Supporting information

Investigating the Anticancer Activity of Isatin/Dihydropyrazole Hybrids

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Chemistry

General methods

Reagents and solvents were obtained from commercial suppliers and were used without further purification. All melting points were determined by the capillary method on a Stuart SMP30 Digital Advanced apparatus and are uncorrected.

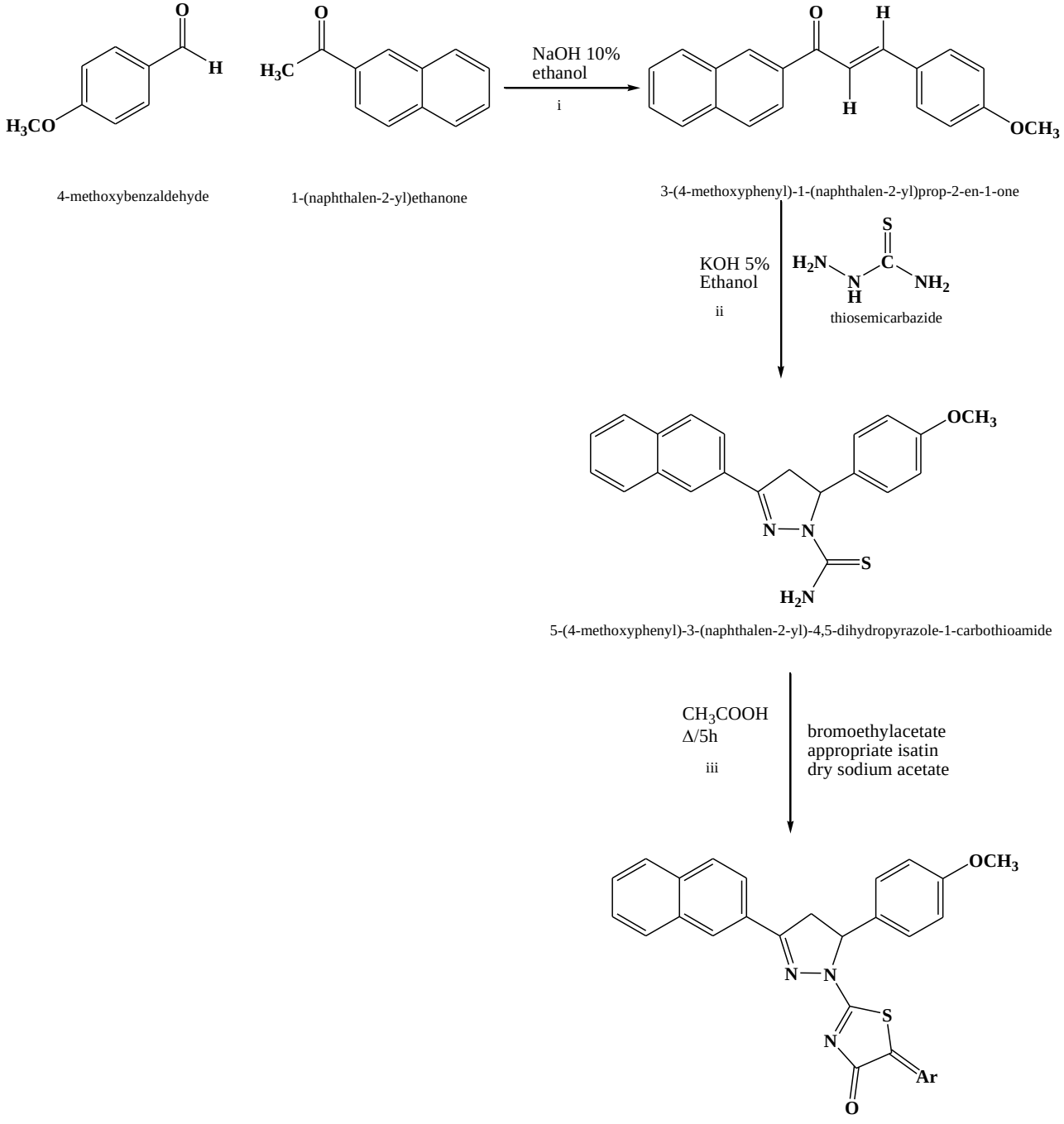
Mass spectra were registered on a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Germany). Compounds were initially dissolved in dimethylsulfoxide (DMSO) at 1 mg/mL concentration. Stock solutions were then diluted 100-fold in ethanol: water 4:1 containing 0.1% of formic acid with exception of the compound **EMAC4000**, which was initially dissolved in DMSO and chloroform (3:1, v/v) at 1 mg/mL concentration stock solution. Mass spectra were acquired on a Q-Exactive mass spectrometer (Thermo Fisher Scientific) via a nano-electrospray interface operating in positive ion mode. Ion transfer tube temperature was 250 °C, whereas S-lens value was 50 units. Full MS spectra were acquired at a resolution of 140,000, in the m/z range 200-800, using an in-source CID of 15 eV in order to minimize the presence of adducts with DMSO. Mass spectra are reported in Figures S2-S13.

^{1}H-NMR and ^{13}C-NMR chemical shifts of compounds EMAC are reported and spectra are depicted in Figures S14-S36. All samples were measured in DMSO-d6 $CDCl_3$-d at 278.1 K temperature on a Bruker 400 MHz or on a Varian 500 MHz spectrometer. Chemical shifts are reported in ppm. Coupling constants J are expressed in hertz (Hz).

TLC chromatography was performed using silica gel plates (Merck F 254), spots were visualized by UV light.

Synthesis and characterization

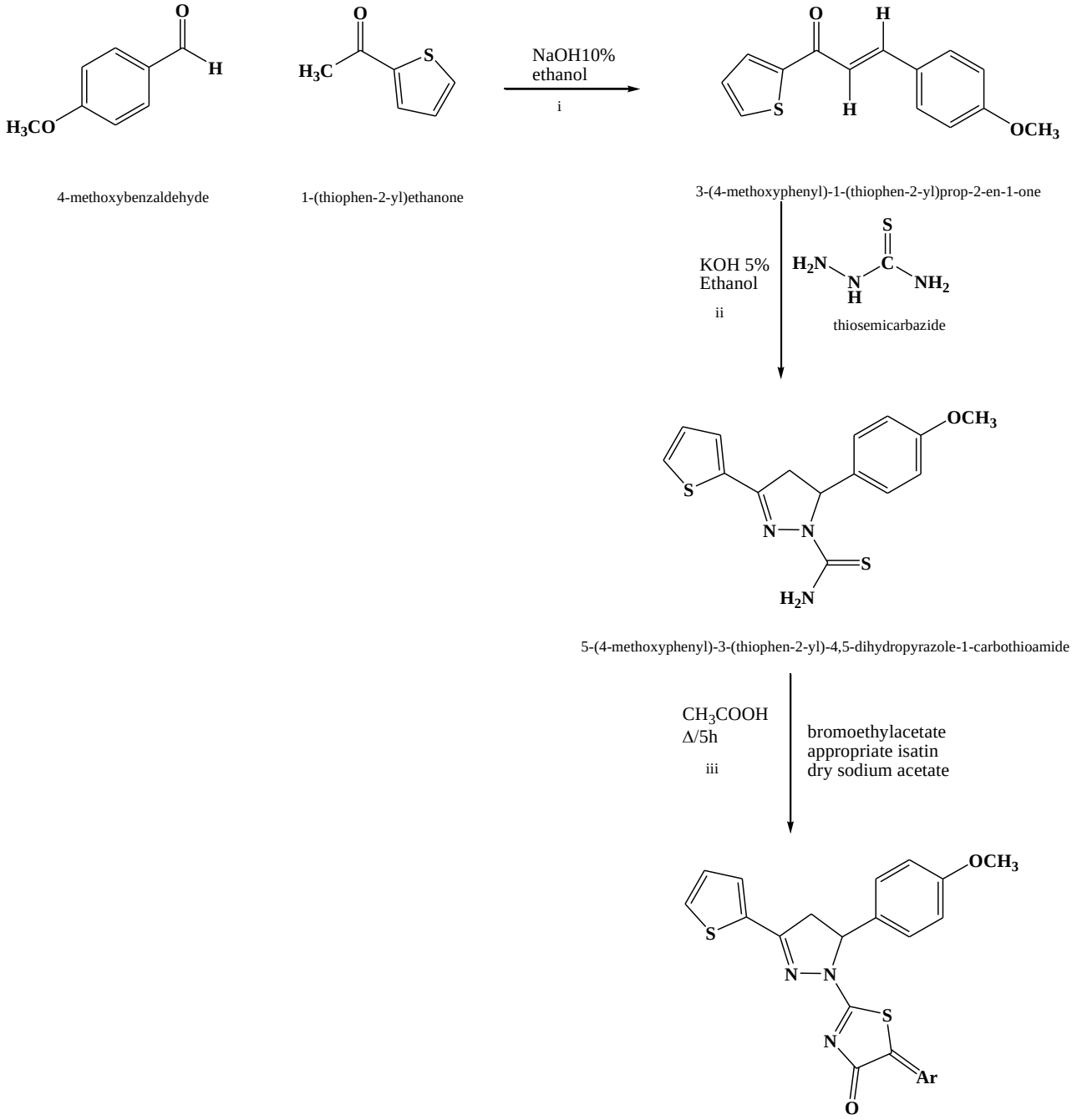
Scheme S1: synthetic pathway for compounds EMAC4000-01-03-05-07-08



EMAC 4000-01-03-05-07-08

Ar: 7-Br-isatin; 5-Cl-isatin;5,7-diCH_3-isatin; 7-F-isatin; 5-OCH_3-isatin; 5-CH_3-isatin.

Scheme S2: synthetic pathway for compounds EMAC4011-12-14-15-18-19



4-methoxybenzaldehyde

1-(thiophen-2-yl)ethanone

3-(4-methoxyphenyl)-1-(thiophen-2-yl)prop-2-en-1-one

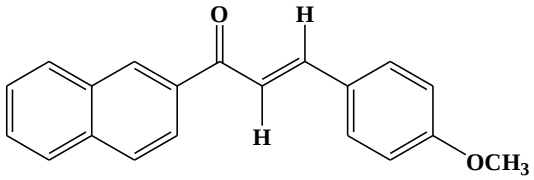
5-(4-methoxyphenyl)-3-(thiophen-2-yl)-4,5-dihydropyrazole-1-carbothioamide

EMAC 4011-12-14-15-18-19

Ar: 7-Br-isatin; 5-Cl-isatin;5,7-diCH_3-isatin; 5-F-isatin; 5-OCH_3-isatin; 5-CH_3-isatin.

Procedures

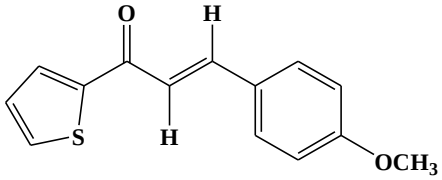
Synthesis of 3-(4-methoxy-phenyl)-1-(naphtalen-2-yl)-2-propen-1-one



An aqueous solution of NaOH 10% (1.2 mmol; 480 μL) was slowly added to a solution of 2-acetyl-naphtalene (1 mmol; 170 mg) in ethanol. The mixture was vigorously stirred, until to obtain a cloudy solution. Then, a solution in ethanol of 4-methoxybenzaldehyde (1.2 mmol; 163 mg) was added dropwise, obtaining a light-yellow suspension. The obtained solid was filtered, washed with water and cold ethanol to obtain a light-yellow solid. This crude product was crystallized from ethanol. The whole reaction was carried out keeping the temperature around 0°C and its progression monitored with TLC, using ethyl acetate/petroleum ether 1:1 as eluent.
Yellow solid; Yield: 88%; MW: 288.34 g/mol; Mp: 90-92°C.

^{1}H NMR (500 MHz, DMSO-d_6): δ 8.92 (s, 1H, -CH aromatic), 8.17 (d, 1H, -CH aromatic, J_o=8), 8.14 (dd, 1H, -CH aromatic, J_o=8.5; J_m=2), 8.07 (d, 1H, -CH aromatic, J_o=8.5), 8.03 (d, 1H, -CH aromatic, J_o=8), 8.00 (d, 1H, -CH propen, J=15.5), 7.91 (d, 2H, -CH aromatic, J_o=8.5), 7.80 (d, 1H, -CH propen, J=15.5), 7.70 (td, 1H, -CH aromatic, J_o=8.5; J_m=2), 7.66 (td, 1H, -CH aromatic, J_o=8.5; J_m=2), 7.06 (d, 2H, -CH aromatic, J_o=8.5), 3.85 (s, 3H, -OCH_3)

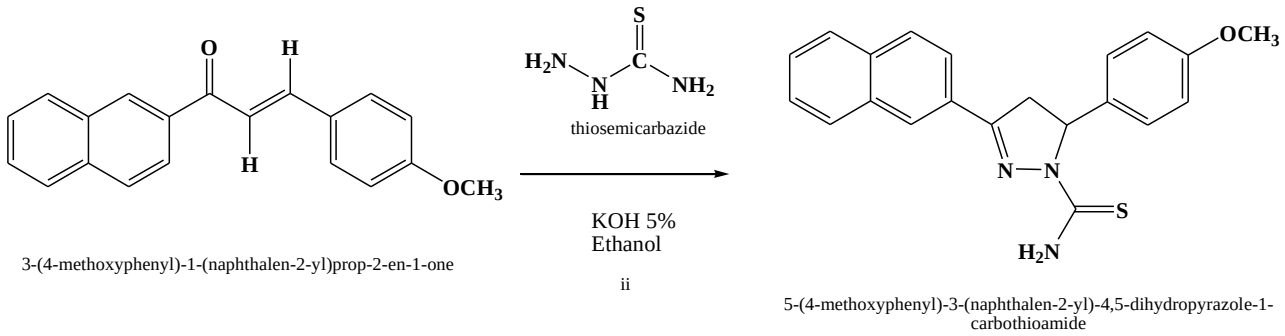
Synthesis of 3-(4-methoxyphenyl)-1-(thiophen-2-yl)-2-propen-1-one.



An aqueous solution of NaOH 10% (1.2 mmol; 480 μL) was slowly added to a solution of 2-acetyl-thiophene (1 mmol; 130 mg) in ethanol. The mixture was vigorously stirred, until to obtain a cloudy solution. Then, a solution in ethanol of 4-methoxybenzaldehyde (1.2 mmol; 163 mg) was added dropwise, obtaining a light-yellow solution. The whole reaction was carried out keeping the temperature around 0°C and its progression monitored with TLC, using ethyl acetate/petroleum ether 1:1 as eluent. Since the reaction product is soluble in ethanol, the solution is concentrated in vacuum to halve the volume and poured in ~ 20 g of chipped ice, obtaining a yellow precipitate. The solid was filtered off and washed with fresh water, obtaining a light-yellow powder that crystallized from isopropanol.
Yellow solid; Yield: 86%; MW: 244.31 g/mol; M.p.: 70°C.

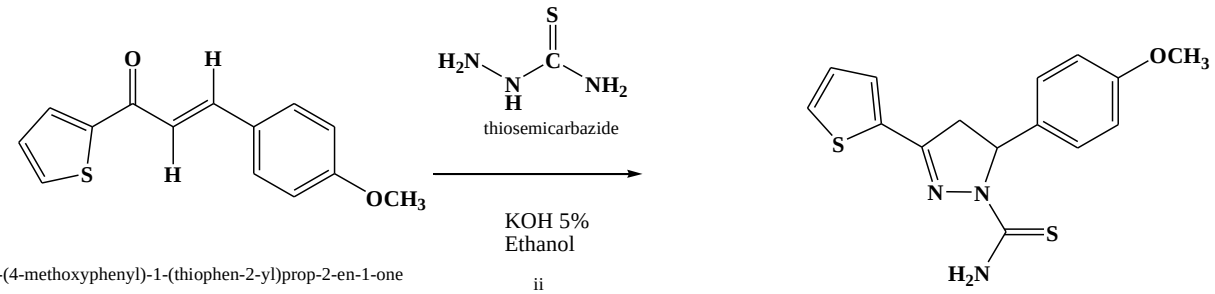
^{1}H NMR (500 MHz, chloroform-d): δ 7.87 (d, 1H, -CH thioph., J=3.5), 7.85 (d, 1H, -CH propen, J=15.5), 7.65 (d, 1H, -CH thioph., J=5), 7.63 (d, 2H, -CH aromatic, J_o=8.5), 7.33 (d, 1H, -CH propen, J=15.5), 7.20 (t, 1H, -CH thioph., J=3.5; J=5), 6.97 (d, 2H, -CH aromatic, J_o=8.5), 3.88 (s, 3H, -OCH_3).

Scheme S3: synthetic pathway for the synthesis of 5-(4-methoxyphenyl)-3-(aryliden-2-yl)-4,5-dihydropyrazole-1-carbothioamide



3-(4-methoxyphenyl)-1-(naphthalen-2-yl)prop-2-en-1-one

5-(4-methoxyphenyl)-3-(naphthalen-2-yl)-4,5-dihydropyrazole-1-carbothioamide



3-(4-methoxyphenyl)-1-(thiophen-2-yl)prop-2-en-1-one

5-(4-methoxyphenyl)-3-(thiophen-2-yl)-4,5-dihydropyrazole-1-carbothioamide

The reaction of pyrazoline formation is not enantioselective, both enantiomers have been obtained. Therefore, each final compound exists as a couple of enantiomers. The structure of each compound has been elucidated by ^{1}H-NMR spectroscopy. The 3,5-diaryl carbothioamide pyrazolines were detected by -CH_2 and -CH protons of the ring and, when detectable, by the -NH_2 signal of thiocarbamoil group that appears as a broad singlet at δ 7.2-7.1 ppm. In fact, the group –CH_2CH-Ar is part of a AMX system that gives raises to a characteristic set of three doublets of doublets. The AMX system is depicted in the Figure S1, where an enlargement of the NMR spectrum shows the coupling effect on the pyrazoline system. The -CH_2 resonates as a pair of doublets of doublets at δ 3.63-3.73 ppm (HA), 4.24-4.31 ppm (HM). The -CH proton appears as a doublet of doublets at δ 5.95-5.99 (HX) ppm due to vicinal coupling with two magnetically non-equivalent protons of the methylene group at position 4 of the pyrazoline ring (J_{AM} 18.00 Hz, J_{AX} 4 Hz, J_{MX} 11 Hz).

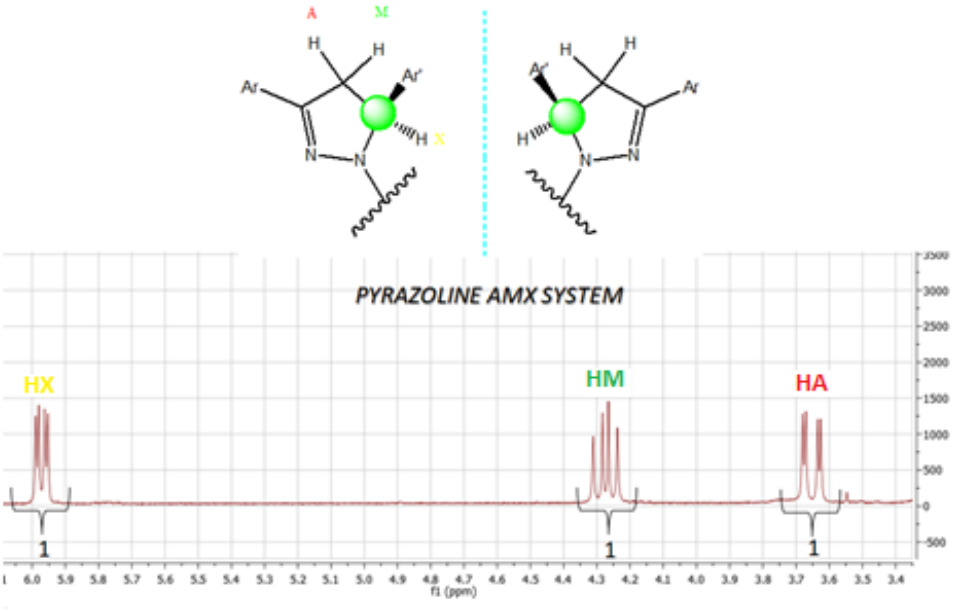
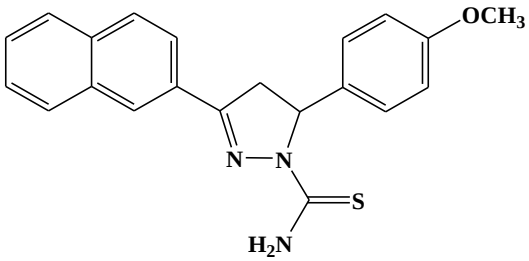


Figure S1: Coupling effect on the pyrazoline system.

Procedures

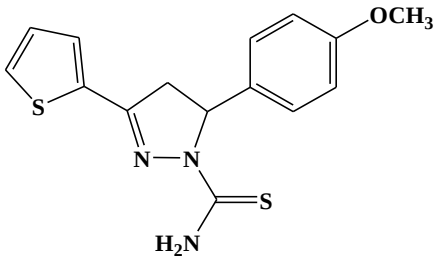
Synthesis of 5-(4-methoxyphenyl)-3-(naphthalen-2-yl)-4,5-dihydropyrazole-1-carbothioamide



A freshly prepared solution of alcoholic KOH 5% (1.2 mmol; 1.3 mL) was added dropwise to a mixture of 3-(4-methoxy-phenyl)-1-(naphtalen-2-yl)-2-propenone (1 mmol, 288 mg) and thiosemicarbazide (1.2 mmol; 110 mg) in ethanol. This solution was refluxed until reaction completion, checking the progression with TLC. This solution was cooled at room temperature, obtaining a yellow suspension. The solid was filtered out, washed with water and crystallized from ethanol.
Yellow solid; Yield:74%; MW: 361.43 g/mol; Mp 210-211°C.

