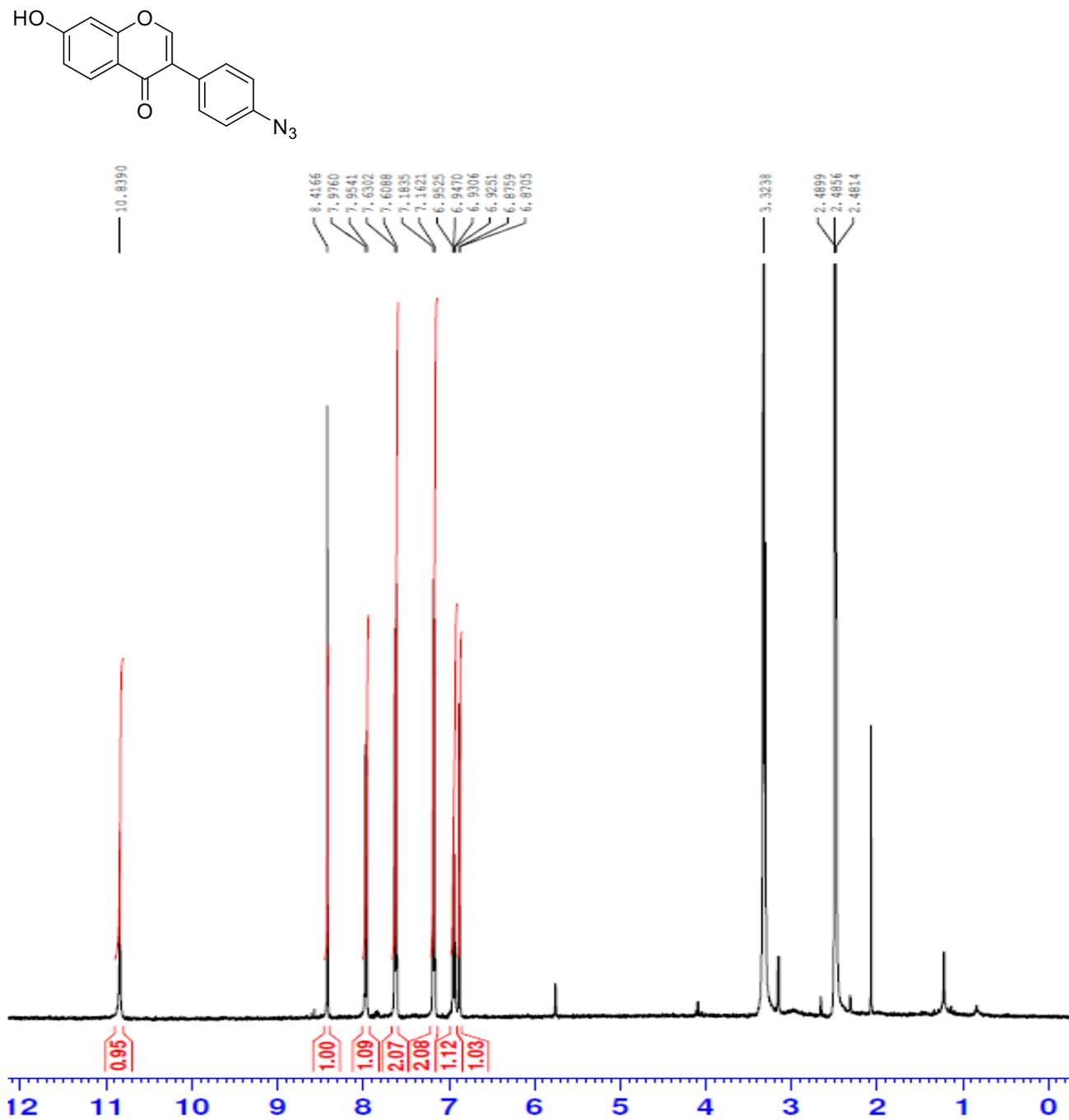
The 100  $\mu\text{L}$  of BV2 microglia cells culture medium collected from the 96-well plates before performing the XTT Assay were centrifuged and used to determine the NO production by Griess Assay. If no cell culture medium was available, the BV2 cells were seeded out, grown and treated as reported for the XTT Assay. After, the collected cell supernatant was centrifuged at 2500 rpm for 5 min at 4 °C.

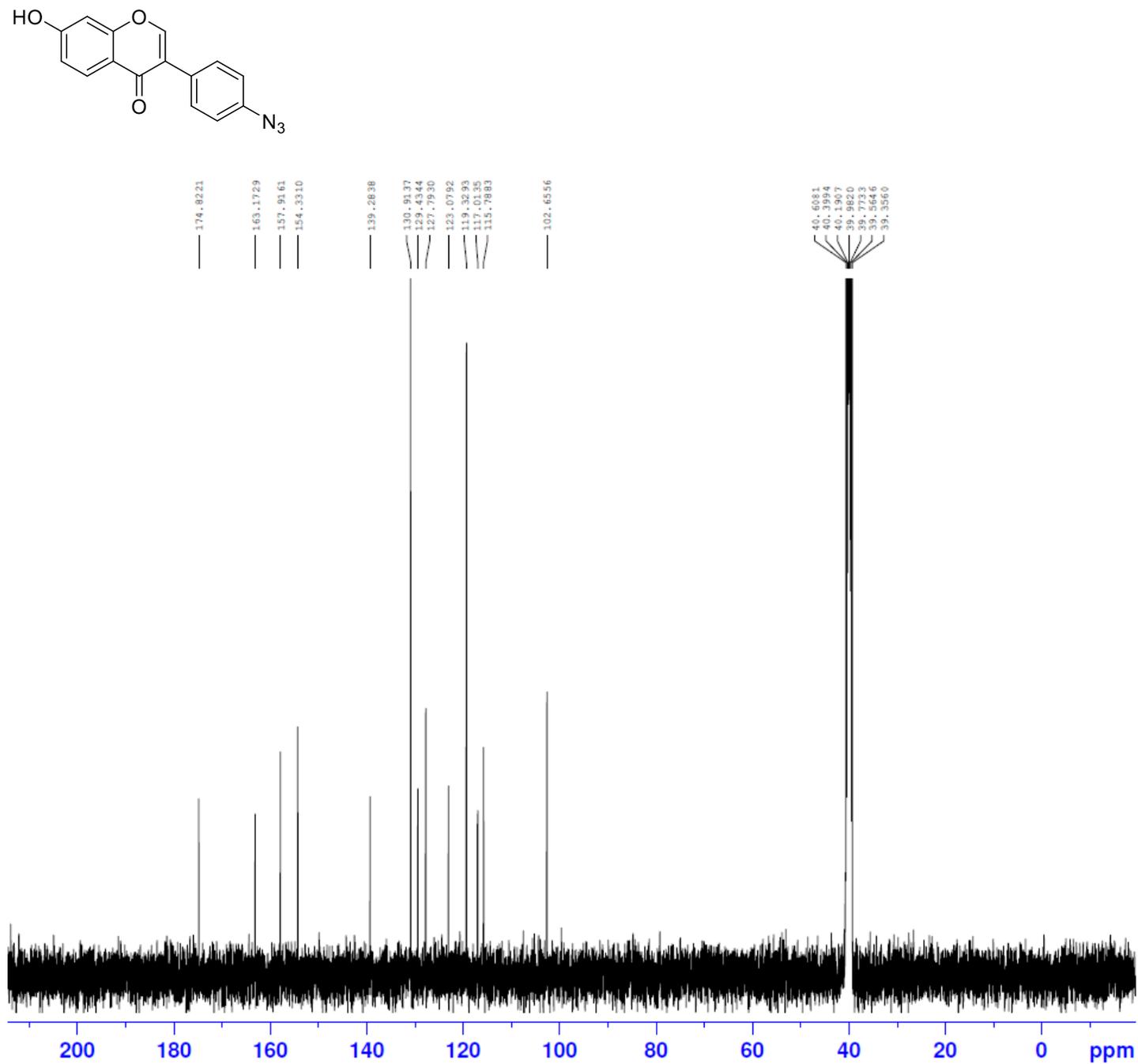
Griess Assay was conducted according to the supplier's protocol Griess Assay System by Promega. 50  $\mu\text{L}$  of the corresponding centrifuged cell culture medium were dispensed into each well of a 96-well plate. Each well was treated with 50  $\mu\text{L}$  of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) and the plate was incubated in the dark at room temperature for 10 min. Subsequently, 50  $\mu\text{L}$  of NED solution (0.1% *N*-1-naphthylethylenediamine dihydrochloride in water) was added, and the plate was incubated again in the dark for 10 min. After, the absorbance was read at 540 nm.

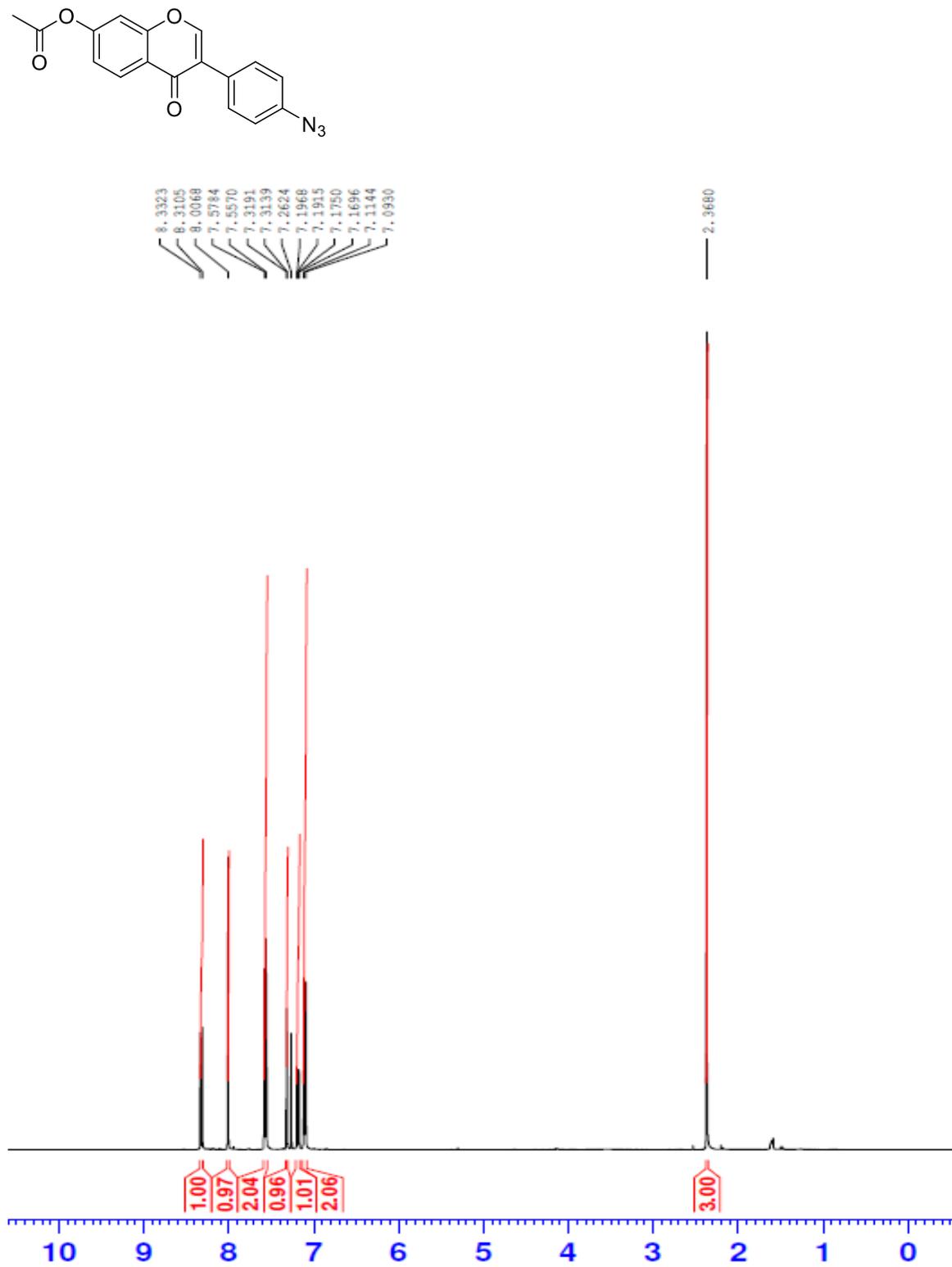
Nitric oxide (NO) was indirectly measured through nitrite  $\text{NO}_2^-$ , a stable product of NO oxidation in aqueous media. The Griess assay detects  $\text{NO}_2^-$  *via* a diazotization reaction that gives a purple coloured compound quantified spectrophotometrically.<sup>43</sup>

