

## Synthesis, *in vitro* and *in vivo* anticancer activity of novel 1-(4-imino-1-substituted-1H-pyrazolo [3,4-d]pyrimidin-5(4H)-yl)urea derivatives

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### 1. Synthetic methodology of design hybrids (28-40)

#### Chemicals

All the chemicals and reagents were obtained from Sigma Aldrich (St. Louis, MO, USA), Alfa Aesar (Massachusetts), S.D Fine Chemicals (India) and Merck (Darmstadt, Germany) and solvents for reaction medium were dried by standard methods. Melting points were determined in open capillaries using model KSP11, KRUSS, (Germany). The infrared (IR) spectra (KBr, thin film) were recorded on a Nicolet iS5 IR spectrophotometer (Thermo scientific, United States). The nuclear magnetic resonance (NMR) spectra were obtained on high resolution Jeol-400MHz NMR spectrophotometer (USA). Mass spectra were recorded on an Agilent 6310 Ion trap LC/MS and elemental analysis (C, H and N) was carried on Elementar analysensysteme.

#### **Procedure for the synthesis of 2-(ethoxymethylene) malononitrile (1)**

Equimolar amount of malononitrile (660mg, 10 mmol) and triethylorthoformate(1.65ml, 10 mmol) were heated at 130<sup>0</sup>C in acetic anhydride for 5 h. After completion of reaction, acetic anhydride was evaporated under reduced pressure and the crude product was purified by column chromatography using chloroform as eluent to yield pure 2-(ethoxymethylene) malononitrile (1).

#### ***2-(ethoxymethylene) malononitrile (1)***

Yield: 90%; pale yellow liquid,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  1.42 (t, 3H,  $\text{CH}_3$ ,  $J = 7.2\text{Hz}$ ), 4.35 (q, 2H,  $\text{CH}_2$ ,  $J = 7.1\text{Hz}$ ), 7.62 (s, =CH).

**Procedure for the synthesis of 5-amino-1-substituted phenyl-1H-pyrazole-4-carbonitrile (2-14)**<sup>1,2</sup>

An equimolar ratio of substituted phenylhydrazine (10mmol) and 2-(ethoxymethylene) malononitrile **1** (1220mg, 10mmol) was heated at  $100^\circ\text{C}$  for 8-12 h in ethanol. The reaction mixture was poured in to chilled water. Appeared precipitate was filtered, dried and washed with hexane to give pure intermediates **2-14**.

***5-amino-1-phenyl-1H-pyrazole-4-carbonitrile (2)***

Yield: 75%; creamish solid; mp:  $124\text{-}126^\circ\text{C}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  4.60 (s, 2H,  $\text{NH}_2$ ), 7.44-7.56 (m, 5H, Ar), 7.60 (s, 1H, Ar); LC-MS: m/z, 185 (M+1).

***5-amino-1-(3-fluorophenyl)-1H-pyrazole-4-carbonitrile (3)***

Yield: 78%; yellow solid; mp:  $140\text{-}142^\circ\text{C}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  4.68 (s, 2H,  $\text{NH}_2$ ), 7.14-7.19(m, 1H, Ar), 7.28-7.34 (m, 2H, Ar), 7.49-7.54 (m, 1H, Ar), 7.65 (s, 1H, Ar); LC-MS: m/z, 203 (M+1).

***5-amino-1-(4-fluorophenyl)-1H-pyrazole-4-carbonitrile (4)***

Yield: 79%; yellow solid; mp:  $161\text{-}163^\circ\text{C}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  4.57 (s, 2H,  $\text{NH}_2$ ), 7.21-7.26(m, 2H, Ar), 7.48-7.51(m, 2H, Ar), 7.64 (s, 1H, Ar); LC-MS: m/z, 203 (M+1).

***5-amino-1-(3-chlorophenyl)-1H-pyrazole-4-carbonitrile (5)***

Yield: 72%; yellow solid; mp:  $165\text{-}167^\circ\text{C}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  4.68 (2H,  $\text{NH}_2$ ), 7.41-7.50 (m, 3H, Ar), 7.56-7.57 (m, 1H, Ar), 7.65 (s, 1H, Ar); LC-MS: m/z, 219 (M+1).

***5-amino-1-(4-chlorophenyl)-1H-pyrazole-4-carbonitrile (6)***

Yield: 70%; orange solid; mp: 159-161<sup>0</sup>C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz ): δ 4.61 (s, 2H, NH<sub>2</sub>), 7.45-7.52 (m, 4H, Ar), 7.64 (s, 1H, Ar); LC-MS: m/z, 219 (M+1).

***5-amino-1-(3-bromophenyl)-1H-pyrazole-4-carbonitrile (7)***

Yield: 75%; yellow solid; mp: 163-165<sup>0</sup>C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz ): δ 4.61 (s, 2H, NH<sub>2</sub>), 7.41 (t, 1H, Ar, *J*= 8.0 Hz), 7.48 (d, 1H, Ar, *J*= 8.3Hz), 7.59 (d, 1H, Ar, *J*= 8.4 Hz), 7.65 (s, 1H, Ar), 7.71 (s, 1H, Ar); LC-MS: m/z, 262 (M+1), 263 (M+2).

***5-amino-1-(4-bromophenyl)-1H-pyrazole-4-carbonitrile (8)***

Yield: 65%; yellow solid; mp: 160-162<sup>0</sup>C; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz ): δ 6.79 (s, 2H, NH<sub>2</sub>), 7.46 (d, 2H, Ar, *J*= 8.4 Hz), 7.71(d, 2H, Ar, *J*= 8.4 Hz), 7.80 (s, 1H, Ar), LC-MS: m/z, 262 (M+1), 263 (M+2).

***5-amino-1-(4-cyanophenyl)-1H-pyrazole-4-carbonitrile (9)***

Yield: 68%; white solid; mp: 163-165<sup>0</sup>C; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz ): δ 6.98 (s, 2H, NH<sub>2</sub>), 7.73 (d, 2H, Ar, *J*= 8.4Hz), 7.87 (s, 1H, Ar), 7.99 (d, 2H, Ar, *J*= 8.4 Hz); LC-MS: m/z, 210 (M+1).

***5-amino-1-(3-nitrophenyl)-1H-pyrazole-4-carbonitrile (10)***

Yield: 63%; yellow solid; mp: 122-124<sup>0</sup>C; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 7.01 (s, 2H, NH<sub>2</sub>), 7.80 (t, 1H, *J*= 8.0 Hz), 7.87 (s, 1H, Ar), 7.98 (d, 1H, Ar, *J*= 8.4Hz), 8.25 (d, 1H, Ar, *J*= 8.4 Hz), 8.29 (s, 1H, Ar); LC-MS: m/z, 230 (M+1).

***5-amino-1-(4-nitrophenyl)-1H-pyrazole-4-carbonitrile (11)***

Yield: 71%; yellow solid; mp: 140-142<sup>0</sup>C; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 4.65 (s, 2H, NH<sub>2</sub>), 7.65 (s, 1H, Ar), 7.76 (d, 2H, Ar, *J*= 9.1Hz), 8.34 (d, 2H, Ar, *J*= 9.1Hz); LC-MS: m/z, 230 (M+1).

***5-amino-1-(p-tolyl)-1H-pyrazole-4-carbonitrile (12)***

Yield: 76%; creamish-white solid; mp: 118-120<sup>0</sup>C; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 2.35 (s, 3H, CH<sub>3</sub>), 6.61 (s, 2H, NH<sub>2</sub>), 7.33 (q, 4H, Ar, *J*= 8.6Hz), 7.74 (s, 1H, Ar); LC-MS: m/z, 199 (M+1).

***4-amino-1-(3, 4-dimethylphenyl)-1H-pyrazole-5-carbonitrile (13)***

Yield: 72%; yellow solid; mp: 143-145<sup>0</sup>C; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 2.25 (s, 6H, 2CH<sub>3</sub>), 6.59 (s, 1H, NH<sub>2</sub>), 7.16-7.26 (m, 3H, Ar), 7.73 (s, 1H, Ar); LC-MS: m/z, 213 (M+1).

***4-amino-1-(4-methoxyphenyl)-1H-pyrazole-5-carbonitrile (14)***

Yield: 79%; brown solid; mp: 138-140<sup>0</sup>C; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 3.79 (s, 3H, CH<sub>3</sub>), 6.54 (s, 2H, NH<sub>2</sub>), 7.04 (d, 2H, Ar, *J*= 9.1Hz), 7.37 (d, 2H, Ar, *J*= 9.1Hz), 7.72 (s, 1H, Ar), LC-MS: m/z, 215 (M+1).

**Procedure for the synthesis of (E)-ethyl N-(5-cyano-1-substitued phenyl-1H-pyrazol-4-yl)formimidate (15-27)<sup>1,2</sup>**

Carbonitrile derivatives **2–13** (10mmol) and triethylorthoformate (1.65ml, 10mmol) were heated at 130<sup>0</sup>C in acetic anhydride for 6 h. The reaction mixture was diluted with ethyl acetate and washed with water twice. The crude product was purified by column chromatography using chloroform as eluent to offer subsequent imino-ether derivatives (**15-27**).

***(E)-ethyl N-(5-cyano-1-phenyl-1H-pyrazol-4-yl)formimidate (15)***

Yield: 75%; yellow liquid; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): δ 1.36 (t, 3H, CH<sub>3</sub>, *J*= 7.2Hz), 4.32 (q, CH<sub>2</sub>, 2H, *J*= 7.0Hz), 7.37 (t, 1H, Ar, *J*= 7.6Hz), 7.46 (t, 2H, Ar, *J*= 8.0Hz), 7.61 (d, 2H, ArH, *J*= 8.4Hz), 7.81 (s, 1H, Ar), 8.38 (s, 1H, N=CH), LC-MS: m/z, 241(M+1).

***(E)-ethyl N-(5-cyano-1-(3-fluorophenyl)-1H-pyrazol-4-yl)formimidate (16)***

Yield: 78%; yellow liquid; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz ): δ 1.40 (t, 3H, CH<sub>3</sub>, *J*= 6.4Hz), 4.38 (q, 2H, CH<sub>2</sub>, *J*= 7.09Hz), 7.08 (t, 1H, Ar, *J*= 7.6Hz), 7.52-7.39 (m, 3H, Ar), 7.82 (s, 1H, Ar), 8.41 (s, 1H, CH); LC-MS: m/z, 259(M+1).

***(E)-ethyl N-(5-cyano-1-(4-fluorophenyl)-1H-pyrazol-4-yl)formimidate(17)***

Yield: 65%; yellow liquid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  1.32 (t, 3H,  $\text{CH}_3$ ,  $J = 7.2\text{Hz}$ ), 4.29 (q, 2H,  $\text{CH}_2$ ,  $J = 6.8\text{Hz}$ ), 7.08-7.12 (m, 2H, Ar), 7.56-7.59 (m, 2H, Ar), 7.76 (s, 1H, Ar), 8.37 (s, 1H, N=CH), LC-MS: m/z, 259 (M+1).