^{1}H NMR (500 MHz, chloroform-d): δ 8.02 (d, 1H, -CH aromatic, Jo=8.5), 7.98 (s, 1H, -CH aromatic), 7.89-7.84 (m, 3H, -CH aromatic), 7.58-7.52 (m, 2H, -CH aromatic), 7.20 (d, 2H, -CH aromatic, Jo=8), 7.15 (bs, 1H, -NH thiocarbamoyl), 6.87 (d, 2H, -CH aromatic, Jo=8), 6.06 (dd, 1H, -CH_x pyrazoline, J_{AX}=4; J_{MX}=11), 3.94 (dd, 1H -CH_M pyrazoline, J_{MA}=18.5; J_{MX}=11), 3.78 (s, 3H, -OCH_3), 3.36 (dd, 1H, -CH_A pyrazoline, J_{AM}=18.5; J_{AX}=4).

Synthesis of 5-(4-methoxyphenyl)-3-(thiophen-2-yl)-4,5-dihydropyrazole-1-carbothioamide



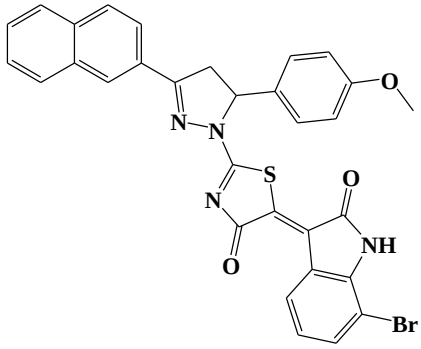
To a mixture of 3-(4-methoxy-phenyl)-1-(thiophen-2-yl)-2-propenone (1 mmol; 244 mg) and thiosemicarbazide (1.2 mmol; 110 mg) in ethanol a freshly prepared solution of alcoholic KOH 5% (1.2 mmol; 1.3 mL) was added dropwise at room temperature. After 1h the temperature was gradually increased to 50°C and maintained at this level until reaction completion. The reaction was monitored with TLC, using ethyl acetate/petroleum ether 1:1 as eluent. The obtained pyrazoline was crystallized from ethanol, obtaining a light-yellow solid.
Yellow solid; Yield:74%; MW: 317.43 g/mol; Mp 150-152 °C.

^{1}H NMR (500 MHz, chloroform-d): δ 7.50 (d, 1H, -CH thioph., $J_{4\text{-}5}$=5), 7.28 (solvent peak and -CH thioph. signal) , 7.19 (d, 2H, -CH aromatic, Jo=8.5), 7.11 (t, 1H, -CH thioph., J=5; J=4) , 7.04 (bs, 2H, -NH_2 thiocarbamoil), 6.88 (d, 2H, -CH aromatic, Jo=8.5), 6.01 (dd, 1H, -CH_X pyrazoline, J_{XA}=3.5; J_{XM}=11.5), 3.86 (dd, 1H, -CH_M pyrazoline, J_{MX}=11.5; J_{MA}=18.5), 3.80 (s, 3H, -OCH_3), 3.20 (dd, 1H, -CH_A pyrazoline, J_{AM}=18.5; J_{AX}=3.5).

General method for the synthesis of EMAC4000,-01,-03,-05,-07,-08 and EMAC4011,-12,-14,-15,-18,-19

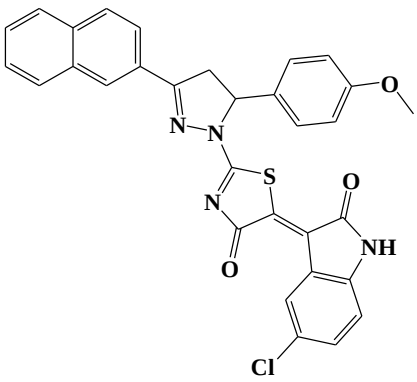
A mixture of 5-(4-methoxyphenyl)-3-(naphtalen-2-yl)-1-thiocarbamoyl-2-pyrazoline or 5-(4-methoxyphenyl)-3-(thiophen-2-yl)-1-thiocarbamoyl-2-pyrazoline (1.0 mmol), ethyl bromoacetate (1.0 mmol), appropriate isatin (1.2 mmol), and anhydrous sodium acetate (2.0 mmol) was refluxed in glacial acetic acid (5 mL) until reaction completion (TLC ethyl acetate/petroleum ether 1:1). The mixture was cooled to room temperature and the obtained precipitate was filtered, washed with water and crystallized from methanol/water.

7-bromo-3-{2-[5-(4-methoxyphenyl)-3-(naphtalen-2-yl)-4,5-dihydropyrazol-1-yl]}-4-oxothiazol-5(4H)-ylidene)-indolin-2-one (EMAC4000)



^{1}H-NMR: (400 MHz, DMSO) δ: 11.46 (s, 1H, -NH isatin), 8.96 (d, 1H, -CH aromatic, J=8), 8.43 (s, 1H, -CH aromatic), 8.14 -8.02 (m, 4H, -CH aromatic), 7.68-7.56 (m, 2H, -CH aromatic) , 7.55 (d, 1H, -CH aromatic, J=8), 7.27 (d, 2H, -CH aromatic, J=8), 7.02 (t, 1H, -CH aromatic, J=8), 6.95 (d, 2H, -CH aromatic, J=8), 5.99 (dd, 1H, $-CH_X$ pyrazoline, J_{XA}=4; J_{XM}=11), 4.29 (dd, 1H, $-CH_M$ pyrazoline, J_{MA}=18.5; J_{MX}=11), 3.73 (s, -3H, $-OCH_3$), 3.69 (dd, 1H, $-CH_A$ pyrazoline, J_{AM}=18.5; J_{AX}=4).

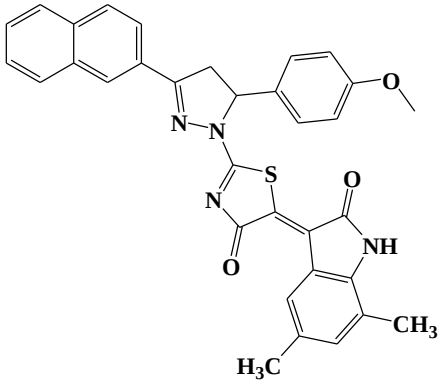
5- chloro-3-{2-[5-(4-methoxyphenyl)-3-(naphtalen-2-yl)-4,5-dihydropyrazol-1-yl]}-4-oxothiazol-5(4H)-ylidene)-indolin-2-one (EMAC4001)



^{1}H-NMR: (400 MHz, DMSO) δ: 11.30 (s, 1H, -NH isatin), 8.97 (s, 1H,-CH aromatic), 8.42 (s, 1H, -CH aromatic), 8.13- 8.01 (m, 4H, -CH aromatic), 7.67-7.60 (m, 2H, -CH aromatic), 7.40 (dd, 1H, -CH aromatic, J_o=8; J_m=2), 7.28 (d, 2H, -CH aromatic, J_o=8), 6.94 (m, 3H), 5.98 (dd, 1H, $-CH_X$ pyrazoline, J_{AX}=4; J_{MX}=11), 4.29 (dd, 1H, $-CH_M$ pyrazoline, J_{MA}=18.5; J_{MX}=11), 3.74 (s, -3H, $-OCH_3$), 3.67 (dd, 1H, $-CH_A$ pyrazoline, J_{AM}=18.5; J_{AX}=4).

^{13}C NMR (101 MHz, DMSO) δ:178.97 (1C), 172.63 (1C), 168.75 (1C), 162.84 (1C), 158.99 (1C), 141.85 (1C), 138.89 (1C), 134.29 (1C), 132.54 (1C), 131.89 (1C), 130.99 (1C), 129.37 (1C), 128.86 (1C), 128.71 (1C), 128.17 (1C), 127.82 (1C), 127.36 (2C), 127,25 (1C), 127.12 (1C), 126.99 (1C), 125.62 (1C), 124.86 (1C), 123.23 (1C), 121.63 (1C), 114.27 (2C), 63.63 (1C), 55.11 (2C), 43.32 (1C).

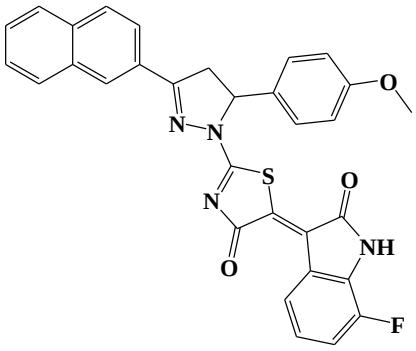
5,7-dimethyl-3-{2-[5-(4-methoxyphenyl)-3-(naphtalen-2-yl)-4,5-dihydropyrazol-1-yl]}-4-oxothiazol-5(4H)-ylidene)-indolin-2-one (EMAC4003)



^{1}H-NMR: (400 MHz, DMSO) δ: 11.08 (s, 1H, -NH isatin), 8.63 (s, 1H, -CH aromatic), 8.43 (s, 1H, -CH aromatic), 8.13 (d, 1H, -CH aromatic, J=8.4), 8.09-8.01 (m, 3H, -CH aromatic) 7.67- 7.60 (m, 2H, -CH aromatic), 7.27 (d, 2H, -CH aromatic, J=8.4), 7.01 (s, 1H,-CH aromatic), 6.94 (d, 2H, -CH aromatic, J=8.4), 5.97 (dd, 1H, -CH_x pyrazoline, J_{AX}=4; J_{MX}=11) , 4.27 (dd, 1H -C_{HM} pyrazoline, J_{MA}=18.5; J_{MX}=11), 3.73 (s, 3H, -OCH_3), 3.65 (dd, 1H, -CH_A pyrazoline, J_{AM}=18.5; J_{AX}=4), 2.25 (s, 3H, -CH_3), 2.20 (s, 3H, -CH_3).

^{13}C NMR (101 MHz, DMSO) δ:178.96 (1C), 172.90 (1C), 169.47 (1C), 162.25 (1C), 158.93 (1C), 139.53 (1C), 136.43 (1C), 134.25 (1C), 133.69 (1C), 132.56 (1C), 132.02 (1C), 130.27 (1C), 129.21 (1C), 128.84 (1C), 128.67 (1C), 128.11 (1C), 127.81 (2C), 127.29 (1C), 127.11 (1C), 126.74 (1C), 125.88 (1C), 123.24 (1C), 120.18 (1C), 118,98 (1C), 114.26 (2C), 63.45 (1C), 55.10 (2C), 43.26 (1C), 20.83 (1C), 16.23 (1C).

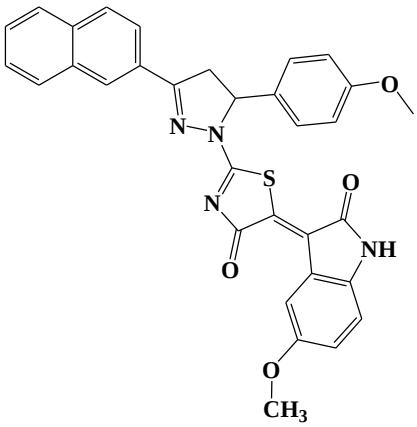
7-fluoro-3-{2-[5-(4-methoxyphenyl)-3-(naphtalen-2-yl)-4,5-dihydropyrazol-1-yl]}-4-oxothiazol-5(4H)-ylidene)-indolin-2-one (EMAC4005)



^{1}H-NMR: (400 MHz, DMSO) δ: 11.68 (s, 1H, -NH isatin), 8.77 (d, 1H, -CH aromatic, J=7.6), 8.41 (s, 1H, -CH aromatic), 8.13 -8.00 (m, 4H, -CH aromatic), 7.67-7.60 (m, 2H, -CH aromatic) , 7.28 (m, 3H, -CH aromatic), 7.08-7.02 (m, 1H, -CH aromatic), 6.95(d, 1H, -CH aromatic, J=8.8), 5.97 (dd, 1H, -CH_X pyrazoline, J_{XA}=4; J_{XM}=11), 4.28 (dd, 1H, -CH_M pyrazoline, J_{MA}=18.5; J_{MX}=11), 3.73 (s, 3H, -OCH_3), 3.66 (dd, 1H, -CH_A pyrazoline, J_{AM}=18.5; J_{AX}=4).

^{13}C NMR (101 MHz, DMSO) δ:178.74 (1C), 172.55 (1C), 168.83 (1C), 162.66 (1C), 158.97 (1C), 147.71 (1C), 145.31 (1C), 138.86 (1C), 134.27 (1C), 132.53 (1C), 131.95 (1C), 129.33 (1C), 128.84 (1C), 128.68 (1C), 128.14 (1C), 127.81 (1C), 127.28 (2C), 127.09 (1C), 127.00 (1C), 125,16 (1C), 123.92 (1C), 123.21 (1C), 123,03 (1C), 122.31 (1C), 114.29 (2C), 63.58 (1C), 55.10 (2C), 43.36 (1C).

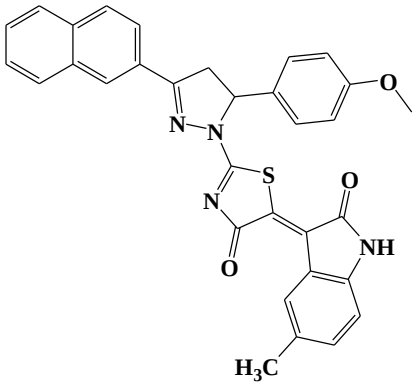
5-methoxy-3-{2-[5-(4-methoxyphenyl)-3-(naphtalen-2-yl)-4,5-dihydropyrazol-1-yl]}-4-oxothiazol-5(4H)-ylidene)-indolin-2-one (EMAC4007)



^{1}H-NMR: (400 MHz, DMSO) δ: 10.98 (s, 1H, -NH isatin), 8.65 (s, 1H, -CH aromatic), 8.42 (s, 1H, -CH aromatic), 8.13-8.01 (m, 4H, -CH aromatic), 7.67-7.61 (m, 2H, -CH aromatic), 7.27 (d, 2H, -CH aromatic, J=8.8), 6.98-6.93 (m, 3H, -CH aromatic), 6.84 (d, 1H, aromatic, J=8.4), 5.97 (dd, 1H, -CH_X pyrazoline, J_{XA}=4; J_{XM}=11), 4.28 (dd, 1H, -CH_M pyrazoline, J_{MA}=18.5; J_{MX}=11) 3.74 (s, 3H, -OCH_3), 3.73 (s, 3H, -OCH_3), 3.66 (dd, 1H, -CH_A pyrazoline, J_{AM}=18.5; J_{AX}=4).

^{13}C NMR (101 MHz, DMSO) δ: 179.04 (1C), 172.91 (1C), 170.55 (1C), 168.96 (1C), 162.46 (1C), 158.99 (1C), 154.52 (1C), 137.16 (1C), 136.97 (1C), 134.28 (1C), 132.57 (1C), 132.00 (1C), 129.73 (1C), 128.85 (1C), 128.13 (1C), 127.83 (1C), 127.35 (2C), 127.10 (1C), 126.57 (1C), 123.25 (1C), 121.01 (1C), 117.27 (1C), 114.28 (2C), 114.07 (1C), 110.40 (1C), 63.57 (1C), 55.44 (1C), 55.12 (2C), 43.27 (1C).

5-methyl-3-{2-[5-(4-methoxyphenyl)-3-(naphtalen-2-yl)-4,5-dihydropyrazol-1-yl]}-4-oxothiazol-5(4H)-ylidene)-indolin-2-one (EMAC4008)

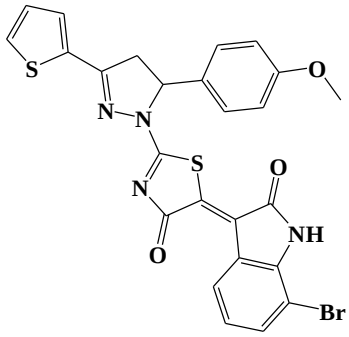


^{1}H-NMR: (400 MHz, DMSO) δ: 11.06 (s, 1H, -NH isatin), 8.76 (s, 1H, -CH aromatic), 8.42 (s, 1H, -CH aromatic), 8.13-8.01 (m, 4H, -CH aromatic), 7.67-7.60 (m, 2H, -CH aromatic), 7.27 (d, 2H , -CH aromatic, J=8.8), 7.17 (d, 1H, -CH aromatic, J=8), 6.95 (d, 2H, -CH aromatic, J=8.8), 6.82 (d, 1H, -CH aromatic, J=8),

5.98 (dd, 1H, -CH_X pyrazoline, J_{XA}=4; J_{XM}=11), 4.27 (dd, 1H, -CH_M pyrazoline, J_{MA}=18.5; J_{MX}=11), 3.73 (s, 3H, -OCH_3), 3.68 (dd, 1H, -CH_A pyrazoline, J_{AM}=18.5; J_{AX}=4), 2.28 (s, 3H, -CH_3).

^{13}C NMR (101 MHz, DMSO) δ:178.98 (1C), 172.63 (1C), 169.02 (1C), 162.33 (1C), 158.95 (1C), 140.95 (1C), 136.65 (1C), 134.25 (1C), 132.56 (1C), 132.17 (1C), 132.02 (1C), 130.43 (1C), 129.23 (1C), 128.84 (1C), 128.69 (1C), 128.39 (1C), 127.82 (1C), 127.31 (2C), 127.09 (1C), 126.29 (1C), 123.23 (1C), 122.75 (1C), 120.41 (1C), 114.26 (2C), 109.82 (1C), 63.48 (1C), 55.10 (2C), 40.17 (1C), 20.97 (1C).

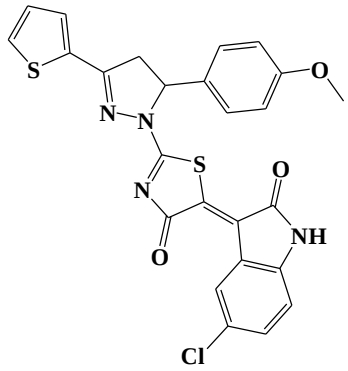
7-bromo-3-{2-[5-(4-methoxyphenyl)-3-(thiophen-2-yl)-4,5-dihydropyrazol-1-yl]}-4-oxothiazol-5(4H)-ylidene)-indolin-2-one (EMAC4011)



^{1}H-NMR: (400 MHz, DMSO) δ: 11.43 (s, 1H, -NH isatin), 8.95 (d, 1H, -CH aromatic, J=8), 7.97 (d, -1H, -CH thioph., J=4.4), 7.73 (d, 1H, -CH thioph., J=2.8), 7.55 (d, 1H, -CH aromatic, J=8), 7.26-7.21 (m, 3H, -CH aromatic), 7.02 (t, 1H, -CH aromatic, J=8), 6.94 (d, 2H, -CH aromatic, J=8.4), 5.92 (dd, 1H, -CH_X pyrazoline, J_{XA}=3.5; J_{XM}=11.5), 4.18 (dd, 1H, -CH_M pyrazoline, J_{MX}=11.5; J_{MA}=18.5), 3.73 (s, 3H, -OCH_3), 3.52 (dd, 1H, -CH_A pyrazoline, J_{AM}=18.5; J_{AX}=3.5).

^{13}C NMR (101 MHz, DMSO) δ:178.63 (1C), 172.05 (1C), 168.90 (1C), 158.97 (1C), 158.04 (1C), 142.08 (1C), 139.06 (1C), 133.34 (1C), 132.38 (1C), 132.26 (1C), 131.72 (1C), 128.62 (1C), 127.19 (1C), 126.80 (2C), 125.46 (1C), 123.31 (1C), 122,12 (1C), 114.31 (2C), 102.4 (1C), 63.57 (1C), 55.11 (2C), 43.91 (1C).

5-chloro-3-{2-[5-(4-methoxyphenyl)-3-(thiophen-2-yl)-4,5-dihydropyrazol-1-yl]}-4-oxothiazol-5(4H)-ylidene)-indolin-2-one (EMAC4012)

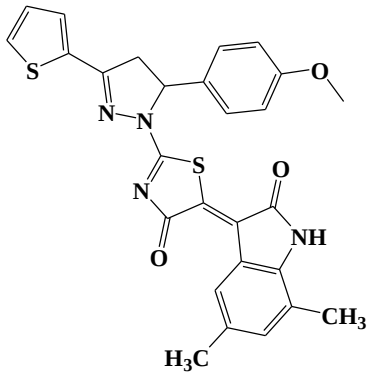


^{1}H-NMR: (400 MHz, DMSO) δ: 11.28 (s, 1H, -NH isatin), 8.96 (s, 1H, -CH aromatic), 7.97 (d, -1H, -CH thioph., J=4.8), 7.74 (d, 1H, -CH thioph., J=4), 7.38 (dd, 1H, -CH aromatic, J=8, J=2), 7.26- 7.21 (m, 3H, -CH aromatic), 6.95-6.91 (m, 3H, -CH aromatic), 5.92 (dd, 1H, -CH_X pyrazoline, J_{XA}=3.5; J_{XM}=11.5), 4.18 (dd, 1H,

-CH_M pyrazoline, J_{MX}=11.5; J_{MA}=18.5), 3.73 (s, 3H, -OCH_3), 3.53 (dd, 1H, -CH_A pyrazoline, J_{AM}=18.5; J_{AX}=3.5)

^{13}C NMR (101 MHz, DMSO) δ:178.83 (1C), 172.09 (1C), 168.72 (1C), 158.99 (1C), 158.08 (1C), 141.81 (1C), 138.85 (1C), 133.34 (1C), 132.39 (1C), 132.24 (1C), 131.67 (1C), 130.94 (1C), 128.59 (1C), 127.26 (2C), 125.60 (1C), 124.75 (1C), 121,61 (1C), 114.28 (2C), 111.48 (1C), 63.59 (1C), 55.11 (2C), 43.88 (1C).