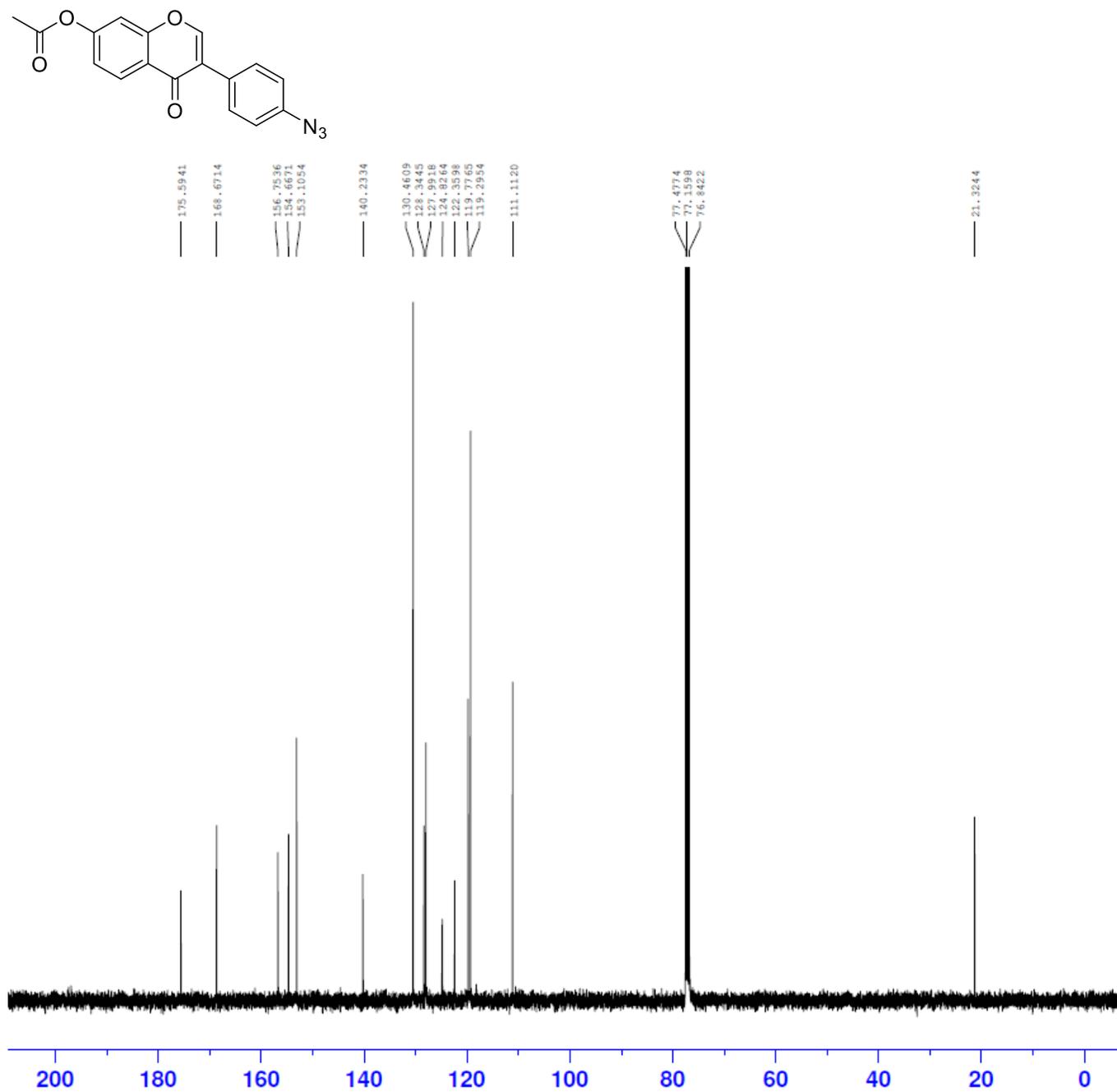
### 3. NMR Spectra for Previously Unreported Compounds

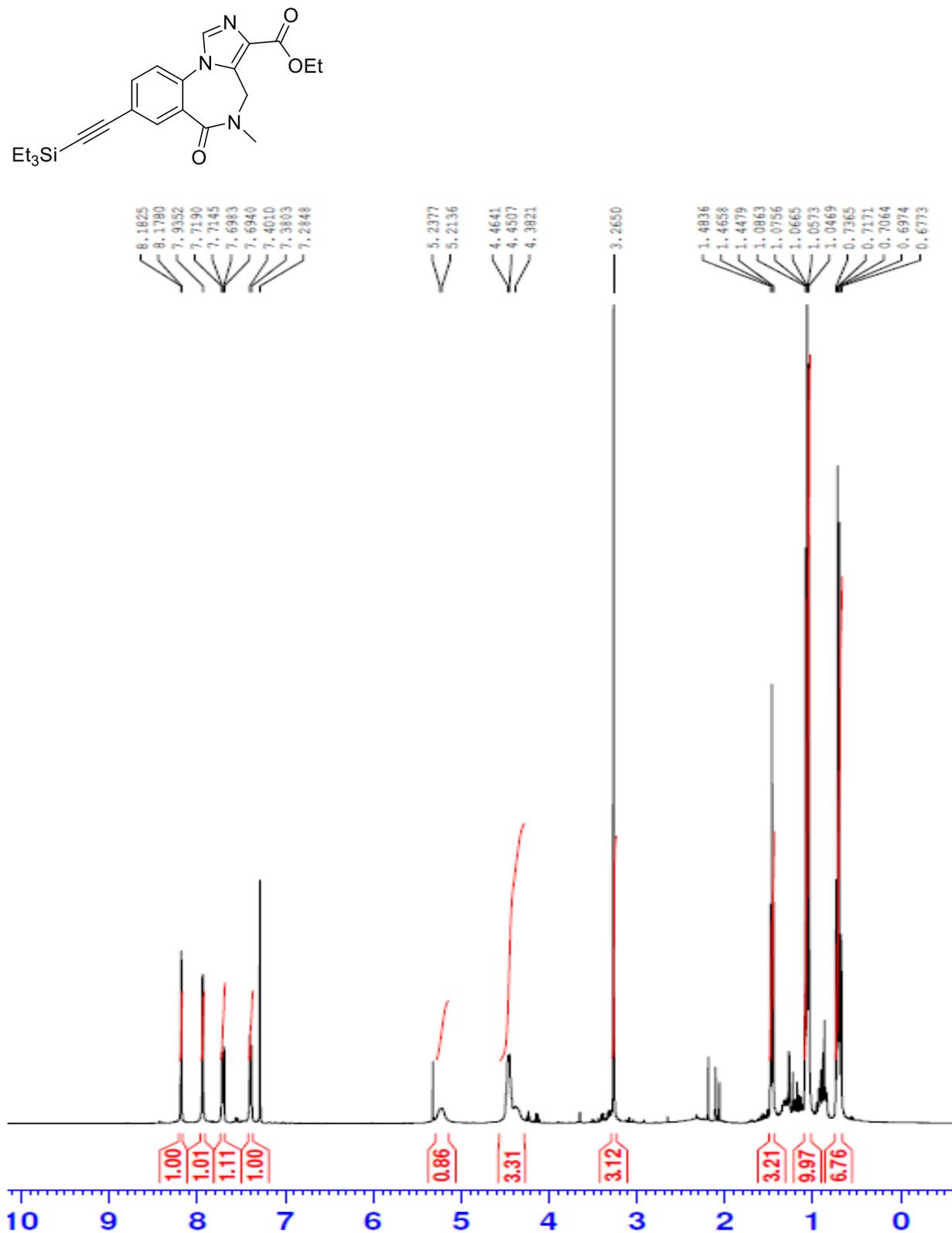
Compound **10**  $^1\text{H}$  NMR Spectrum (DMSO)

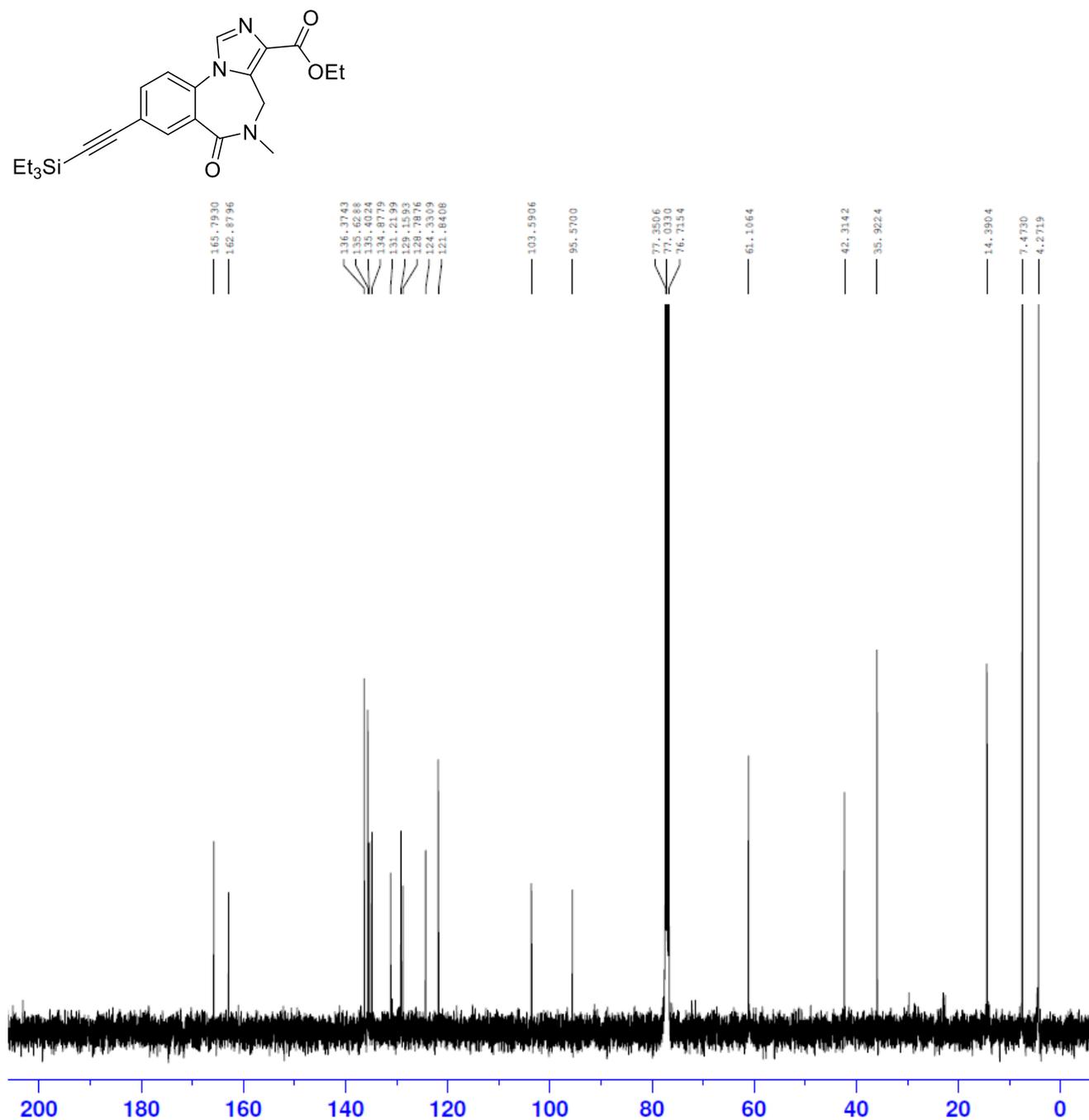


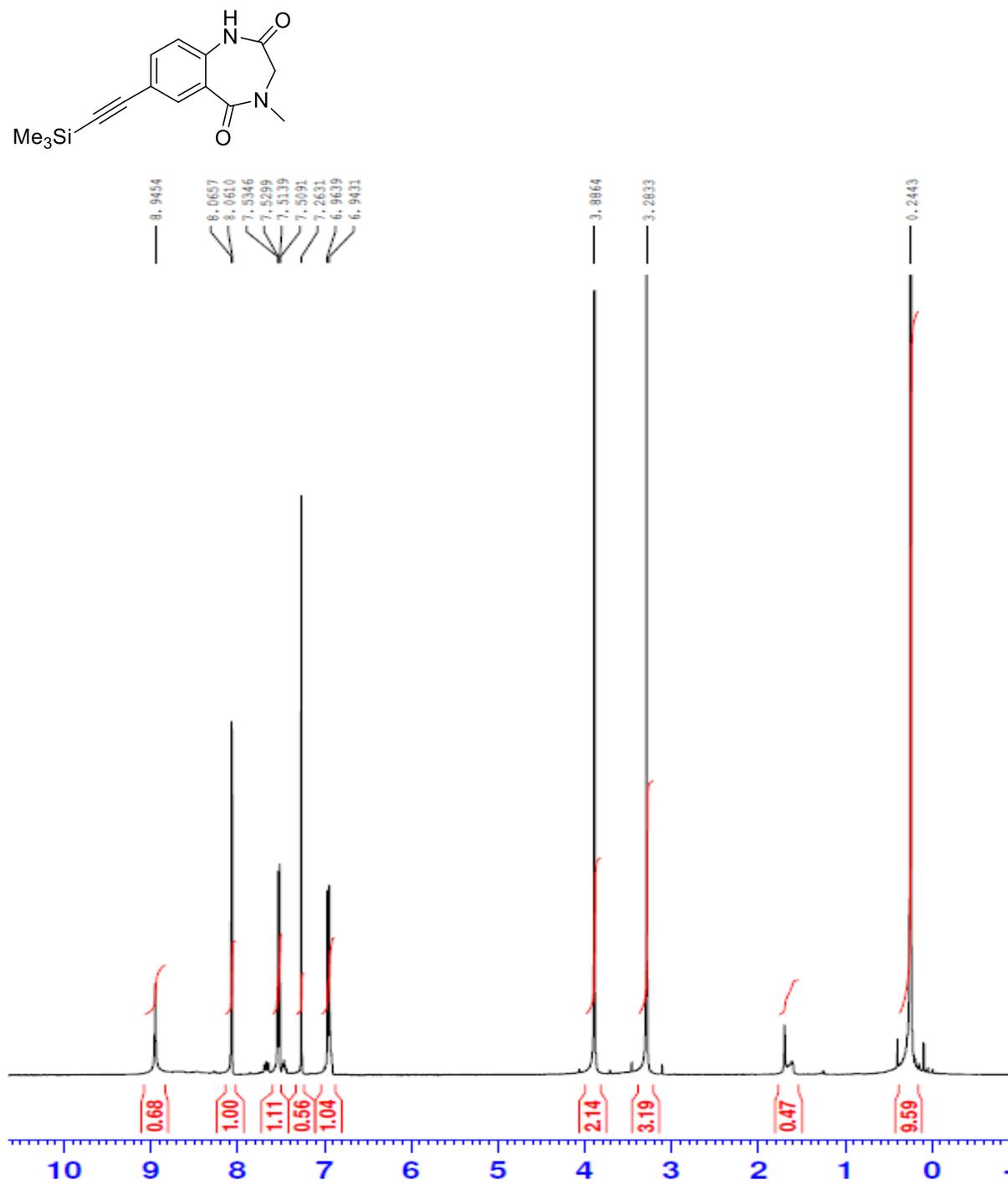
Compound **10**  $^{13}\text{C}$  NMR Spectrum (DMSO)