***(E)-ethyl N-(1-(3-chlorophenyl)-5-cyano-1H-pyrazol-4-yl)formimidate (18)***

Yield: 62%; yellow liquid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  1.40 (t, 3H,  $\text{CH}_3$ ,  $J = 6.8\text{Hz}$ ), 4.37 (q, 2H,  $\text{CH}_2$ ,  $J = 7.0\text{Hz}$ ), 7.33-7.41 (m, 2H, Ar), 7.59 (d, 1H, Ar,  $J = 7.6\text{Hz}$ ), 7.75 (s, 1H, Ar), 7.81 (s, 1H, Ar), 8.41 (s, 1H, N=CH), LC-MS: m/z, 248 (M+1).

***(E)-ethyl N-(1-(4-chlorophenyl)-5-cyano-1H-pyrazol-4-yl)formimidate (19)***

Yield: 64%; yellow liquid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  1.38 (t, 3H,  $\text{CH}_3$ ,  $J = 6.8\text{Hz}$ ), 4.34 (q, 2H,  $\text{CH}_2$ ,  $J = 6.9\text{Hz}$ ), 7.43 (d, 2H, Ar,  $J = 8.4\text{Hz}$ ), 7.62 (d, 2H, Ar,  $J = 9.1\text{Hz}$ ), 7.81 (s, 1H, Ar), 8.41 (s, 1H, N=CH), LC-MS: m/z, 248 (M+1).

***(E)-ethyl N-(1-(3-bromophenyl)-5-cyano-1H-pyrazol-4-yl)formimidate (20)***

Yield: 70%; yellow liquid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  1.40 (t, 3H,  $\text{CH}_3$ ,  $J = 7.2\text{Hz}$ ), 4.38 (q, 2H,  $\text{CH}_2$ ,  $J = 7.1\text{Hz}$ ), 7.33 (t, 1H, Ar,  $J = 8.0\text{Hz}$ ), 7.58-7.65 (m, 3H, Ar), 7.81 (s, 1H, Ar), 8.41 (s, 1H, CH), LC-MS: m/z, 318 ( $\text{M}^+$ ), 320 (M+2).

***(E)-ethyl N-(1-(4-bromophenyl)-5-cyano-1H-pyrazol-4-yl)formimidate (21)***

Yield: 63%; yellow liquid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  1.38 (t, 3H,  $\text{CH}_3$ ,  $J = 7.2\text{Hz}$ ), 4.34 (q, 2H,  $\text{CH}_2$ ,  $J = 7.0\text{Hz}$ ), 7.54-7.60 (m, 4H, Ar), 7.81 (s, 1H, Ar), 8.41 (s, 1H, N=CH), LC-MS: m/z, 318 ( $\text{M}^+$ ), 320 (M+2).

***(E)-ethyl N-(5-cyano-1-(4-cyanophenyl)-1H-pyrazol-4-yl)formimidate (22)***

Yield: 76%; yellow liquid;  $^1\text{H}$  NMR ( $\text{DMSO-d}_6$ , 400 MHz):  $\delta$  1.30 (t, 3H,  $\text{CH}_3$ ,  $J = 6.8\text{Hz}$ ), 4.32 (q, 2H,  $\text{CH}_2$ ,  $J = 7.08\text{Hz}$ ), 7.90 (d, 2H, Ar,  $J = 8.4\text{Hz}$ ), 8.01 (d, 2H, Ar,  $J = 8.4\text{Hz}$ ), 8.26 (s, 1H, Ar), 8.57 (s, 1H, N=CH), LC-MS: m/z, 266 (M+1).

***(E)-ethyl N-(5-cyano-1-(3-nitrophenyl)-1H-pyrazol-4-yl)formimidate (23)***

Yield: 74%; yellow liquid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  1.36 (t, 3H,  $\text{CH}_3$ ,  $J= 7.2\text{Hz}$ ), 4.36 (2, 2H,  $\text{CH}_2$ ,  $J= 7.3\text{Hz}$ ), 7.59 (t, 1H, Ar,  $J= 8.7\text{Hz}$ ), 7.79 (s, 1H, Ar), 8.06-8.10 (m, 1H, Ar), 8.14-8.17 (m, 1H, Ar), 8.44 (s, 1H,  $\text{N}=\text{CH}$ ), 8.74 (t, 1H, Ar), LC-MS:  $m/z$ , 286 (M+1).

***(E)-ethyl N-(5-cyano-1-(4-nitrophenyl)-1H-pyrazol-4-yl)formimidate (24)***

Yield: 68%; yellow liquid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  1.32 (t, 3H,  $\text{CH}_3$ ,  $J= 6.8\text{Hz}$ ), 4.37 (q, 4H,  $\text{CH}_2$ ,  $J= 7.0\text{Hz}$ ), 8.00 (d, 2H, Ar,  $J= 9.1\text{Hz}$ ), 8.29 (s, 1H, Ar), 8.38 (s, d, 2H, Ar,  $J= 9.1\text{Hz}$ ), 8.60 (s, 1H,  $\text{N}=\text{CH}$ ), LC-MS:  $m/z$ , 286 (M+1).

***(E)-ethyl N-(5-cyano-1-(p-tolyl)-1H-pyrazol-4-yl)formimidate (25)***

Yield: 78%; yellow liquid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  1.36 (t, 3H,  $\text{CH}_3$ ,  $J= 6.8\text{Hz}$ ), 2.40 (s, 3H,  $\text{CH}_3$ ), 4.31 (q, 2H,  $\text{CH}_2$ ,  $J= 7.0\text{Hz}$ ), 7.34 (d, 2H, Ar,  $J= 7.4\text{Hz}$ ), 7.48 (d, 2H, Ar,  $J= 8.4\text{Hz}$ ), 7.79 (s, 1H, Ar), 8.37 (s, 1H,  $\text{N}=\text{CH}$ ), LC-MS:  $m/z$ , 255 (M+1).

***(E)-ethyl N-(5-cyano-1-(3,4-dimethylphenyl)-1H-pyrazol-4-yl)formimidate (26)***

Yield: 69%; yellow liquid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  1.29 (t, 3H,  $\text{CH}_3$ ,  $J= 7.2\text{Hz}$ ), 2.23 (s, 6H, 2 $\text{CH}_3$ ), 4.26 (q, 2H,  $\text{CH}_2$ ,  $J= 7.0\text{Hz}$ ), 7.12 (d, 1H, Ar,  $J= 7.6\text{Hz}$ ), 7.25 (d, 1H, Ar,  $J= 7.6\text{Hz}$ ), 7.33 (s, 1H, Ar), 7.72 (s, 1H, Ar), 8.29 (s, 1H,  $\text{N}=\text{CH}$ ), LC-MS:  $m/z$ , 269 (M+1).

***(E)-ethyl N-(5-cyano-1-(4-methoxyphenyl)-1H-pyrazol-4-yl)formimidate (27)***

Yield: 62%; yellow liquid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  1.36 (t, 3H,  $\text{CH}_3$ ,  $J= 7.2\text{Hz}$ ), 3.85 (s, 3H,  $\text{CH}_3$ ), 4.31 (q, 2H, Ar,  $J= 7.0\text{Hz}$ ), 6.96 (d, 2H, Ar,  $J= 8.4\text{Hz}$ ), 7.51 (d, 2H, Ar,  $J= 9.1\text{Hz}$ ), 7.79 (s, 1H, Ar), 8.37 (s, 1H,  $\text{N}=\text{CH}$ ), LC-MS:  $m/z$ , 271 (M+1).

**Procedure for the synthesis of (4-Imino-1-substitued phenyl-1, 4-dihydro-pyrazolo [3, 4-d] pyrimidin-5-yl)-urea derivatives (28-40)**

A mixture of imino-ether derivatives **15-27** (10mmol), semicarbazide hydrochloride (1110mg, 10mmol), and TEA (1–2 ml) in ethanol was stirred at 25-30 $^{\circ}\text{C}$  for 12 h. Emerged precipitate was filtered, washed with absolute ethanol and then with water followed by recrystallization in mixture of acetonitrile and methanol to give pyrazolo-pyrimidine-urea derivatives (**28-40**).

***(4-Imino-1-phenyl-1, 4-dihydro-pyrazolo [3, 4-d] pyrimidin-5-yl)-urea (28)***

Yield: 78%; White solid; mp: 285-287<sup>0</sup>C; IR (KBr, cm<sup>-1</sup>): 3374.7, 3174.8, 1659.7, 1534.0, 1261.6, 1152.5, 975.8, 871.6, 848.5, 768.8, 688.2; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 6.48 (brs, 2H, NH<sub>2</sub>), 7.37 (t, 1H, Ar, *J*= 8.0Hz), 7.51-7.55 (m, 2H, Ar), 7.97 (d, 2H, Ar, *J*= 7.6 Hz), 8.02-8.03 (m, 2H, Ar+NH), 8.29 (s, 1H, Ar), 8.92 (brs, 1H, NH). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 100 MHz): 106.5, 121.6, 126.9, 136.2, 138.2, 147.1, 152.8, 158.0. LC-MS: m/z, 270 (M+1); Anal. Calcd for. C<sub>12</sub>H<sub>11</sub>N<sub>7</sub>O: C, 53.53; H, 4.12; N, 36.41; Found: C, 53.64; H, 4.24; N, 36.20

***[1-(3-Fluoro-phenyl)-4-imino-1, 4-dihydro-pyrazolo [3, 4-d] pyrimidin-5-yl]-urea (29)***

Yield: 85%; White solid; mp: 299-301<sup>0</sup>C; IR (KBr, cm<sup>-1</sup>): 3355.7, 3290.2, 3161.1, 1661.3, 1536.8, 1282.1, 1163.2, 993.3, 869.4, 779.3, 630.0; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 6.51(s, 2H, NH<sub>2</sub>), 7.22 (t, 1H, Ar, *J*= 8.3Hz), 7.58 (q, 1H, Ar, *J*= 7.6Hz), 7.88-7.92 (m, 2H, Ar), 8.07 (s, 1H, Ar), 8.16 (brs, 1H, NH), 8.34 (s, 1H, Ar), 8.83 (brs, 1H, NH); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 100 MHz): δ 106.8, 108.2, 113.4, 117.0, 131.1, 136.6, 139.7, 147.4, 153.1, 160.7, 163.2; LC-MS: m/z, 288(M+1); Anal. Calcd for. C<sub>12</sub>H<sub>10</sub>FN<sub>7</sub>O: C, 50.17; H, 3.51; N, 34.13; Found: C, 50.32; H, 3.42; N, 34.41.