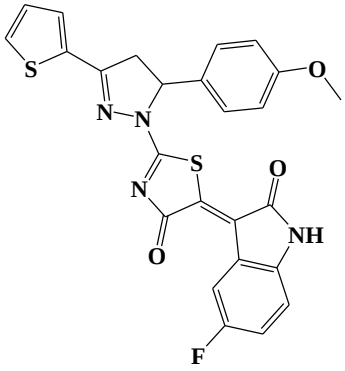
5,7-dimethyl-3-{2-[5-(4-methoxyphenyl)-3-(thiophen-2-yl)-4,5-dihydropyrazol-1-yl]}-4-oxothiazol-5(4H)-ylidene)-indolin-2-one (EMAC4014)



^{1}H-NMR: (400 MHz, DMSO) δ: 11.08 (s, 1H, -NH isatin), 8.45 (s, 1H,-CH isatin), 7.71 (d, 1H, -CH thioph., J=4), 7.26-7.21 (m, 3H), 7.03-6.93 (m, 4H), 5.90 (dd, 1H, -CH_X pyrazoline, J_{AX}=3,5; J_{MX}=11,5), 4.15 (dd, 1H, -CH_M pyrazoline, J_{MA}=18,5; J_{MX}=11,5), 3.73 (s, 3H, -OCH_3), 3.51 (dd, 1H, -CH_A pyrazoline, J_{AM}=16; J_{AX}=3,5), 2.26 (s, 3H, -CH_3), 2.24 (s, 3H, -CH_3).

^{13}C NMR (101 MHz, DMSO) δ:178.83 (1C), 172.39 (1C), 168.76 (1C), 158.95 (1C), 157.53 (1C), 140.07 (1C), 136.36 (1C), 134.34 (1C), 133.10 (1C), 132.38 (1C), 131.83 (1C), 130.45 (1C), 130.23 (1C), 127.21 (2C), 127.03 (1C), 126.58 (1C), 125.74 (1C), 119.24 (1C), 114.26 (2C), 63.40 (1C), 55.10 (2C), 43.81 (1C), 20.87 (1C), 20.81 (1C)

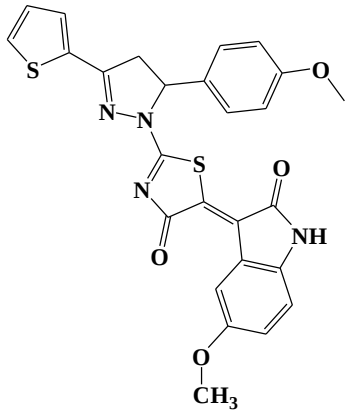
5-fluoro-3-{2-[5-(4-methoxyphenyl)-3-(thiophen-2-yl)-4,5-dihydropyrazol-1-yl]}-4-oxothiazol-5(4H)-ylidene)-indolin-2-one (EMAC4015)



^{1}H-NMR: (400 MHz, DMSO) δ: 11.17 (s, 1H, -NH isatin), 8.73 (dd, 1H, -CH aromatic, J=2.8; J=10.4), 7.97 (dd, 1H, -CH thioph., J=4.8, J=0.4), 7.71 (d, 1H, -CH thioph., J=3.2), 7.26-7.19 ((m, 4H, -CH aromatic), 6.95-6.89 (m, 3H, -CH aromatic), 5.91 (dd, 1H, -CH_X pyrazoline, J_{XA}=3.5; J_{XM}=11.5), 4.17 (dd, 1H, -CH_M pyrazoline, J_{MX}=11.5, J_{MA}=18.5), 3.73 (s, 3H, -OCH_3), 3.53 (dd, 1H, -CH_A pyrazoline, J_{AM}=18.5; J_{AX}=3.5)

^{13}C NMR (101 MHz, DMSO) δ:178.89 (1C), 172.16 (1C), 168.94 (1C), 158.99 (1C), 158.07 (1C), 156.24 (1C), 139.50 (1C), 138.57 (1C), 133.33 (1C), 132.25 (1C), 132.38 (1C), 131.69 (1C), 128.60 (1C), 127.25 (2C), 125.39 (1C), 121.03 (1C), 118.08 (1C), 114.41 (2C), 110.76 (1C), 63.59 (1C), 55.11 (2C), 43.82 (1C)

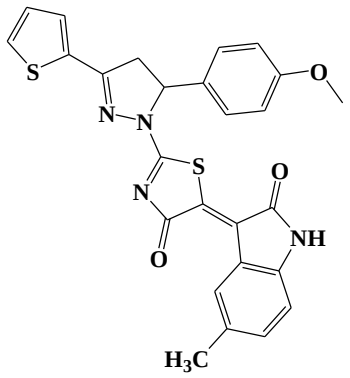
5-methoxy-3-{2-[5-(4-methoxyphenyl)-3-(thiophen-2-yl)-4,5-dihydropyrazol-1-yl]}-4-oxothiazol-5(4H)-ylidene)-indolin-2-one (EMAC4018)



^{1}H-NMR: (400 MHz, DMSO) δ: 10.94 (s, 1H, -NH isatin), 8.63 (d, 1H, -CH aromatic, J=2.8), 7.95 (d, 1H, -CH thioph., J=5.2), 7.70 (d, 1H, -CH thioph., J=3.6), 7.25-7.21 (m, 3H), 6.95-6.92 (m, 3H), 6.81 (d, 1H, -CH aromatic, J=8.4), 5.89 (dd, 1H, -CH_X pyrazoline, J_{XA}=3.5; J_{XM}=11.5), 4.15 (dd, 1H, -CH_M pyrazoline, J_{MX}=11.5; J_{MA}=18.5), 3.73 (s, 3H, -OCH3), 3.72 (s, 3H, -OCH3), 3.51 (dd, 1H, -CH_A pyrazoline, J_{AM}=18.5; J_{AX}=3.5)

^{13}C NMR (101 MHz, DMSO) δ:178.91 (1C), 172.33 (1C), 168.92 (1C), 158.98 (1C), 157.72 (1C), 154.46 (1C), 137.11 (1C), 136.90 (1C), 133.16 (1C), 132.34 (1C), 132.23 (1C), 131.78 (1C), 128.58 (1C), 127.26 (2C), 126.42 (1C), 120.97 (1C), 117.14 (1C), 114.26 (2C), 110.35 (1C), 63.51 (1C), 55,37 (1C), 55.10 (2C), 43.31 (1C)

5-methyl- 3-{2-[5-(4-methoxyphenyl)-3-(thiophen-2-yl)-4,5-dihydropyrazol-1-yl]}-4-oxothiazol-5(4H)-ylidene)-indolin-2-one (EMAC4019)



^{1}H-NMR: (400 MHz, DMSO) δ: 11.03 (s, 1H, -NH isatin), 8.74 (s, 1H,-CH isatin), 7.95 (d, 1H, -CH thioph., J=4.8), 7.70 (d, 1H, -CH thioph., J=3.6), 7.26-7.21 (m, 3H, -CH aromatic), 7.14 (d, 1H, -CH isatin, J=7.6), 6.93 (d, 2H, -CH aromatic, J=8.8) 6.80 (d, 1H, -CH isatin, J=7.6), 5.90 (dd, 1H, -CH_X pyrazoline, J_{AX}=3.6 ; J_{MX}=11.2), 4.14 (dd, 1H, -CH_M pyrazoline, JMA=18; J_{MX}=11.2), 3.73 (s, -3H, - OCH_3), 3.50 (dd, 1H, -CHA pyrazoline, J_{AM}=18; J_{AX}=3.6), 2.27 (s, 1H, -CH_3).

^{13}C NMR (101 MHz, DMSO) δ:178.86 (1C), 172.38 (1C), 168.99 (1C), 158.96 (1C), 157.61 (1C), 140.90 (1C), 136.62 (1C), 133.13 (1C), 132.36 (1C), 132.20 (1C), 132.11 (1C), 131.80 (1C), 128.55 (1C), 128.38 (1C), 127.22 (2C), 126.17 (1C), 120.40 (1C), 114.26 (2C), 109.79 (1C), 63.44 (1C), 55.10 (2C), 43.82 (1C), 20.96 (1C).

Table S1: Experimental and analytical data of compound **EMAC4000,-01,-03,-05,-07,-08** and **EMAC4011,-12,-14,-15,-18,-19.**

Compounds	Yield%	CHN calc. / CHN found	M.p. °C	Crist. solv.	Colour
EMAC4000	80.03	C, 61.09; H, 3.47; N, 9.19 / C, 61.11; H, 3.48; N, 8.99	341-342	H_2O and methanol	Light orange solid
EMAC4001	70.36	C, 65.89; H, 3.75; N, 9.92 / C, 65.87; H, 3.74; N, 9.89	314-317	H_2O and methanol	Red Orange solid
EMAC4003	84.11	C, 70.95; H, 4.69; N, 10.03 / C, 70.98; H, 4.70; N, 10.05	335-336	H_2O and methanol	Light orange solid
EMAC4005	79.06	C, 67.87; H, 3.86; N, 10.21 / C, 67.90; H, 3.87; N, 10.20	308-309	H_2O and methanol	Light orange solid
EMAC4007	72.21	C, 68.56; H, 4.31; N, 9.99 / C, 68.53; H, 4.30; N, 10.27	303-305	H_2O and methanol	Brown solid
EMAC4008	74.33	C, 70.57; H, 4.44; N, 10.29 / C, 70.55; H, 4.46; N, 10.25	288-291	H_2O and methanol	Orange solid
EMAC4011	79.09	C, 53.10; H, 3.03; N, 9.91 / C, 52.98; H, 3.02; N, 9.89	323-324	H_2O and methanol	Orange solid
EMAC4012	75	C, 57.63; H, 3.29; N, 10.75 / C, 57.60; H, 3.30; N, 10.77	307-309	H_2O and methanol	Red solid
EMAC4014	83.5	C, 63.02; H, 4.31; N, 10.89 / C, 62.99; H, 4.32; N, 10.92	>350	H_2O and methanol	Brown solid
EMAC4015	77.8	C, 59.51; H, 3.40; N, 11.10 / C, 59.48; H, 3.40; N, 11.10	318-320	H_2O and methanol	Red solid
EMAC4018	73	C, 60.45; H, 3.90; N, 10.85 / C, 60.43; H, 3.90; N, 10.83	283-284	H_2O and methanol	Brown solid
EMAC4019	75.4	C, 62.38; H, 4.03; N, 11.19 / C, 62.40; H, 4.05; N, 11.21	285-286	H_2O and methanol	Red solid

Mass spectra of compounds EMAC4000,-01,-03,-05,-07,-08 and EMAC4011,-12,-14,-15,-18,-19.

Table S2: Calculated and experimental mass values for **EMAC4000,-01,-03,-05,-07,-08** and **EMAC4011,-12,-14,-15,-18,-19**.

Compounds	Molecular Formula	Calculated $[M+H]^+$	Found m/z, $[M+H]^+$	delta m/z (ppm)
EMAC4000	$C_{31}H_{21}BrN_4O_3S$	609.0591	609.0593	0.3
EMAC4001	$C_{31}H_{21}ClN_4O_3S$	565.1096	565.11	0.7
EMAC4003	$C_{33}H_{26}N_4O_3S$	559.1798	559.1801	0.5
EMAC4005	$C_{31}H_{21}FN_4O_3S$	549.1391	549.1395	0.7
EMAC4007	$C_{32}H_{24}N_4O_4S$	561.1591	561.1594	0.5
EMAC4008	$C_{32}H_{24}N_4O_3S$	545.1642	545.1644	0.4
EMAC4011	$C_{25}H_{17}BrN_4O_3S_2$	564.9998	565	0.4
EMAC4012	$C_{25}H_{17}ClN_4O_3S_2$	521.0503	521.0506	0.6
EMAC4014	$C_{27}H_{22}N_4O_3S_2$	515.1206	515.1208	0.4
EMAC4015	$C_{25}H_{17}FN_4O_3S_2$	505.0799	505.0801	0.4
EMAC4018	$C_{26}H_{20}N_4O_4S_2$	517.0999	517.1002	0.6
EMAC4019	$C_{26}H_{20}N_4O_3S_2$	501.105	501.105	0.0