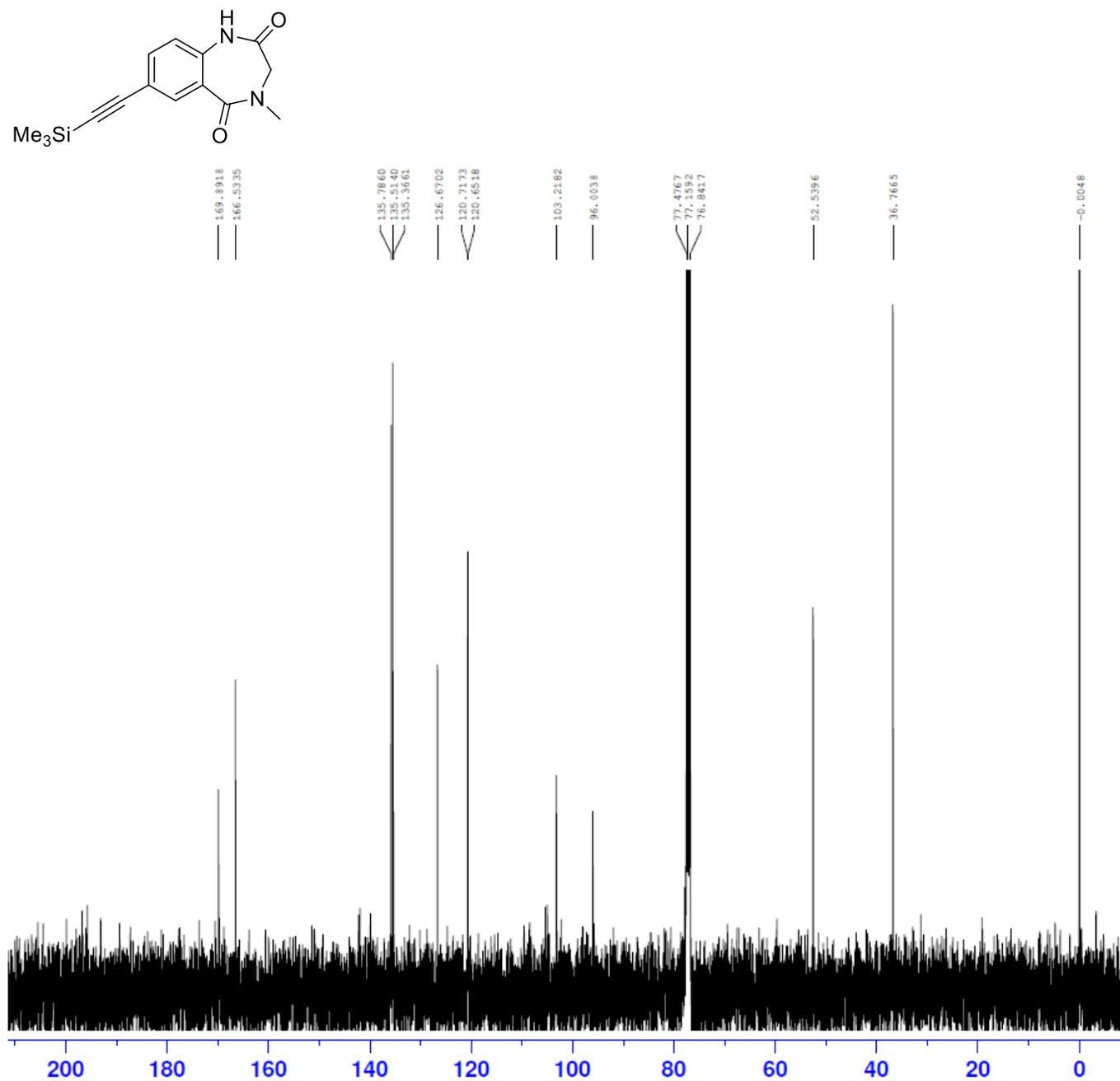
Compound **11**  $^1\text{H}$  NMR Spectrum ( $\text{CDCl}_3$ )

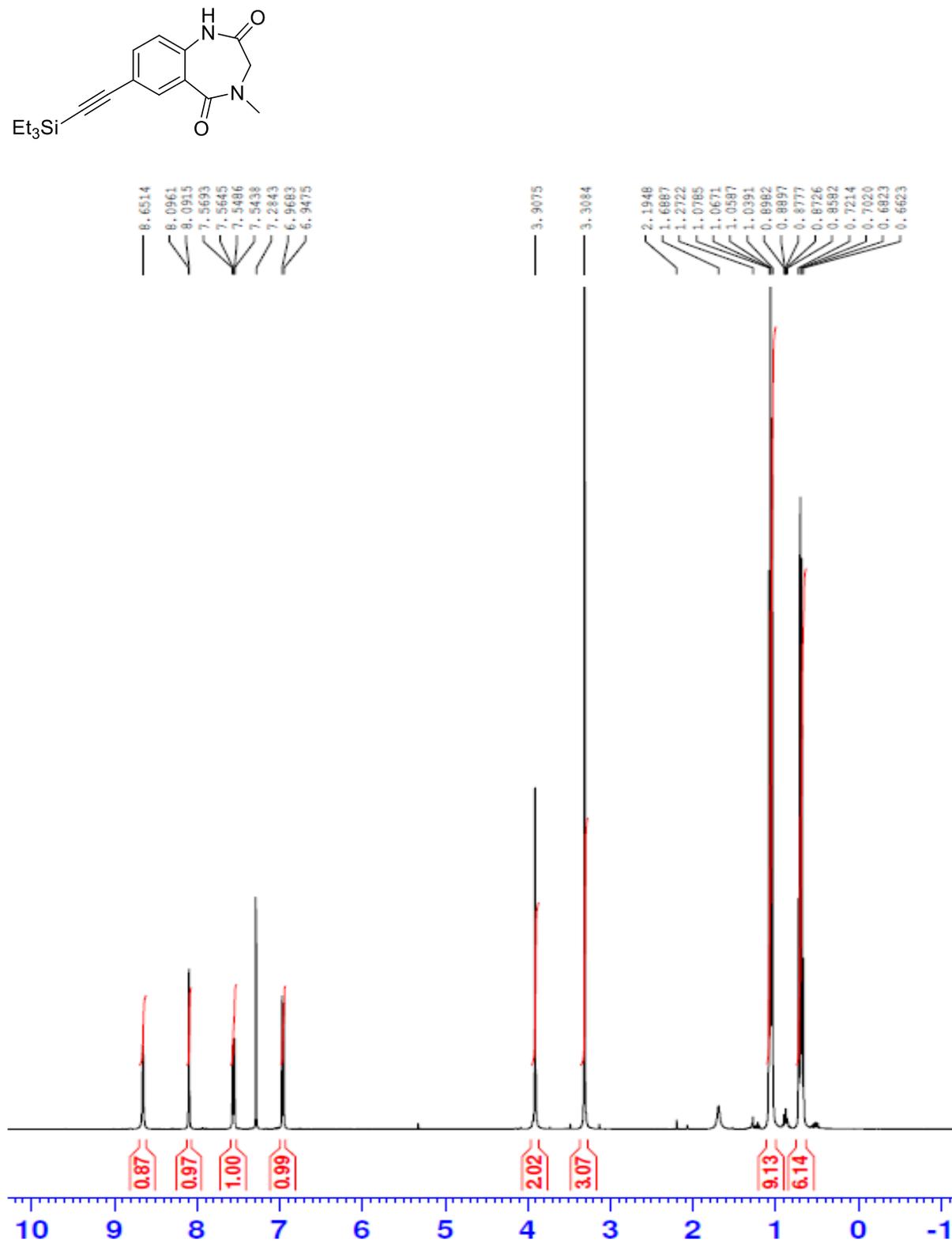
Compound **11**  $^{13}\text{C}$  NMR Spectrum ( $\text{CDCl}_3$ )

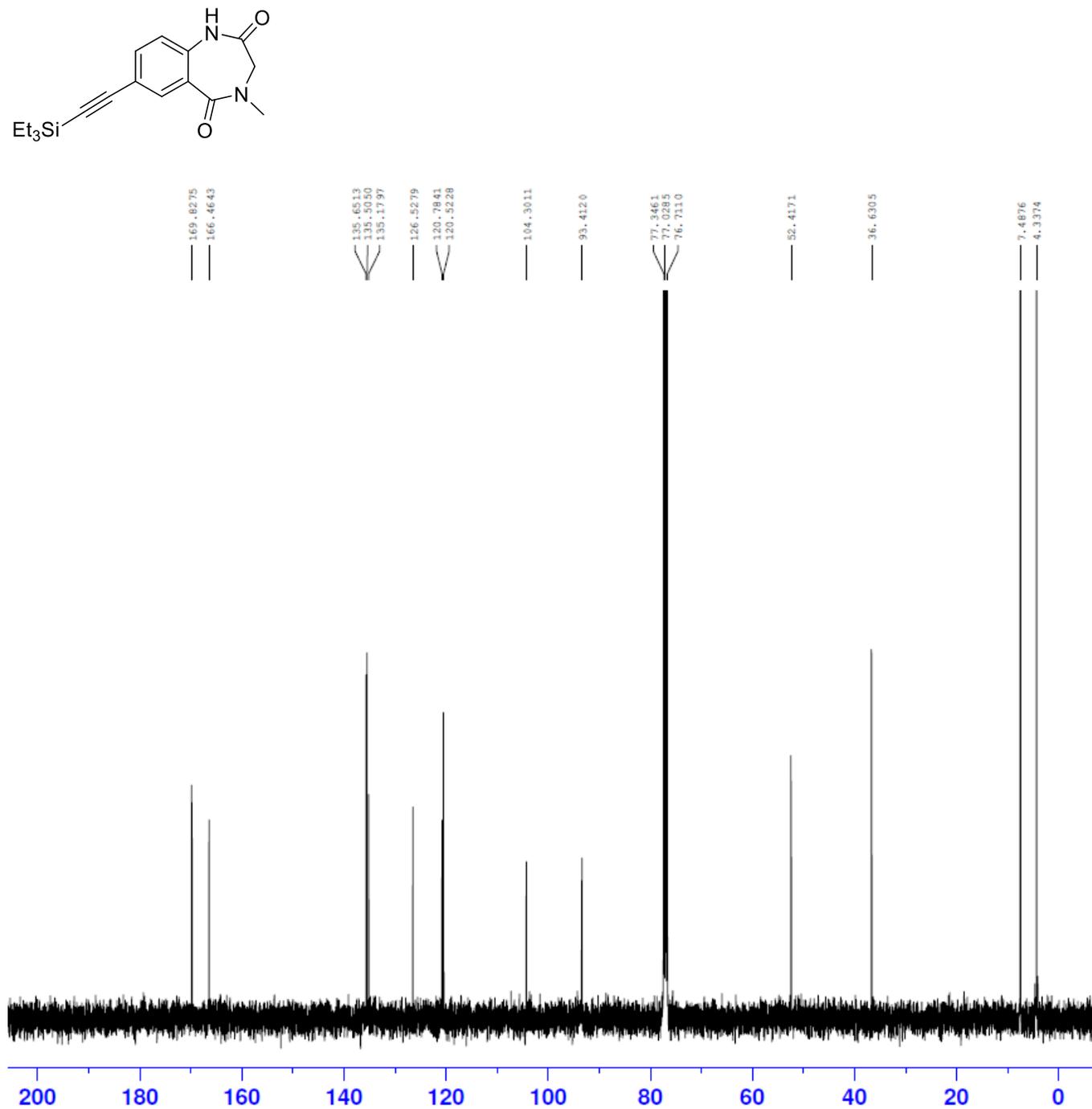
Compound **26**  $^1\text{H}$  NMR Spectrum