***[1-(4-Fluoro-phenyl)-4-imino-1, 4-dihydro-pyrazolo [3, 4-d] pyrimidin-5-yl]-urea (30)***

Yield: 88%; White solid; mp: 304-306<sup>0</sup>C; IR (KBr, cm<sup>-1</sup>): 3357.8, 3310.0, 3176.0, 1657.0, 1534.9, 1264.3, 1158.6, 977.3, 871.3, 817.1, 777.2, 648.5; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 6.51 (s, 2H, NH<sub>2</sub>), 7.39 (t, 2H, *J*= 8.8 Hz), 7.97-7.99 (m, 4H, Ar + NH), 8.30 (s, 1H, Ar), 8.86 (br s, 1H, NH); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 100 MHz): δ 106.4, 115.9, 116.1, 123.7, 134.6, 136.1, 147.0, 152.9, 159.3, 161.7; LC-MS: m/z, 288 (M+1); Anal. Calcd for. C<sub>12</sub>H<sub>10</sub>FN<sub>7</sub>O: C, 50.17; H, 3.51; N, 34.13; Found: C, 50.36; H, 3.64; N, 33.92.

***[1-(3-Chloro-phenyl)-4-imino-1, 4-dihydro-pyrazolo [3, 4-d] pyrimidin-5-yl]-urea (31)***

Yield: 82%; White solid; mp: 302-304<sup>0</sup>C; IR (KBr, cm<sup>-1</sup>): 3353.0, 3278.7, 1660.8, 1567.4, 1269.4, 1163.8, 986.0, 878.9, 853.1, 766.6, 673.4; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 6.51 (brs, 2H, NH<sub>2</sub>), 7.42 (d, 1H, Ar, *J*= 7.6Hz), 7.57 t, 1H, *J*= 8.0Hz), 8.00( d, 1H, Ar, *J*= 6.8 Hz), 8.08 (s, 1H, Ar), 8.13 (t, 1H, Ar), 8.18 (brs, 1H, NH), 8.33 (s, 1H, Ar), 8.89 (brs, 1H, NH); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>,

100 MHz):  $\delta$  106.8, 119.7, 120.7, 126.7, 131.4, 133.4, 136.7, 139.4, 147.5, 153.2, 158.0 LC-MS: m/z, 304 (M+1); Anal. Calcd for. C<sub>12</sub>H<sub>10</sub>ClN<sub>7</sub>O: C, 47.46; H, 3.32; Cl, N, 32.28; Found: C, 47.27; H, 3.48; Cl, N, 32.47.

***[1-(4-Chloro-phenyl)-4-imino-1,4-dihydro-pyrazolo [3,4-d] pyrimidin-5-yl]-urea (32)***

Yield:86%; White solid; mp: 298-300<sup>0</sup>C; IR (KBr, cm<sup>-1</sup>): 3344.9, 3300.8, 1659.5, 1533.3, 1262.0, 1156.7, 974.7, 871.7, 826.7, 748.6, 640.6; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz):  $\delta$  6.48 (s, 2H, NH<sub>2</sub>), 7.60 (d, 2H, Ar, *J*= 7.3 Hz), 8.03-8.05 (4H, Ar + NH), 8.30 (s, 1H, Ar), 8.88 (brs, 1H, NH); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 100 MHz):  $\delta$  106.7, 122.8, 129.2, 131.0, 136.5, 137.1, 147.2, 153.0, 158.1; LC-MS: m/z, 304 (M+1); Anal. Calcd for. C<sub>12</sub>H<sub>10</sub>ClN<sub>7</sub>O: C, 47.46; H, 3.32; N, 32.28; Found: C, 47.71; H, 3.16; N, 32.09.

***[1-(3-Bromo-phenyl)-4-imino-1,4-dihydro-pyrazolo[3,4-d]pyrimidin-5-yl]-urea (33)***

Yield:80%; White solid; mp: 303-305<sup>0</sup>C; IR (KBr, cm<sup>-1</sup>): 3348.9, 3291.6, 3156.6, 1665.8, 1538.8, 1283.0, 1135.7, 982.1, 880.5, 769.2, 710.7, 653.3; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz):  $\delta$  6.52 (brs, 2H, NH<sub>2</sub>), 7.51 (t, 1H, Ar, *J*=8.0 Hz), 7.57 (d, 1H, Ar, *J*=9.1 Hz), 8.02-8.08 (m, 3H, Ar+NH), 8.25 (s, 1H, Ar-H), 8.32 (s, 1H, Ar), 8.88 (brs, 1H NH). LC-MS: m/z, 348 (M+1), 349 (M+2); Anal. Calcd for. C<sub>12</sub>H<sub>10</sub>BrN<sub>7</sub>O: C, 41.40; H, 2.90; N, 28.16; Found: C, 41.54; H, 3.08; N, 27.95.

***[1-(4-Bromo-phenyl)-4-imino-1,4-dihydro-pyrazolo[3,4-d]pyrimidin-5-yl]-urea (34)***

Yield:80%; White solid; mp: 304-306<sup>0</sup>C; IR (KBr, cm<sup>-1</sup>): 3347.0, 3360.5, 3174.3, 1661.0, 1532.2, 1263.2, 1156.2, 973.1, 871.6, 823.8, 768.4, 695.0; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz):  $\delta$  6.50 (s, 2H, NH<sub>2</sub>), 7.73 (d, 2H, Ar, *J*= 8.4Hz), 7.98 (d, 2H, Ar, *J*= 8.4Hz), 8.04 (s, 1H, Ar), 8.15 (brs, 1H, NH), 8.31 (s, 1H, Ar), 8.84 (brs, 1H, NH); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 100MHz):  $\delta$  106.4, 119.4, 123.1, 132.1, 136.7, 137.5, 147.1, 153.0, 157.9; LC-MS: m/z, 348 (M+1), 349 (M+2); Anal. Calcd for. C<sub>12</sub>H<sub>10</sub>BrN<sub>7</sub>O: C, 41.40; H, 2.90; N, 28.16; Found: 41.64; H, 2.68; N, 28.39.

***[1-(4-Cyano-phenyl)-4-imino-1,4-dihydro-pyrazolo[3,4-d]pyrimidin-5-yl]-urea (35)***



Yield:79%; White solid; mp: 305-307<sup>0</sup>C; IR (KBr, cm<sup>-1</sup>): 3393.0, 3305.8, 3250.7, 2228.6, 1650.2, 1535.6, 1292.1, 1196.7, 983.7, 875.9, 801.1, 754.5, 675.1; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 6.50 (s, 2H, NH<sub>2</sub>), 8.01 (d, 2H, Ar, *J*= 9.2Hz), 8.10 (s, 1H, Ar), 8.25-8.31 (m, 3H, Ar +NH), 8.38 (s, 1H, Ar), 8.91 (brs, 1H, NH); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 100 MHz): δ 107.2, 108.7, 118.6, 121.0, 133.6, 137.4, 141.7, 147.9, 153.4, 157.9; LC-MS: m/z, 295 (M+1); Anal. Calcd for. C<sub>13</sub>H<sub>10</sub>N<sub>8</sub>O: C, 53.06; H, 3.43; N, 38.08; Found: C, 53.27; H, 3.59; N, 37.93.

***[4-Imino-1-(3-nitro-phenyl)-1,4-dihydro-pyrazolo[3,4-d]pyrimidin-5-yl]-urea (36)***

Yield:86%; White solid; mp: 299-301<sup>0</sup>C; IR (KBr, cm<sup>-1</sup>): 3382.2, 3309.9, 3254.4, 1655.8, 1521.4, 1288.6, 1196.4, 999.2, 880.6, 828.7, 757.8, 660.7; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 6.52 (brs, 2H, NH<sub>2</sub>), 7.84 (t, 1H, Ar, *J*= 10.0Hz), 8.15 (s, 1H, Ar), 8.20-8.26 (m, 2H, Ar+NH), 8.39 (s, 1H, Ar), 8.52 (d, 1H, Ar, *J*= 7.6Hz), 8.84-8.98 (m, 2H, Ar + NH); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 100 MHz): δ 107.1, 115.2, 121.1, 126.7, 131.0, 137.1, 139.0, 147.8, 148.1, 153.5, 157.9 LC-MS: m/z, 315 (M+1); Anal. Calcd for. C<sub>12</sub>H<sub>10</sub>N<sub>8</sub>O<sub>3</sub>: C, 45.86; H, 3.21; N, 35.66; Found: C, 45.64; H, 3.34; N, 35.83.

***[4-Imino-1-(4-nitro-phenyl)-1,4-dihydro-pyrazolo[3,4-d]pyrimidin-5-yl]-urea (37)***

Yield:88%; White solid; mp: 301-303<sup>0</sup>C; IR (KBr, cm<sup>-1</sup>): 3392.1, 3312.4, 1675.8, 1520.3, 1230.9, 1198.5, 982.0, 873.3, 799.4, 749.0, 674.3; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 6.53 (brs, 2H, NH<sub>2</sub>), 8.13 (s, 1H, Ar), 8.37-8.42 (m, 6H, Ar + NH), 8.94 (brs, 1H, NH); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 100 MHz): δ 107.4, 120.9, 125.1, 137.7, 143.2, 145.0, 148.1, 153.7, 158.0; LC-MS: m/z, 315 (M+1); Anal. Calcd for. C<sub>12</sub>H<sub>10</sub>N<sub>8</sub>O<sub>3</sub>: C, 45.86; H, 3.21; N, 35.66; Found: C, 45.63; H, 3.39; N, 35.49.

***(4-Imino-1-p-tolyl-1,4-dihydro-pyrazolo[3,4-d]pyrimidin-5-yl)-urea (38)***

Yield: 92%; White solid; mp: 285-287<sup>0</sup>C; IR (KBr, cm<sup>-1</sup>): 3348.6, 3307.9, 3169.5, 2915.5, 2863.4, 1656.6, 1561.4, 1277.9, 1183.7, 976.1, 870.4, 814.9, 777.5, 651.8; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 2.34 (s, 3H, CH<sub>3</sub>), 6.48 (brs, 2H, NH<sub>2</sub>), 7.32 (d, 2H, Ar, *J*= 8.4Hz), 7.83 (d, 2H, *J*= 8.4Hz), 7.99 (s, 1H, Ar), 8.06 (s, 1H, NH), 8.26 (s, 1H, Ar), 8.81 (brs, 1H, NH); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 100 MHz): δ 20.6, 106.3, 121.5, 129.5, 135.8, 135.9, 136.3, 146.8, 152.5, 158.1; LC-MS: m/z, 284

(M+1); Anal. Calcd for. C<sub>13</sub>H<sub>13</sub>N<sub>7</sub>O: C, 55.12; H, 4.63; N, 34.61; Found: C, 55.37; H, 4.51; N, 34.39.

