Supporting Information

Squaramides with cytotoxic activity against human gastric carcinoma against HGC-27. Synthesis, biological evaluation and mechanism study as novel anticancer agents

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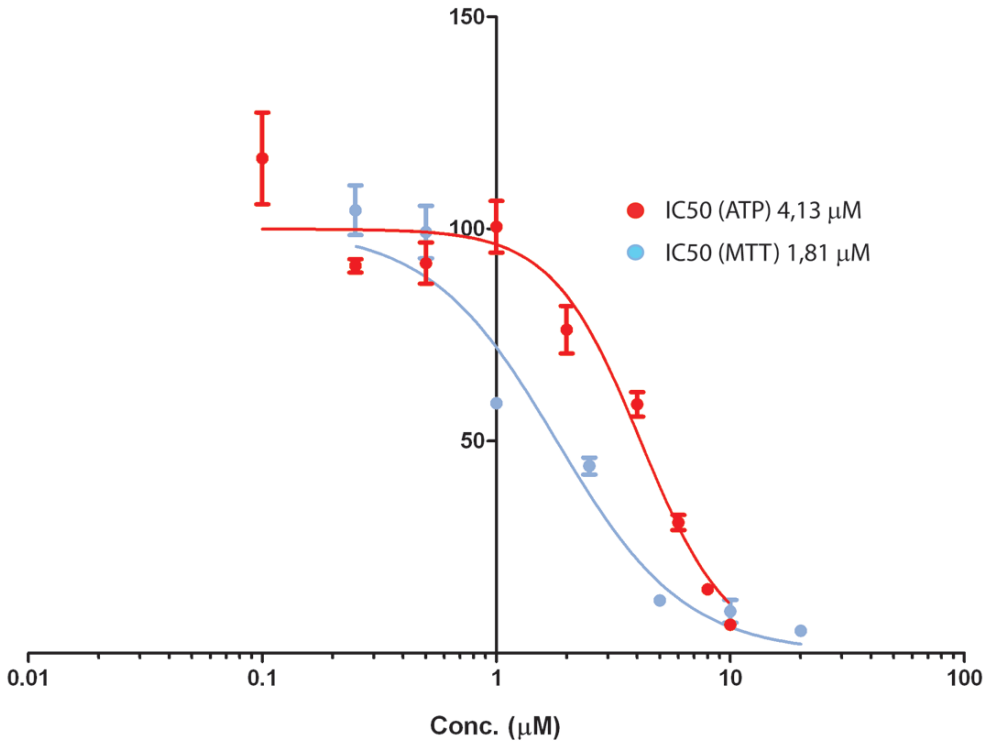
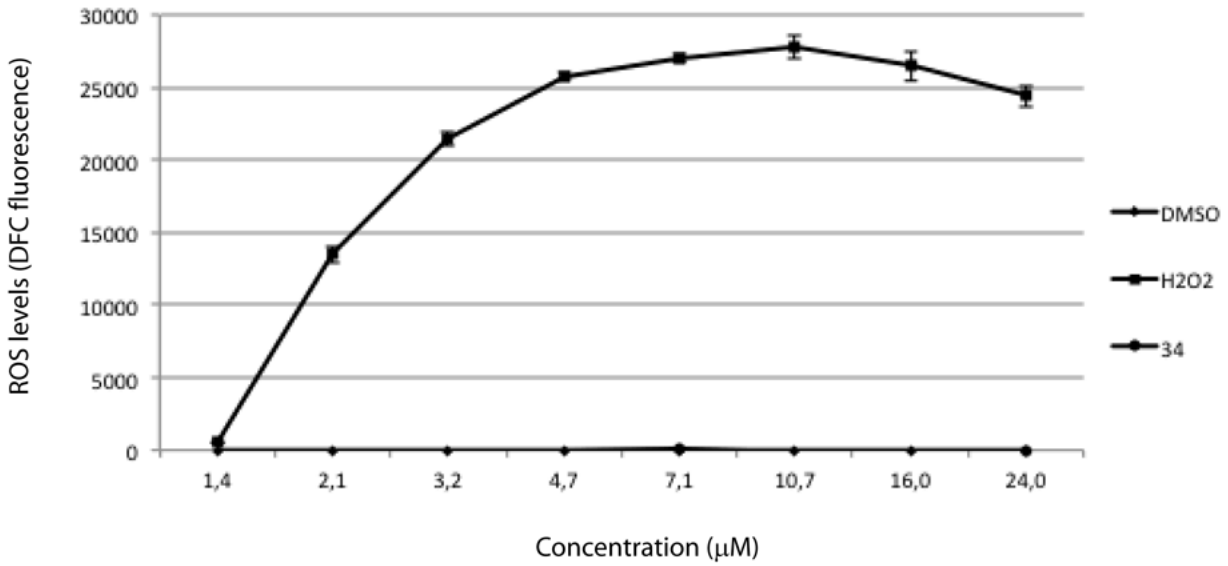
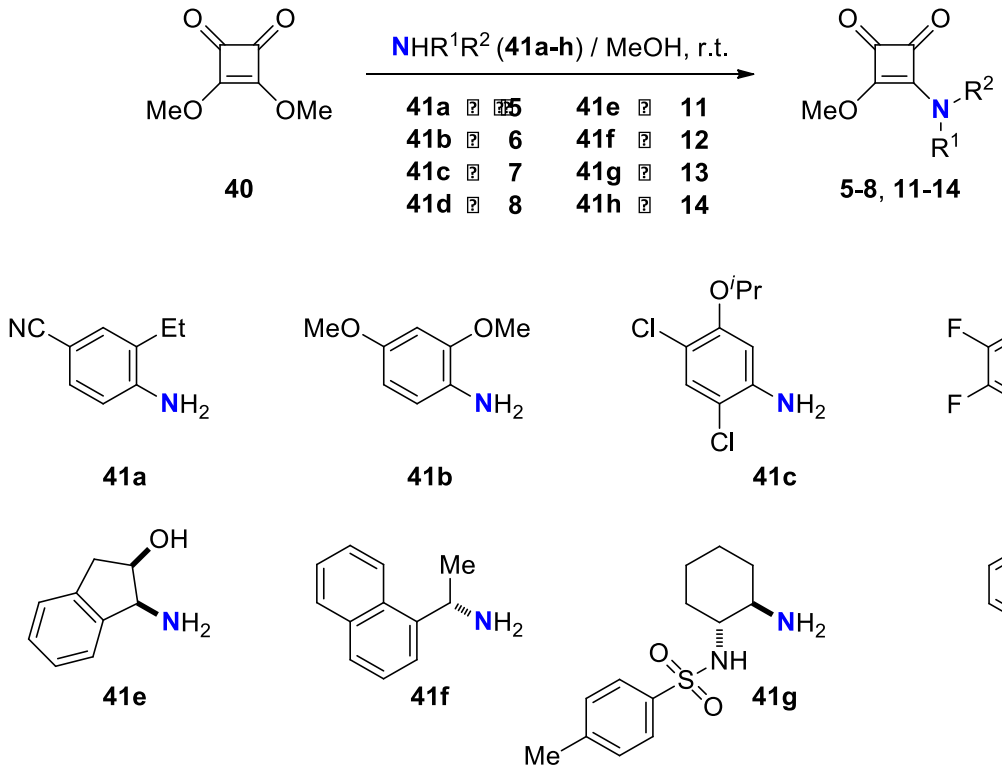


Supp Fig. 1 Dose-dependent curves obtained with an ATP-based assay or a MTT assay show the difference of cell viability at given concentrations, thus supporting the need of a 8 µM concentration of **34** to correlate a decrease in cell viability with the effects on cell death induction.



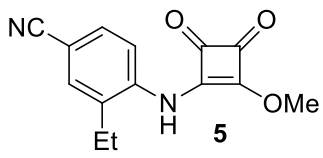
Supp Fig. 2 Detection of intracellular ROS levels in HGC-27 cells exposed to increased concentrations of H_2O_2 and **34**.