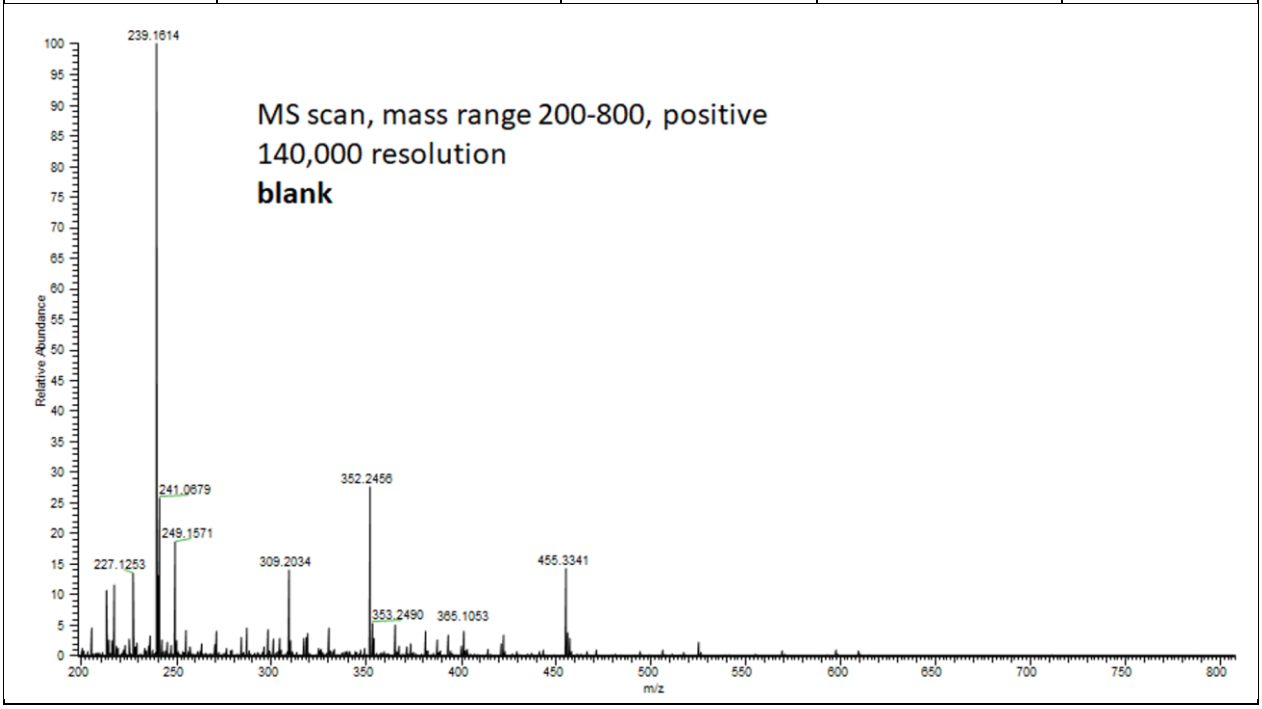


Figure S2: Full Mass Spectrum of compound **EMAC4000.**

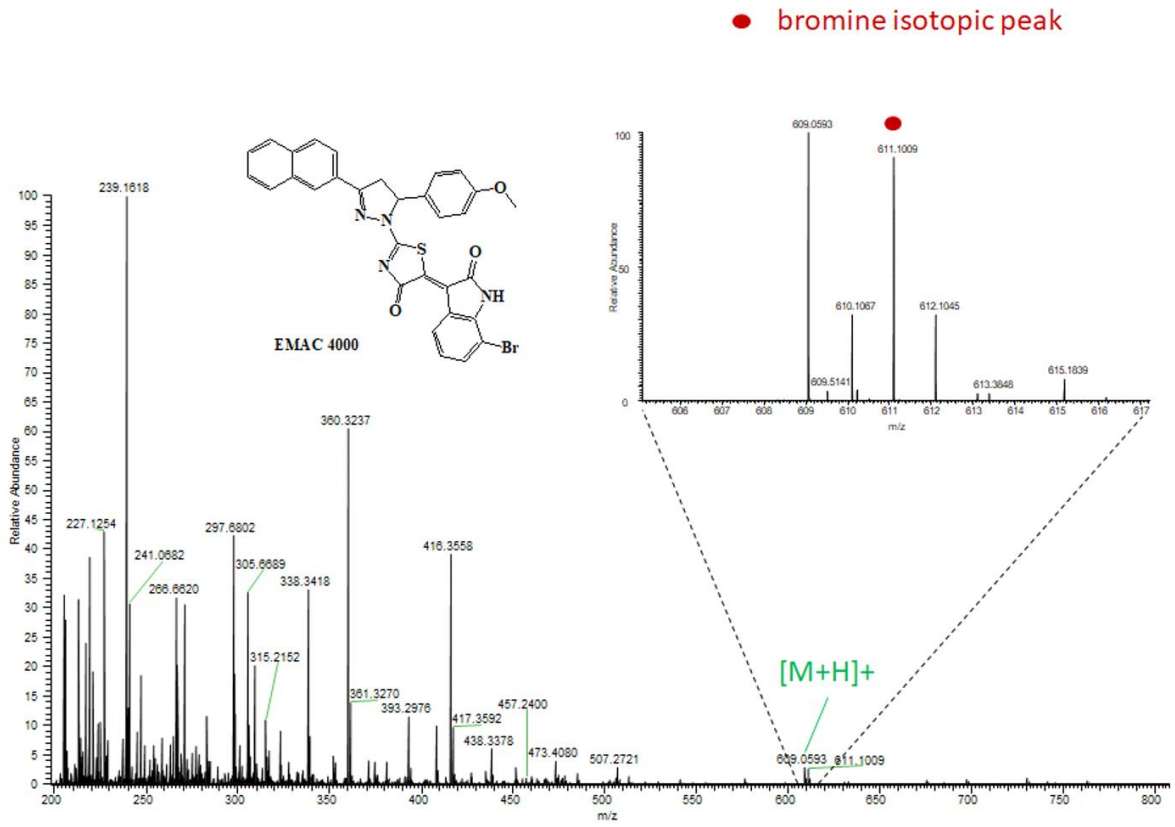


Figure S3: Full Mass Spectrum of compound **EMAC4001**

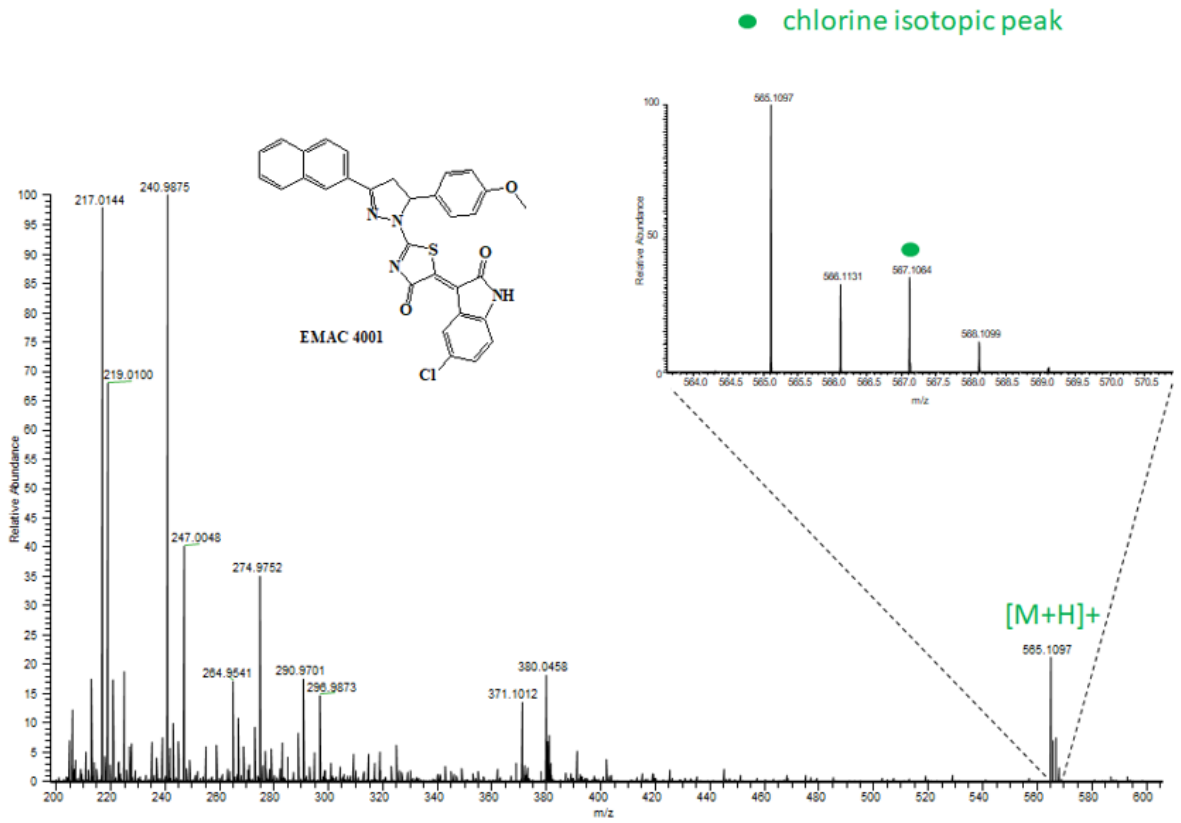


Figure S4: Full Mass Spectrum of compound **EMAC4003**

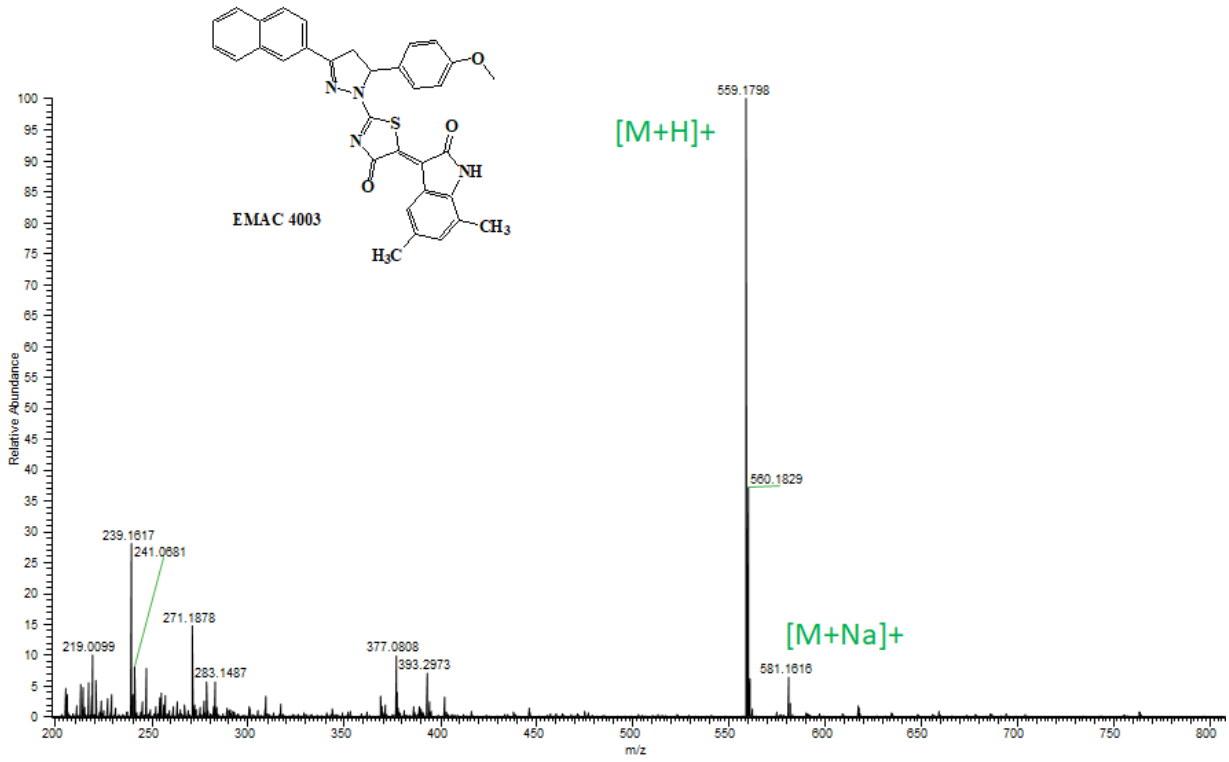


Figure S5: Full Mass Spectrum of compound **EMAC4005**

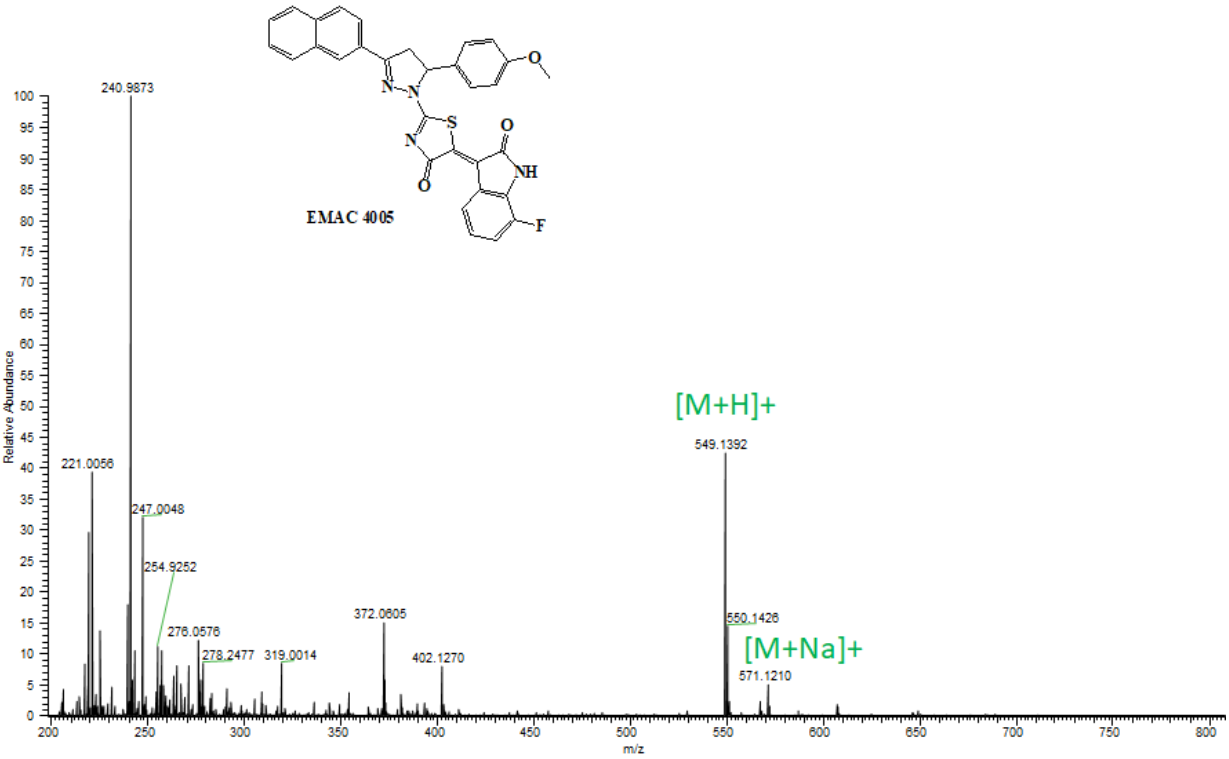


Figure S6: Full Mass Spectrum of compound **EMAC4007**

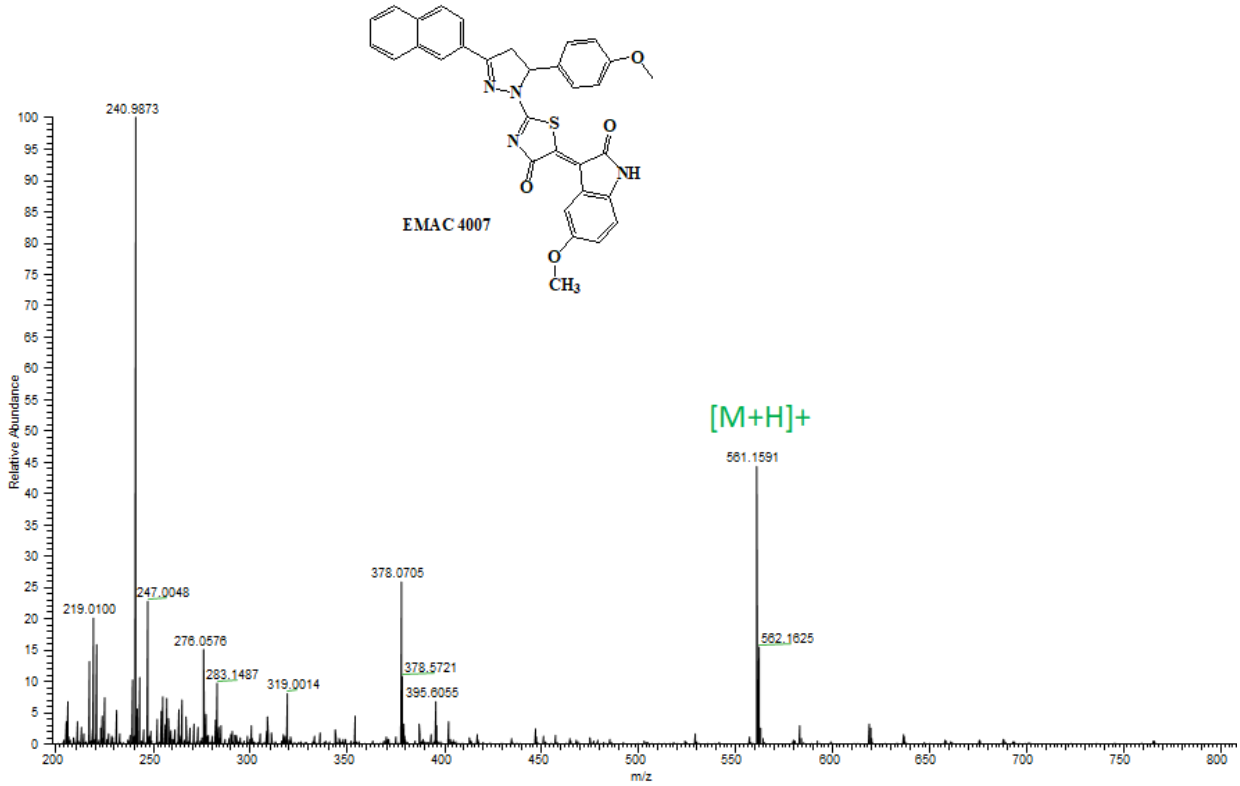


Figure S7: Full Mass Spectrum of compound EMAC4008

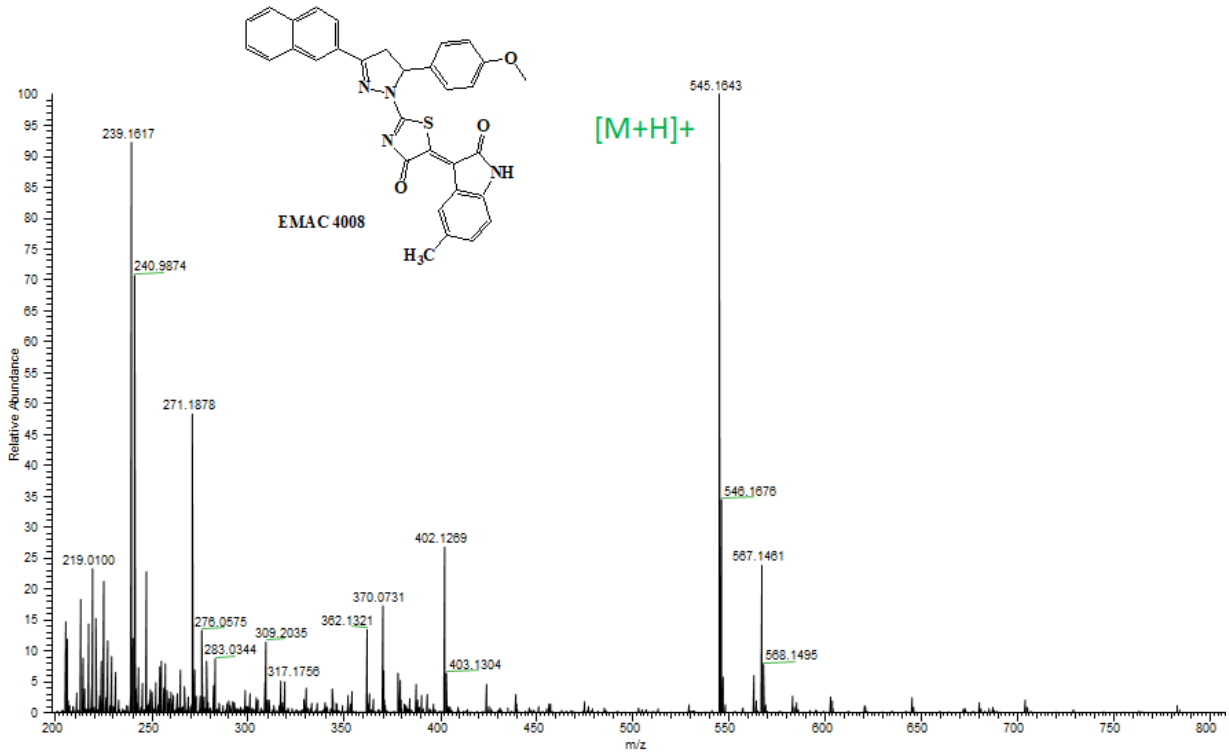


Figure S8: Full Mass Spectrum of compound **EMAC4011**

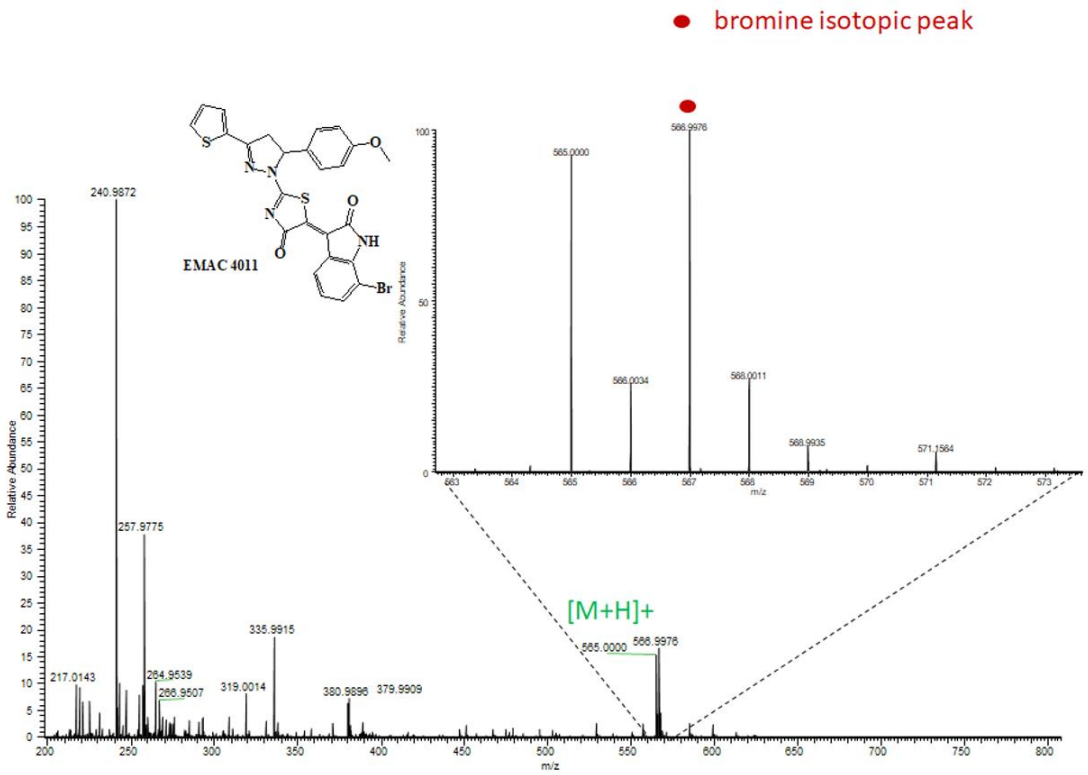


Figure S9: Full Mass Spectrum of compound **EMAC4012**

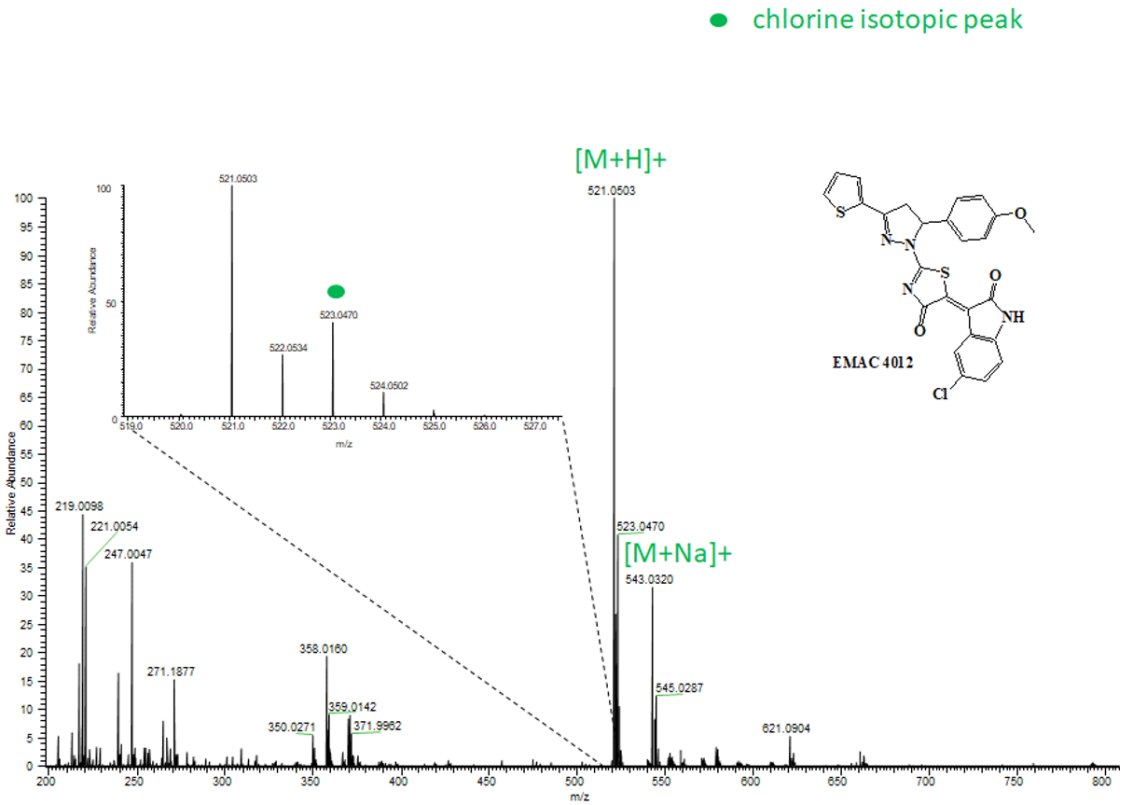


Figure S10: Full Mass Spectrum of compound **EMAC4014**

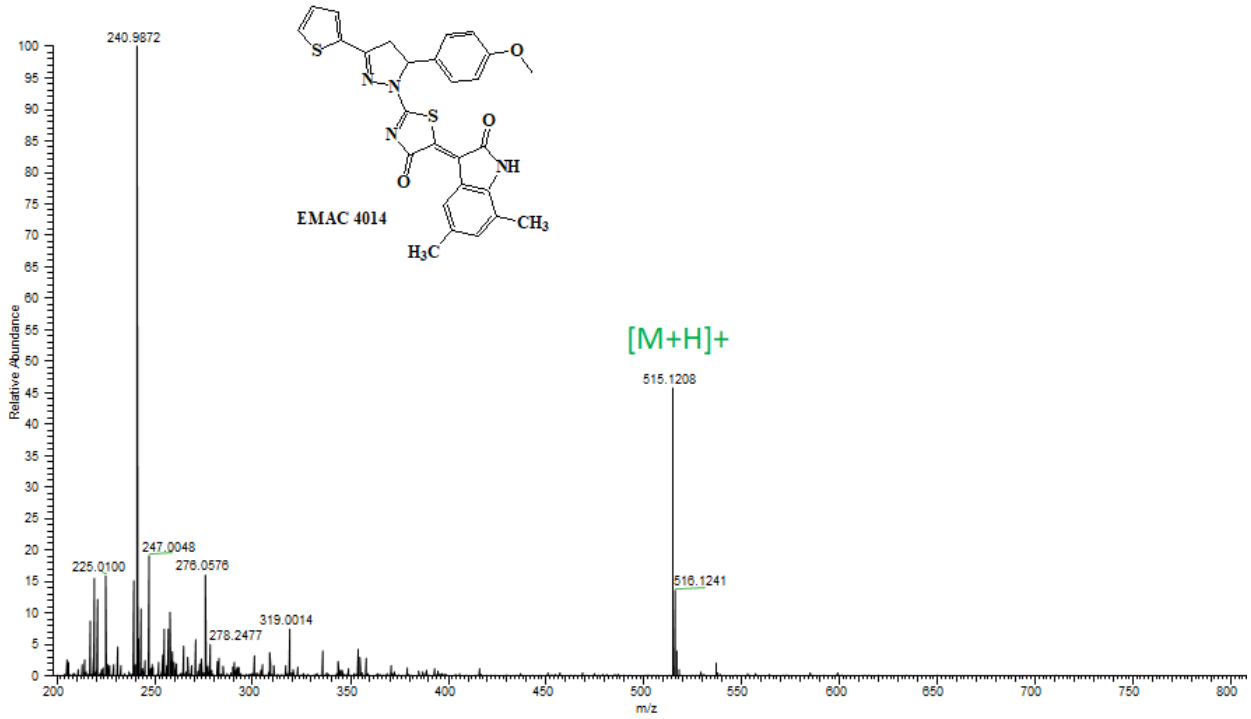


Figure S11: Full Mass Spectrum of compound **EMAC4015**

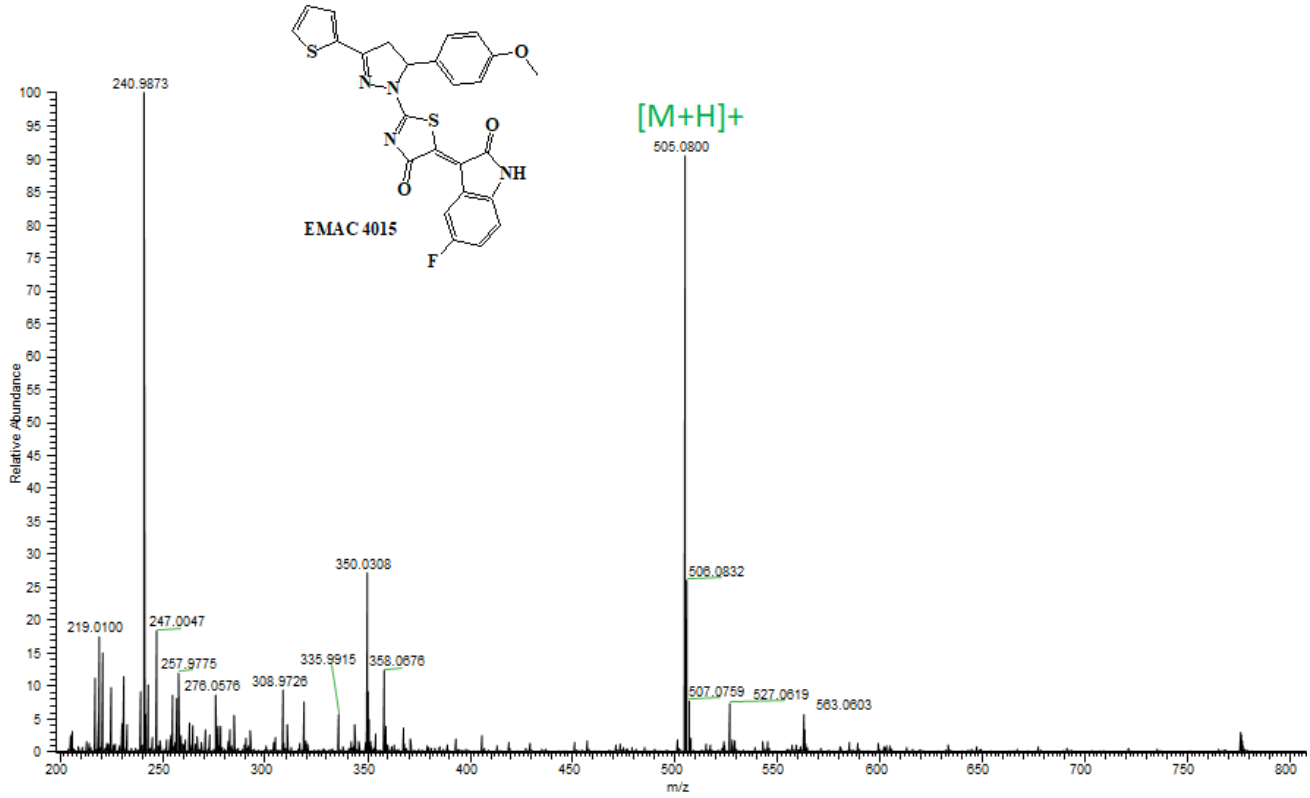


Figure S12: Full Mass Spectrum of compound **EMAC4018**

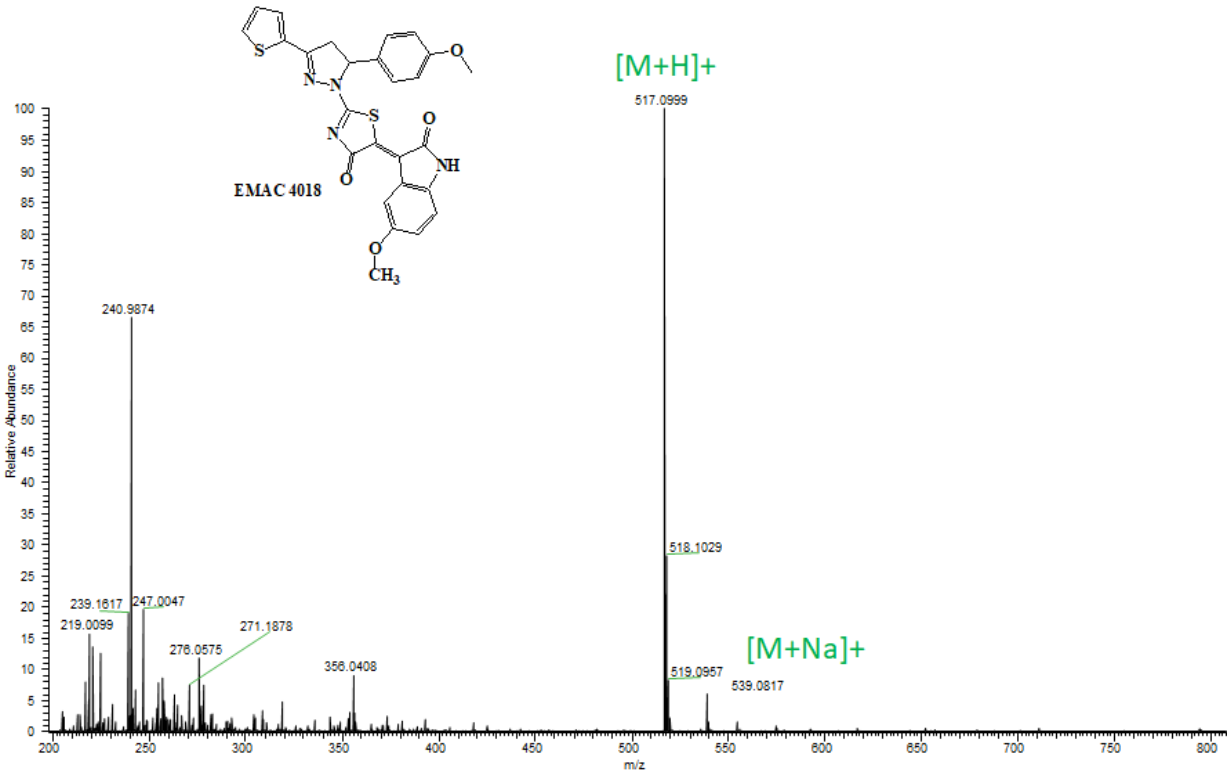
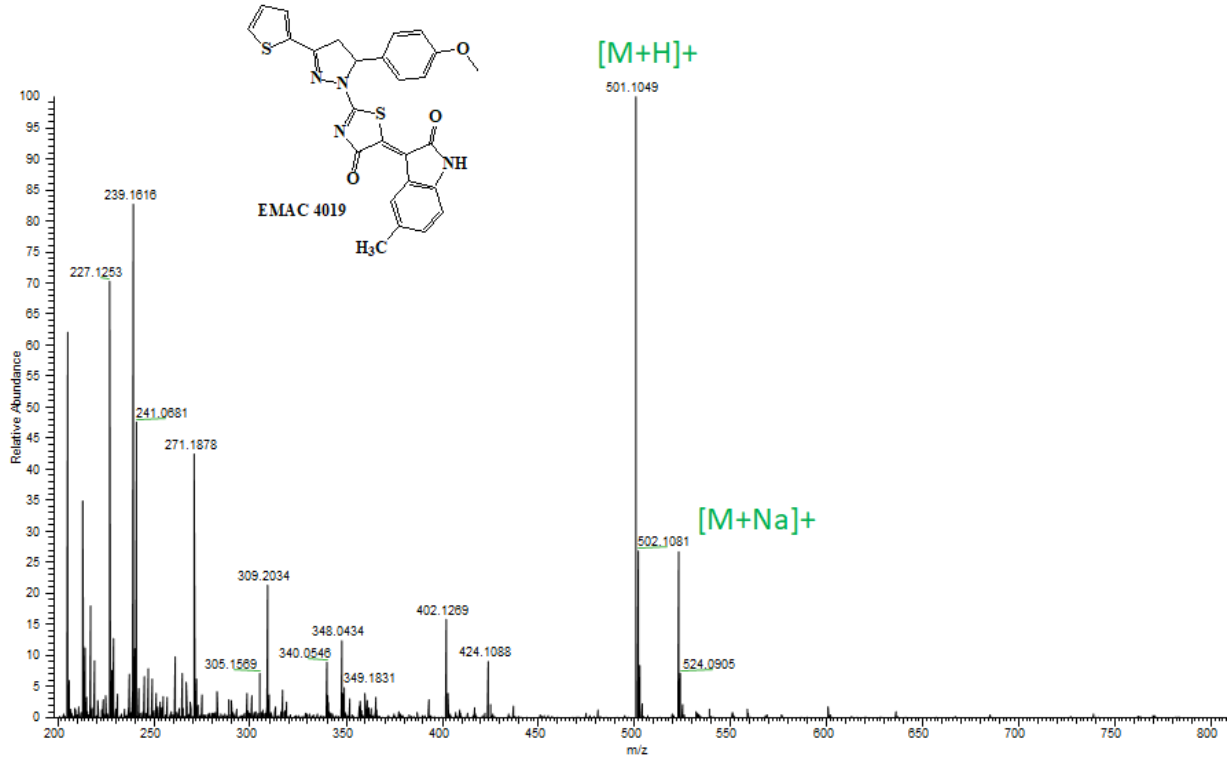


Figure S13: Full Mass Spectrum of compound **EMAC4019**



^{1}H NMR of compounds EMAC4000,-01,-03,-05,-07,-08 and EMAC4011,-12,-14,-15,-18,-19