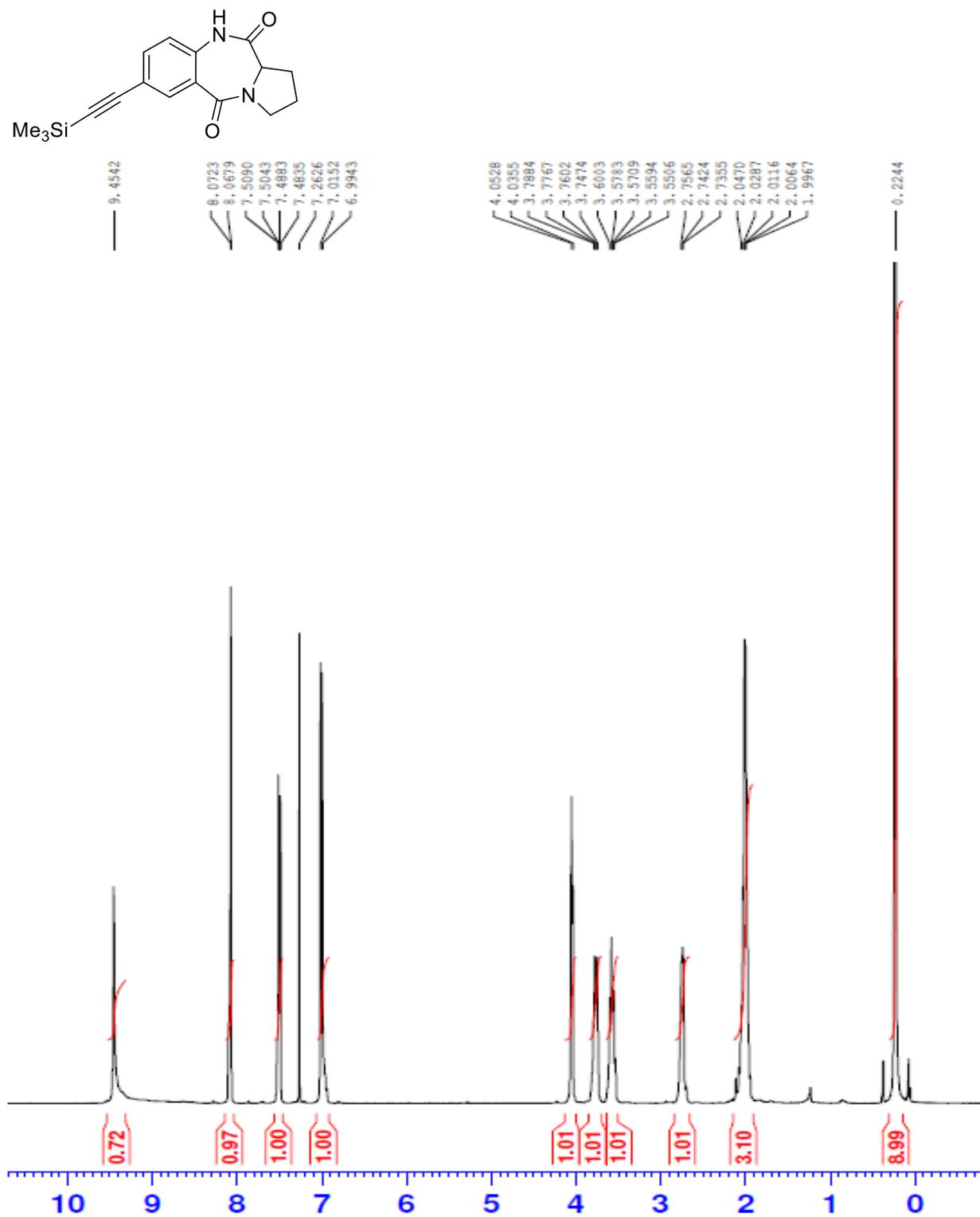
Compound **26**  $^{13}\text{C}$  NMR Spectrum ( $\text{CDCl}_3$ )

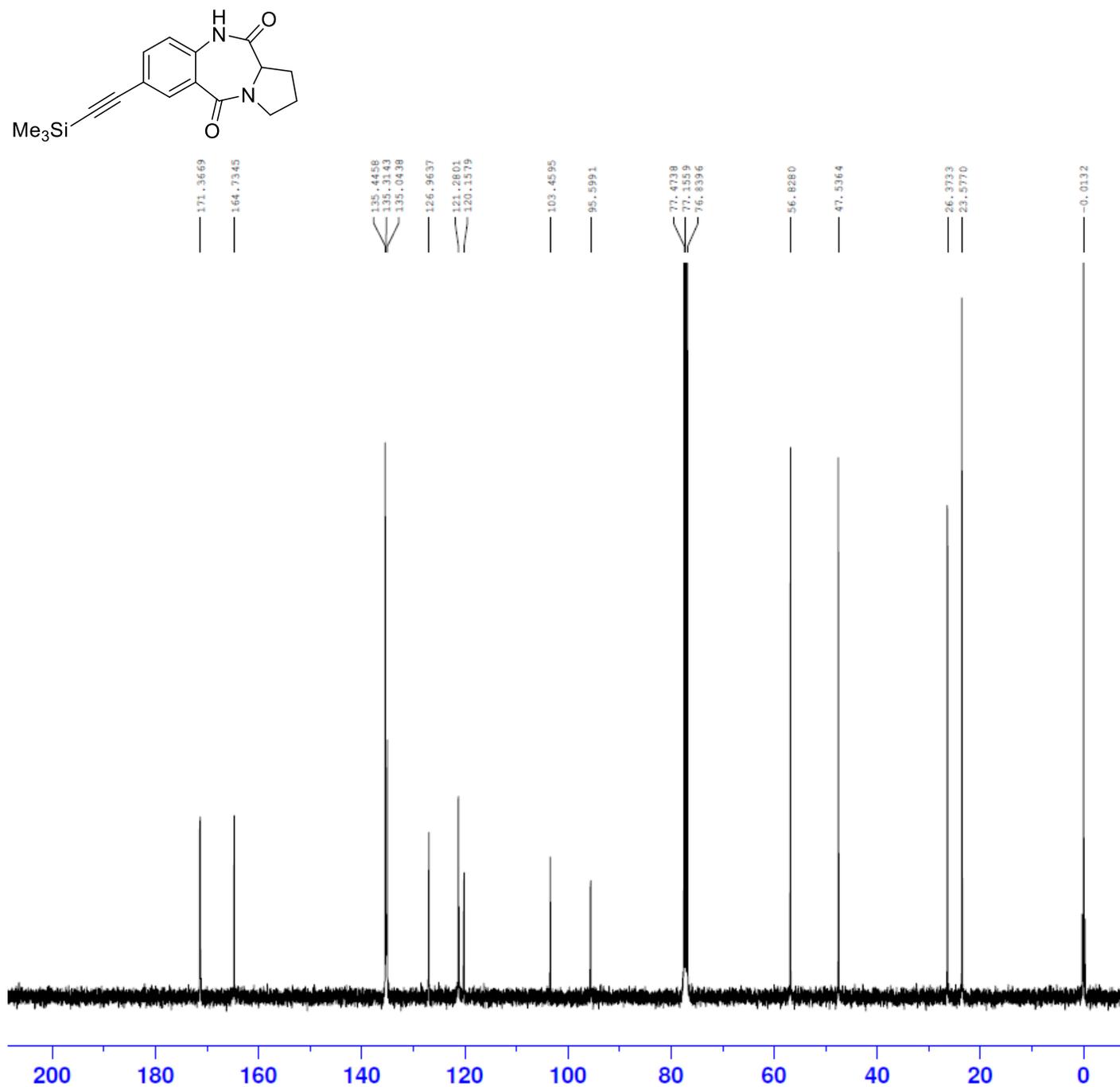
Compound **27**  $^1\text{H}$  NMR Spectrum ( $\text{CDCl}_3$ )

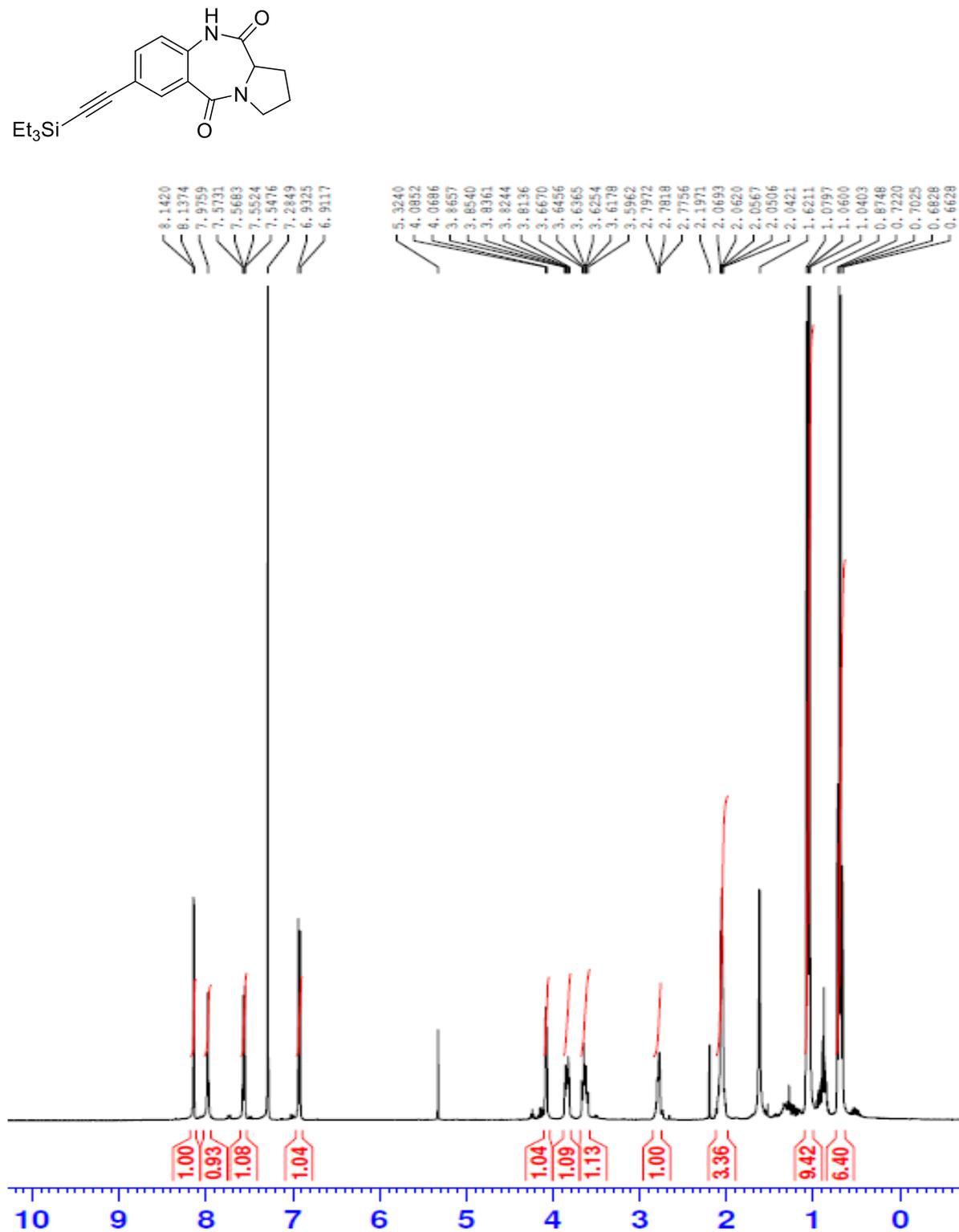
Compound **27**  $^{13}\text{C}$  NMR Spectrum ( $\text{CDCl}_3$ )