***[1-(3,4-Dimethyl-phenyl)-4-imino-1,4-dihydro-pyrazolo[3,4-d]pyrimidin-5-yl]-urea (39)***

Yield: 93%; White solid; mp: 310-312<sup>0</sup>C; IR (KBr, cm<sup>-1</sup>): 3327.3, 3180.8, 3136.7, 2862.9, 2772.9, 1654.1, 1209.0, 1017.5, 950.6, 843.1, 734.4, 654.6; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 2.25 (s, 3H, CH<sub>3</sub>), 2.28 (s, 3H, CH<sub>3</sub>), 6.49 (brs, 2H, NH<sub>2</sub>), 7.27 (d, 1H, Ar, *J*= 8.4Hz), 7.63 (d, 1H, Ar, *J*= 9.1Hz), 7.70 (s, 1H, Ar), 7.99 (s, 2H, Ar + NH), 8.25 (s, 1H, Ar), 8.83 (brs, 1H, NH); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 100 MHz): δ 18.9, 19.6, 106.2, 119.2, 122.7, 129.8, 135.2, 135.7, 136.1, 137.1, 146.8, 152.6, 158.1; LC-MS: m/z, 298 (M+1), Anal. Calcd for. C<sub>14</sub>H<sub>15</sub>N<sub>7</sub>O: C, 56.56; H, 5.09; N, 32.98; Found: C, 56.75; H, 5.25; N, 32.74

***[4-Imino-1-(4-methoxy-phenyl)-1, 4-dihydro-pyrazolo [3,4-d]pyrimidin-5-yl]-urea (40)***

Yield: 85%; White solid; mp: 303-305<sup>0</sup>C; IR (KBr, cm<sup>-1</sup>): 3319.5, 3190.5, 3148.3, 2995.9, 2837.8, 1653.9, 1535.6, 1251.5, 1174.0, 978.3, 879.3, 800.6, 767.4, 652.2; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400 MHz): δ 3.80 (s, 3H, OCH<sub>3</sub>), 6.48 (s, 2H, NH<sub>2</sub>), 7.08 (d, 2H, Ar, *J*= 8.4Hz), 7.81 (d, 2H, Ar, *J*= 7.6Hz), 7.98 (s, 1H, Ar), 8.05 (brs, 1H, NH), 8.24 (s, 1H, Ar), 8.88 (brs, 1H, NH); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 100 MHz): δ 55.4, 105.7, 114.2, 123.4, 130.8, 135.6, 146.8, 152.5, 157.8; LC-MS: m/z, 300 (M+1); Anal. Calcd for. C<sub>13</sub>H<sub>13</sub>N<sub>7</sub>O<sub>2</sub>: C, 52.17; H, 4.38; N, 32.76; Found: C, 51.94; H, 4.47; N, 32.54.

**2. Cell lines and culture conditions:** The human cancer cell lines HepG-2, A549, MCF-7 and Hela were purchased from ATCC (Manassas, VA). HepG-2 cells were grown in ATCC-formulated Eagle's Minimum Essential Medium (EMEM, Catalog No. 30-2003) while MCF-7, A549 and Hela cells were cultured in DMEM supplemented with 10% of fetal bovine serum (FBS, Hyclone), 2 mmol/ liter glutamine, and 100 units/ml penicillin and streptomycin (complete medium). The cells were kept at 37 °C in a humidified 95% air, 5% CO<sub>2</sub> atmosphere incubator designated as culture at a steady-state condition.

3. **Cytotoxicity assay:** Suspended cells (100  $\mu$ l) at density of 4,000 cells/well were seeded in 96-well plate (Nunc™, Wiesbaden, Germany). After 24 h of recovery the cells were incubated in humid atmosphere at 37°C and 5% CO<sub>2</sub> and treated with synthesized compounds **28-40**. The cells were treated with varying concentrations of compounds **28-40** and Doxorubicin (10 $\mu$ M) separately. After 24 h incubation with compounds **28-40** and Doxorubicin, 25 $\mu$ L of PBS containing 2.5 mg/ml of MTT was added to each well. After 4 h, medium was discarded and 100  $\mu$ L DMSO was added to dissolve the formazan crystals and then cells were incubated for 4 h at 37°C and 5% CO<sub>2</sub>. The light absorbance was determined at 590 nm by the Model 680 microplate-reader (Bio-Rad, Berkeley, CA, USA).

3. **Hoechst 33258 staining:** Apoptotic morphological changes in the nuclear chromatin of the A549 cells were stained by Hoechst 33258. The cells were seeded in sterile 6-well plates (Nunc Nunclon Delta) and then incubated with 5  $\mu$ M and 10  $\mu$ M of CBS-1 and Doxorubicin (10 $\mu$ M) for 24 h. Later, the cells were washed with 1X phosphate-buffered saline (10X PBS Gibco, Life Technologies™, USA) and were fixed with 4% paraformaldehyde for 10 min followed by incubation with 50  $\mu$ M Hoechst 33258 staining solution for 5 min at 25<sup>0</sup>C. After three washes with cold PBS, the cells were observed under a fluorescence microscope (Olympus, IX-70, and Japan).

4. **Apoptosis assay by flow cytometry:** Harvested cells followed by trypsinization (0.2 % EDTA-Trypsin), the cells were suspended in 4 ml fresh complete media and propagated in T25 flask and incubated overnight. Then, **CBS-1** at 5 and 10  $\mu$ M final concentrations was added and incubated for 24 h. After 24 h, cells were harvested and washed twice with cold PBS followed by re-suspension in 1X binding buffer at a concentration of 1x10<sup>6</sup> cells/ml. 100  $\mu$ l of such solution (1x10<sup>6</sup> cells) was mixed with 5  $\mu$ l of Annexin-V-FITC and 5  $\mu$ l of Propidium iodide (PI, BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. The mixed solution was incubated at 25°C in the dark for 15 min. Then 400  $\mu$ l of 1X dilution buffer was added to each tube. Analysis was performed by Beckman Coulter FC500 Flow Cytometry System with CXP Software (BeckmanCoulter, Fullerton, CA, USA) within 1 h of the treatment of cells with dilution buffer.

**5. Cell cycle analysis:** The analysis of cell cycle was performed by PI staining and analysis was carried out by flow cytometry using a fluorescence-activated cell sorting (FACS) caliber (Becton-Dickinson). Following to the treatment with 5  $\mu$ M and 10  $\mu$ M concentrations of **CBS-1** and Doxorubicin for 24 h, the A549 cells were harvested at concentration of  $1 \times 10^6$  cells/ml. The cells were fixed with 70% ethanol and incubated at 4°C overnight. The fixed cells were washed twice with cold PBS and incubated for 30 min with Ribonuclease A (#R-5125, Sigma, 8  $\mu$ g/ml) and PI (10  $\mu$ g/ml). Then, the cell samples were transferred to meshed blue capped tubes (BD Falcon™ Tubes #352235). Then after, the fluorescent signals were distinguished through the FL2 channel and the proportion of DNA level that was present in the various phases was analyzed using ModfitLT Version 3.0 (Verity Software House, Topsham, ME, USA).

**6. Immunofluorescence staining:** A549 cells were seeded in 4-well chambered slide with glass coverslips (SPL Life Sciences Co., Ltd., Korea 30104 PS/Glass/PP). The cells were incubated with **CBS-1** and Doxorubicin (5 and 10 $\mu$ M) and non-treated incubated with DMEM high glucose medium for 24 h. After incubation cells were washed with cold 1X PBS and were fixed with 4% paraformaldehyde for 15 min and cells were permeabilized with 0.2% Triton X-100 for 10 min. After that, cells were washed with PBS and blocked with 1% bovine serum albumin (BSA) for 30 min. Further, the cells were incubated with the primary antibodies anti-NF- $\kappa$ B antibody, Casp-3 was detected using 1:200 anti-casp-3 antibody (rabbit IgG). Afterward, the cells were incubated with Alexa Fluor 488 (donkey anti-rabbit) secondary antibodies for 1 h at 25°C in the dark. All of the antibodies used were procured from Santa Cruz Biotechnology, Inc. CA. Cells were then washed with cold PBS and were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen) for 5min. Once again cells were washed with cold 1X PBS and 1 ml mounting media was added and cells were observed under a fluorescence microscope (Olympus, Milan, Italy) with adaptable filter consistent with Alexa Fluor 488, FITC or PE.

**7. Quantitative real-time polymerase chain reaction (qPCR):** The cells were separated by 0.5% trypsin-EDTA (Gibco) and total RNA was extracted by easy Blue (Intron Biotech, Seongnam-si, Gyeonggi-do, Korea). Purified RNA (1 $\mu$ g) was subjected to first strand cDNA synthesis using Superscript III first-strand cDNA synthesis kit and Oligo dT primer (Invitrogen). The cDNA was employed to qPCR for the quantification of the relative transcript levels of NF-

$\kappa$ B (p65), inflammatory cytokine IL-6 as well as pro-apoptotic gene/anti-apoptotic gene caspase-3 (Casp-3) using the specific primers (Table I).  $\beta$  - actin opted as endogenous control.

Table 1. Primer sequence used in quantitative real-time PCR

Gene	Sequence	T <sub>m</sub> °C	Accession number
<b><i><math>\beta</math> - actin</i></b>	F- GGACTTCGAGCAAGAGATGG	57.50	NM_001101
	R- AGCACTGTGTTGGCGTACAG		
<b><i>NF-<math>\kappa</math>B</i></b>	F- CTGAACCAGGGCATACTGT	56.45	NM_001243984
	R- GAGAAGTCCATGTCCGCAAT		
<b><i>IL-6</i></b>	F - TACCCCCAGGAGAAGATTCC	55.45	NM_000600.3
	R - TTTTCTGCCAGTGCCTCTTT		
<b><i>Casp-3</i></b>	F- ACTGGACTGTGGCATTGAGA	54.40	XM_011532301
	R- GCACAAAGCGACTGGATGAA		

8. **Western blotting:** Total proteins were isolated by ice-cold RIPA buffer PMSF (Sigma-Aldrich Corp., USA) and prepared to use protein inhibitor cocktails (Fermentas, Thermo Scientific, Rockford, IL, USA). Concentration of protein was determined by using Pierce® BCA Protein Assay kit (Thermo Scientific) following the manufacturer's instructions. 16 $\mu$ g protein were loaded per well and separated on 12% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (PVDF, Sigma-Aldrich Corp.) in the Bio-Rad western blotting system (Bio-Rad, Berkeley, CA, USA). The membrane was incubated with the primary antibodies  $\beta$ -actin (1:1,000), 1:200 diluted anti-NF- $\kappa$ B antibody, Pro-apoptotic protein Casp-3, anti-human-IL-6 antibody (rabbit IgG), was detected using 1:200 (rabbit IgG). All the antibodies used were

procured from Santa Cruz Biotechnology. The proteins of interest were identified by an enhanced chemiluminescence detection kit using LAS4000 machine (GE Healthcare).

**9. A549-Xenograft lung adenocarcinoma animal model:** 5-week-old SPF/VAF immunodeficient nude mice were purchased from KS HI-TEC, Inc Korea. They were housed under suitable environmental and nutritional conditions. Mice were sacrificed at 10-, 11-, and 12-week-age according to the standard protocols of Jeju National University. The research proposal and the relevant experimental procedures were approved by the institutional review board of the Department of Animal Biotechnology, Jeju National University, Jeju Special Self-Governing Province, Korea. To study the tumorigenesis and metastasis, the mice were grouped into four groups. Four groups of animals were established and each group consist five animals. First and second group of animals used for studies tumorocidal effect of CBS-1 at 5mg and 10 mg/kg dose. Third group of animals used for positive control (Doxorubicin at 10 mg/kg) and fourth group of animals were served as negative control. To induce A549 mediated tumorigenesis,  $1 \times 10^6$  A549 cells/ml concentrations of cells into lower right flanks of nude mouse by micro-needle syringe (29 gauge x 1/2" 12.7 mm needle, Ultra-Thin Plus™ Korea). After generation of successful tumor model CBS-1 (5 and 10mg/kg) and Doxorubicin (10mg/kg) treatment were provided up to 45 days. Then animals sacrificed and tumors were removed from all animals and weight.