Synthesis of squaramates 5-8 and 11-14



To a mixture of 3,4-dimethoxy-3-cyclobutene-1,2-dione (**40**) (0.2 mmol) in MeOH (0.75-1.5 ml), amine **41a-h** (0.2 mmol) was added at room temperature. After the corresponding reaction time, the product was purified either by filtration, washing with MeOH at –25 ºC, or by column chromatography.

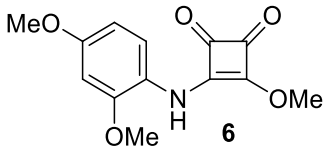
3-Ethyl-4-(2-methoxy-3,4-dioxocyclobut-1-enylamino)benzonitrile (5)



Following the general procedure, using 0.75 ml MeOH and after 5 days, **5** was obtained by filtration as a yellow solid in 60% yield. M.p. 173–175 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.54 (br s, 1H), 7.75-7.63 (m, 2H), 7.30 (d, J = 8.2 Hz, 1H), 4.33 (s, 3H), 2.73 (q, J = 7.5 Hz, 2H), 1.14 (t, J = 7.5 Hz, 3H). ^{13}C-NMR-APT (100 MHz, DMSO-d_6) δ 188.0 (1C), 184.9 (1C), 179.3 (1C), 170.0 (1C), 139.3 (1C), 137.6 (1C), 132.5 (1C), 130.2 (1C), 124.8 (1C), 118.7 (1C), 108.0 (1C), 60.5 (1C), 23.0 (1C), 13.5 (1C). IR (KBr film) (cm^{-1}) ν 3222, 2924, 2854, 1811, 1703, 1584,

1526, 1492, 1456, 1365, 1261, 840, 448. HRMS (ESI+) calcd $C_{14}H_{12}N_2NaO_3$ 279.0746; found 279.0726 [M + Na].

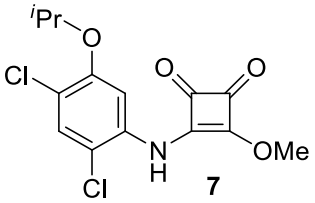
3-((2,4-Dimethoxyphenyl)amino)-4-methoxycyclobut-3-ene-1,2-dione (6)



6

Following the general procedure, using 0.75 ml MeOH and after 1 h, **6** was obtained by filtration as a pale yellow solid in 64% yield. M.p. 186–189 °C. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 10.13 (br s, 1H), 7.09 (d, J = 8.6 Hz, 1H), 6.63 (d, J = 2.6 Hz, 1H), 6.50 (dd, J = 8.6, 2.6 Hz, 1H), 4.26 (s, 3H), 3.78 (s, 3H), 3.77 (s, 3H). ^{13}C-NMR-APT (75 MHz, DMSO-d_6) δ 183.7 (1C), 178.0 (1C), 170.9 (1C), 158.6 (1C), 153.5 (1C), 125.5 (1C), 118.8 (2C), 104.2 (1C), 99.0 (1C), 60.0 (1C), 55.6 (1C), 55.3 (1C). IR (KBr film) (cm^{-1}) ν 3299, 3250, 2923, 2853, 1804, 1708, 1598, 1459, 1419, 1366, 1205, 1048, 928, 865, 801, 454. HRMS (ESI+) calcd $C_{13}H_{13}NNaO_5$ 286.0691; found 286.0706 [M + Na].

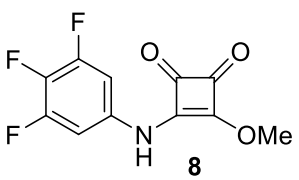
3-((2,4-Dichloro-5-isopropoxyphenyl)amino)-4-methoxycyclobut-3-ene-1,2-dione (7)



7

Following the general procedure, using 0.75 ml MeOH after 61 h, **7** was obtained by filtration as a yellow solid in 50% yield. M.p. 161–164 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.65 (s, 1H), 7.61 (s, 1H), 7.23 (s, 1H), 4.67 (q, J = 6.1 Hz, 1H), 4.30 (s, 3H), 1.29 (d, J = 6.1 Hz, 6H). ^{13}C-NMR-APT (75 MHz, DMSO-d_6) δ 187.9 (1C), 184.9 (1C), 178.9 (1C), 170.0 (1C), 151.9 (1C), 133.9 (1C), 129.7 (1C), 120.0 (1C), 118.0 (1C), 112.7 (1C), 71.8 (1C), 60.4 (1C), 21.6 (2C). IR (KBr film) (cm^{-1}) ν 3268, 2923, 2853, 1801, 1710, 1608, 1583, 1503, 1448, 1405, 1389, 1249, 1088, 879, 442. HRMS (ESI+) calcd $C_{14}H_{13}Cl_2NaNO_4$ 352.0119; found 352.0118 [M + Na].

3-Methoxy-4-((3,4,5-trifluorophenyl)amino)cyclobut-3-ene-1,2-dione (8)

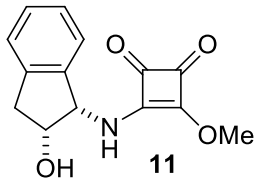


8

Following the general procedure, using 0.75 ml MeOH after 44 h, **8** was obtained by filtration as a pale yellow solid in 68% yield. M.p. 212–213 °C. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 10.94 (s, 1H), 7.29 (dd, J = 9.6, 6.1 Hz, 2H), 4.40 (s, 3H). ^{13}C-NMR-APT (100 MHz, DMSO-d_6) δ 187.4 (1C),

184.1 (1C), 179.5 (1C), 168.9 (1C), 150.4 (ddd, *J* = 245.2, 9.9, 5.0 Hz, 2C), 135.5 (dt, *J* = 190.0 Hz, 13.7 Hz, 1C), 134.1 (1C), 104.0 (d, *J* = 24.4 Hz, 2C), 60.8 (1C). IR (KBr film) (cm^{-1}) *v* 3238, 3069, 2924, 2853, 1816, 1705, 1636, 1595, 1532, 1457, 1362, 1249, 1213, 1111, 1048, 983, 929, 852, 796, 748, 717, 609, 418. HRMS (ESI+) calcd $C_{11}H_6F_3NNaO_3$ 280.0197; found 280.0178 [M + Na].

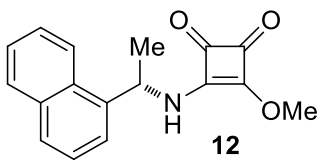
3-(((1*R*,2*S*)-1-Hydroxy-2,3-dihydro-1H-inden-2-yl)amino)-4-methoxycyclobut-3-ene-1,2-dione (11)



11

Following the general procedure, using 0.75 ml MeOH after 42 h, **11** was obtained by column chromatography (SiO_2, using Hex:AcOEt 9:1 to Hex:AcOEt 4:6) as a pale yellow solid in 93% yield. M.p. 86–89 °C. $[\alpha]_D^{26}$ = +107.1 (c 0.12, DMSO). ^{1}H-NMR (300 MHz, DMSO-d_6, 50 ºC) δ 8.59 (br s, 1H), 7.35-7.15 (m, 4H), 5.45 (br s, 0.5H), 5.18 (s, 1H), 4.97 (br s, 0.5H), 4.53-4.45 (m, 1H), 4.32 (s, 3H), 3.06 (dd, *J* = 15.8, 5.9 Hz, 1H), 2.91 (dd, *J* = 16.0, 3.1 Hz, 1H). ^{13}C-NMR-APT (100 MHz, DMSO-d_6) δ 189.8 (1C), 182.9 (1C), 174.4 (1C), 172.7 (1C), 140.9 (1C), 140.7 (1C), 126.5 (2C), 124.6 (2C), 72.3 (1C), 60.9 (1C), 59.8 (1C), 38.8 (1C). IR (KBr film) (cm^{-1}) ν 3337, 2924, 2853, 1805, 1698, 1593, 1461, 1377, 1336, 1049, 933, 740. HRMS (ESI+) calcd $C_{14}H_{13}NNaO_4$ 282,0742; found 282.0728 [M + Na].