Figure S14: ^{1}H NMR spectrum (400 MHz, DMSO-d6) of **EMAC4000**

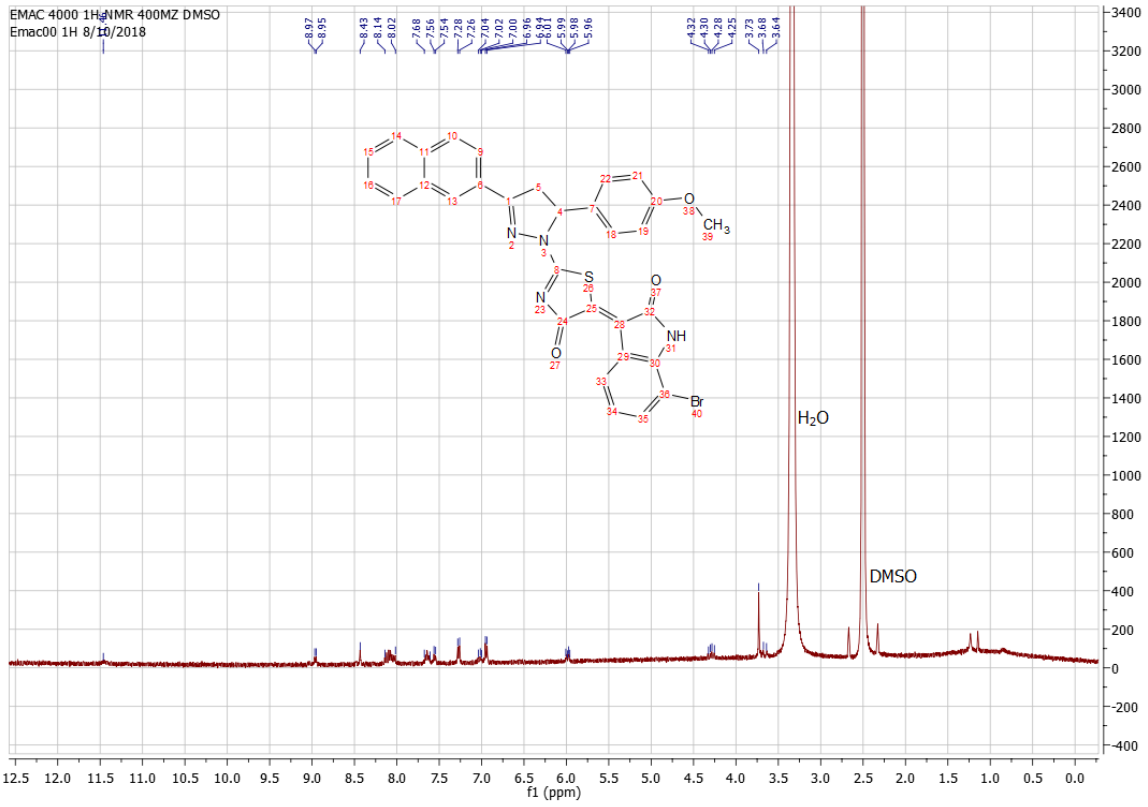


Figure S15: ^{1}H NMR spectrum (400 MHz, DMSO-d6) of **EMAC4001**

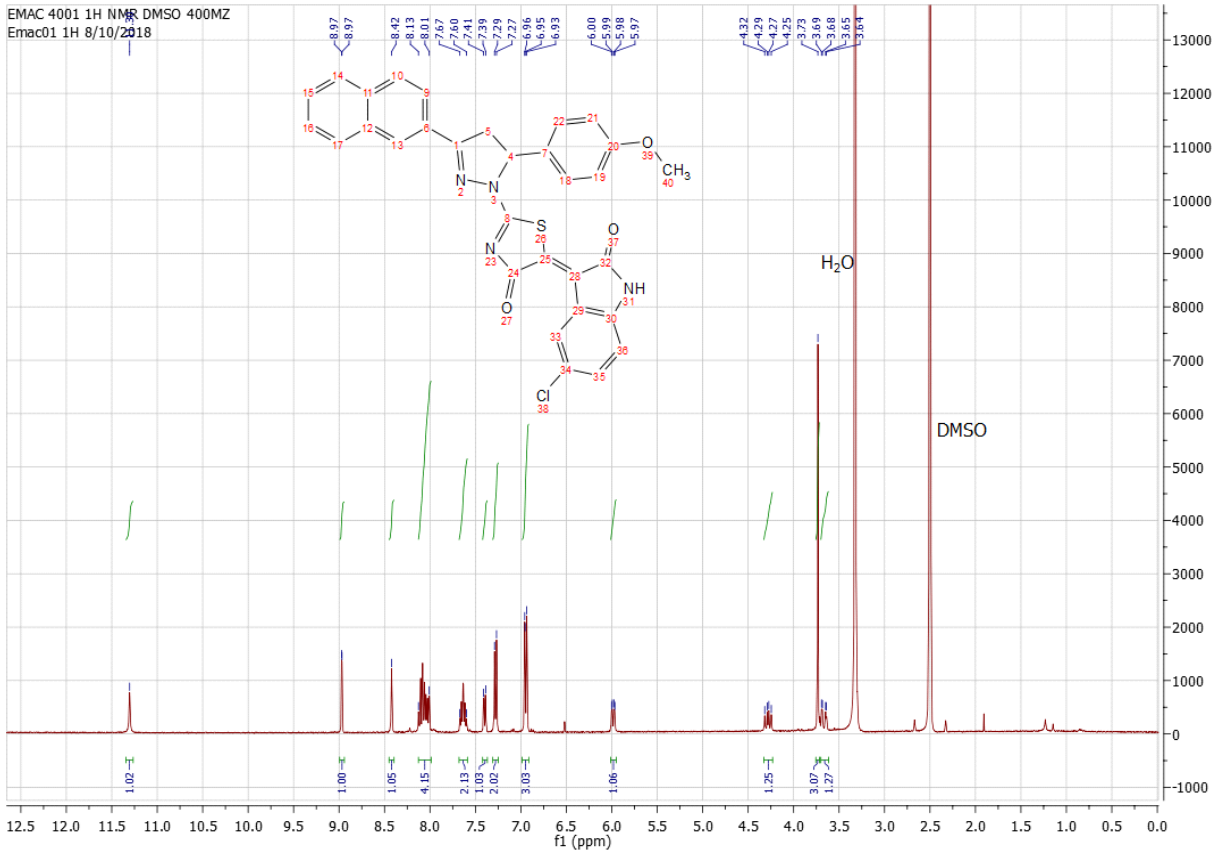


Figure S16: ^{1}H NMR spectrum (400 MHz, DMSO-d6) of **EMAC4003**

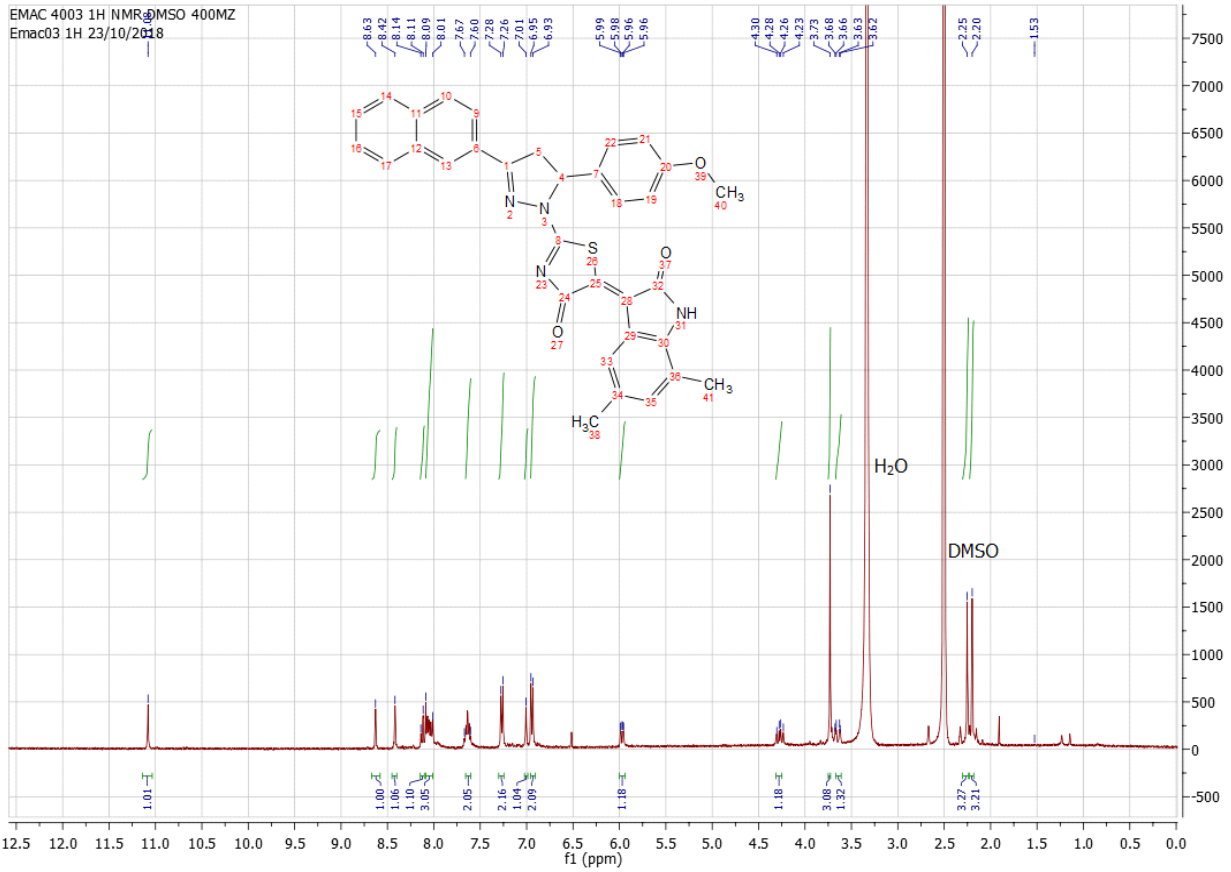


Figure S17: ^{1}H NMR spectrum (400 MHz, DMSO-d6) of **EMAC4005**

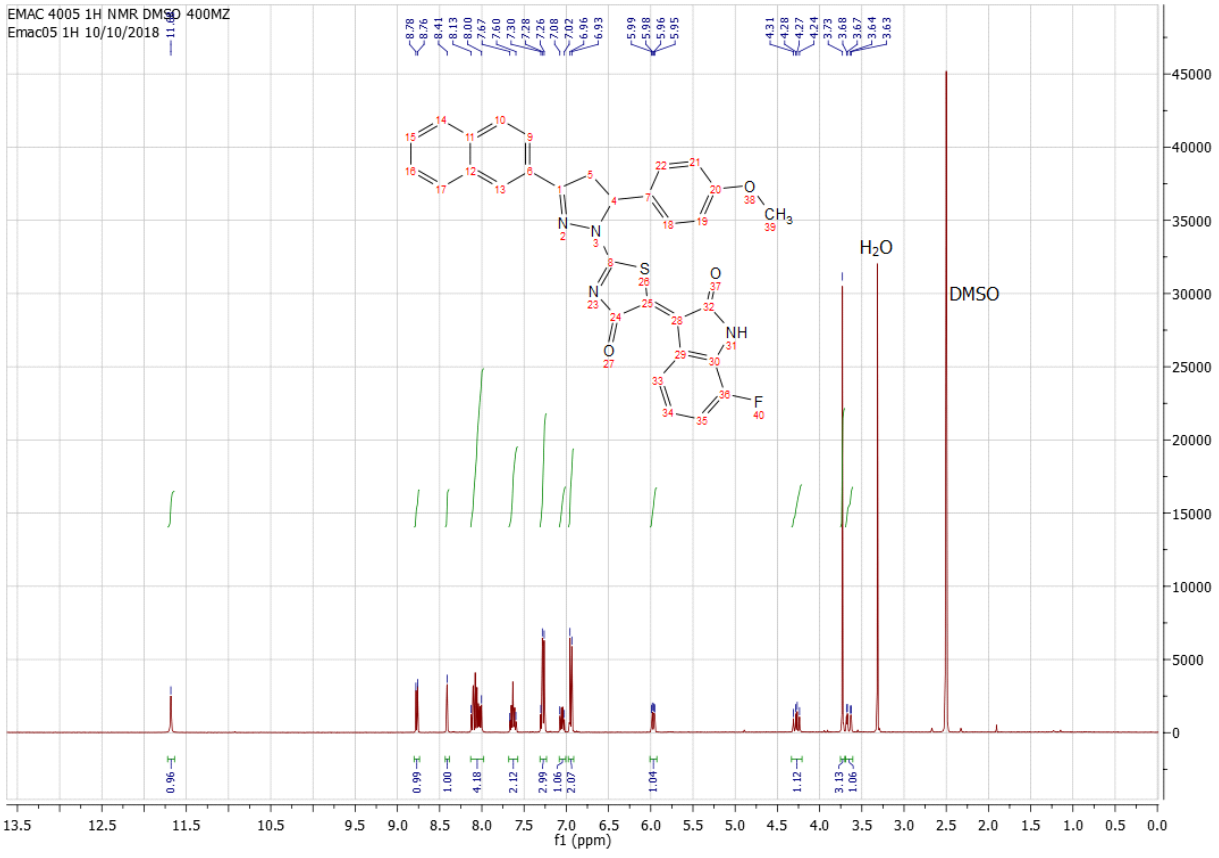


Figure S18: ^{1}H NMR spectrum (400 MHz, DMSO-d6) of **EMAC4007**

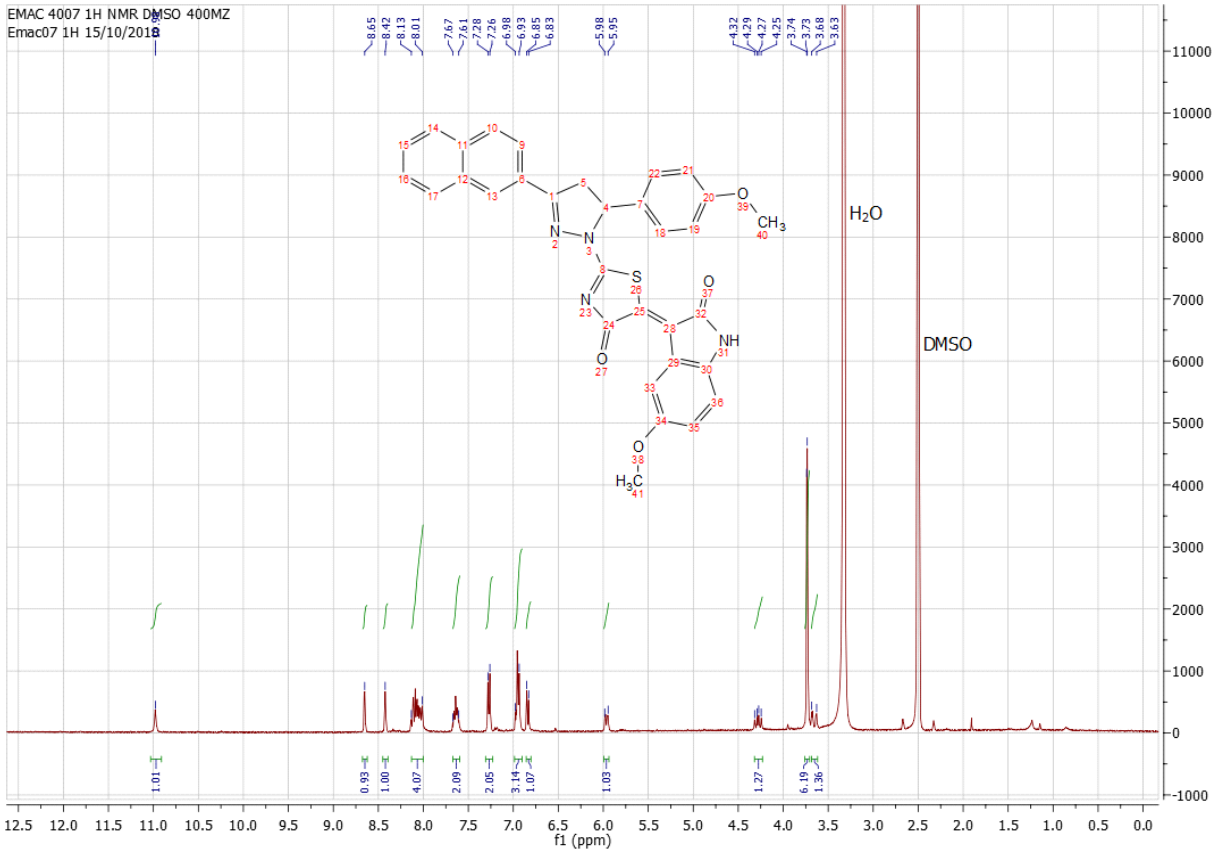


Figure S19: ^{1}H NMR spectrum (400 MHz, DMSO-d6) of **EMAC4008**

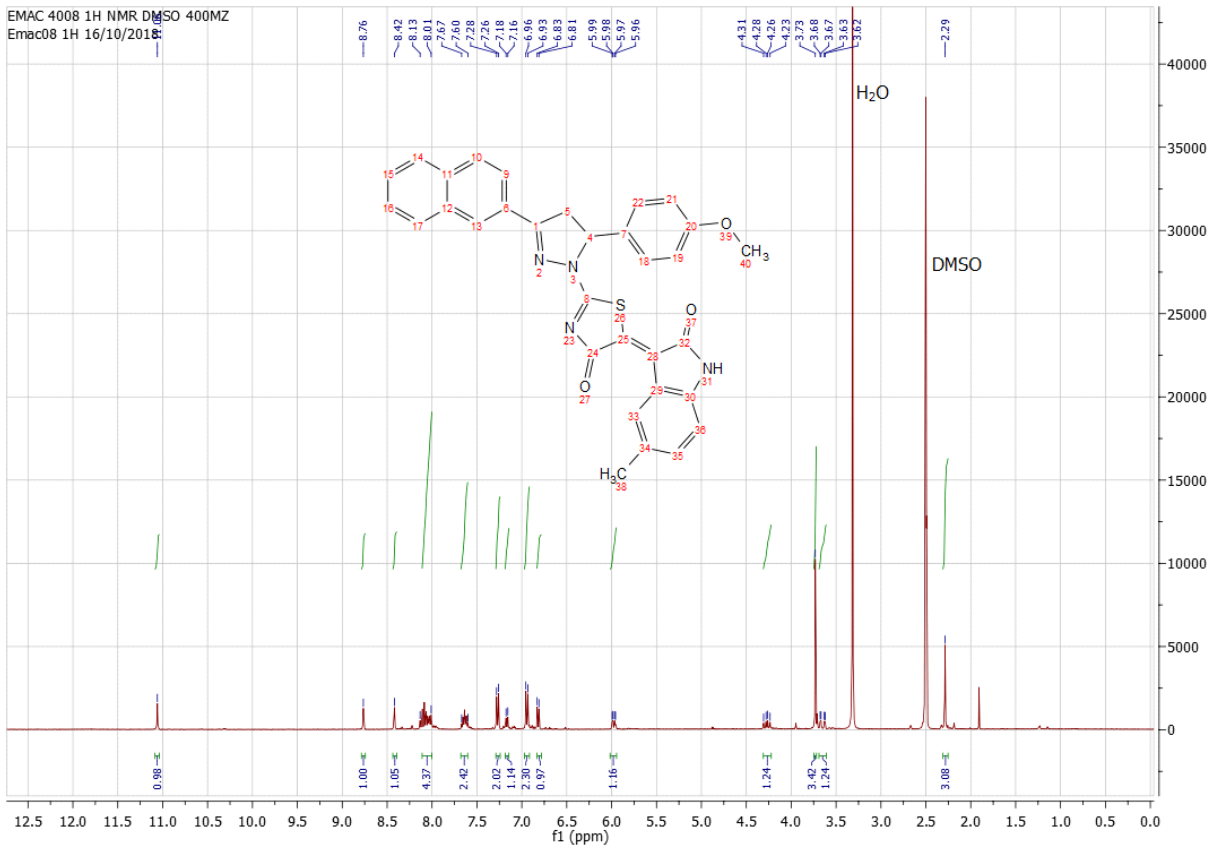


Figure S20: ^{1}H NMR spectrum (400 MHz, DMSO-d6) of **EMAC4011**