**10. Optical probe guided molecular imaging of tumor metastasis:** For pre- and intraoperative tumor localization in real-time resection, we conducted *in vivo* tumor localization assay using IRDye® 800CW 2-DG (2-deoxy-D-glucose) optical probe which was purchased from LI-COR, Biosciences, USA. To evaluate and establish metastatic potential of A549, characterized cells were inoculated subcutaneously to nude mice. After 7 days experimental metastasis was observed followed by observation of distinct ‘spontaneous metastasis’, where the tumor cells were first allowed to form a primary tumor at the site of injection and then to escape into lymphatic or blood circulation. Probe was dissolved in phosphate buffer saline (1X PBS) and was injected into the tail vein of the tumor-bearing nude mice then mice were observed after they were anesthetized with Zoletil 50 (Virbac, Carros, France) 1 ml/kg intraperitoneally and all surgical procedures were performed under general anesthesia at different time intervals.

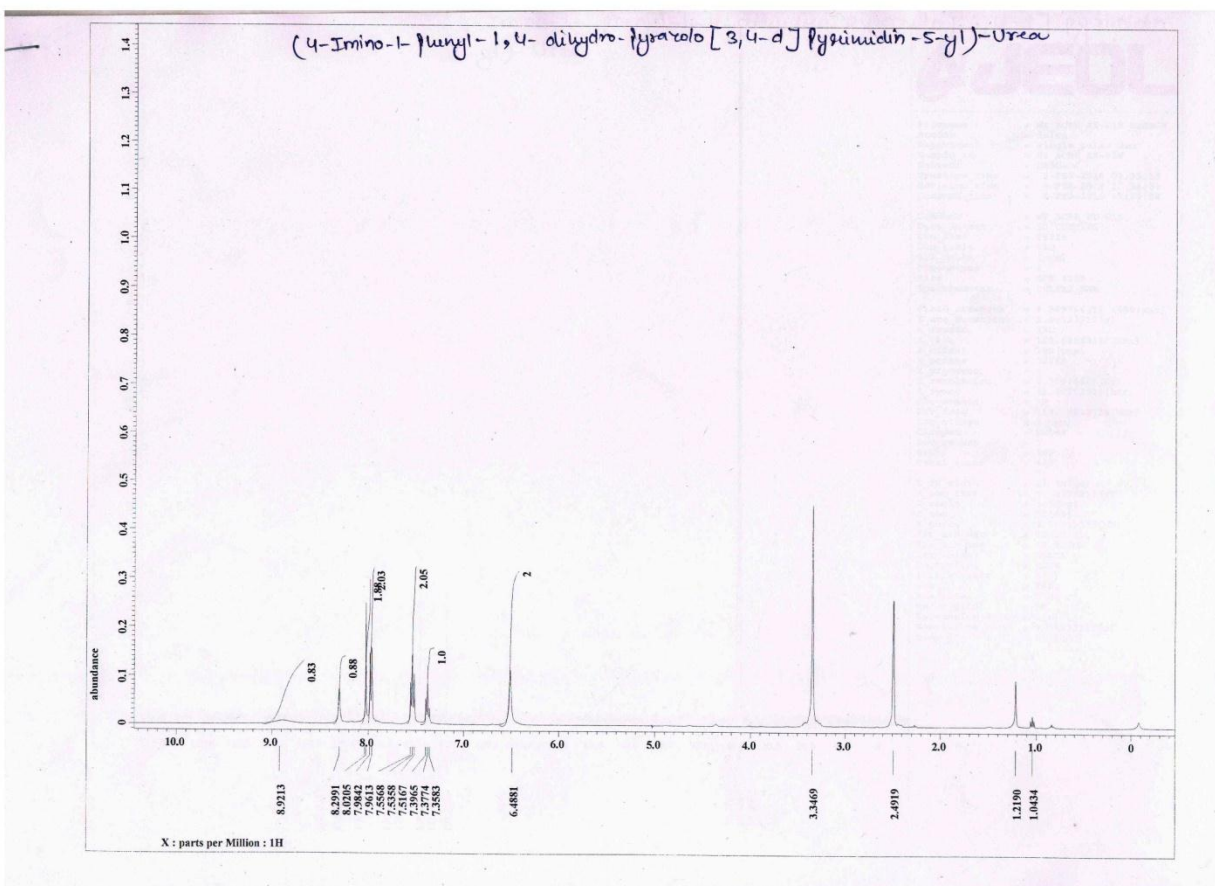
Metastasis was detected using optical molecular imaging, in particular near-infrared fluorescence (NIRF) range.

#### References

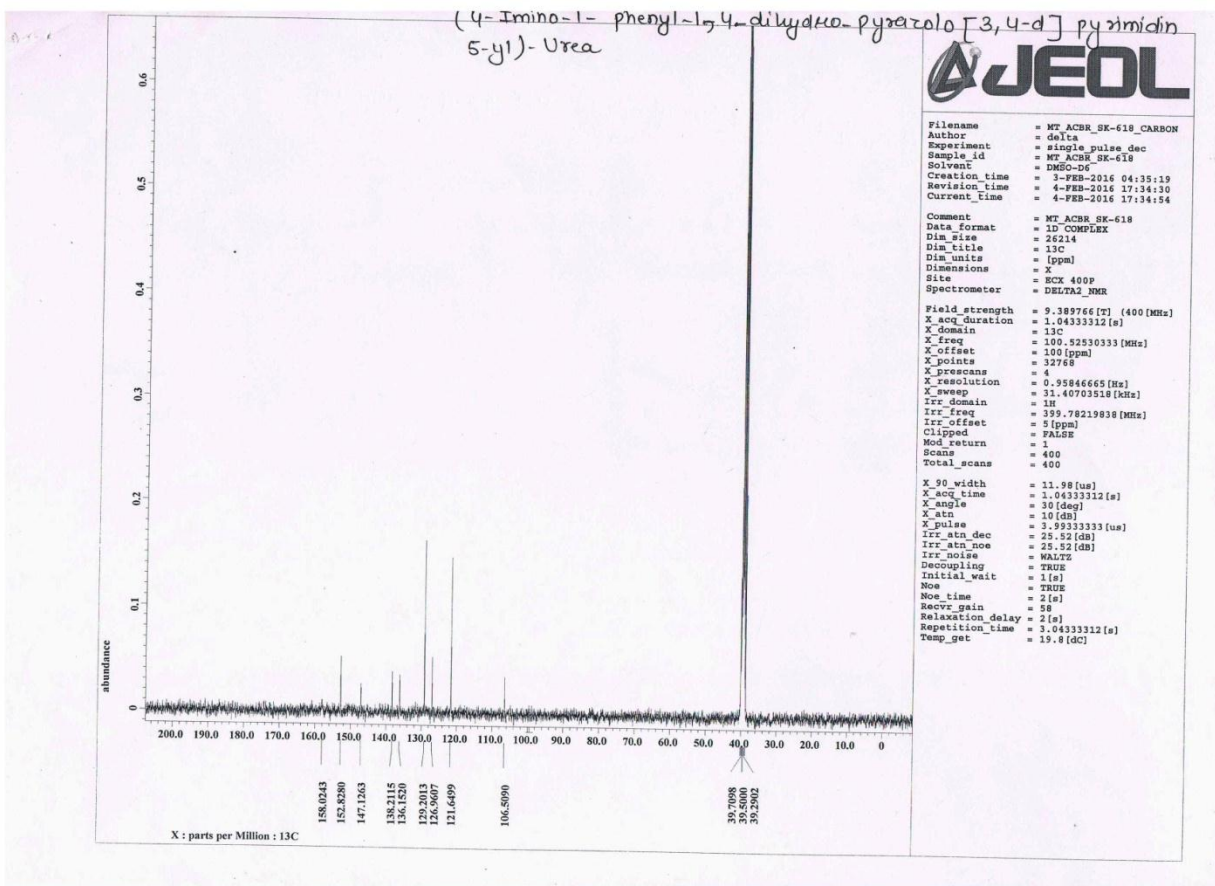
1. Baraldi PG, El-kashef H, Farghaly A, Vanelle P, Fruttarolo F (2004) *Tetrahedron*, 60, 5093-5104.
2. Abdel-Megid M, Awas M.A.A, Seada M, El-Mahdy KM (2008) *Organic chemistry: An Indian Journal*, 4, 259-265.

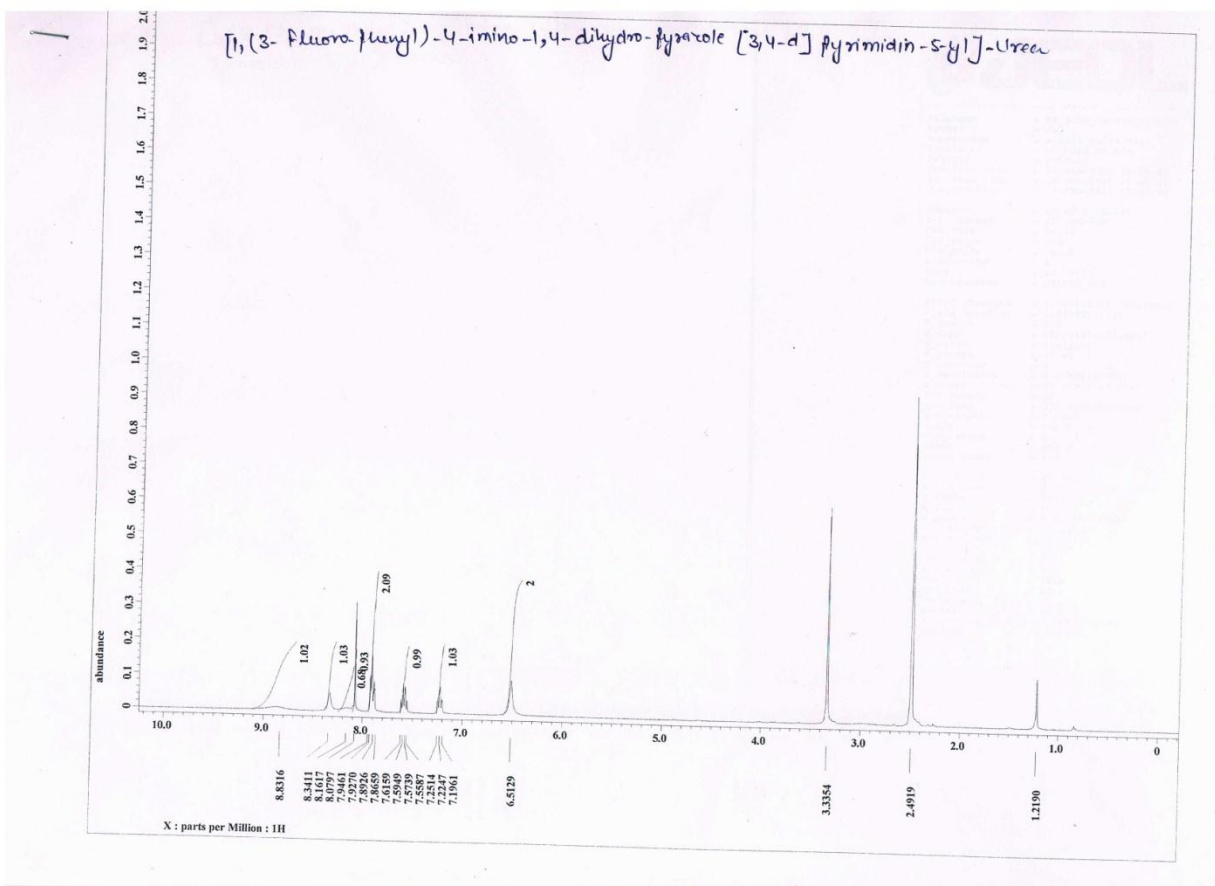
$^1\text{H}$  and  $^{13}\text{C}$  spectra of compounds 28-40