(*S*)-3-Methoxy-4-((1-(naphthalen-1-yl)ethyl)amino)cyclobut-3-ene-1,2-dione (12)

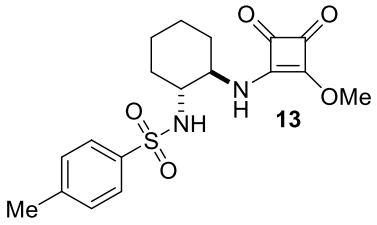


12

Following the general procedure, using 0.75 ml MeOH after 95 h, **12** was obtained by column chromatography (SiO_2, using Hex:AcOEt 9:1 to Hex:AcOEt 5:5) as a pale yellow solid in 95% yield. M.p. 115–117 °C. $[\alpha]_D^{26}$ = +97.6 (c 0.12, DMSO). ^{1}H-NMR (300 MHz, DMSO-d_6, 50 ºC) δ 9.38 (br s, 0.5H), 9.15 (br s, 0.5H), 8.10 (br s, 1H), 7.96 (d, *J* = 8.0 Hz, 1H), 7.86 (d, *J* = 8.1 Hz, 1H), 7.66-7.49 (m, 4H), 6.11 (br s, 0.5H), 5.65 (br s, 0.5H), 4.30 (s, 1.5H), 4.14 (s, 1.5H), 1.64 (d, *J* = 6.8 Hz, 3H). ^{13}C-NMR-APT for the major conformer (100 MHz, DMSO-d_6) δ 189.3 (1C), 182.5 (1C), 177.7 (1C), 171.5 (1C), 139.0 (1C), 133.3 (1C), 129.5 (1C), 128.7 (1C), 127.7 (1C), 126.4 (1C), 125.7 (1C), 125.4 (1C), 122.7 (1C), 122.5 (1C), 60.0 (1C), 50.1 (1C), 22.7

(1C). IR (KBr film) (cm^{-1}) ν 3186, 2923, 2853, 1804, 1693, 1620, 1531, 1462, 1376, 1306, 1074, 939, 781. HRMS (ESI+) calcd $C_{17}H_{15}NNaO_3$ 304.0944; found 304.0938 [M + Na].

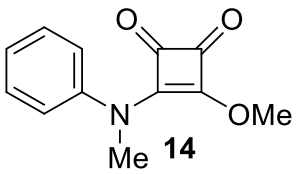
***N*-((1*R*,2*R*)-2-((2-Methoxy-3,4-dioxocyclobut-1-en-1-yl)amino)cyclohexyl)-4-methylbenzenesulfonamide (13)**



13

Following the general procedure, using 1.5 ml MeOH after 96 h, **13** was obtained by filtration as a white solid in 74% yield. M.p. 187–189 °C. $[\alpha]_D^{27}$ = +95.2 (c 0.76, MeOH). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.72 (d, J = 7.3 Hz, 2H), 7.25 (d, J = 7.3 Hz, 2H), 7.12 (d, J = 6.2 Hz, 0.5H), 6.14 (d, J = 7.6 Hz, 0.5H), 5.87 (d, J = 6.6 Hz, 0.5H), 5.56 (d, J = 8.4 Hz, 0.5H), 4.37 (s, 1.7H), 4.32 (s, 1.3H), 3.76-3.55 (m, 0.5H), 3.35-3.00 (m, 1.5H), 2.40 (s, 3H), 2.17-0.92 (m, 8H). ^{13}C-NMR-APT for the major conformer (100 MHz, $CDCl_3$) δ 190.1 (1C), 182.3 (1C), 177.3 (1C), 171.2 (1C), 143.6 (1C), 138.4 (1C), 129.7 (2C), 126.6 (1C), 126.5 (1C), 60.7 (1C), 58.1 (1C), 57.0 (1C), 33.2 (2C), 24.0 (2C), 21.5 (1C). IR (KBr film) (cm^{-1}) ν 3364, 2923, 2853, 1794, 1694, 1572, 1449, 1413, 1376, 1306, 1145, 1089, 946, 915, 834, 819, 720, 706, 662. HRMS (ESI+) calcd $C_{18}H_{22}N_2NaO_5S$ 401.1147; found 401.1140 [M + Na].

3-Methoxy-4-(methyl(phenyl)amino)cyclobut-3-ene-1,2-dione (14)