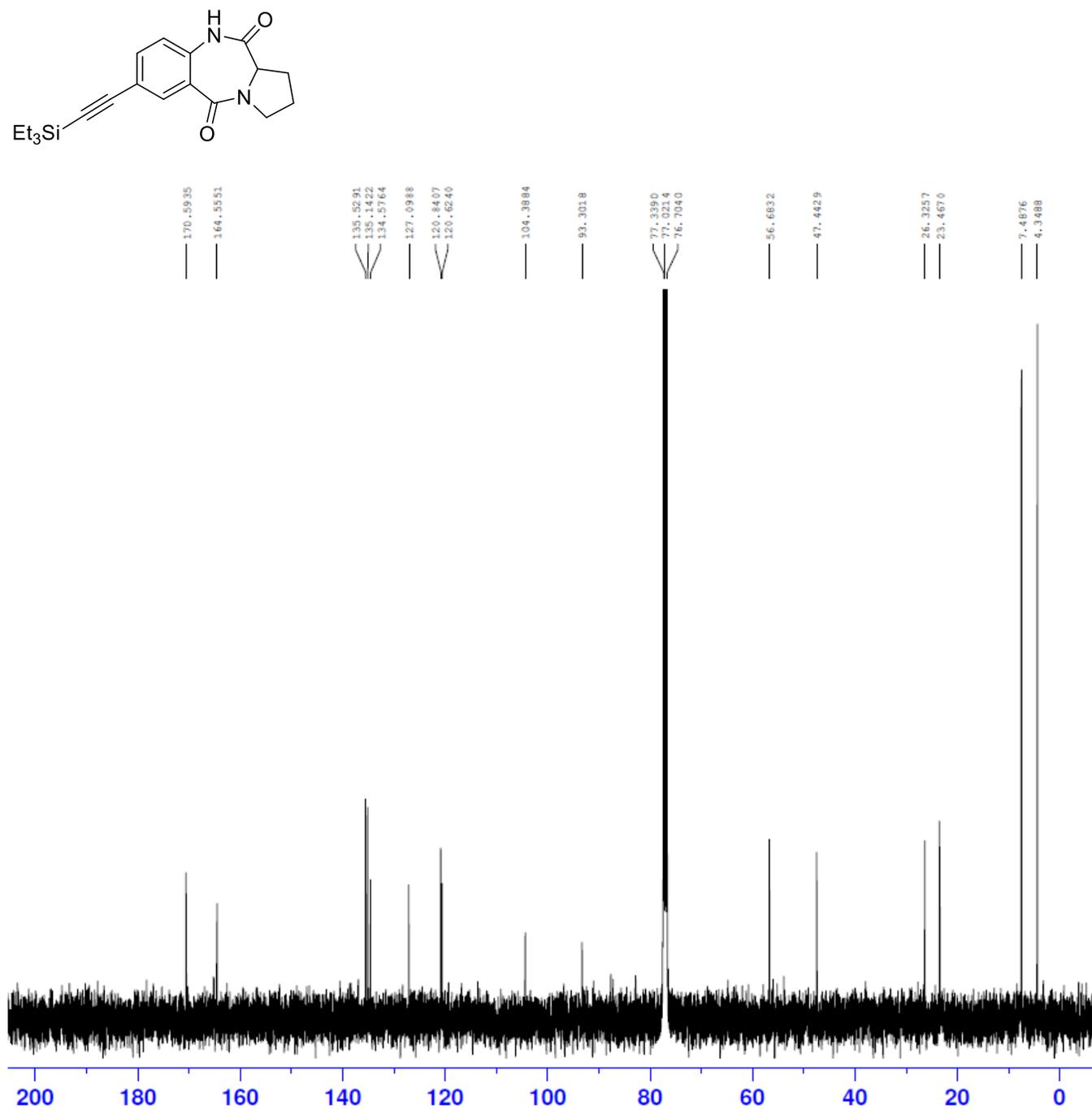
Compound **28**  $^1\text{H}$  NMR Spectrum ( $\text{CDCl}_3$ )

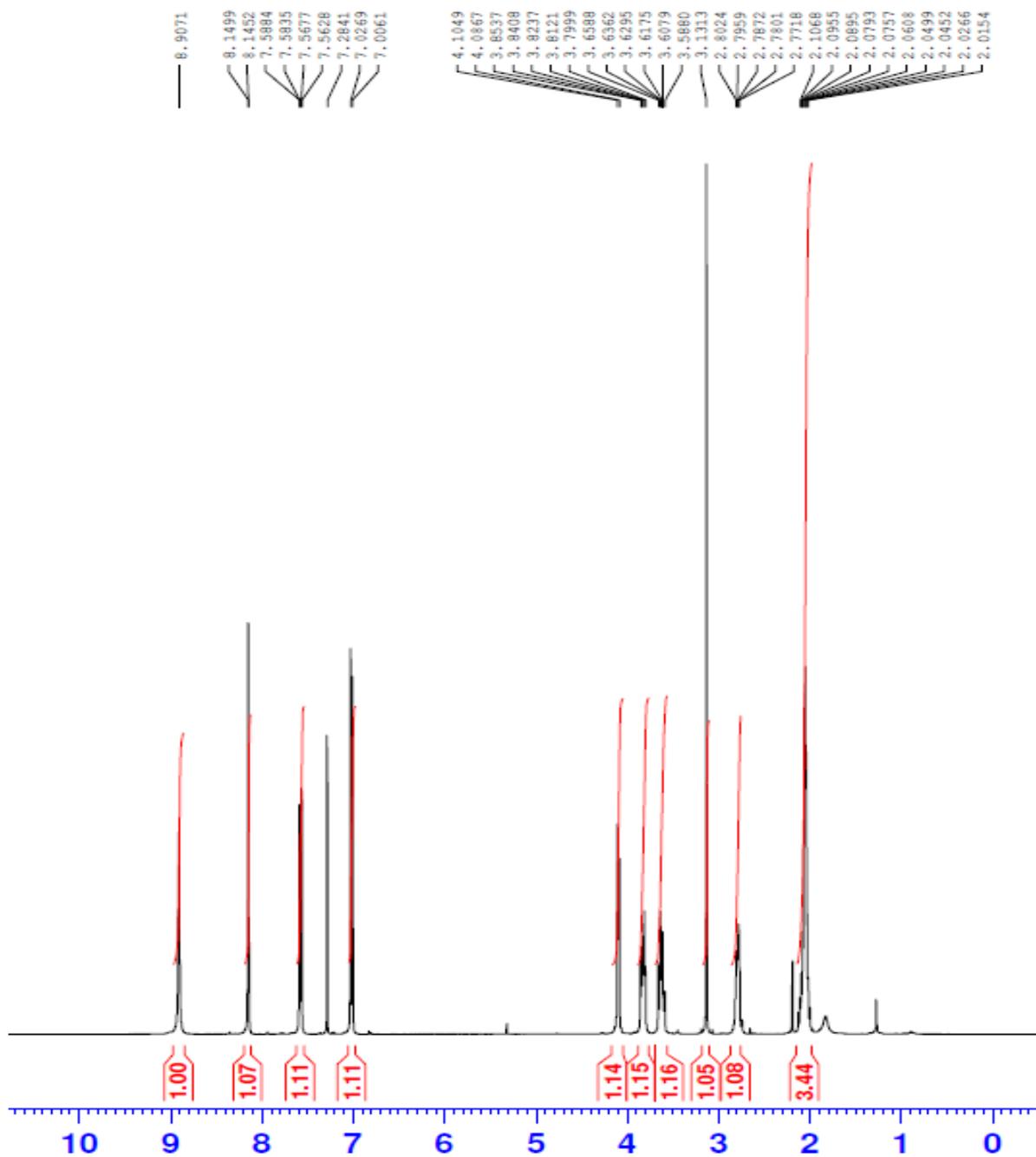
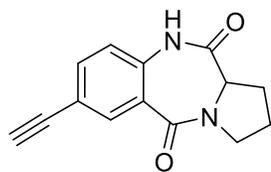
Compound **28**  $^{13}\text{C}$  NMR Spectrum ( $\text{CDCl}_3$ )

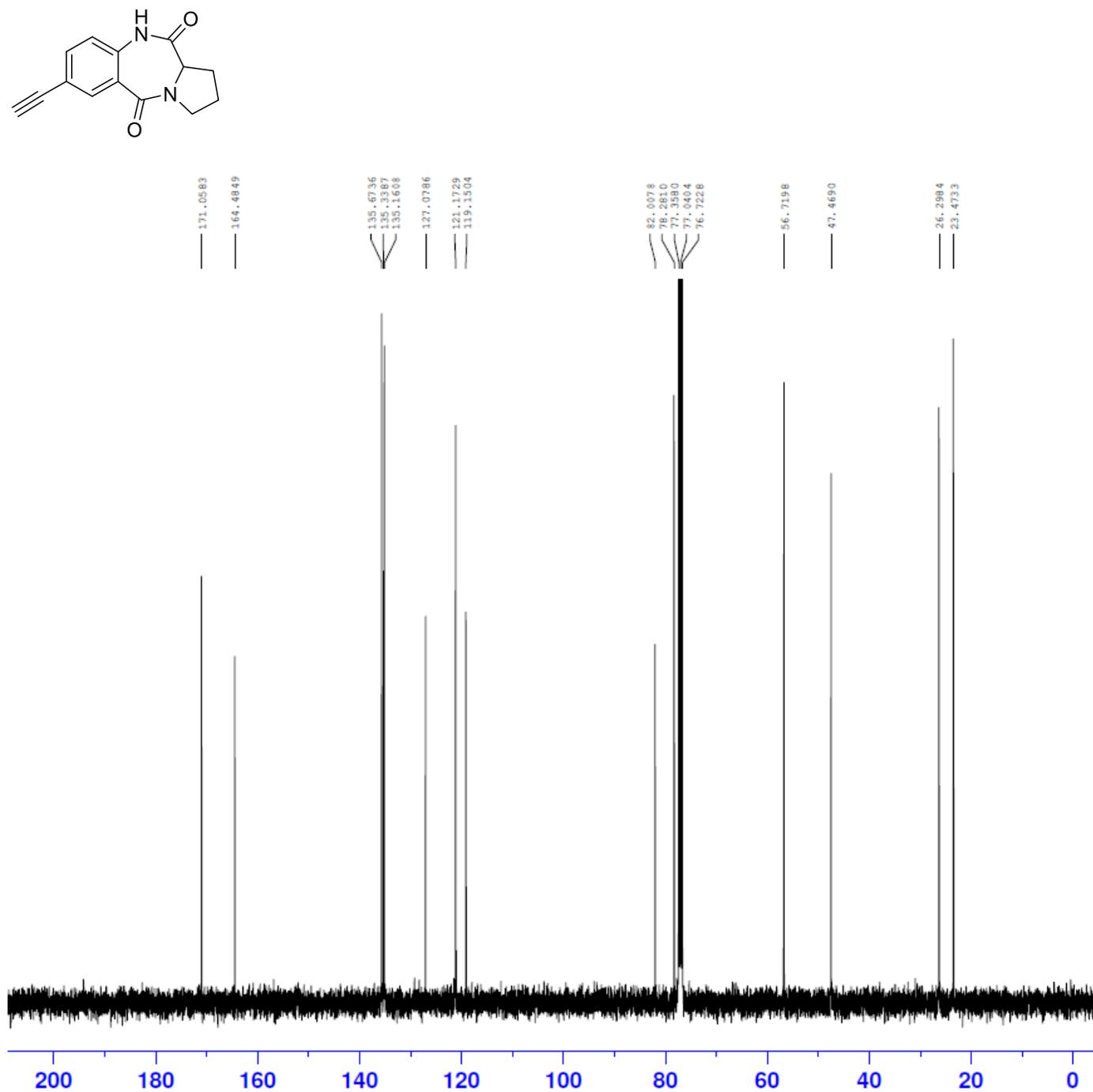
Compound **29**  $^1\text{H}$  NMR Spectrum ( $\text{CDCl}_3$ )

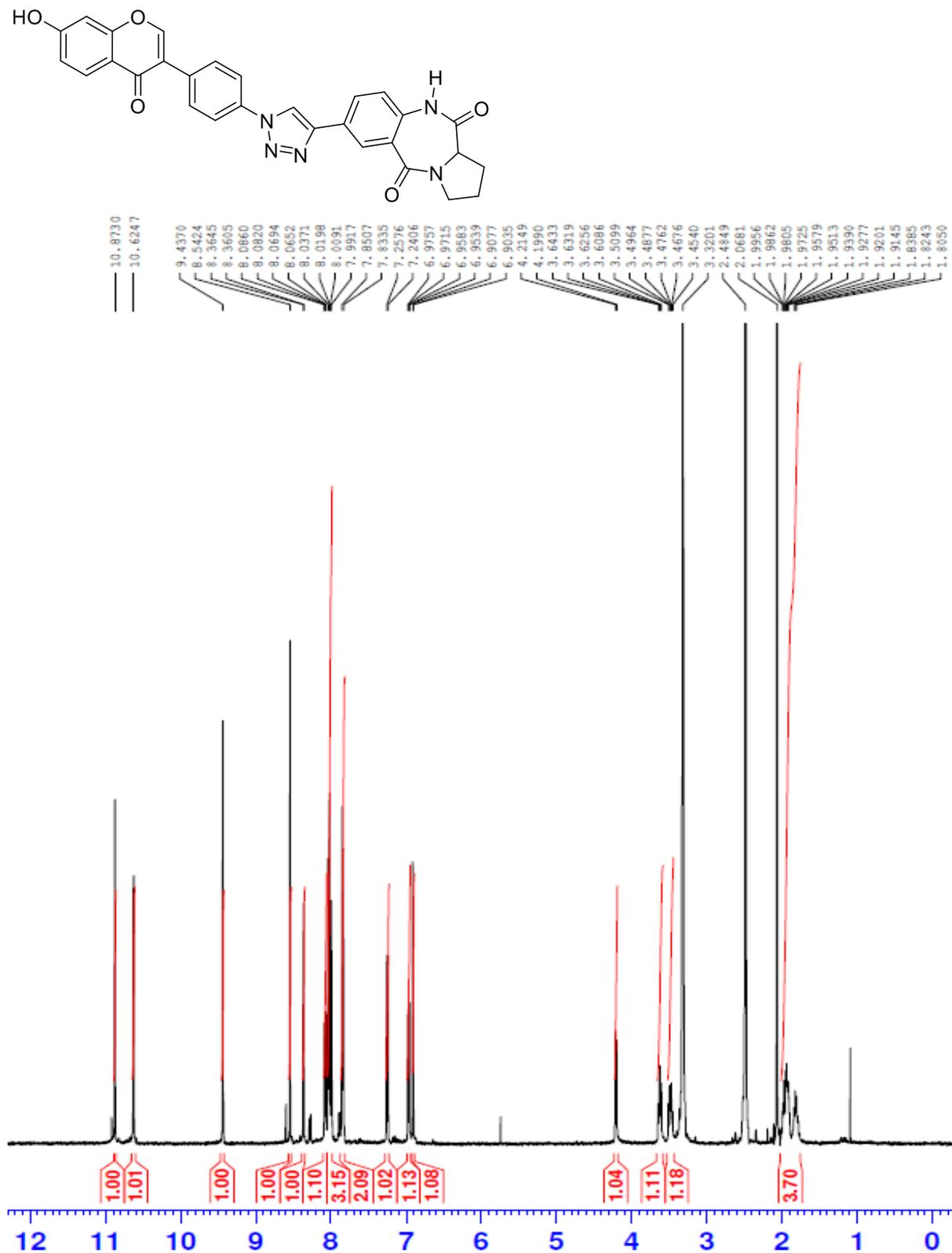
Compound **29**  $^{13}\text{C}$  NMR Spectrum ( $\text{CDCl}_3$ )