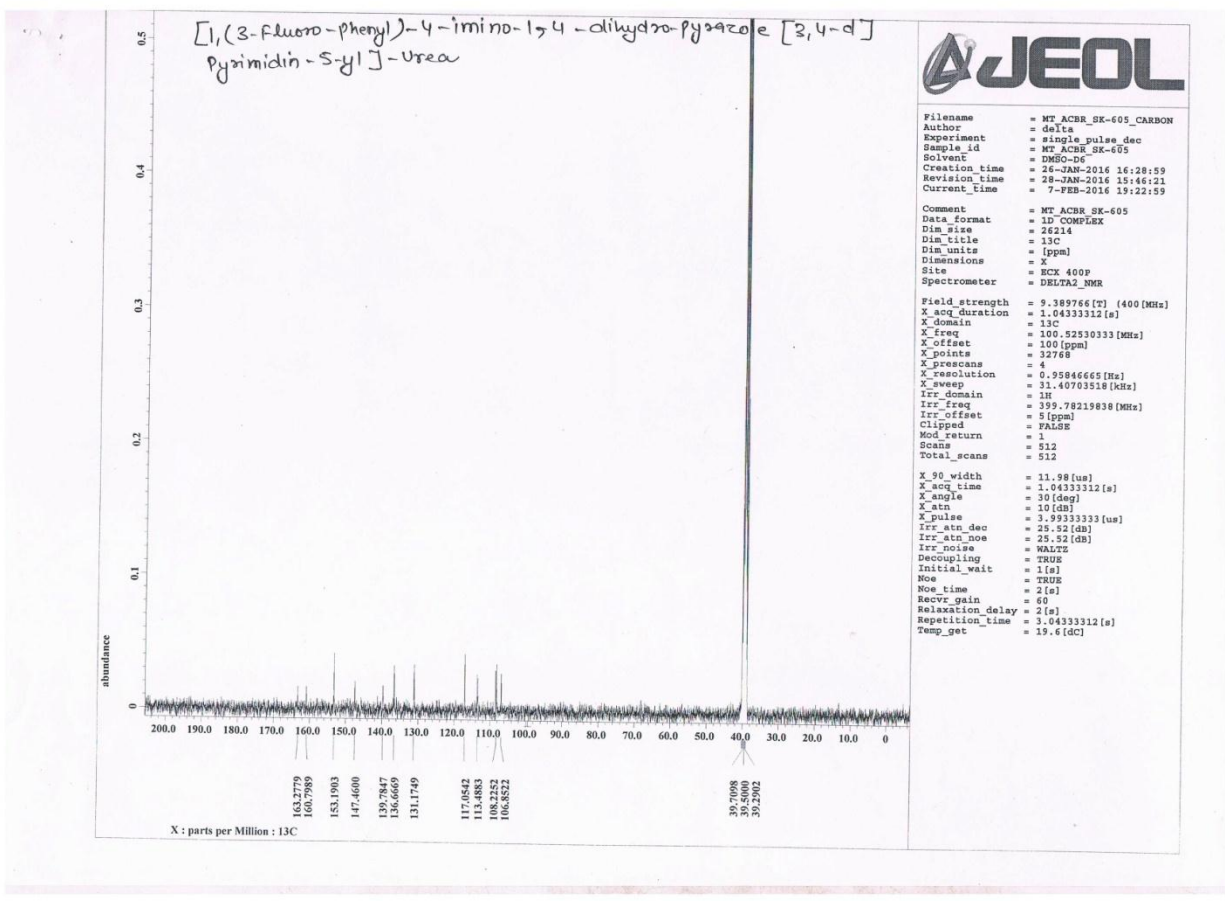
**$^1\text{H}$  of Compound 28**

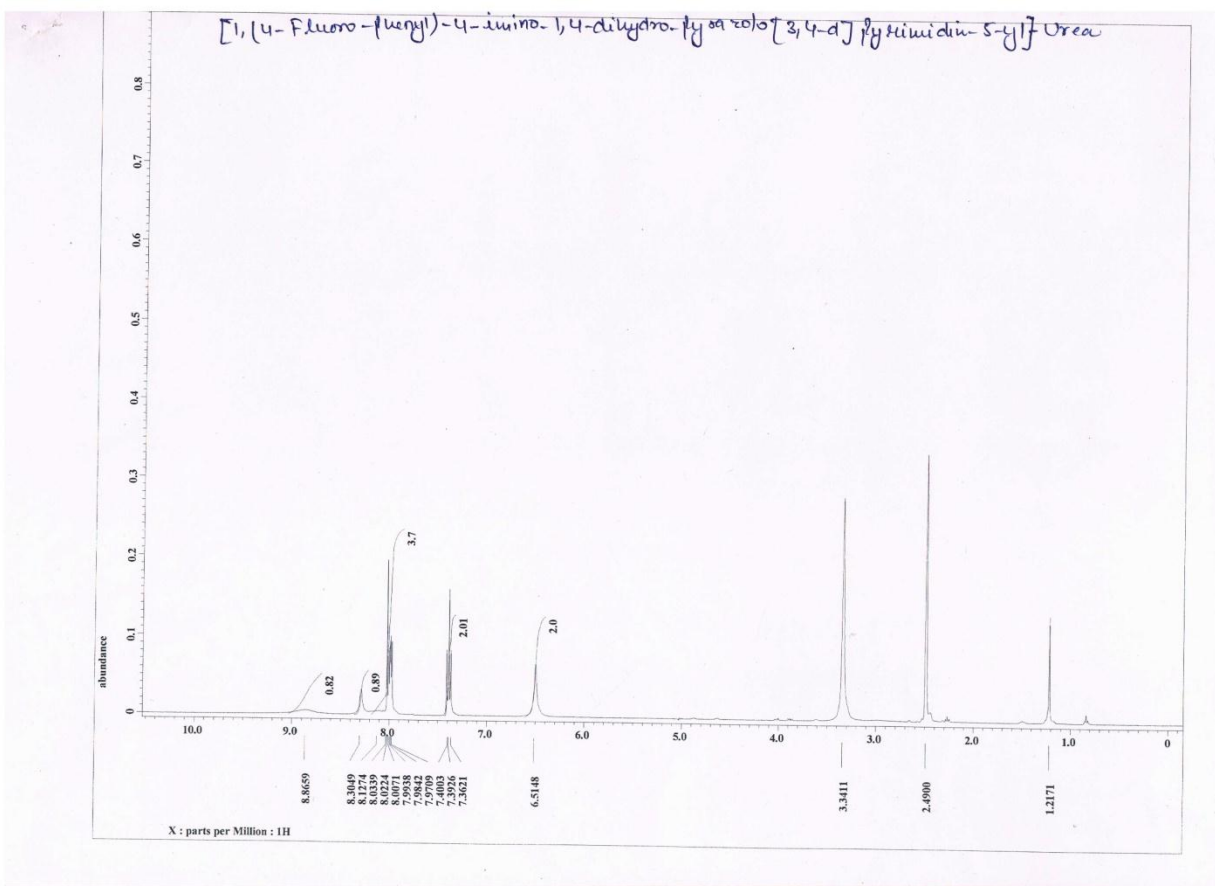


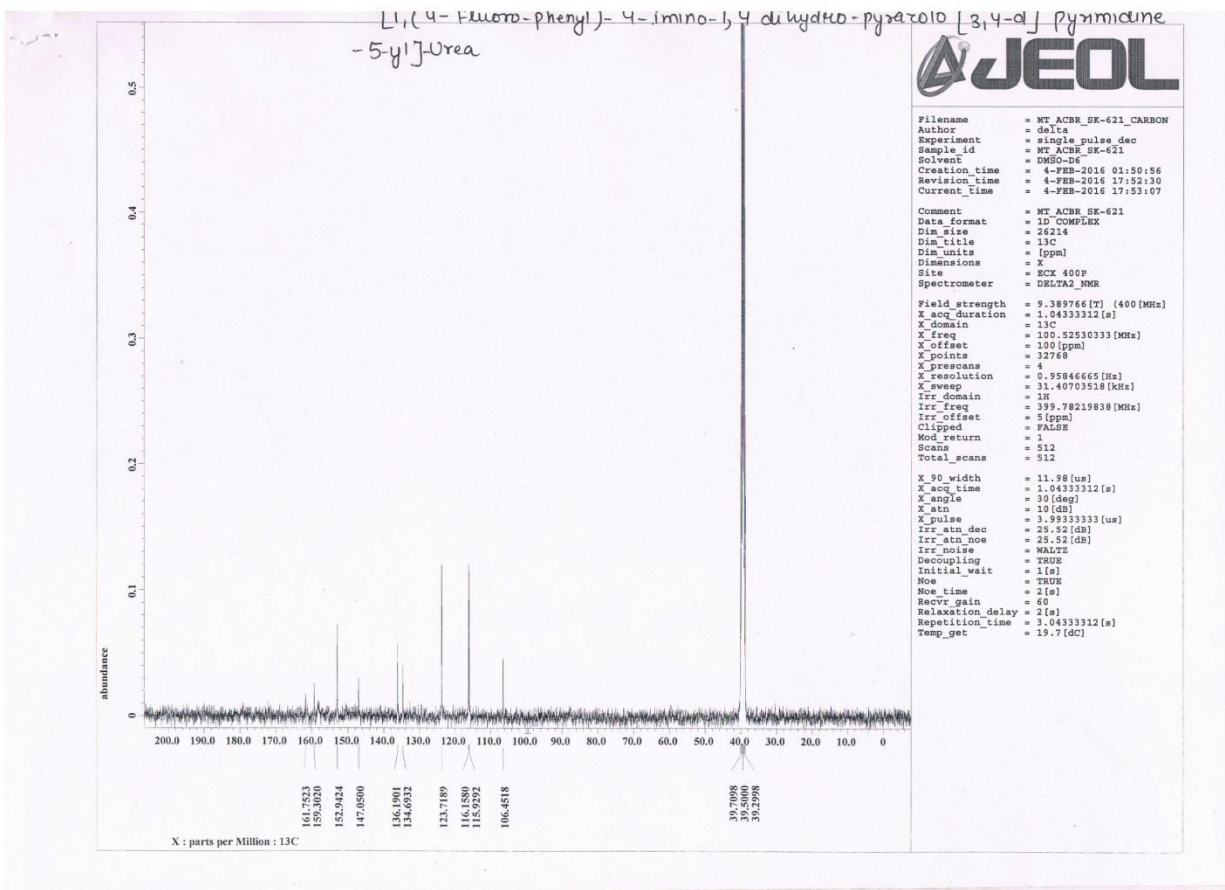


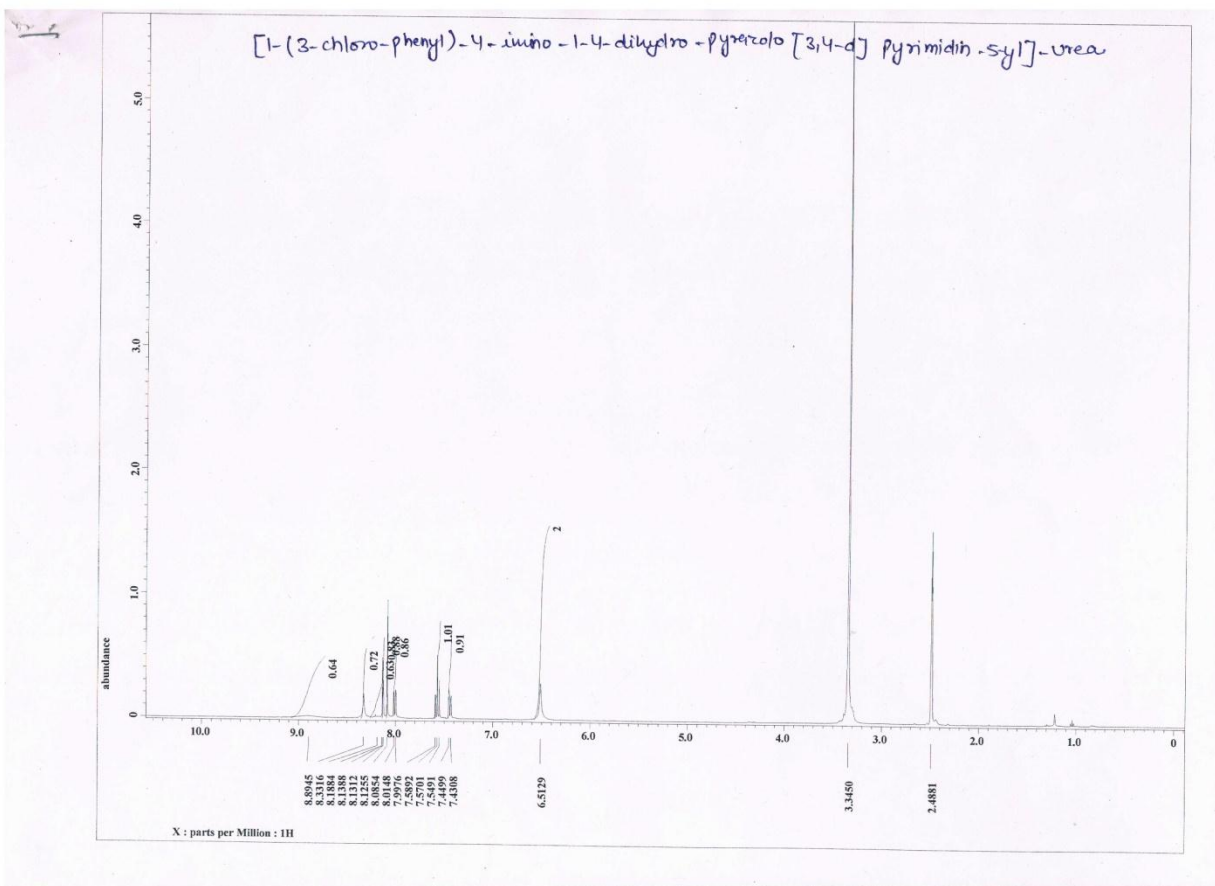
**<sup>13</sup>C of Compound 28**

$^1\text{H}$  of Compound 29