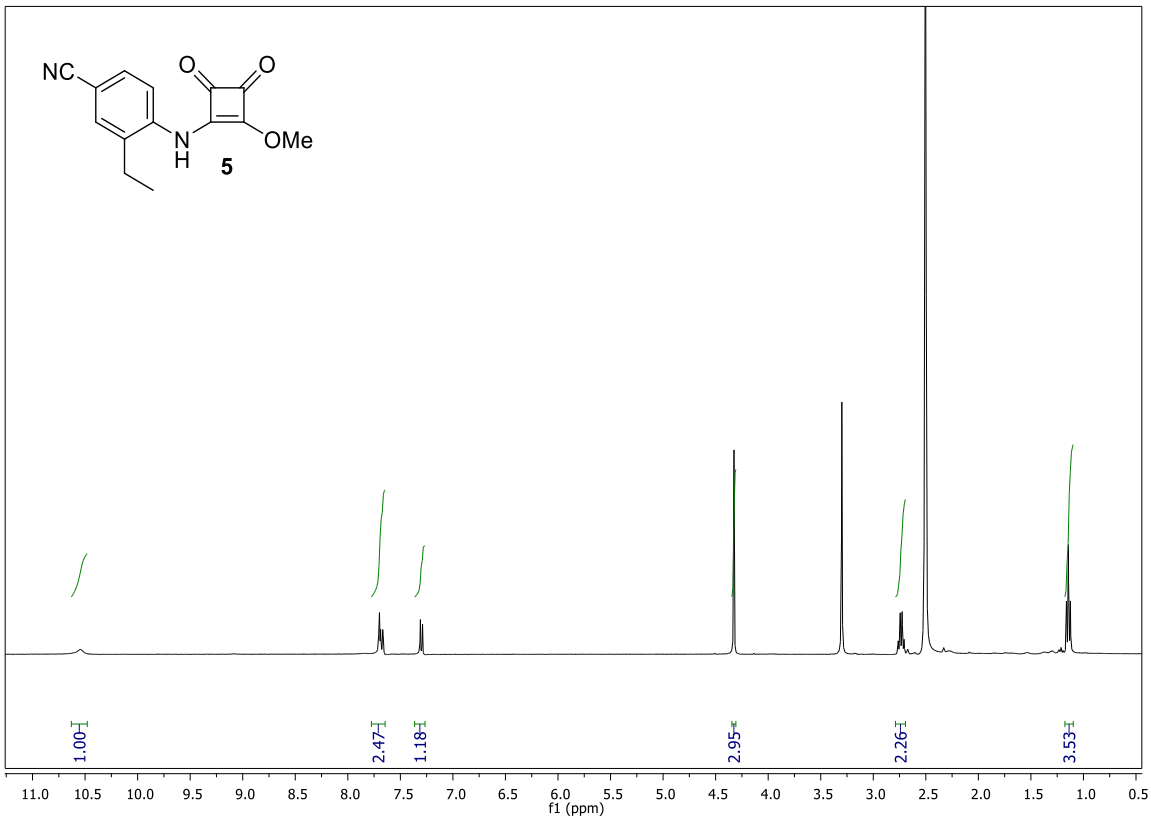


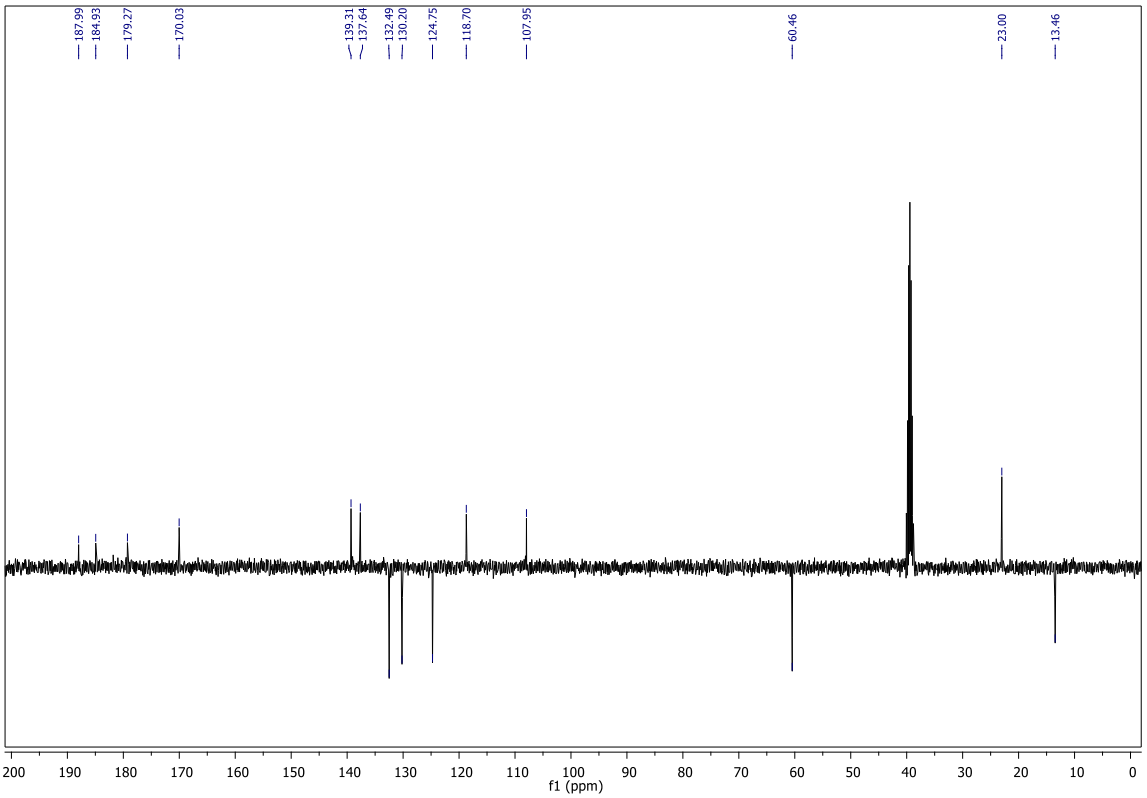
14

Following the general procedure, using 0.75 ml MeOH after 44 h, **14** was obtained by filtration as a pale yellow solid in 32% yield. M.p. 155–158 °C. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.55-7.20 (m, 5H), 4.27 (s, 3H), 3.63 (s, 3H). ^{13}C-NMR-APT (75 MHz, DMSO-d_6) δ 183.7 (1C), 177.9 (1C), 170.3 (1C), 141.4 (1C), 128.7 (2C), 126.4 (1C), 123.1 (2C), 60.4 (1C), 38.7 (1C). IR (KBr film) (cm^{-1}) 3070, 2923, 2853, 1793, 1713, 1603, 1485, 1458, 1431, 1381, 1326, 1170, 1077, 1048, 933, 762, 697, 431. HRMS (ESI+) calcd $C_{12}H_{11}NNaO_3$ 240.0637; found 240.0623 [M + Na].

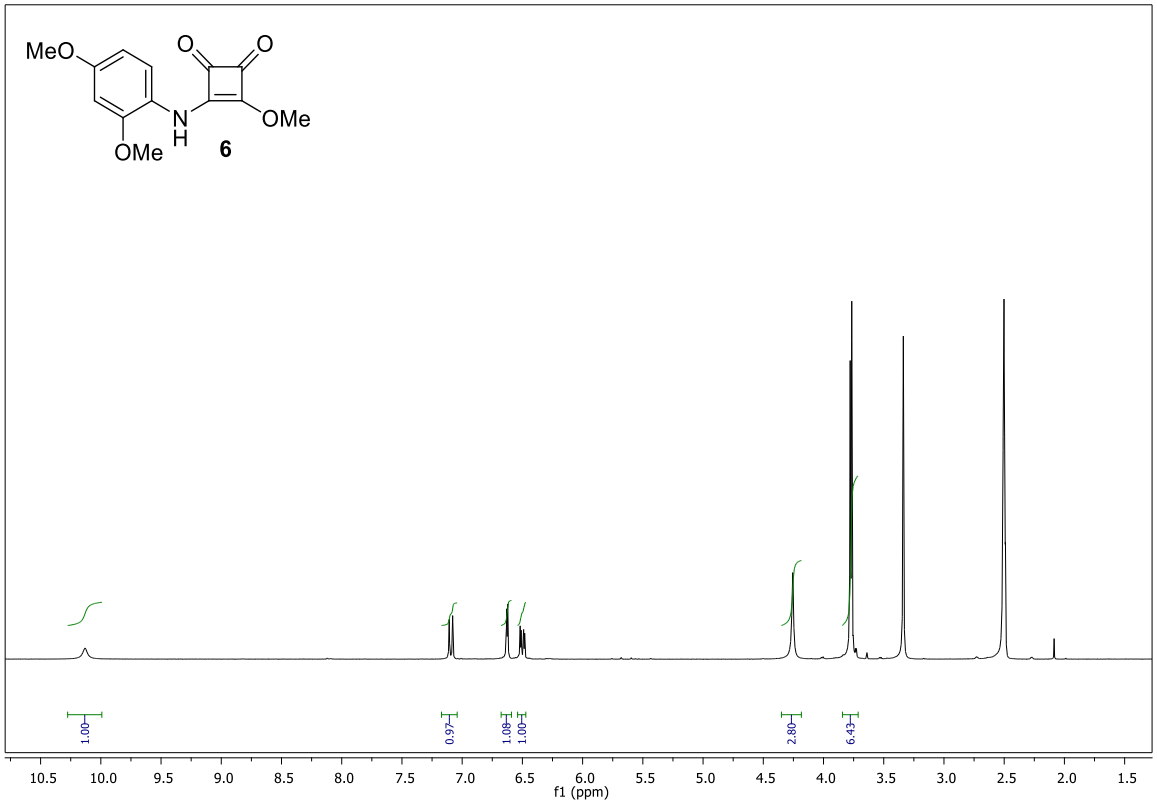
^{1}H-NMR AND ^{13}C-NMR-APT SPECTRA OF SQUARAMATES 5-8 AND 11-14

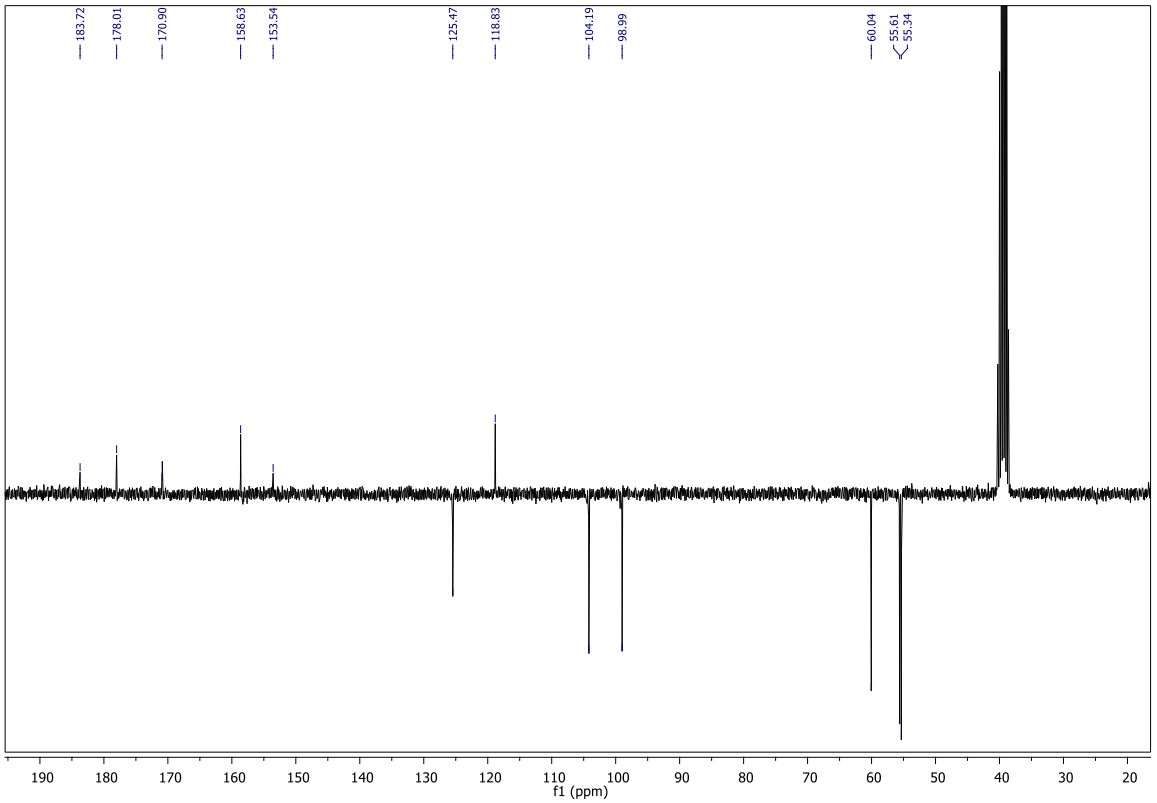
3-Ethyl-4-(2-methoxy-3,4-dioxocyclobut-1-enylamino)benzonitrile (5)