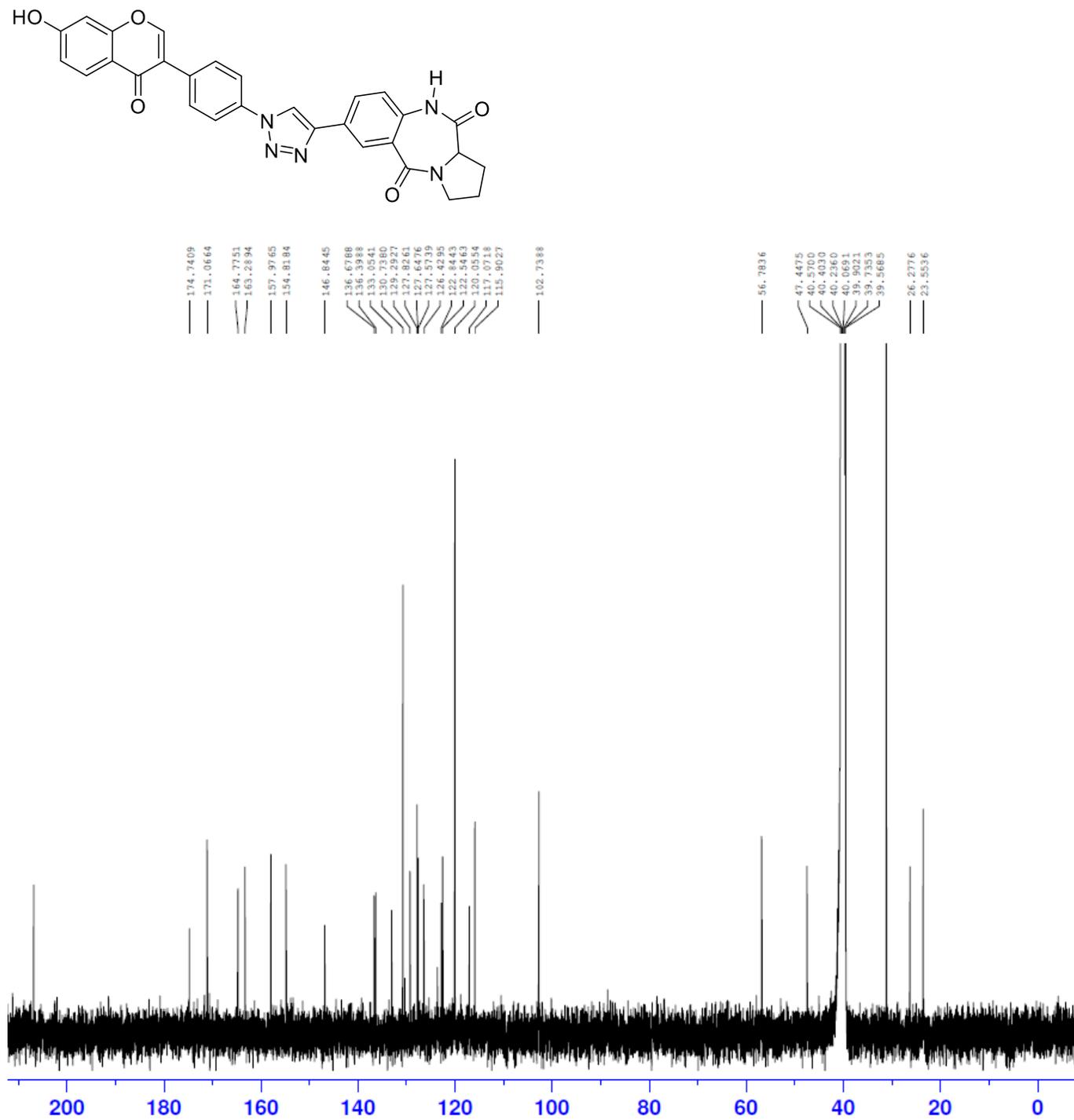
Compound **30**  $^1\text{H}$  NMR Spectrum ( $\text{CDCl}_3$ )

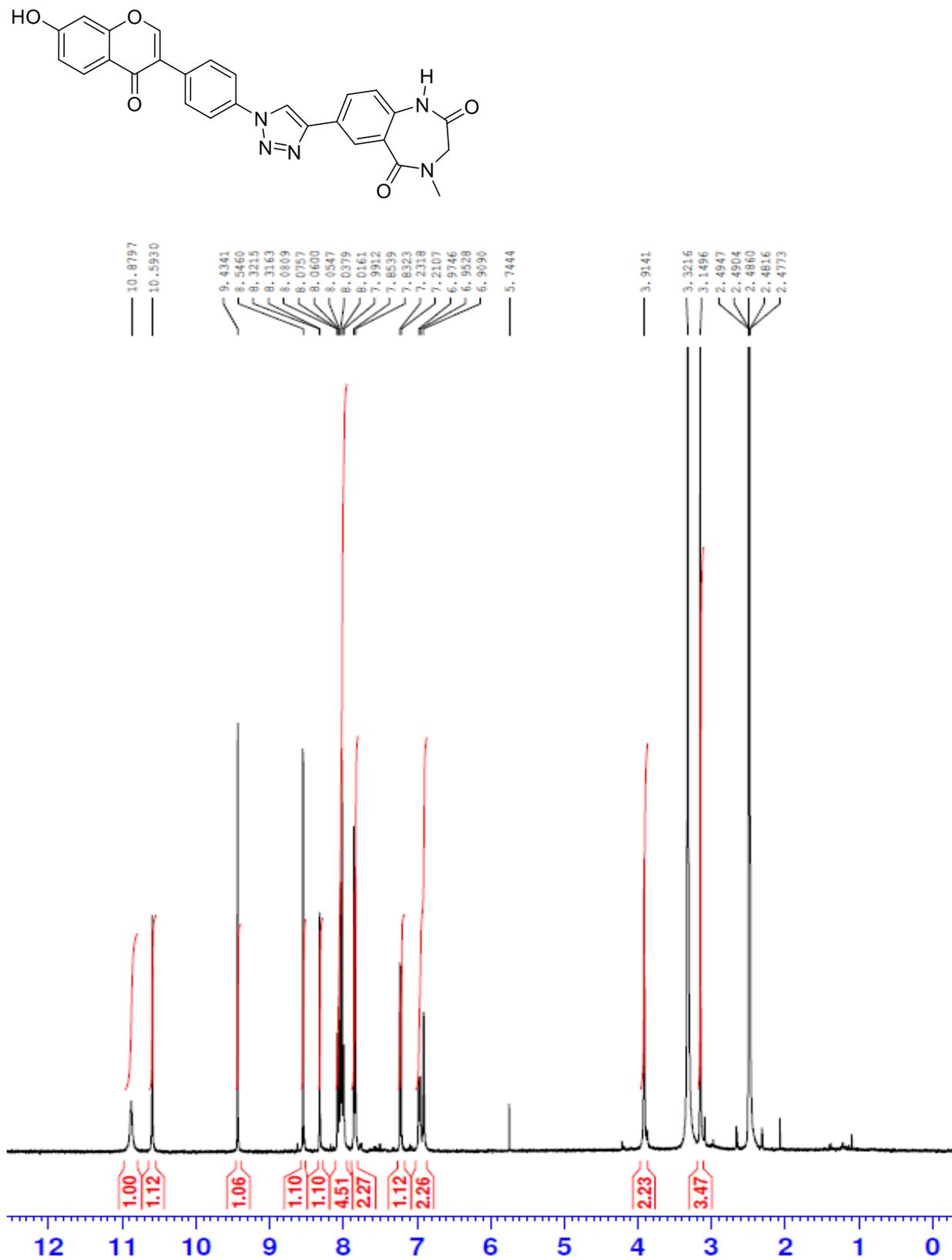
Compound **30**  $^{13}\text{C}$  NMR Spectrum ( $\text{CDCl}_3$ )

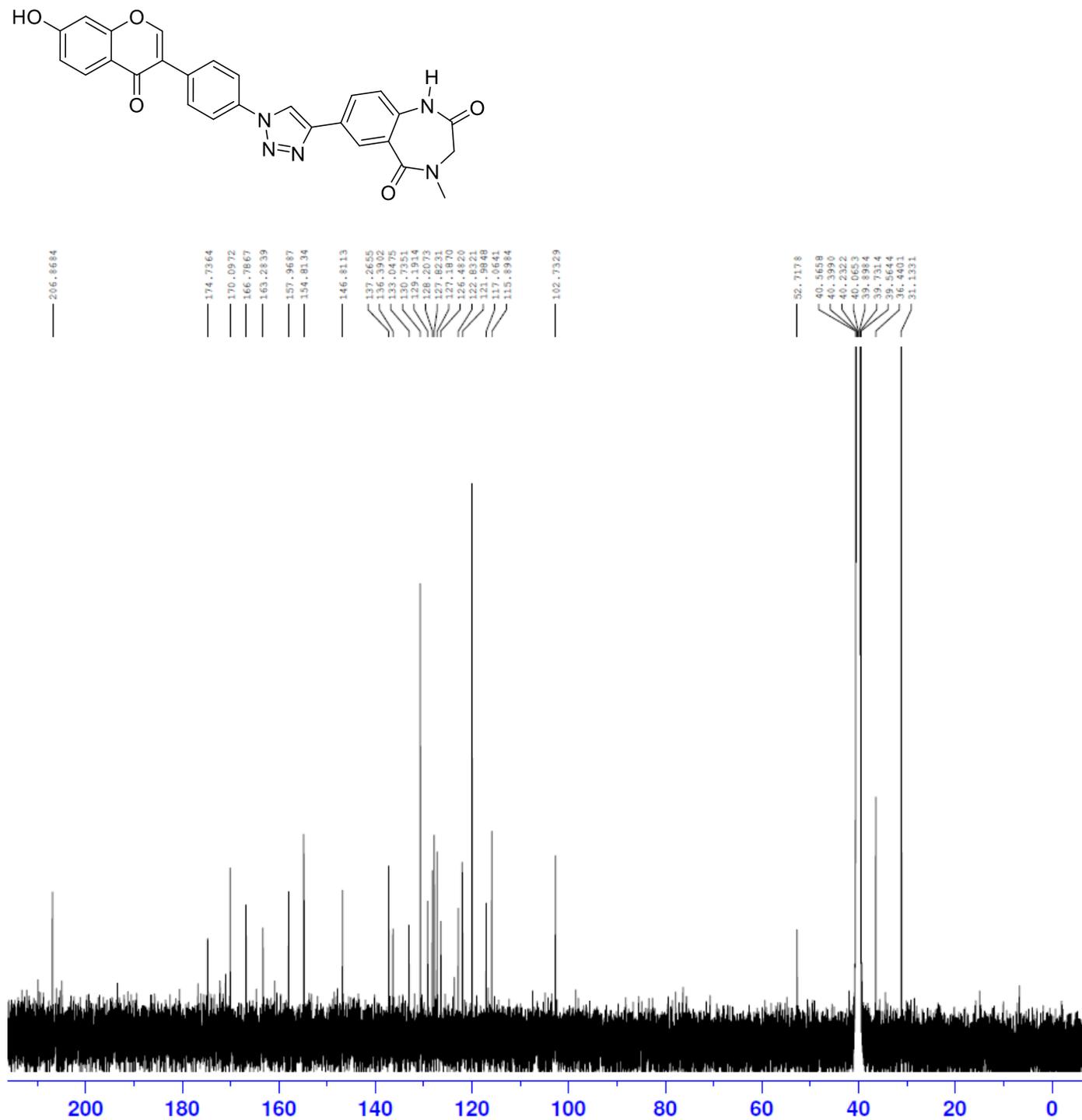
Compound **35**  $^1\text{H}$  NMR Spectrum ( $\text{CDCl}_3$ )