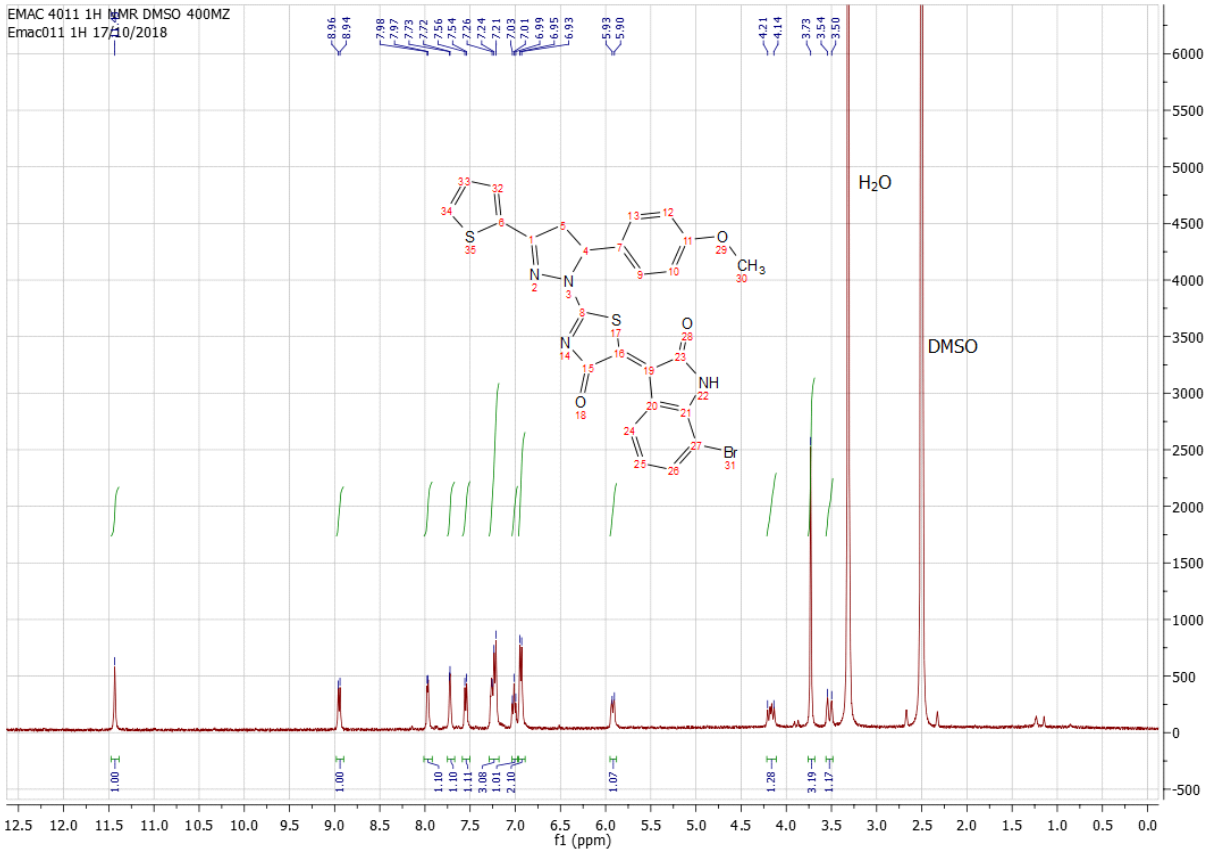


Figure S21: ^{1}H NMR spectrum (400 MHz, DMSO-d6) of **EMAC4012**

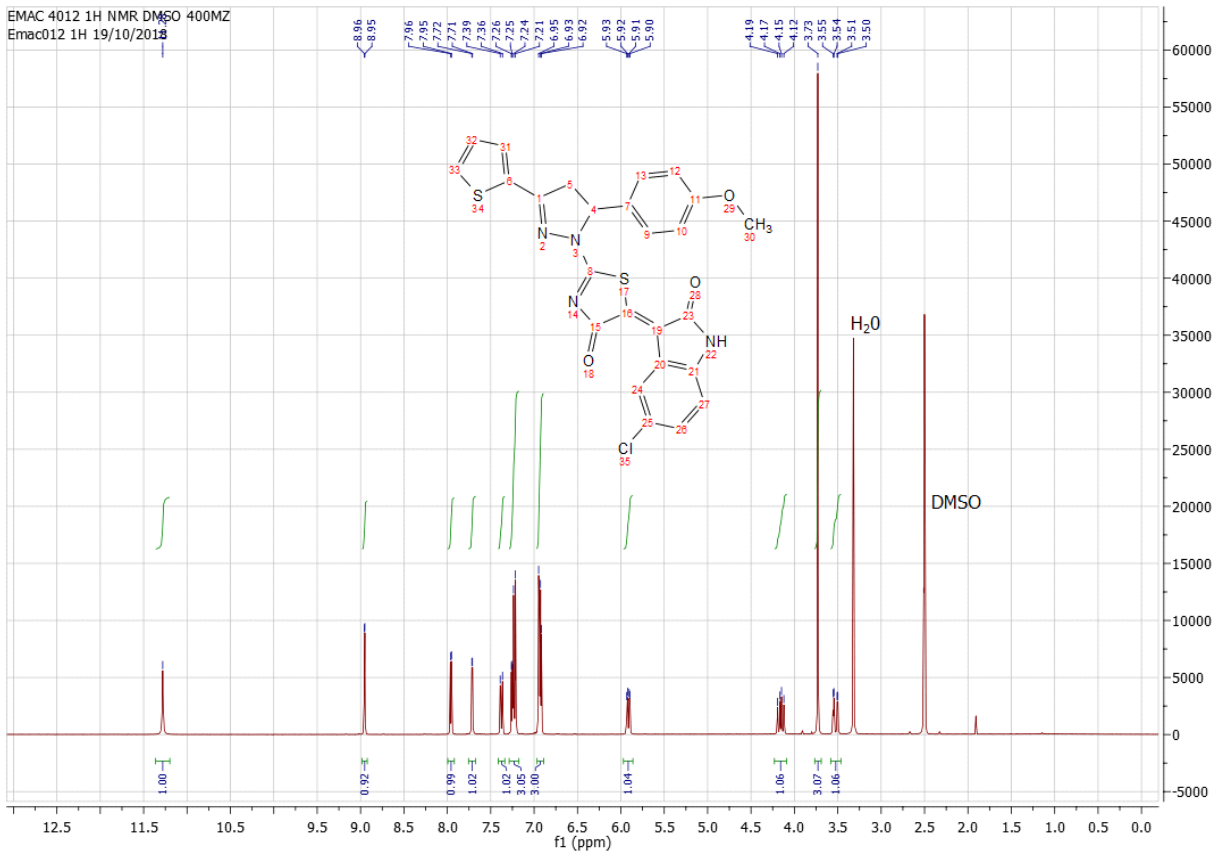


Figure S22: ^{1}H NMR spectrum (400 MHz, DMSO-d6) of **EMAC4014**

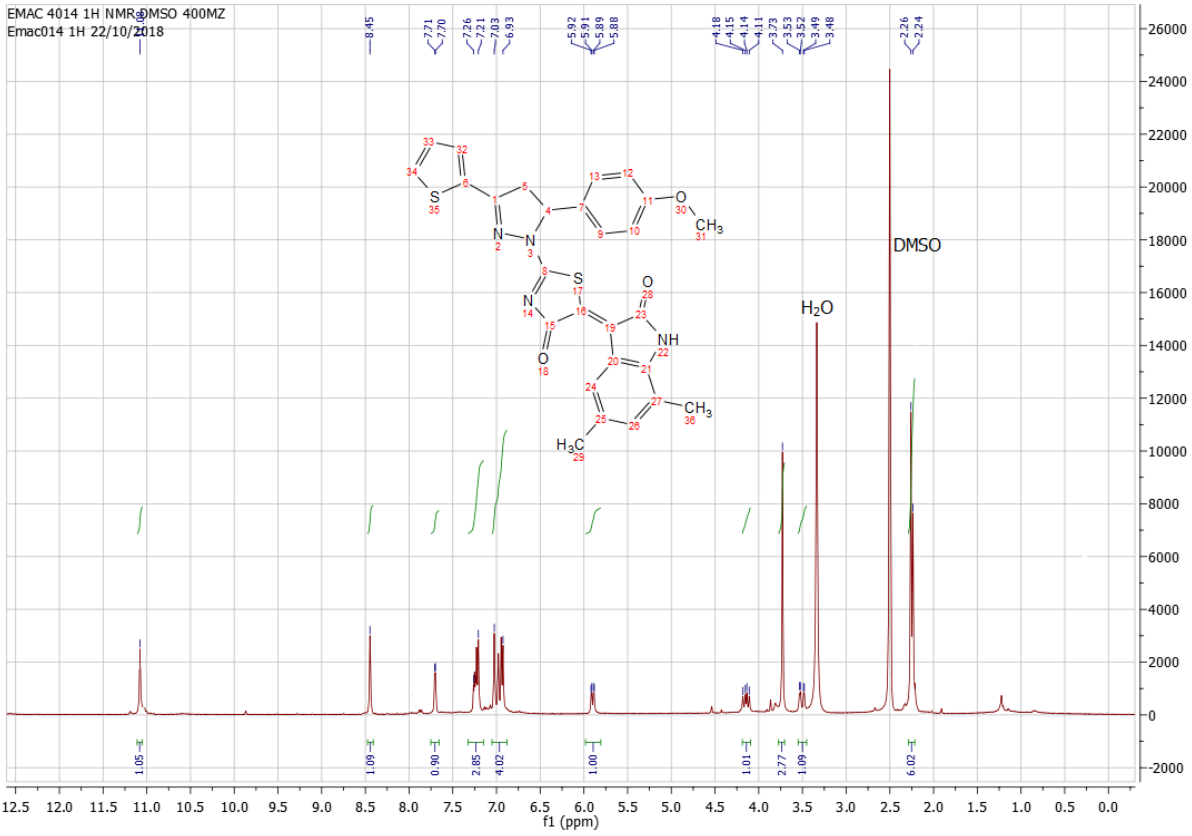


Figure S23: ^{1}H NMR spectrum (400 MHz, DMSO-d6) of **EMAC4015**

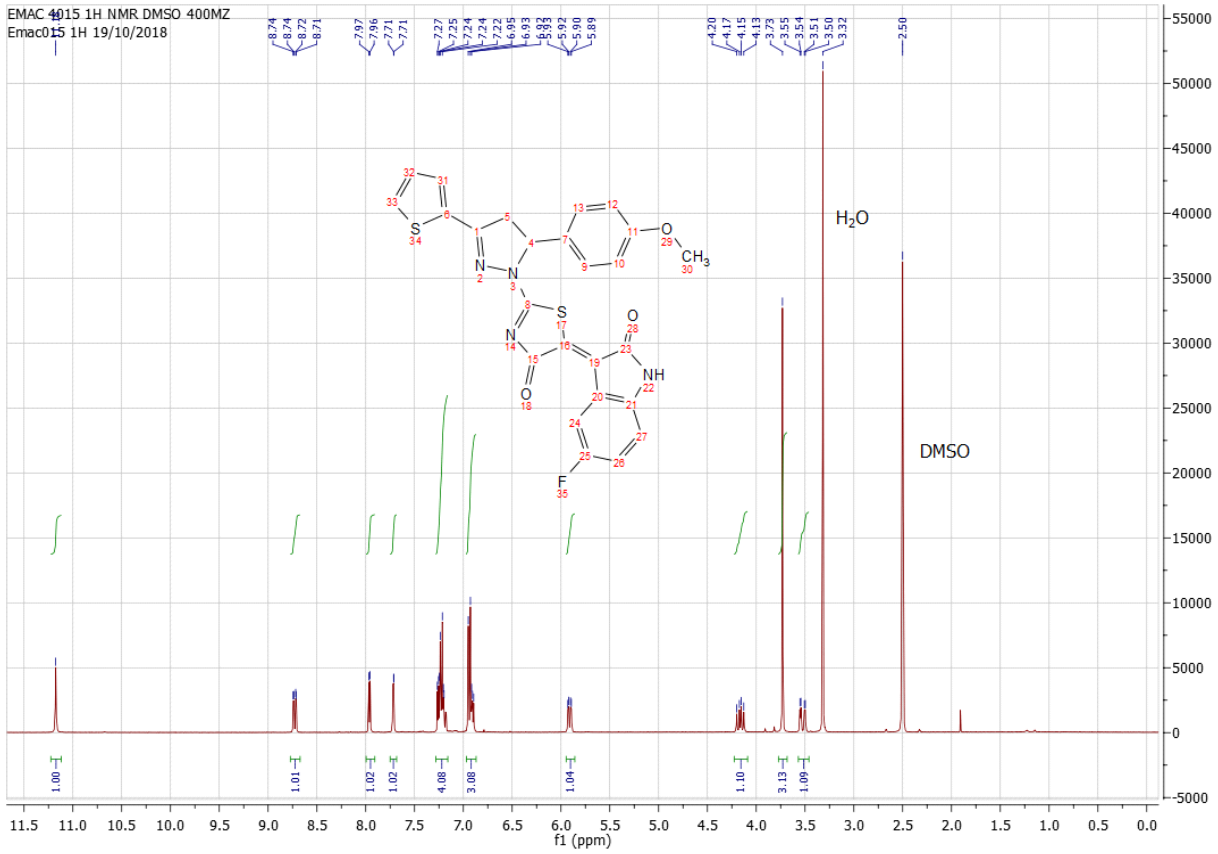


Figure S24: ^{1}H NMR spectrum (400 MHz, DMSO-d6) of **EMAC4018**

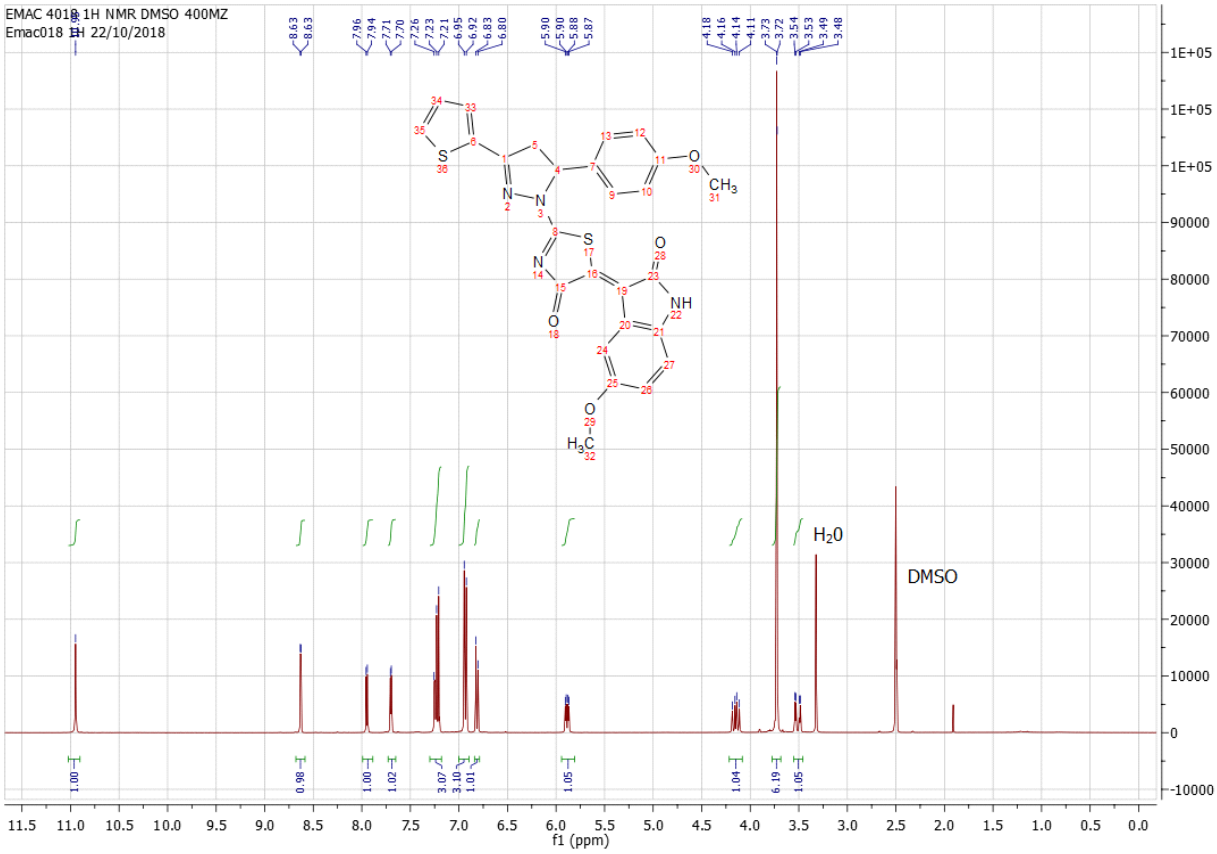
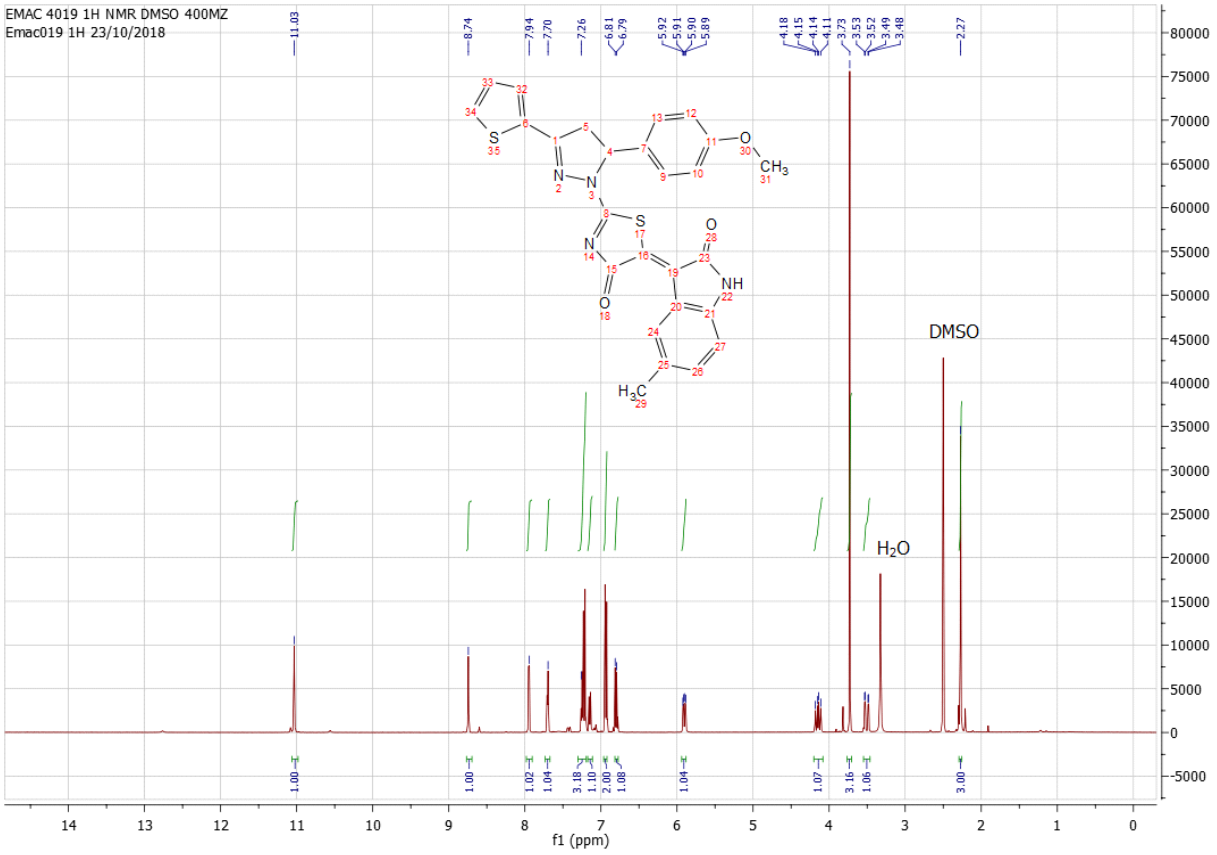


Figure S25: ^{1}H NMR spectrum (400 MHz, DMSO-d6) of **EMAC4019**



^{13}C NMR of compounds **EMAC4000,-01,-03,-05,-07,-08** and **EMAC4011,-12,-14,-15,-18,-19.**