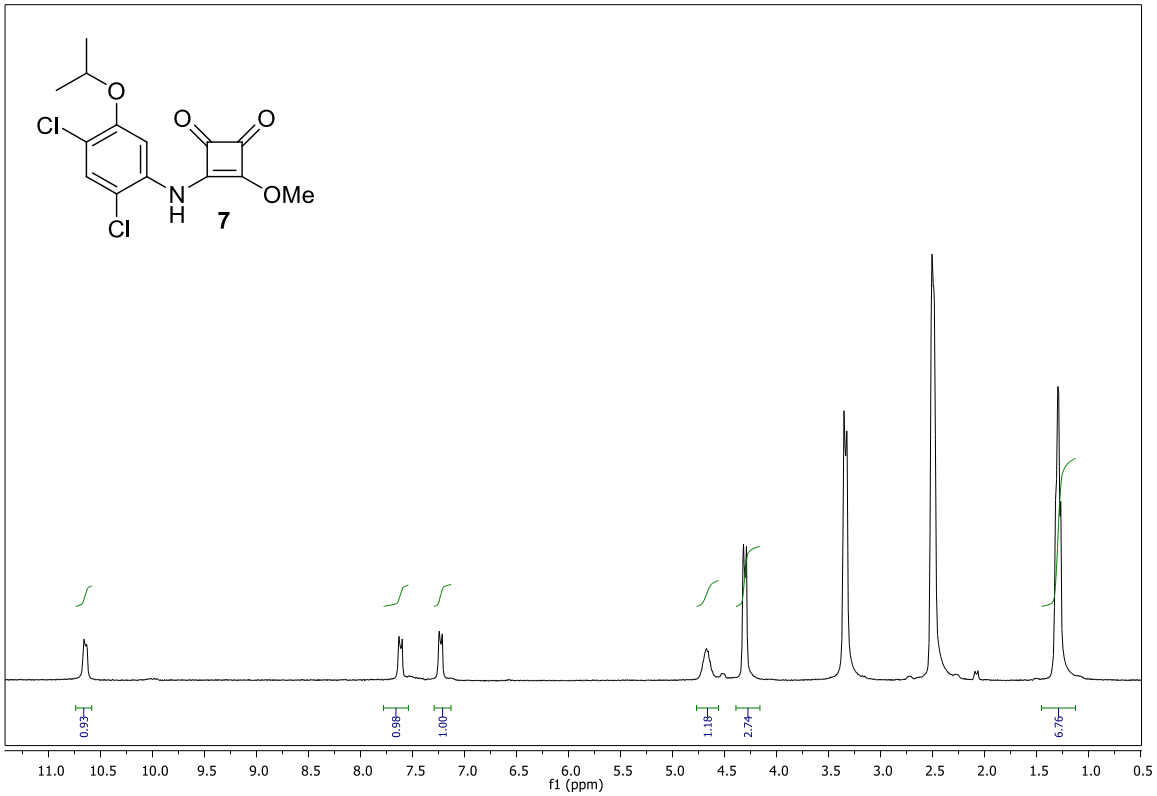


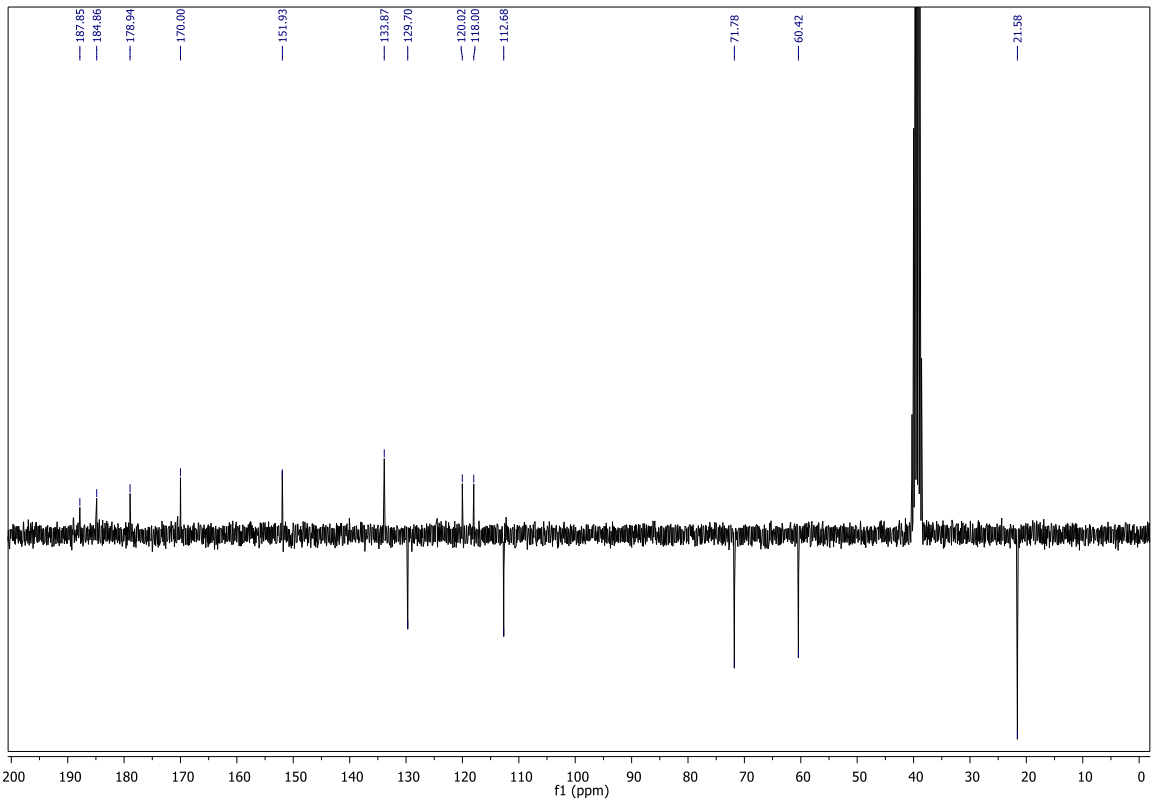
3-((2,4-Dimethoxyphenyl)amino)-4-methoxycyclobut-3-ene-1,2-dione (6)