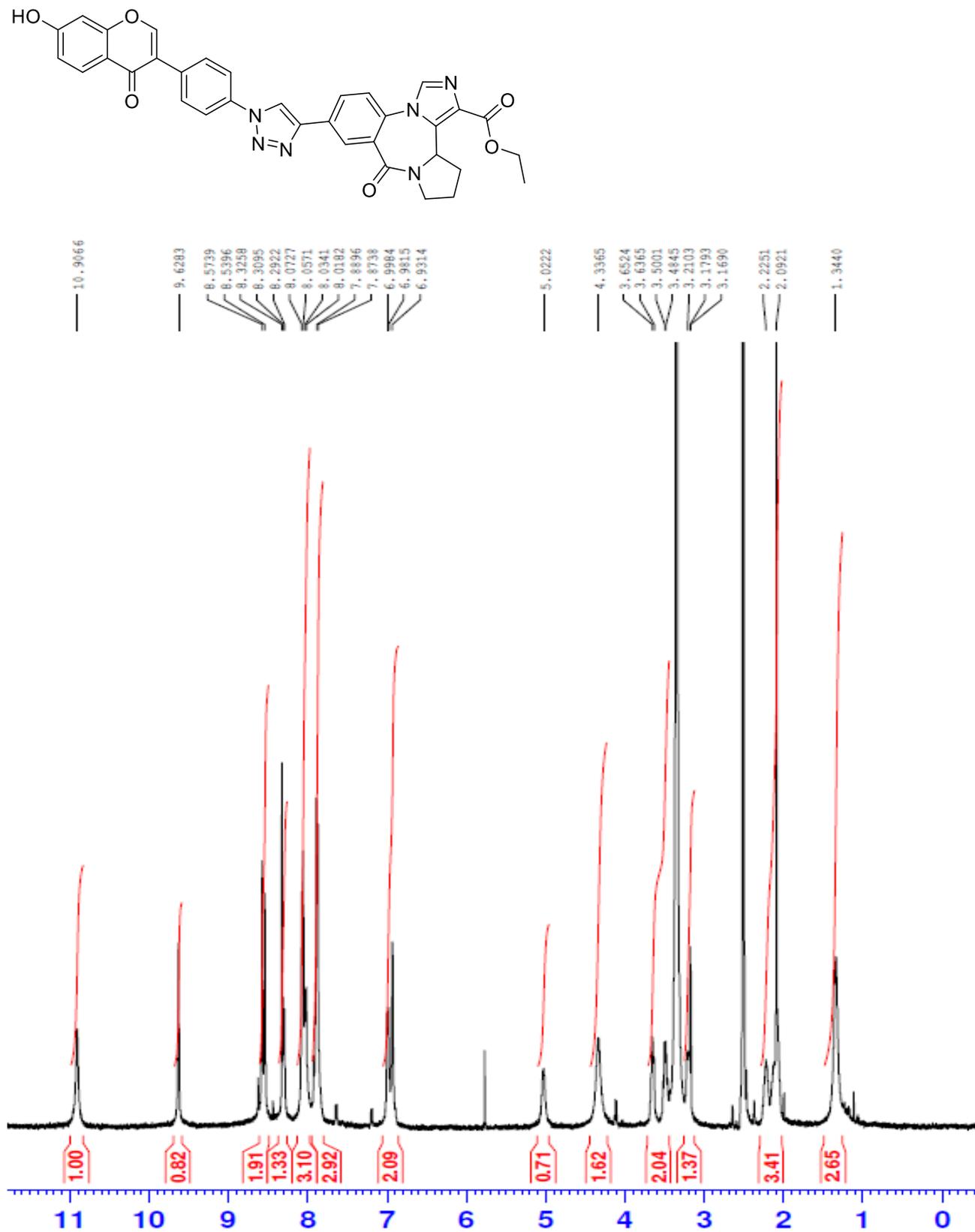
Compound **35**  $^{13}\text{C}$  NMR Spectrum ( $\text{CDCl}_3$ )

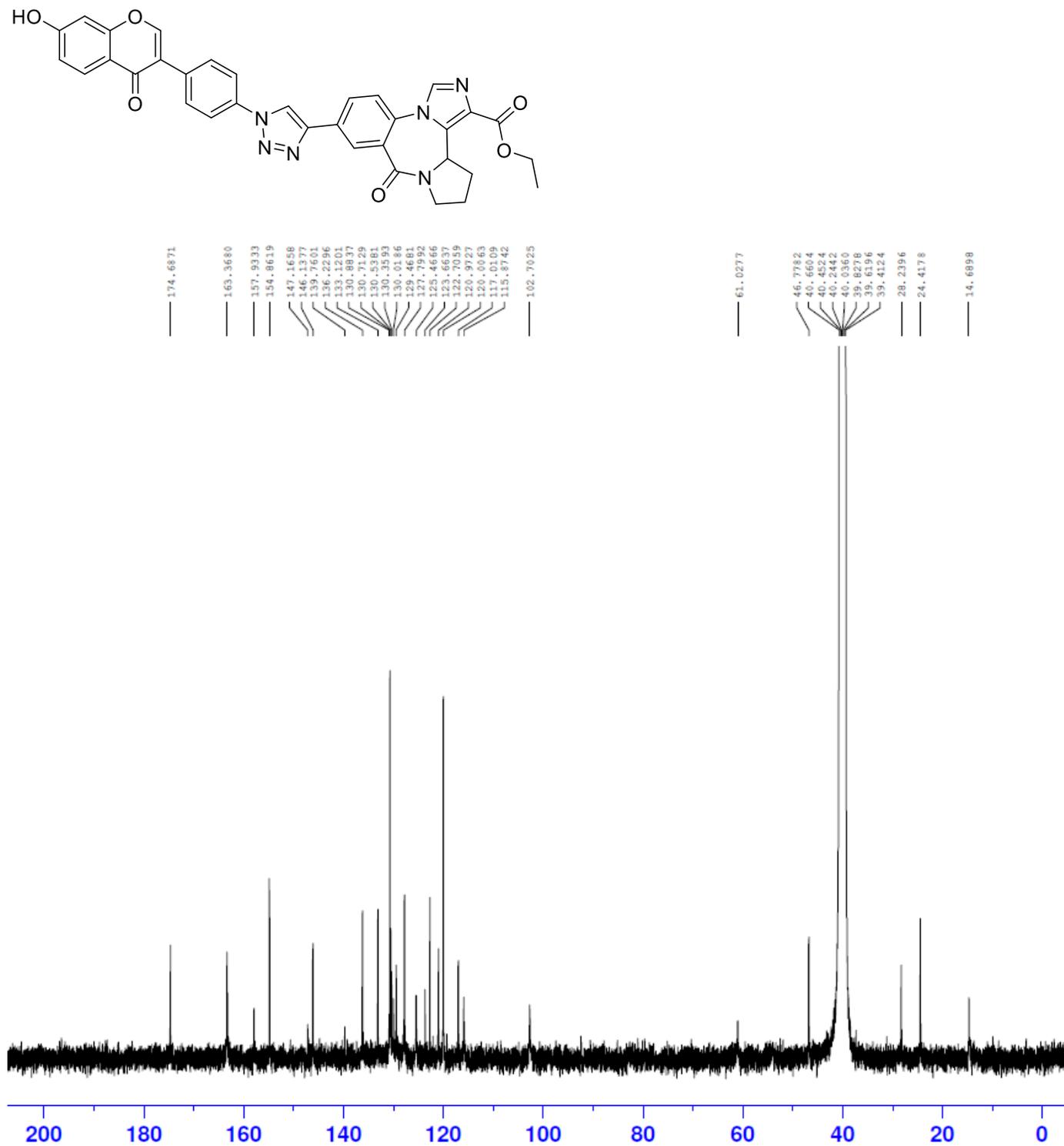
Compound **36**  $^1\text{H}$  NMR Spectrum (DMSO, acetone)

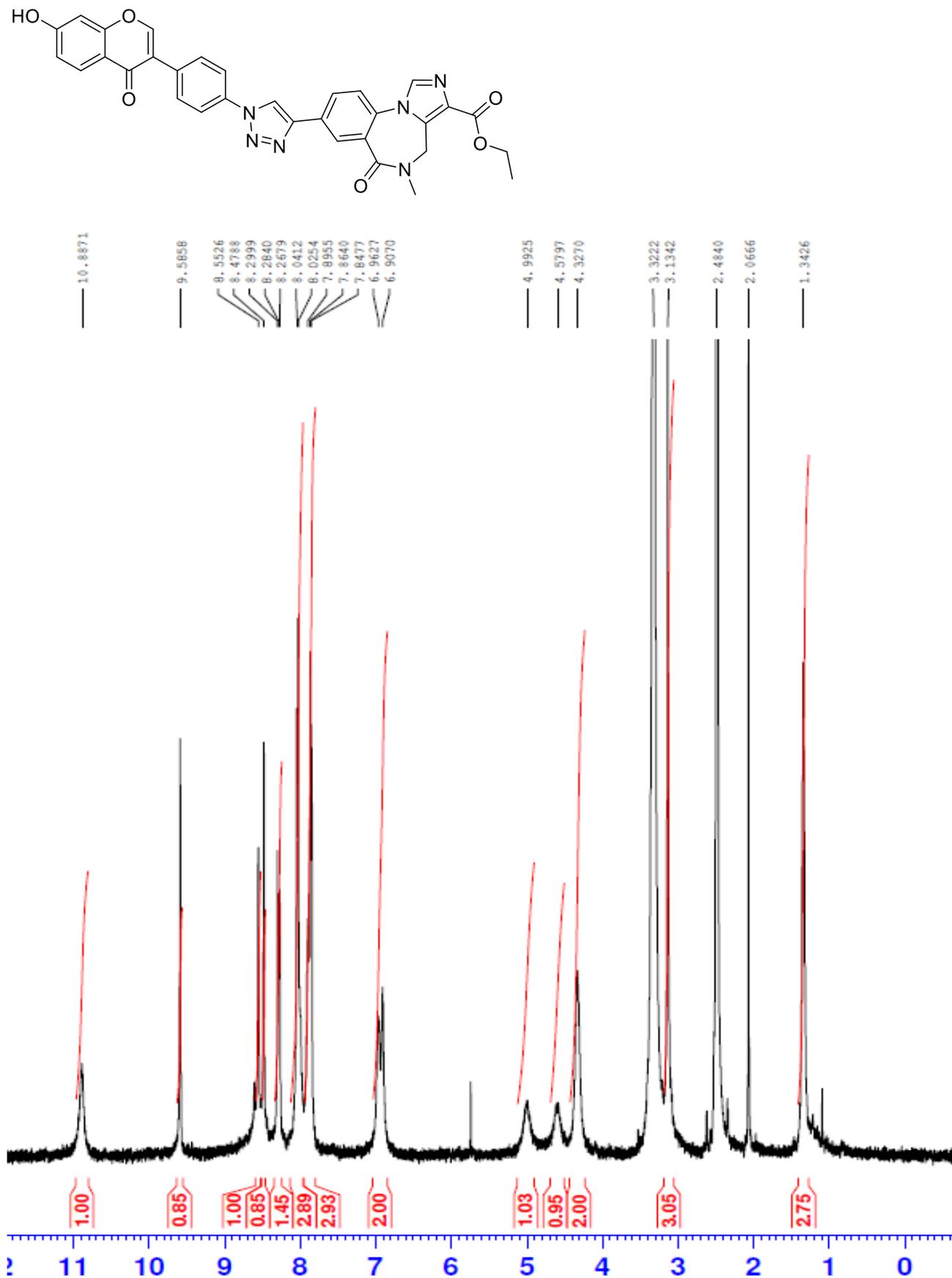
Compound **36**  $^{13}\text{C}$  NMR Spectrum (DMSO, acetone)

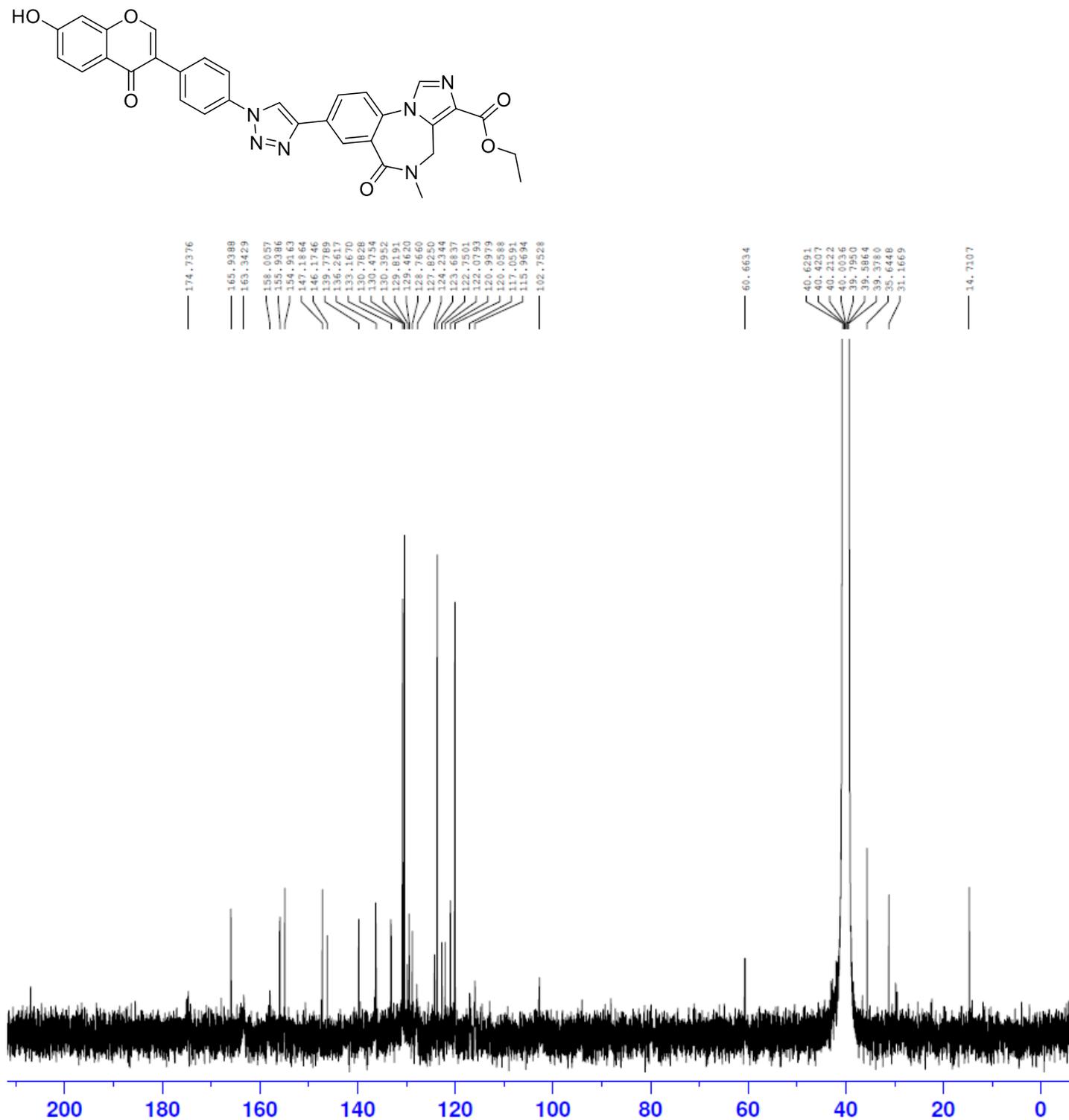
Compound **37**  $^1\text{H}$  NMR Spectrum (DMSO, acetone)