**<sup>13</sup>C of Compound 29**

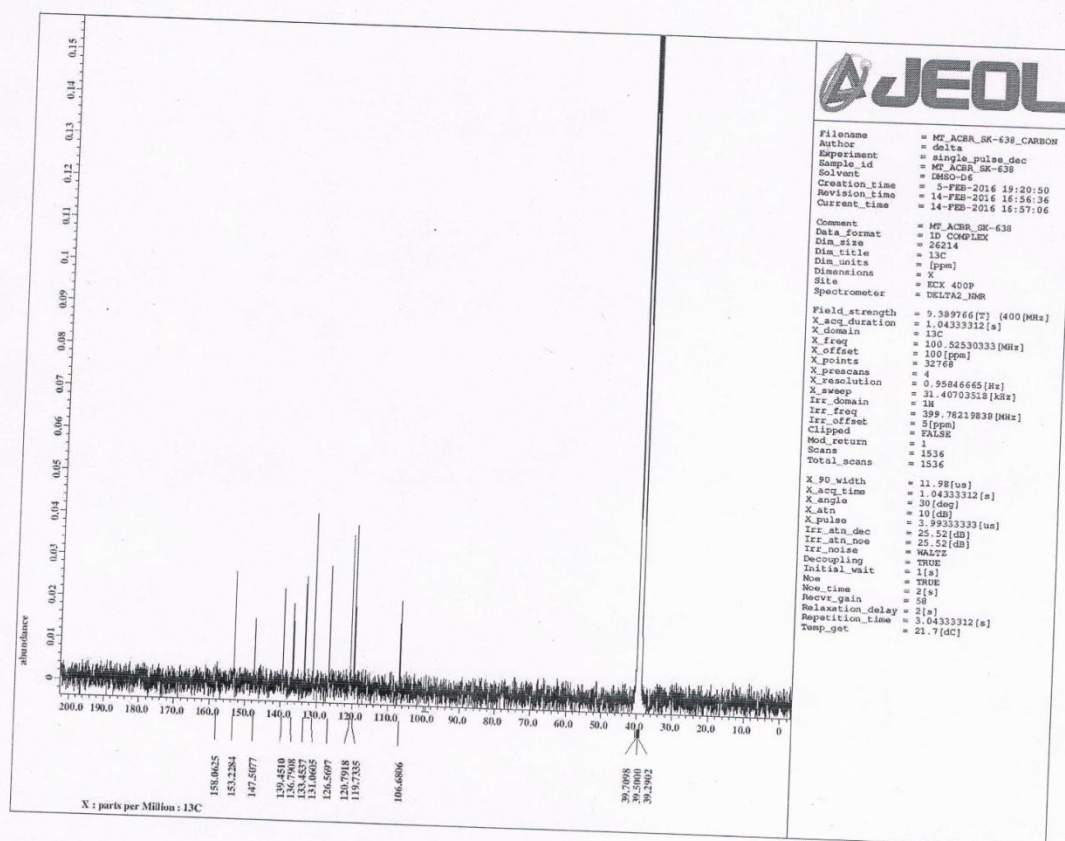
**$^1\text{H}$  of Compound 30**

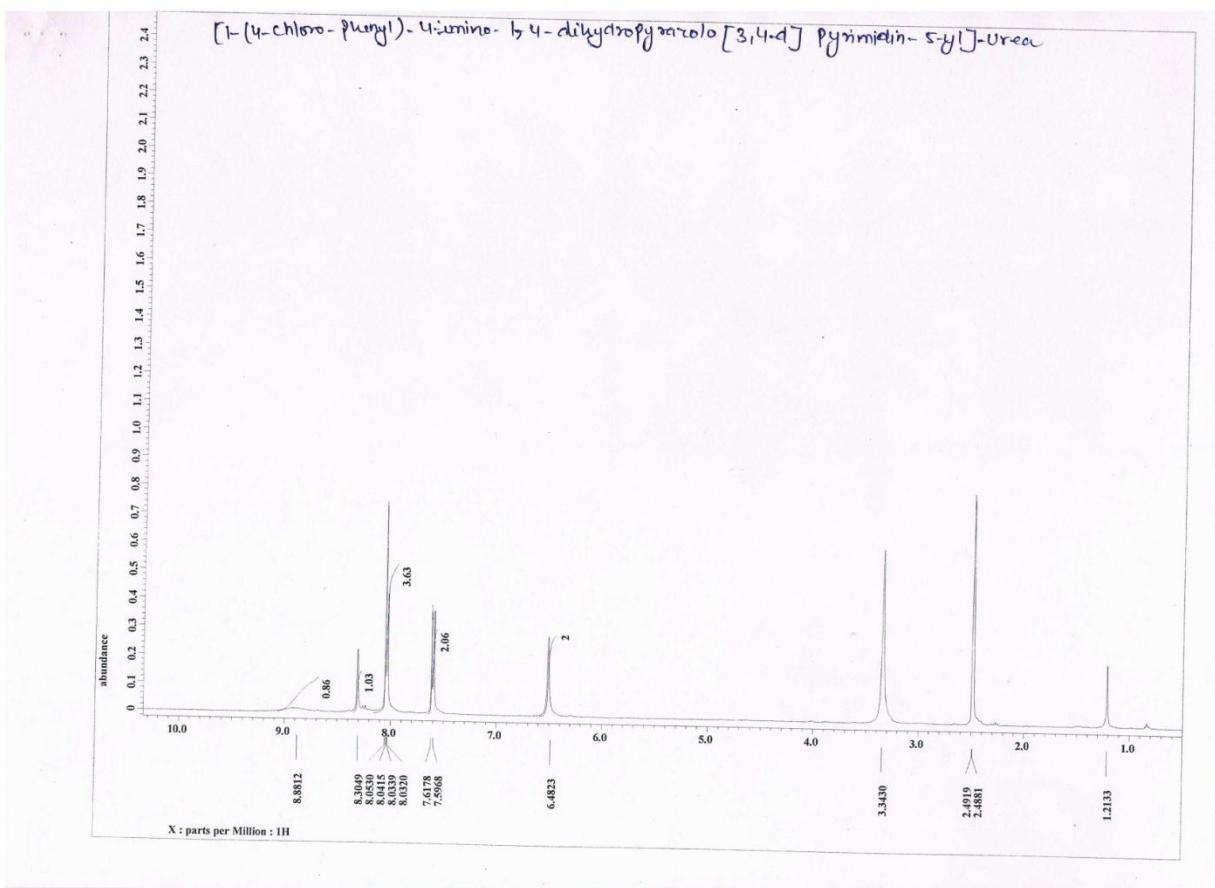
**<sup>13</sup>C of Compound 30**

$^1\text{H}$  of Compound 31