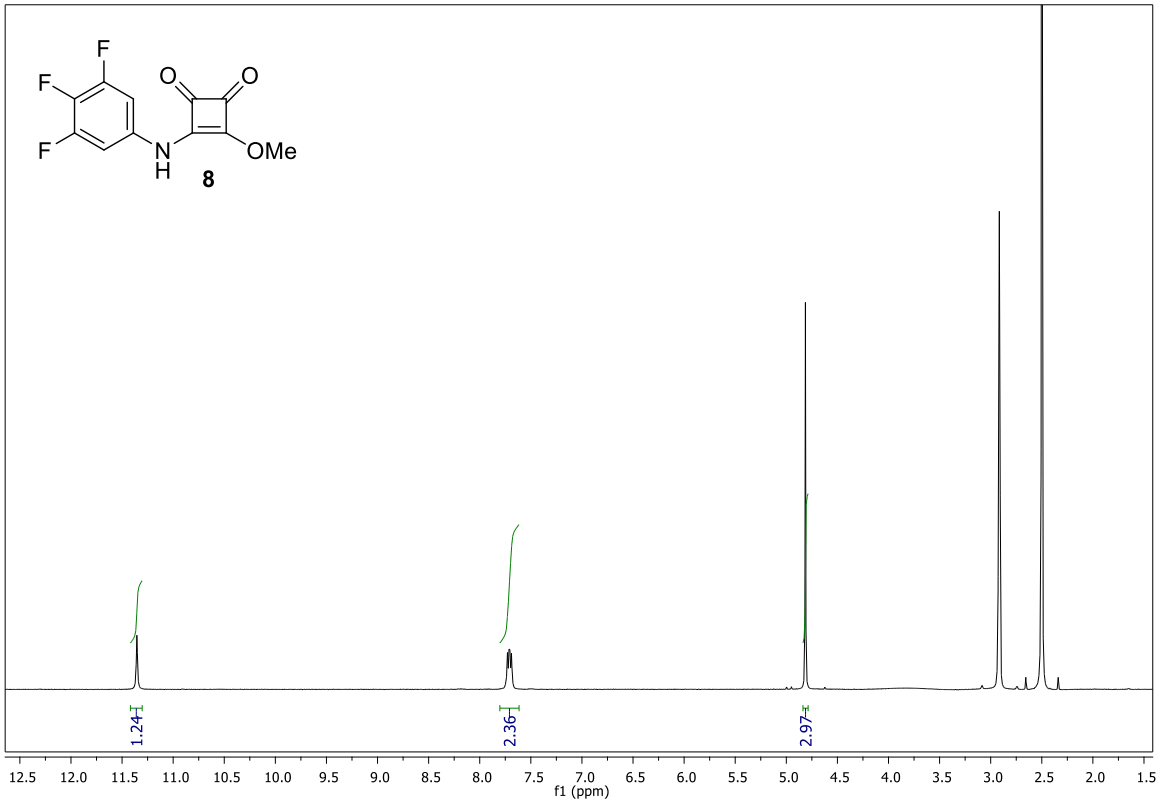


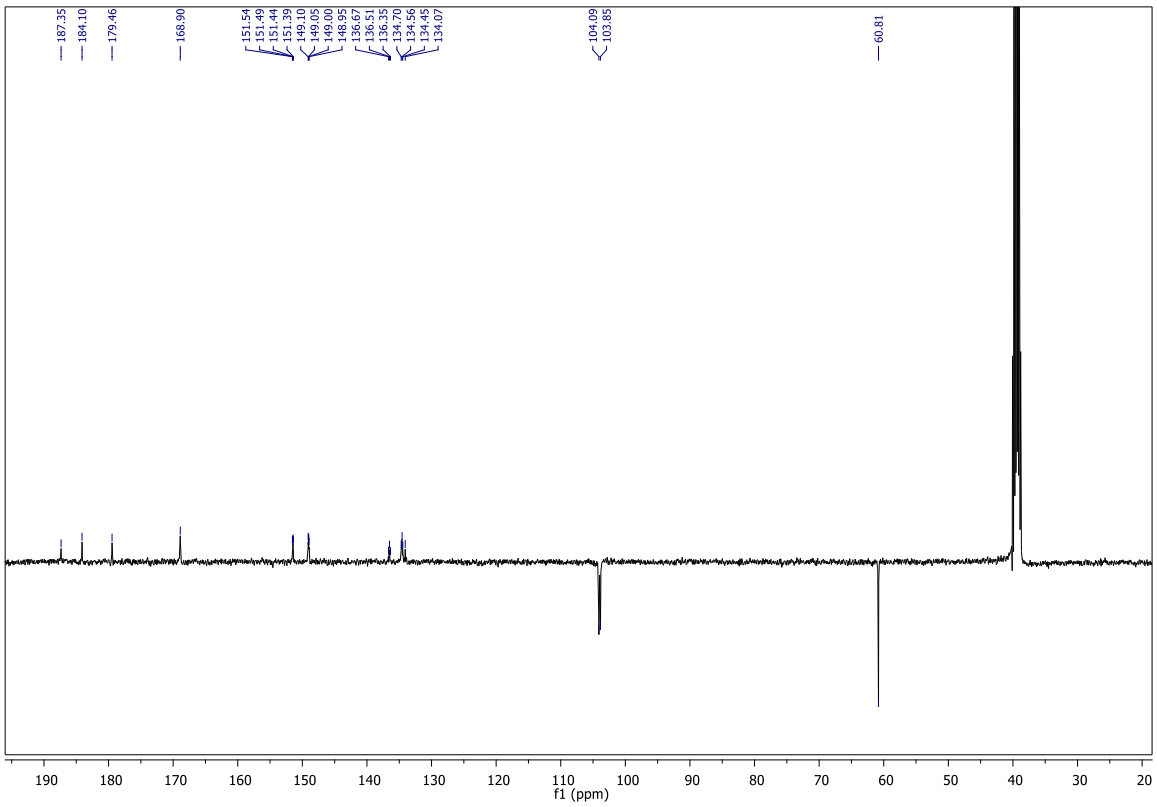
3-((2,4-Dichloro-5-isopropoxyphenyl)amino)-4-methoxycyclobut-3-ene-1,2-dione (7)