Figure S26: ^{13}C NMR spectrum (101 MHz, DMSO-d6) of **EMAC4001**

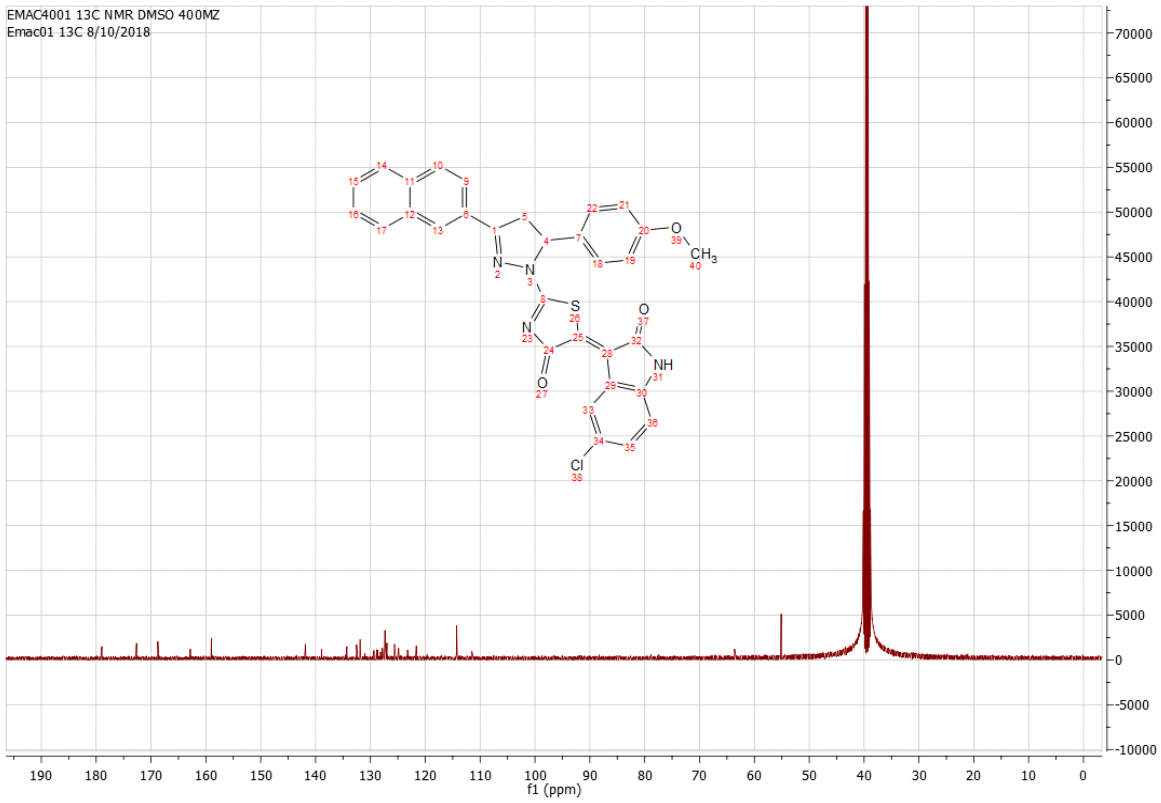


Figure S27: ^{13}C NMR spectrum (101 MHz, DMSO-d6) of **EMAC4003**

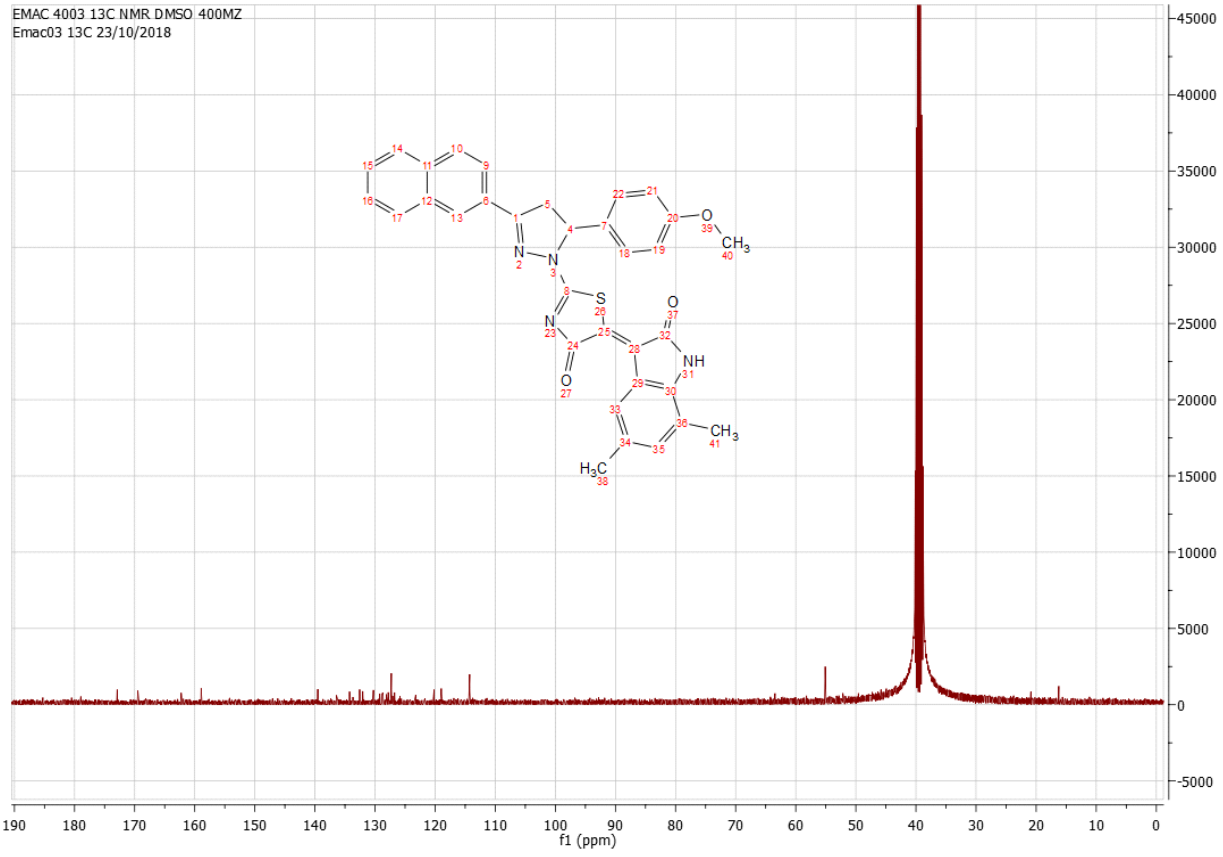


Figure S28: ^{13}C NMR spectrum (101 MHz, DMSO-d6) of **EMAC4005**

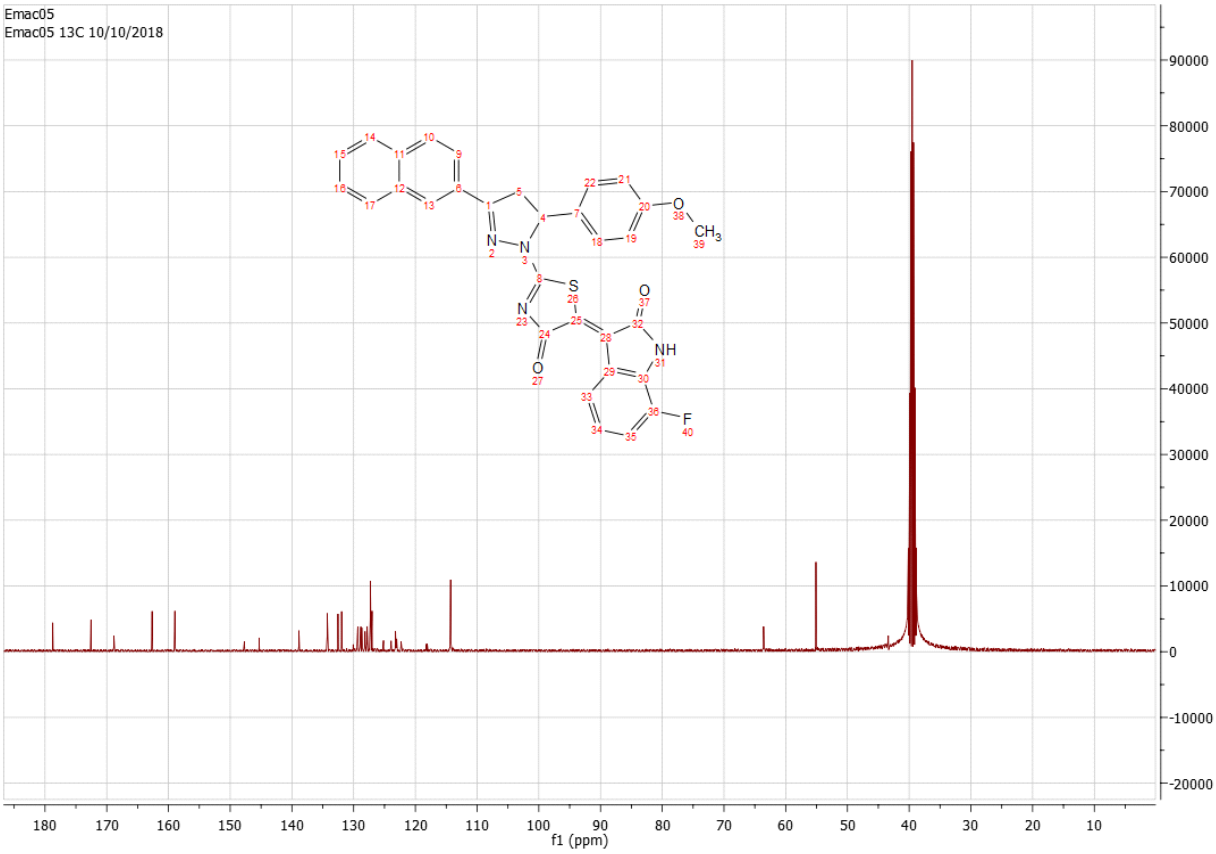


Figure S29: ^{13}C NMR spectrum (101 MHz, DMSO-d6) of **EMAC4007**

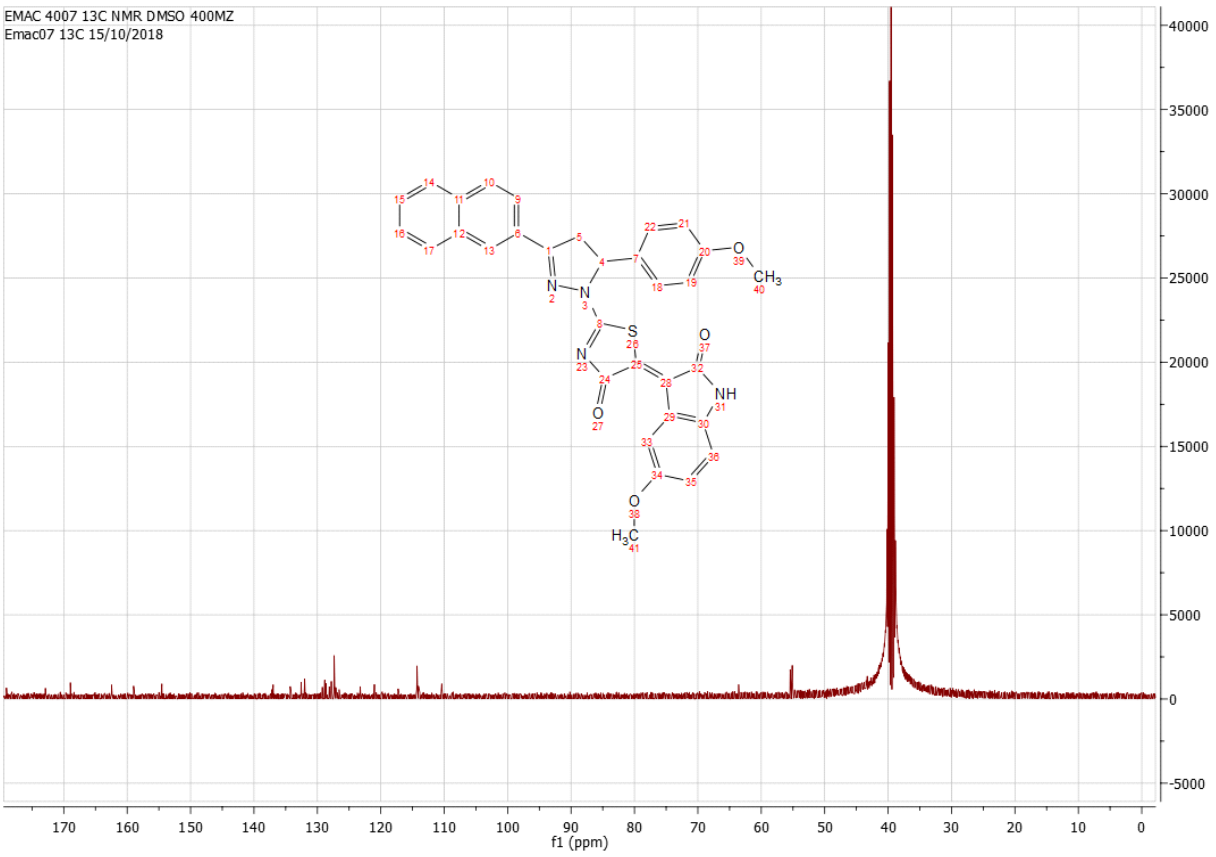


Figure S30: ^{13}C NMR spectrum (101 MHz, DMSO-d6) of **EMAC4008**

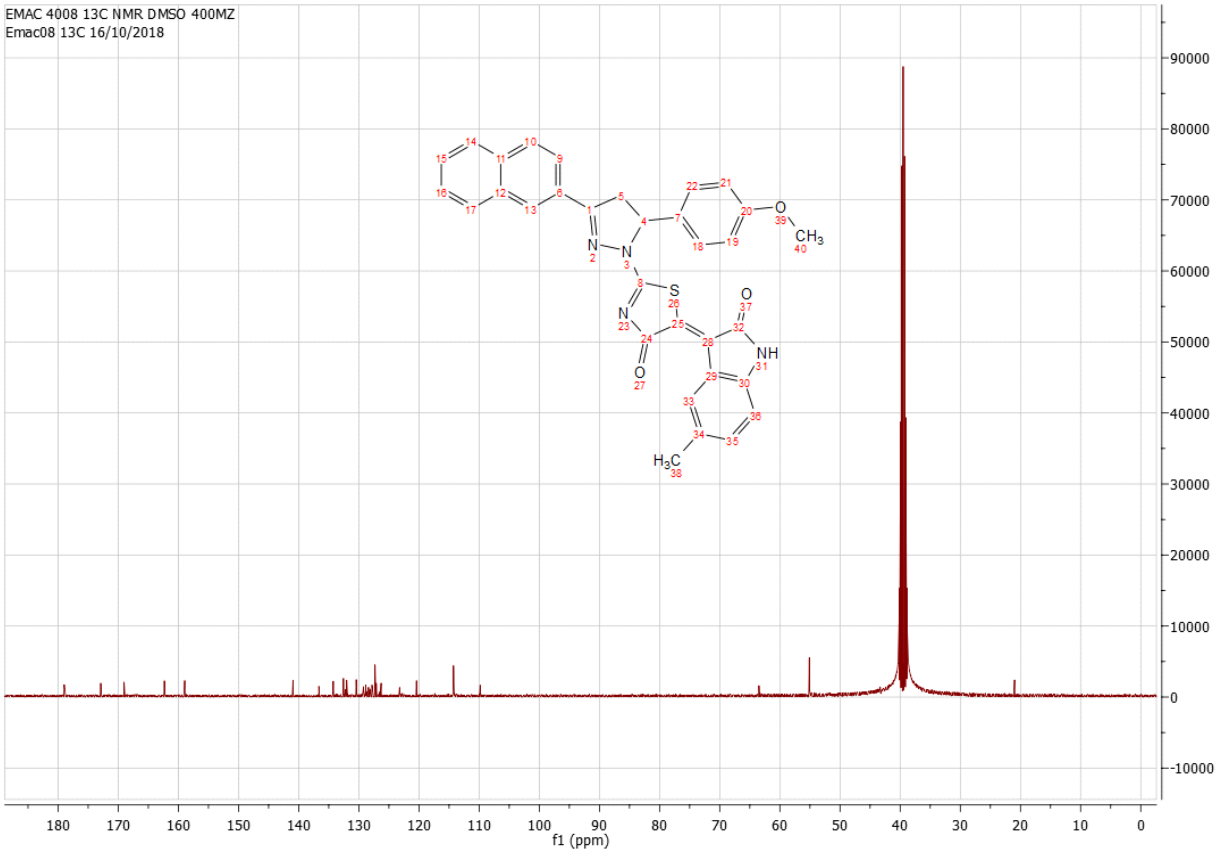


Figure S31: ^{13}C NMR spectrum (101 MHz, DMSO-d6) of **EMAC4011**

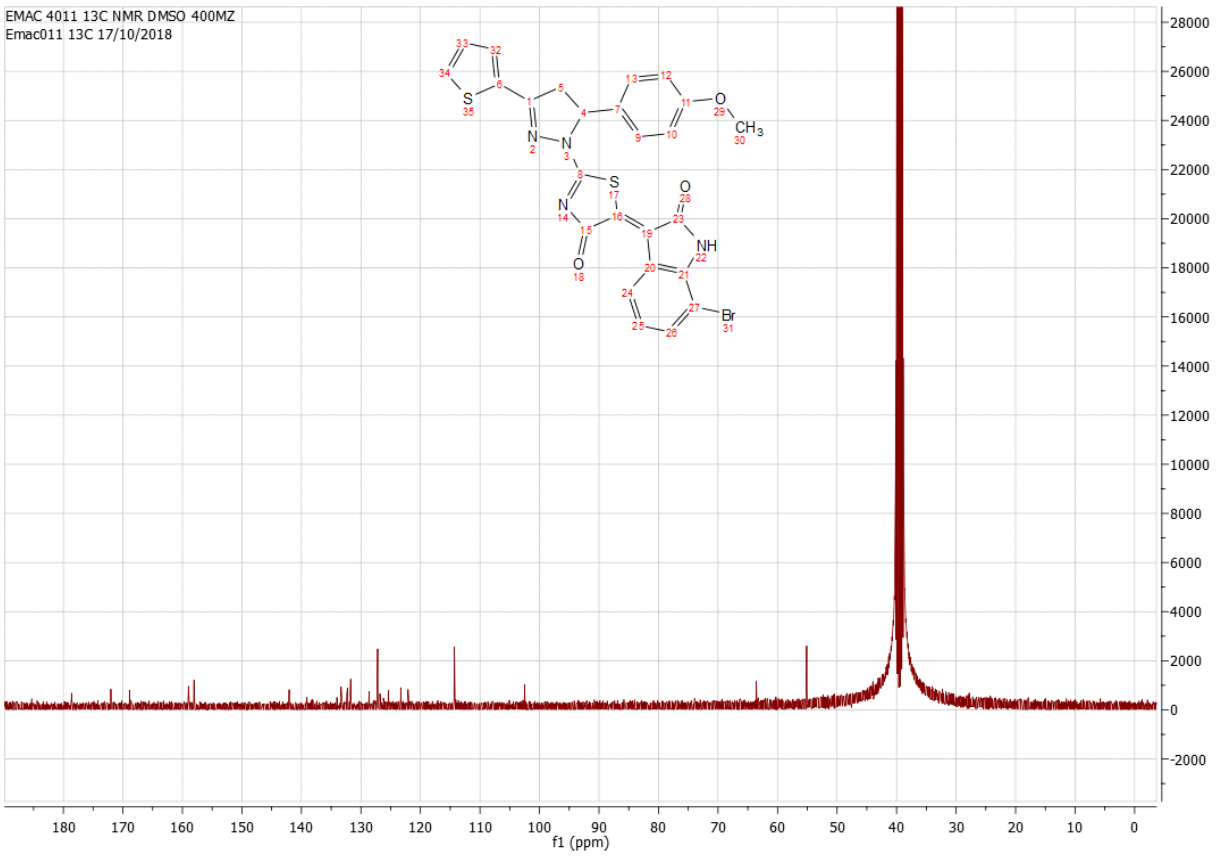


Figure S32: ^{13}C NMR spectrum (101 MHz, DMSO-d6) of **EMAC4012**

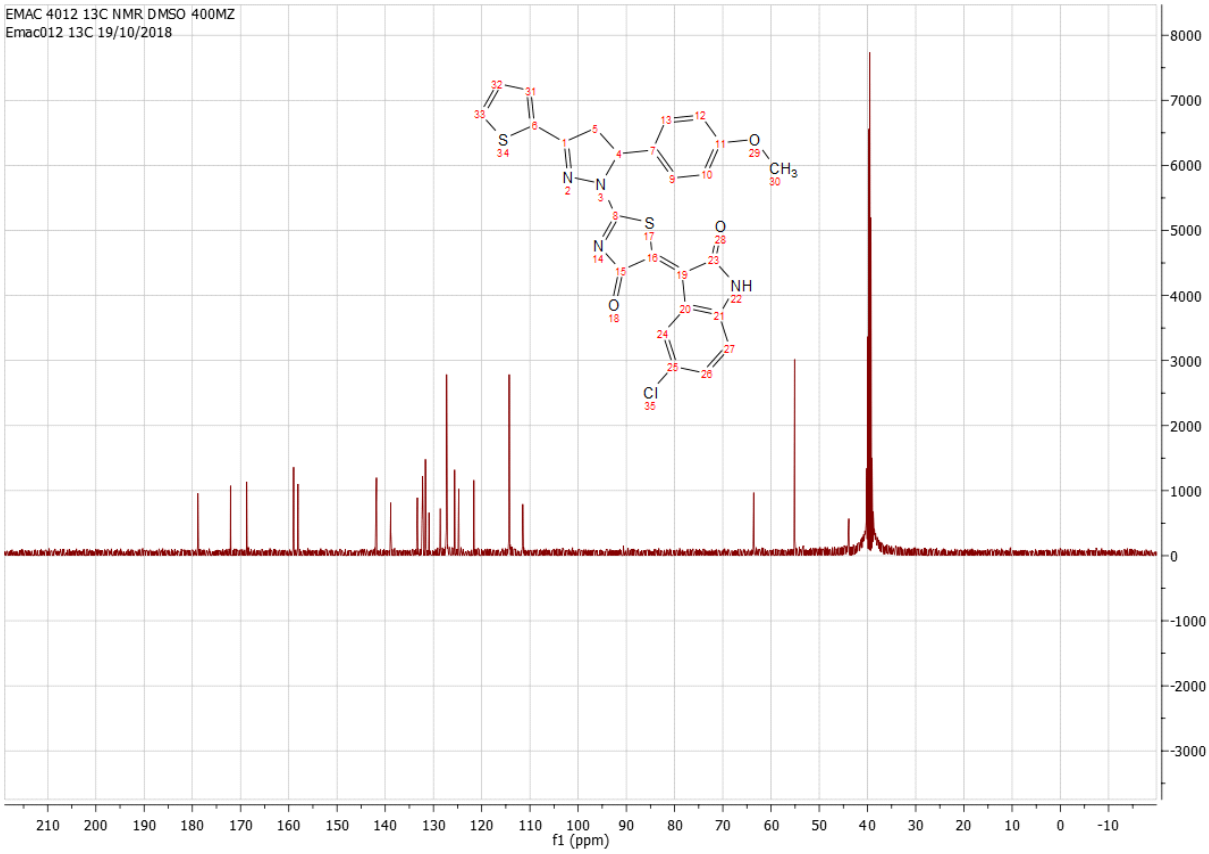


Figure S33: ^{13}C NMR spectrum (101 MHz, DMSO-d6) of **EMAC4014**

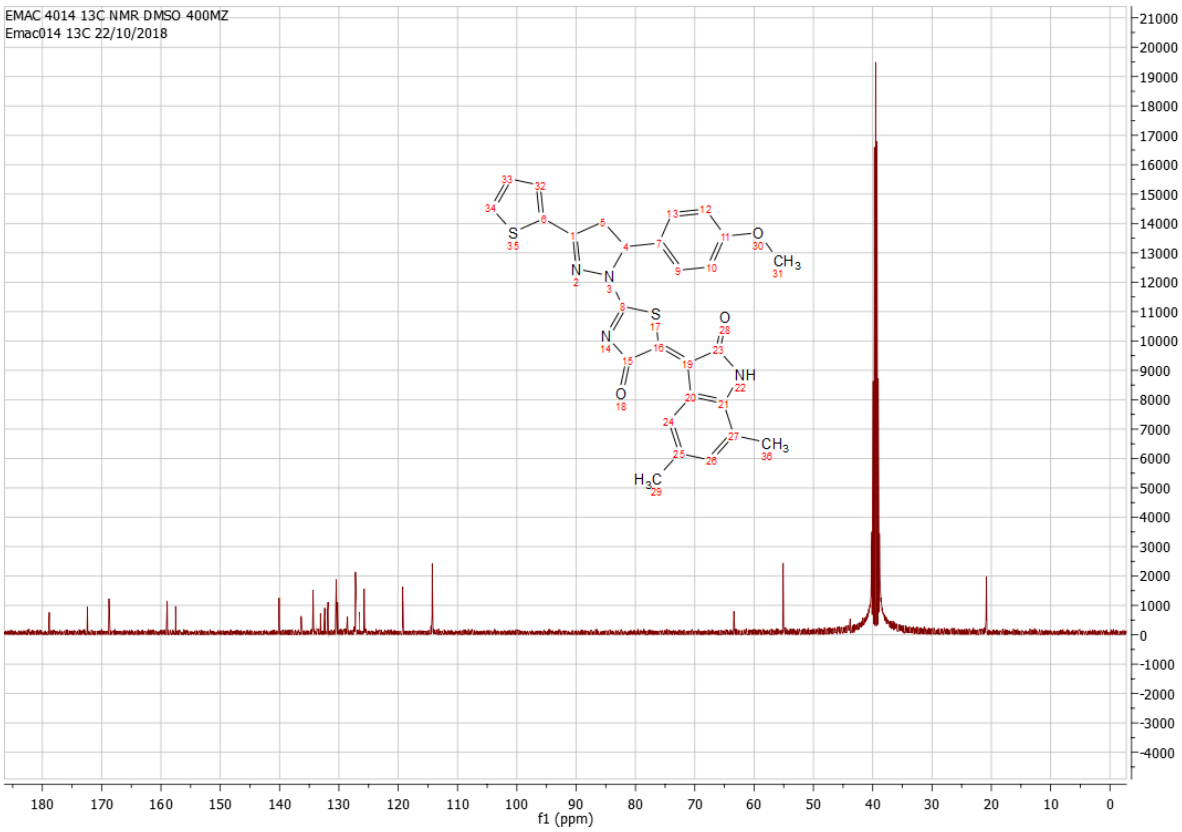


Figure S34: ^{13}C NMR spectrum (101 MHz, DMSO-d6) of **EMAC4015**

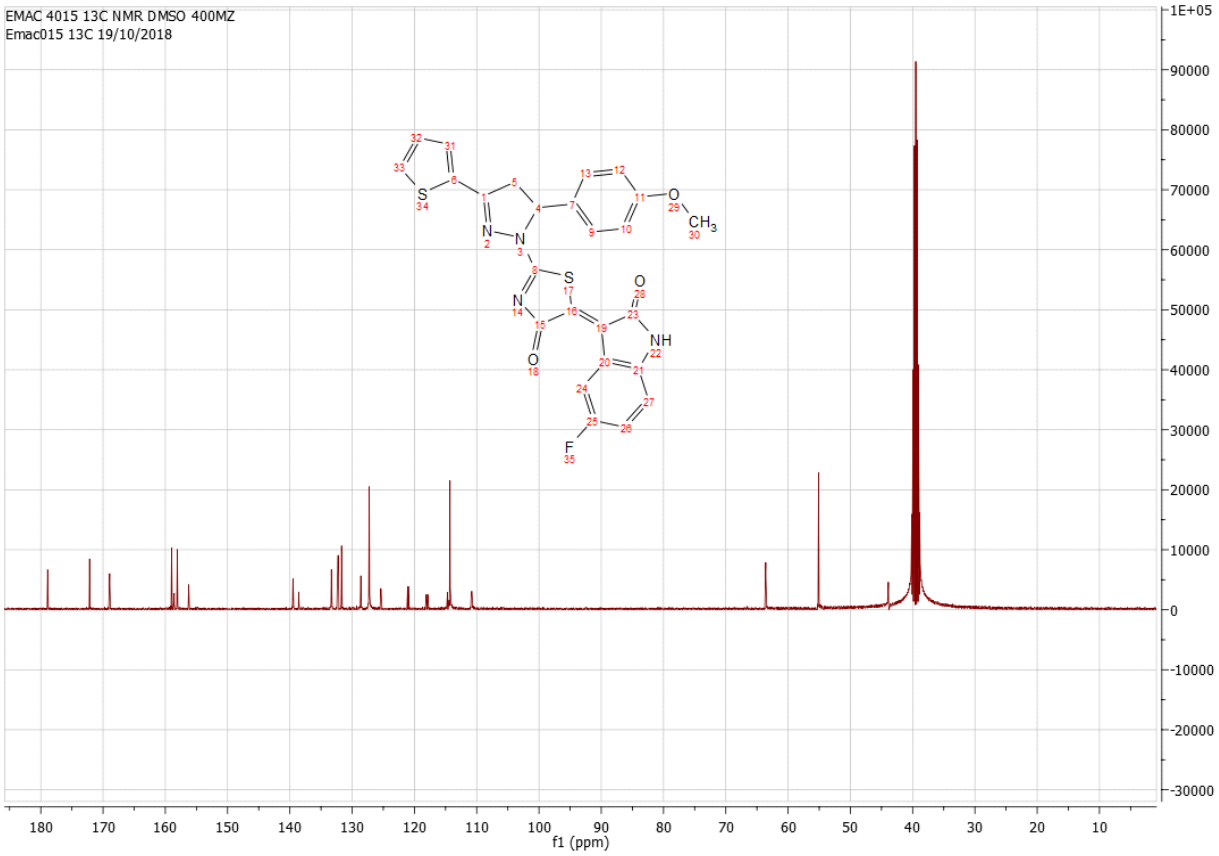


Figure S35: ^{13}C NMR spectrum (101 MHz, DMSO-d6) of **EMAC4018**

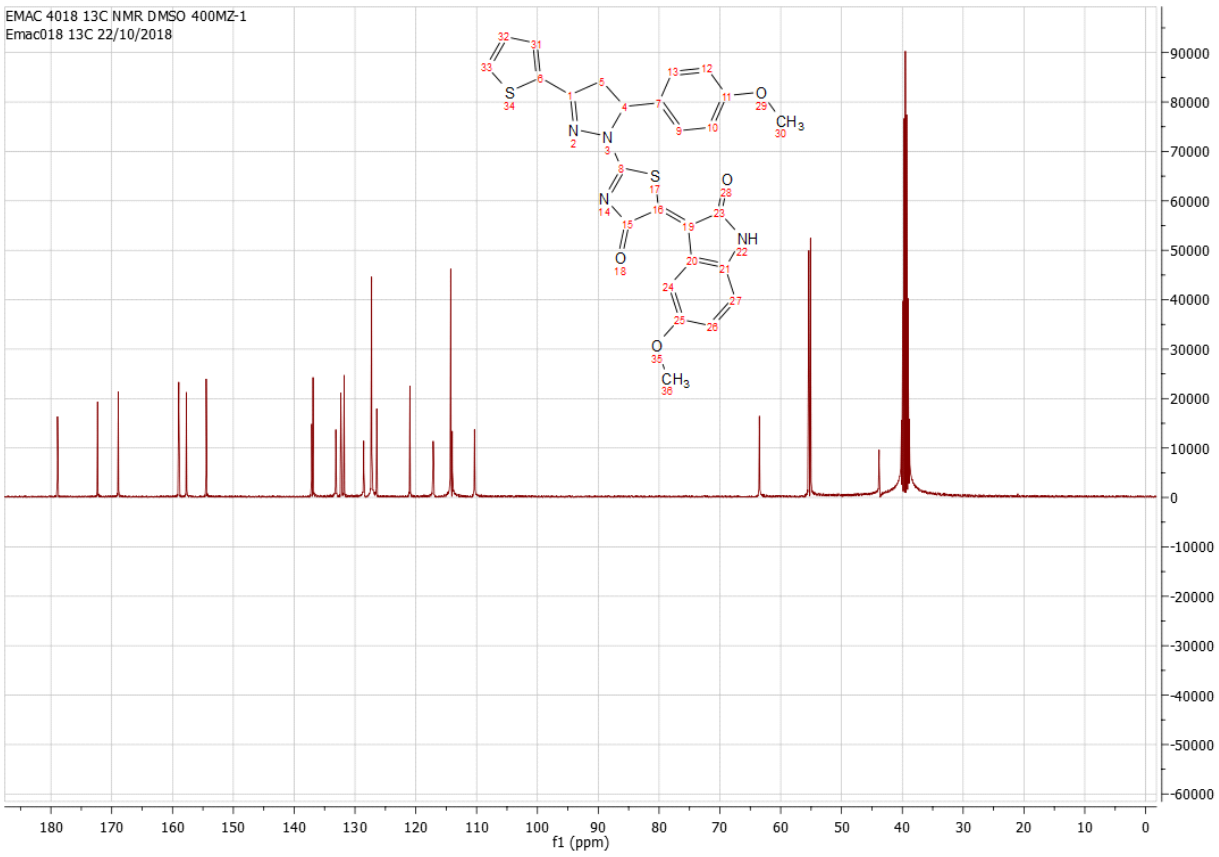
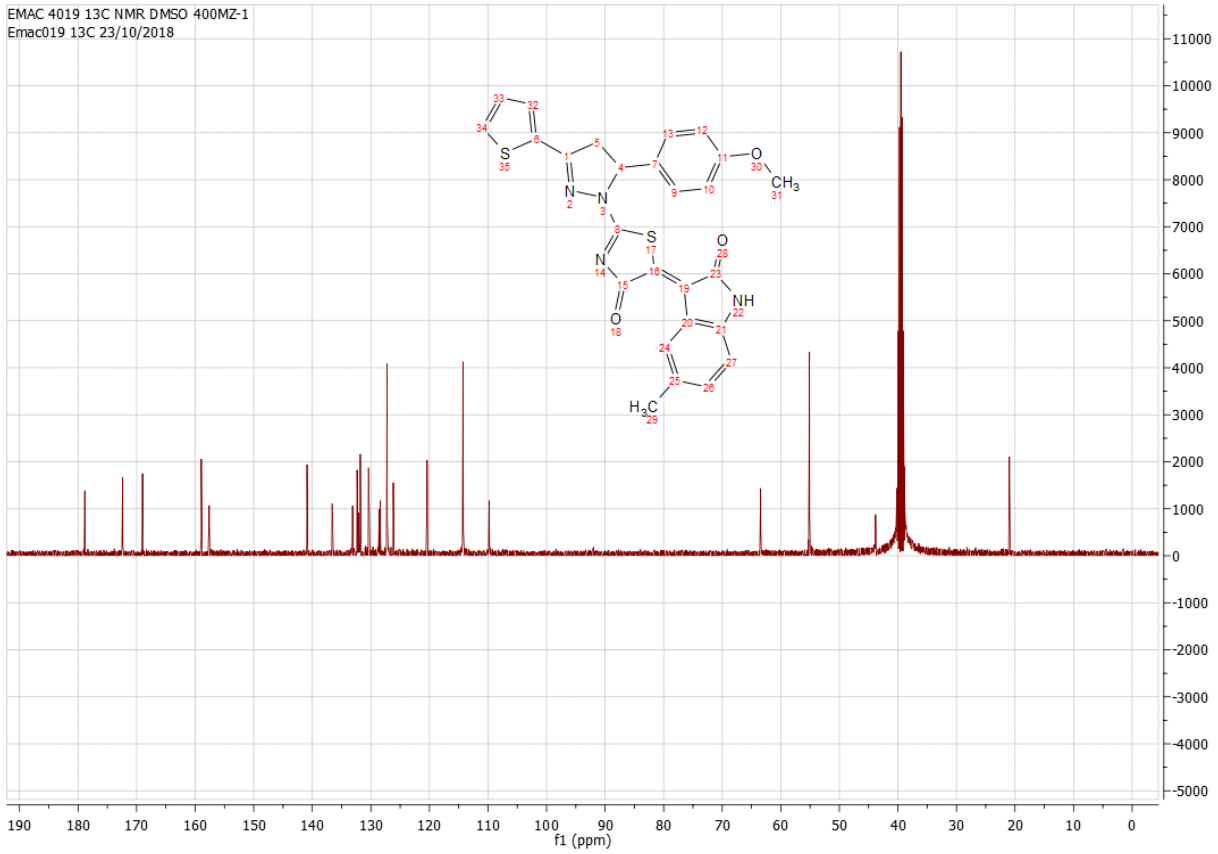


Figure S36: ^{13}C NMR spectrum (101 MHz, DMSO-d6) of **EMAC4019**



Biological evaluation

Materials and methods

Stocks of compounds were prepared in dimethyl sulfoxide (2 mM concentrations) and stored at +4 °C in a refrigerator until biological experiments. Dilutions in media were prepared fresh just before the assays.

Cell culture

Human pancreatic cancer cell line BxPC-3, human lung carcinoma cell line A549, melanoma cell line IGR39, glioblastoma cell line U87, triple-negative breast cancer cell line MDA-MB-231, breast adenocarcinoma cell line MCF-7, invasive ductal carcinoma cell line BT474, non-small cell lung carcinoma cell line H1299, ovarian cancer cell line SKOV-3 were kindly provided by Dr. Manel Esteller, IDIBELL, Spain). Cells were grown in DMEM Glutamax medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum and 1% antibiotic mixture (10,000 U/ml penicillin and 10 mg/ml streptomycin; Gibco). Human foreskin fibroblasts were kindly provided from Prof. Helder Almeida Santos (Helsinki University). Fibroblasts were grown DMEM Glutamax medium containing 20% fetal bovine serum and 1% antibiotic mixture. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and used until passage 20.