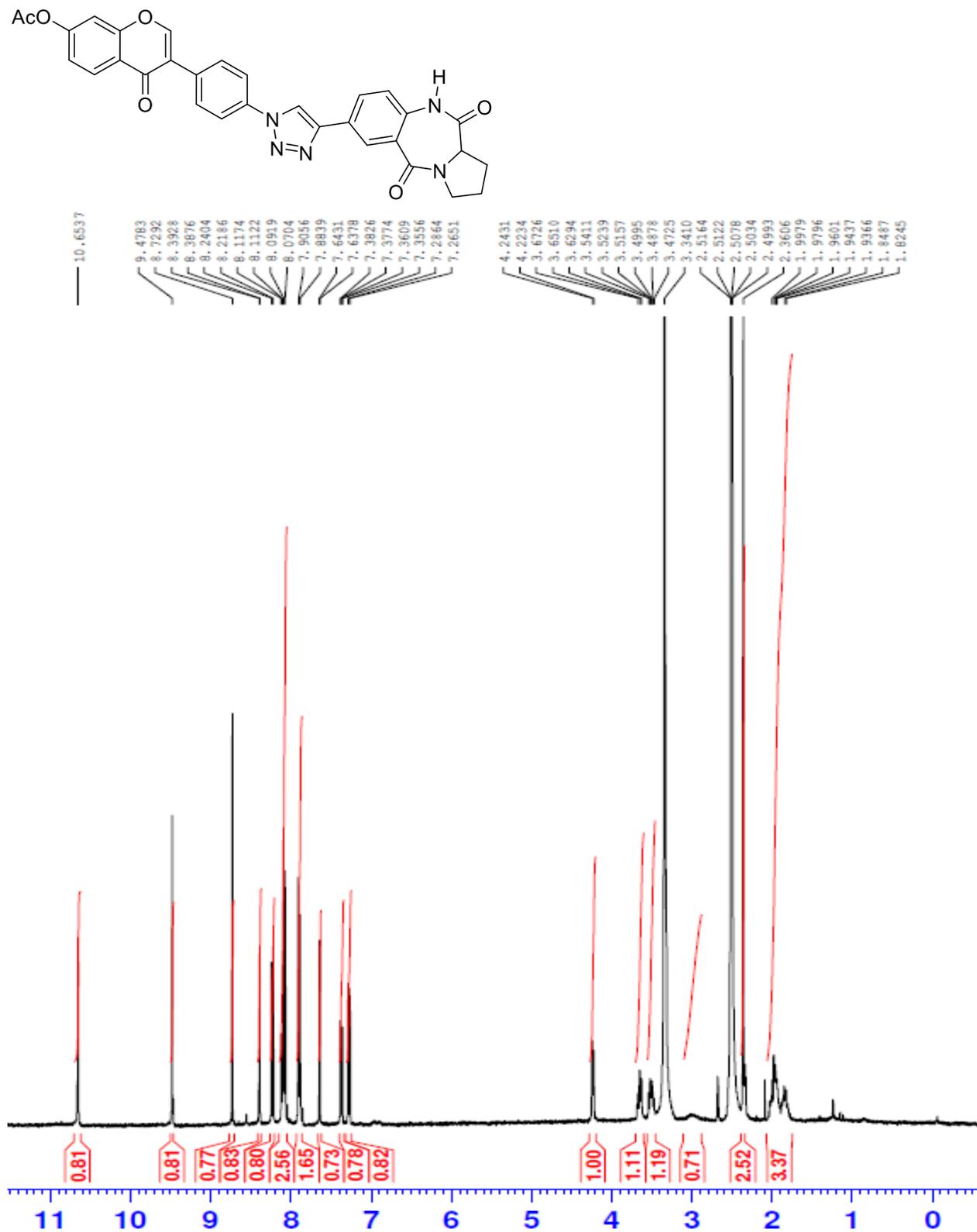
Compound **37**  $^{13}\text{C}$  NMR Spectrum (DMSO, acetone)

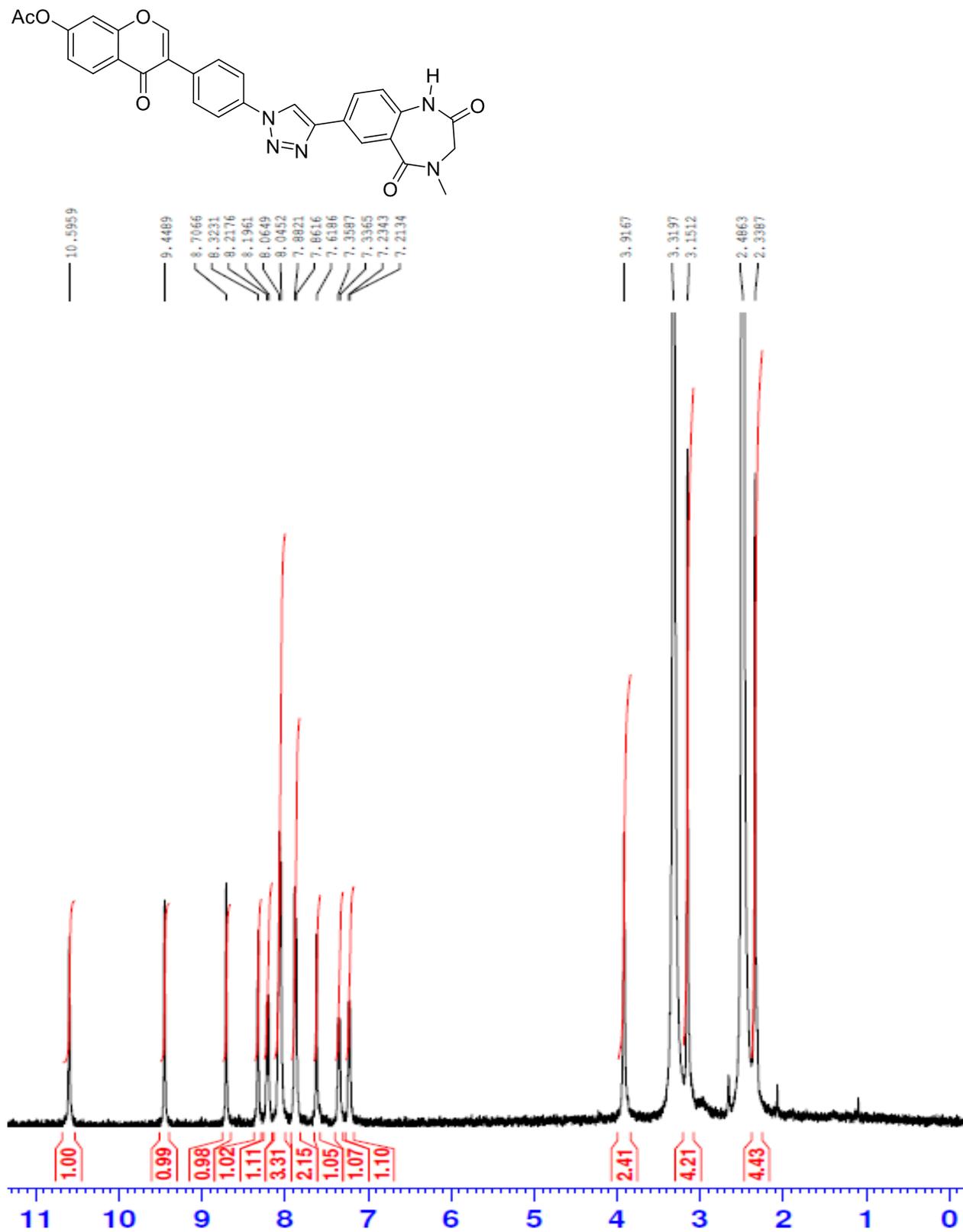
Compound **38**  $^1\text{H}$  NMR Spectrum (DMSO)

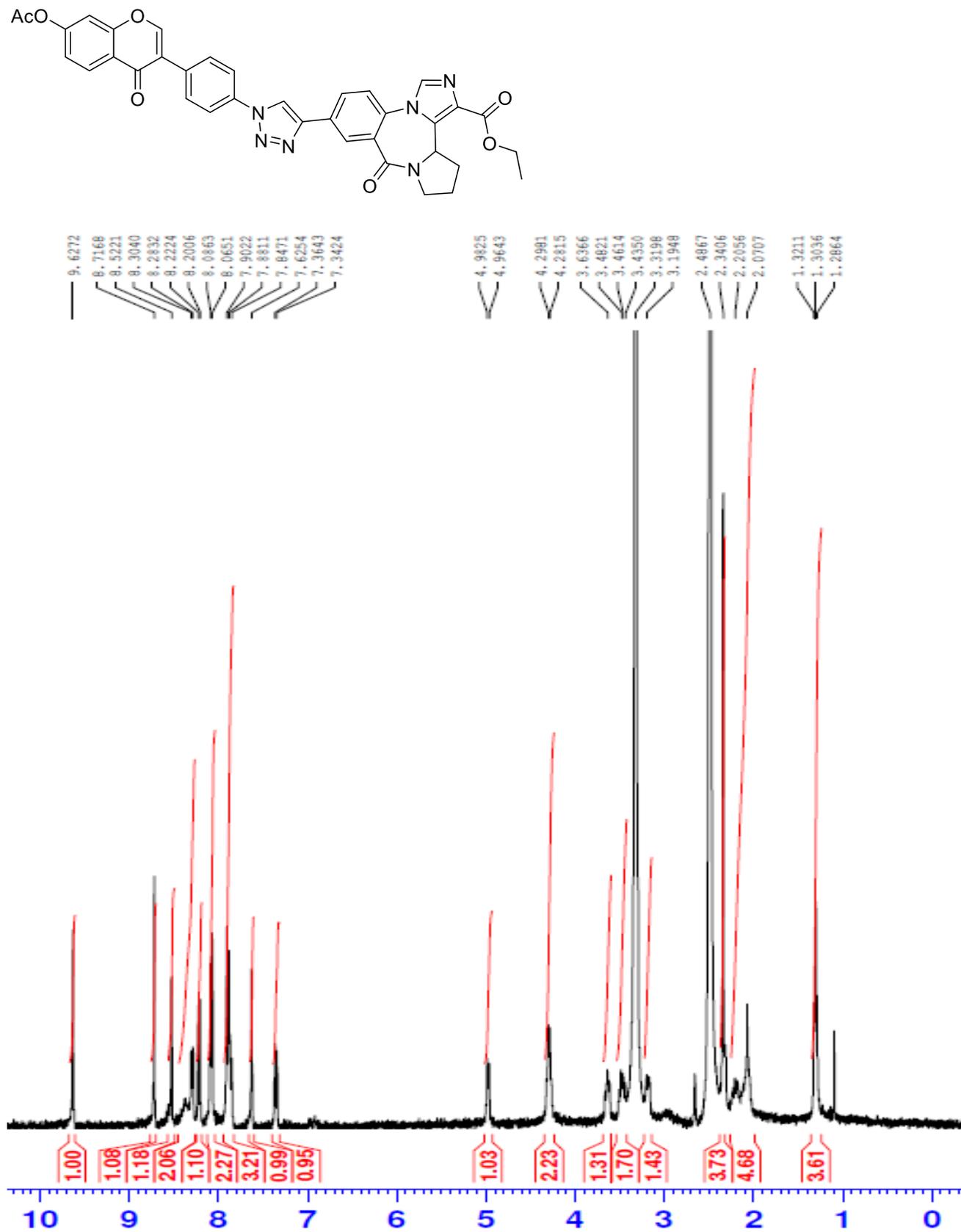
Compound **38**  $^{13}\text{C}$  NMR Spectrum (DMSO)

Compound **39**  $^1\text{H}$  NMR Spectrum (DMSO + acetone)

Compound **39**  $^{13}\text{C}$  NMR Spectrum (DMSO + acetone)

Compound **40**  $^1\text{H}$  NMR Spectrum (DMSO)

Compound **41**  $^1\text{H}$  NMR Spectrum (DMSO)

Compound **42**  $^1\text{H}$  NMR Spectrum (DMSO)

Compound **43**  $^1\text{H}$  NMR Spectrum (DMSO – low solubility)