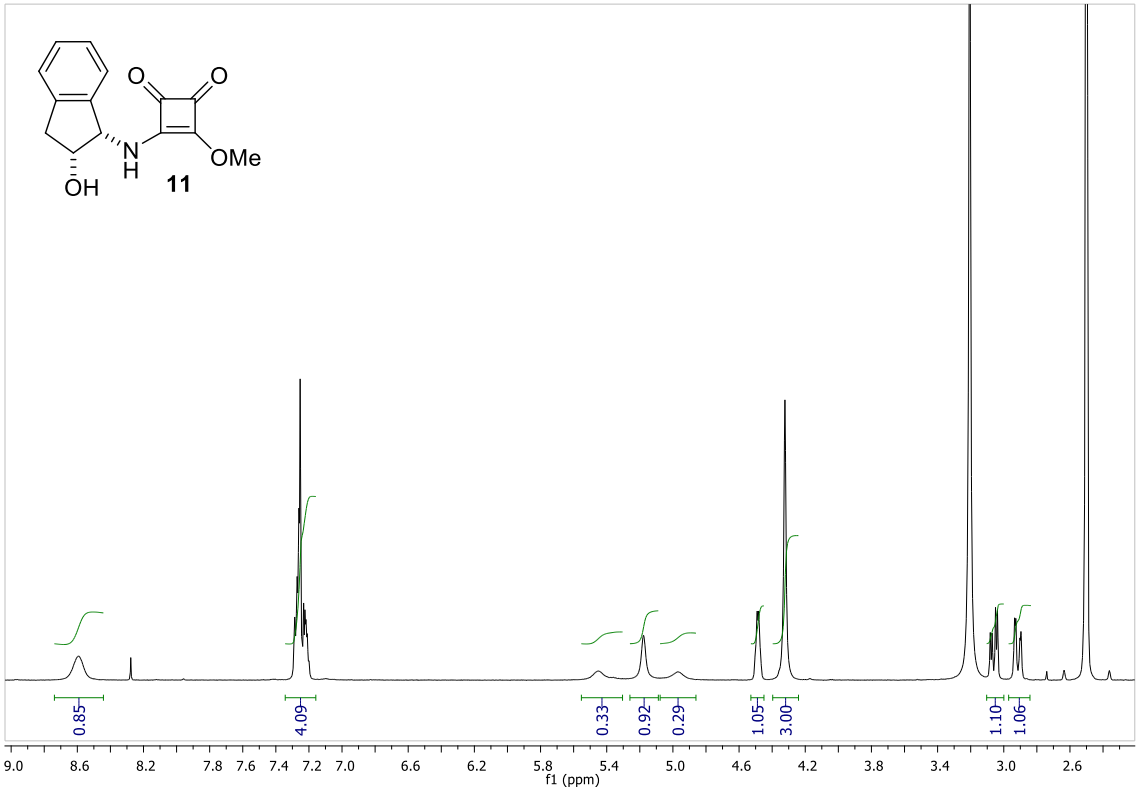


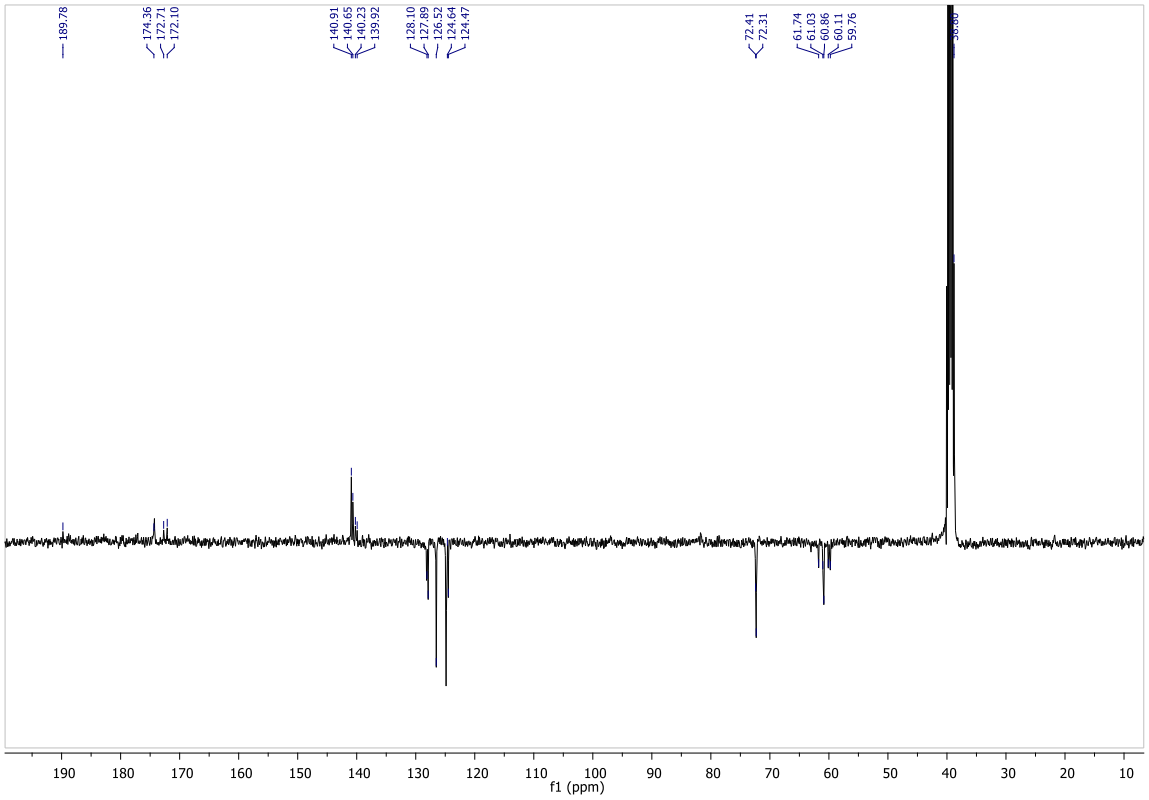
3-Methoxy-4-((3,4,5-trifluorophenyl)amino)cyclobut-3-ene-1,2-dione (8)