Cell viability assay

The viability of cells treated with compounds was determined by MTT (Sigma-Aldrich Co.) assay. Cells were plated (3×10^3 A549, IGR39, U87 cells/well; 5×10^3 BxPC-3, MDA-MB-231, SKOV-3, H1299 cells/well; 10×10^3 MCF-7, BT474) in 96-well plate and incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2.

For the screening purposes, cells were affected by 10 μM of tested compounds. As positive control only medium without cells was used and the medium with 0.5% DMSO (Sigma-Aldrich Co.) served as a negative control. After 72 hours of incubation with compounds, 20 μL of MTT (5 mg/ml) was added into each well and incubated for 4 hours. The medium was removed and 100 μL DMSO was added. The absorbance was measured at 570 nm and 630 nm. The cell growth (%) was calculated by formula (Absorbance of tested compound – Absorbance of negative control)/(Absorbance of negative control – Absorbance of positive control) × 100.
The EC_{50} values were determined for the most active compounds. Experimental procedure was the same as described above, only cells were affected by different concentrations of tested compounds (from 10 μM to 6.4 nM). The experiments were repeated three times independently. Applying Hill fit to compound dose – cell metabolic activity (absorbance) curves, the effective concentration (EC_{50}) values, reducing cell viability by 50%, were calculated.

Apoptosis and necrosis assay

Cells were seeded in 24-well plate (15-30 $\times 10^3$ cells/well) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 24 h 50% EC_{50} of tested compounds were added to the wells and incubated for 72 h. Then 3 μL Hoechst 33342 (1 mg/ml, Invitrogen, Paisley, UK) and 1 μL propidium iodide (1 mg/ml, Invitrogen) were added to each well and cells were incubated for 10 min. Images were taken using inverted fluorescent microscope (Olympus IX73). Apoptotic and necrotic cells were counted and the percentage number of cells was calculated.