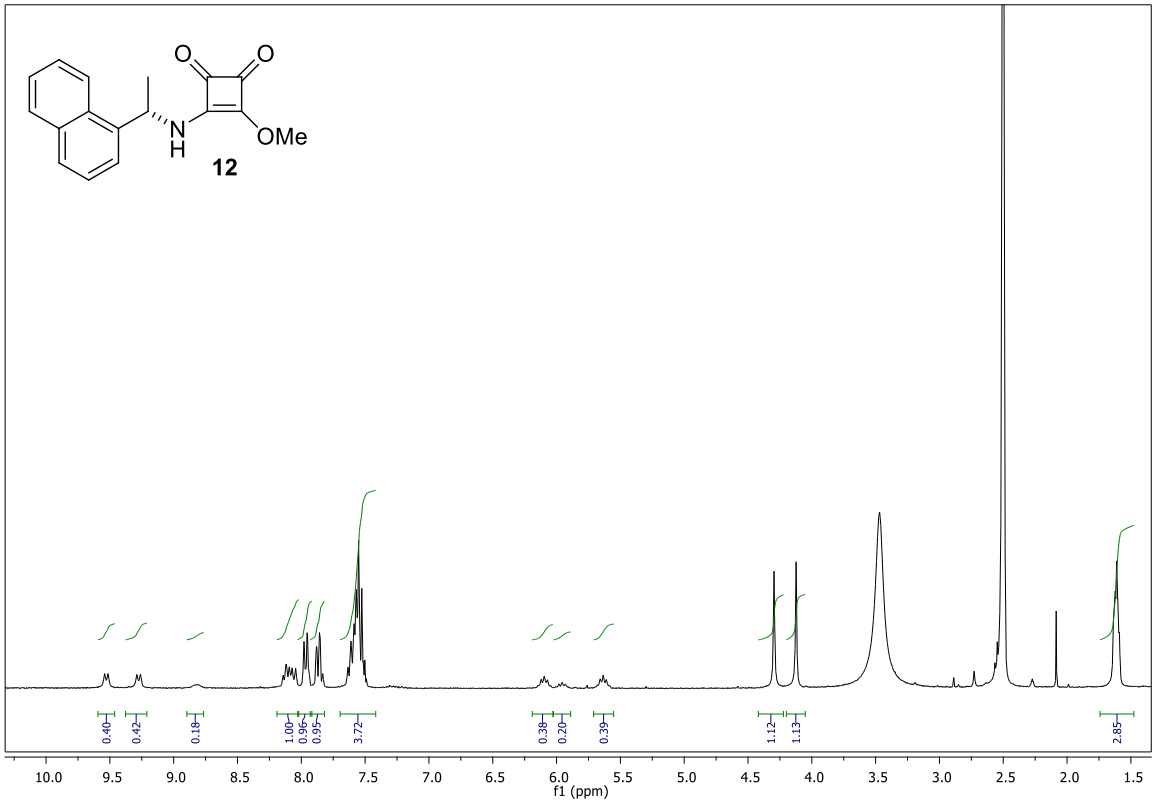


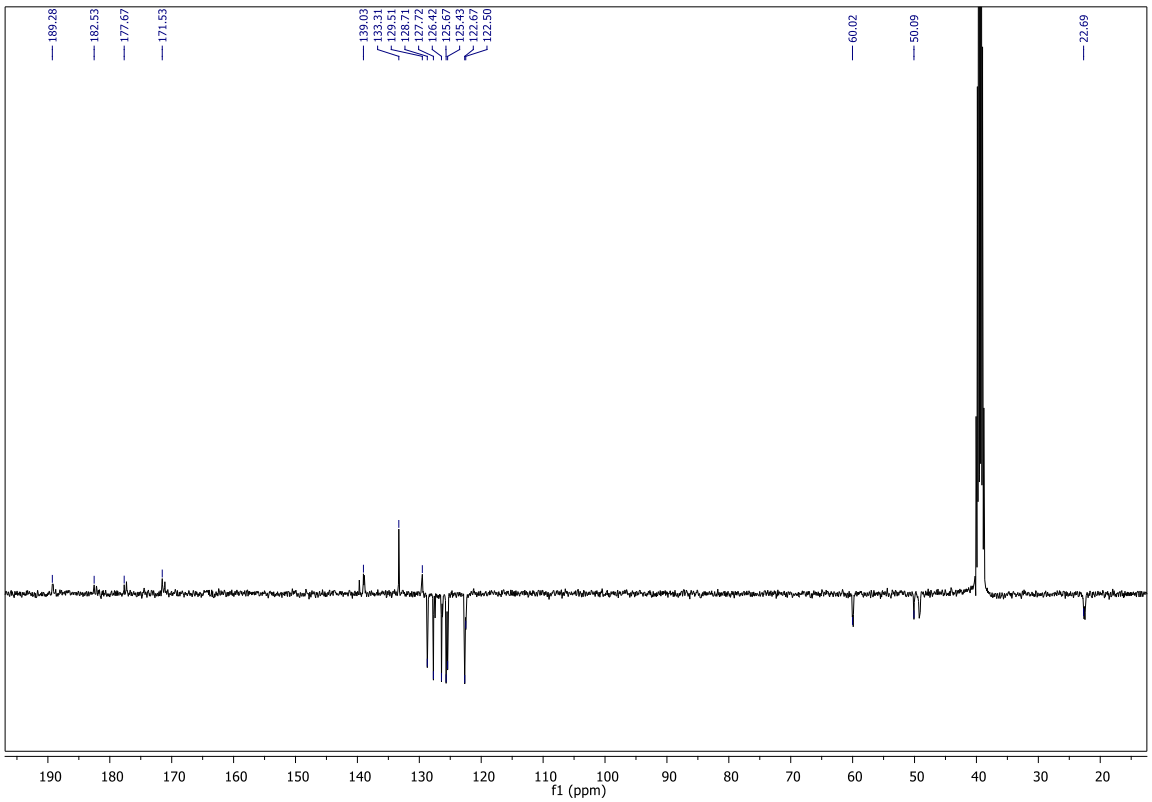
3-(((1*R*,2*S*)-1-Hydroxy-2,3-dihydro-1H-inden-2-yl)amino)-4-methoxycyclobut-3-ene-1,2-dione (11)



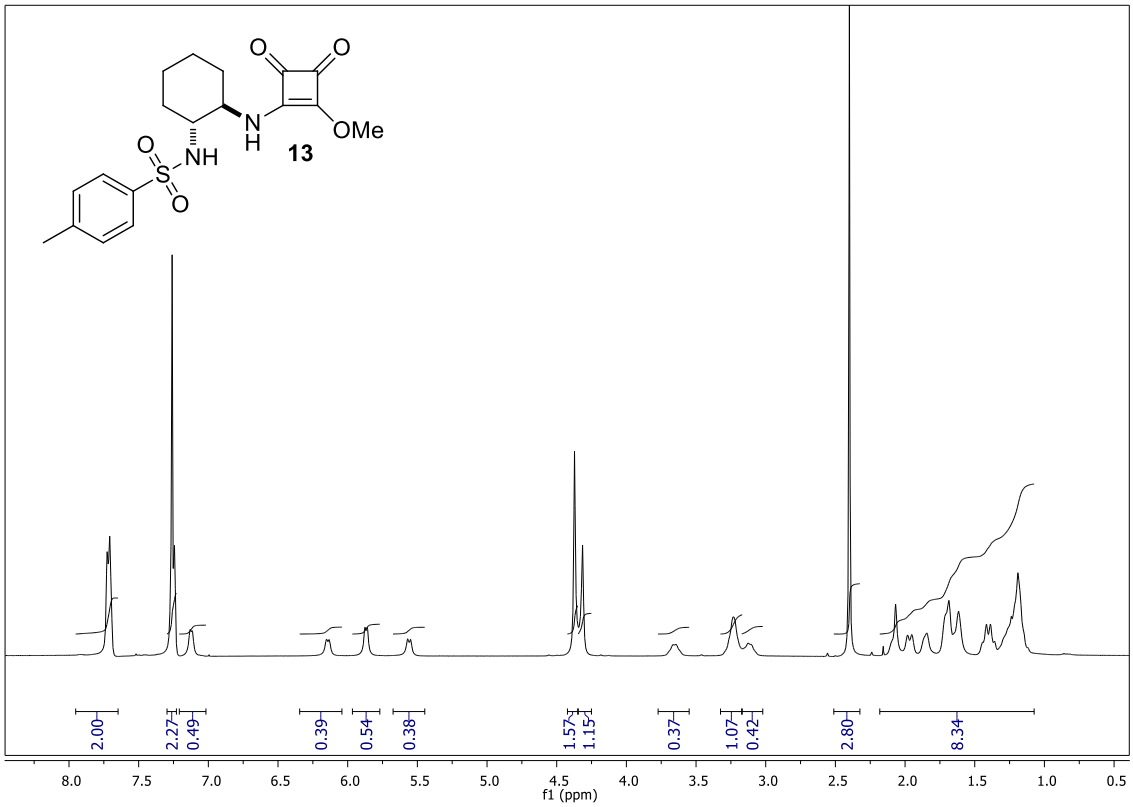


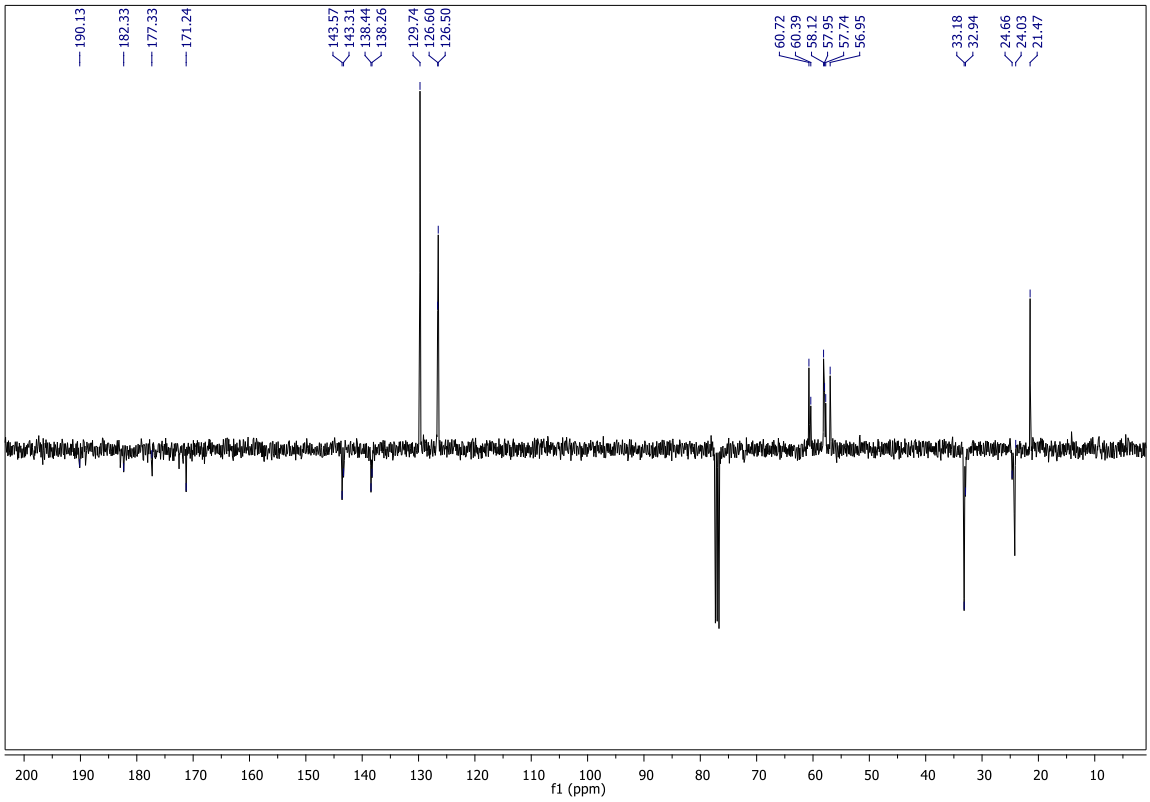
(*S*)-3-Methoxy-4-((1-(naphthalen-1-yl)ethyl)amino)cyclobut-3-ene-1,2-dione (12)





***N*-((1*R*,2*R*)-2-((2-Methoxy-3,4-dioxocyclobut-1-en-1-yl)amino)cyclohexyl)-4-methylbenzenesulfonamide (13)**





3-Methoxy-4-(methyl(phenyl)amino)cyclobut-3-ene-1,2-dione (14)