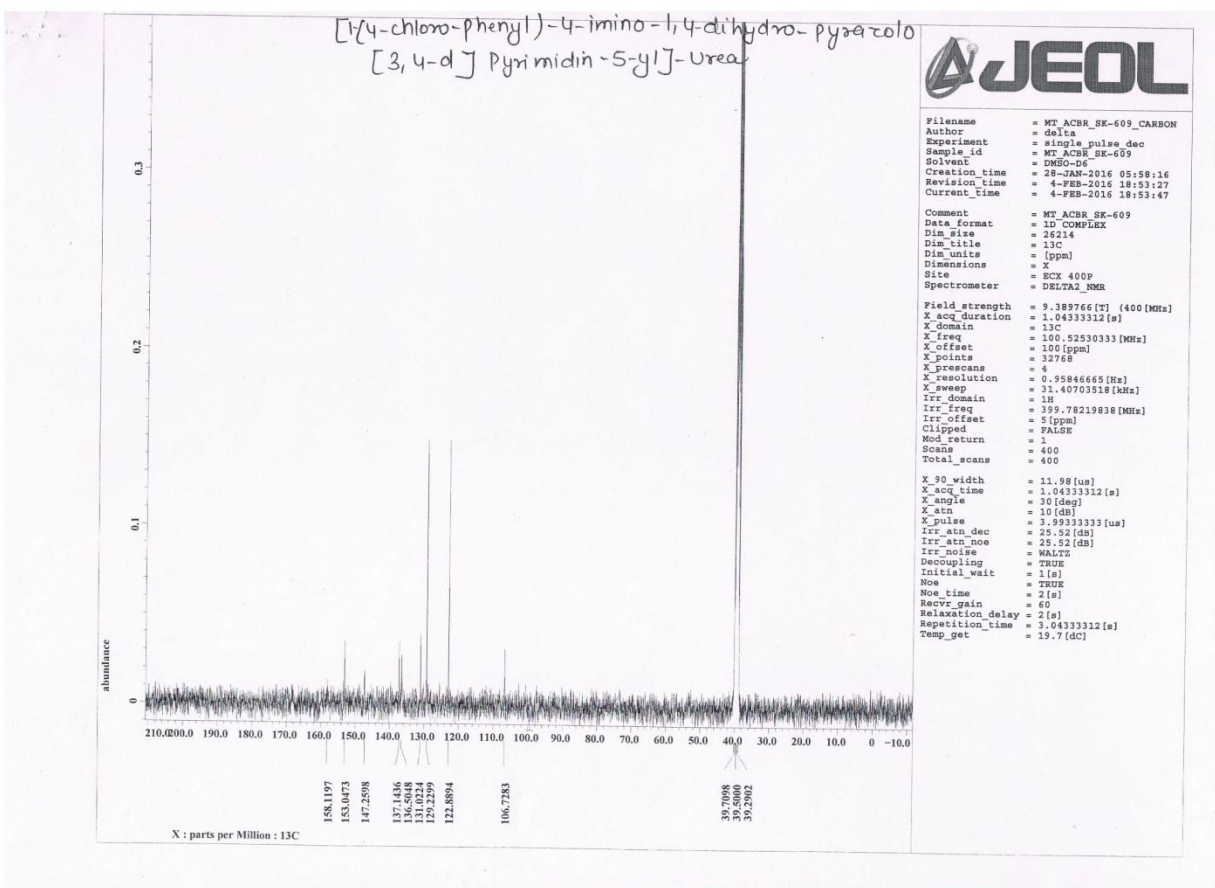
$^{13}\text{C}$  of Compound 31

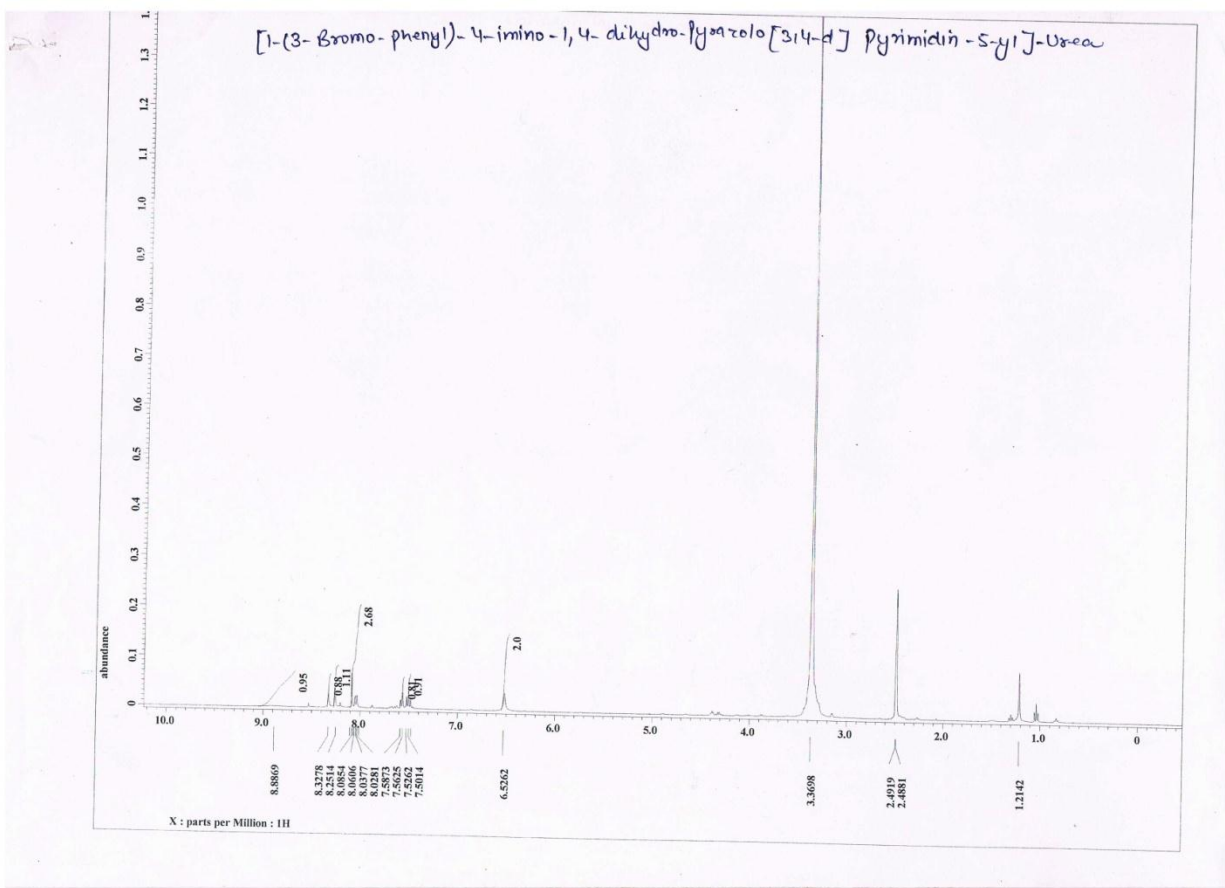
[1-(3-chloro-phenyl)-4-imino-1,4-dihydro-pyrazolo[3,4-d]pyrimidin-5-yl]-Urea

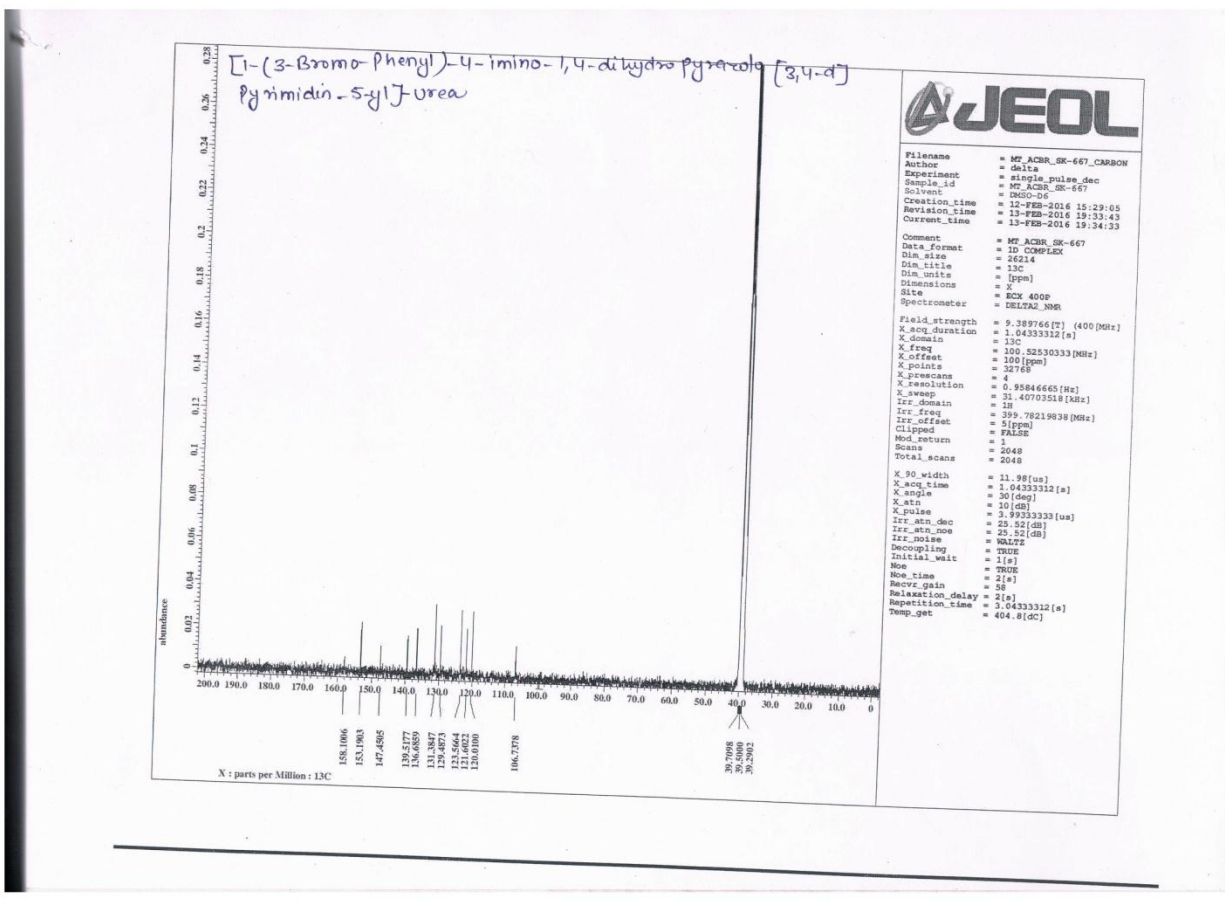


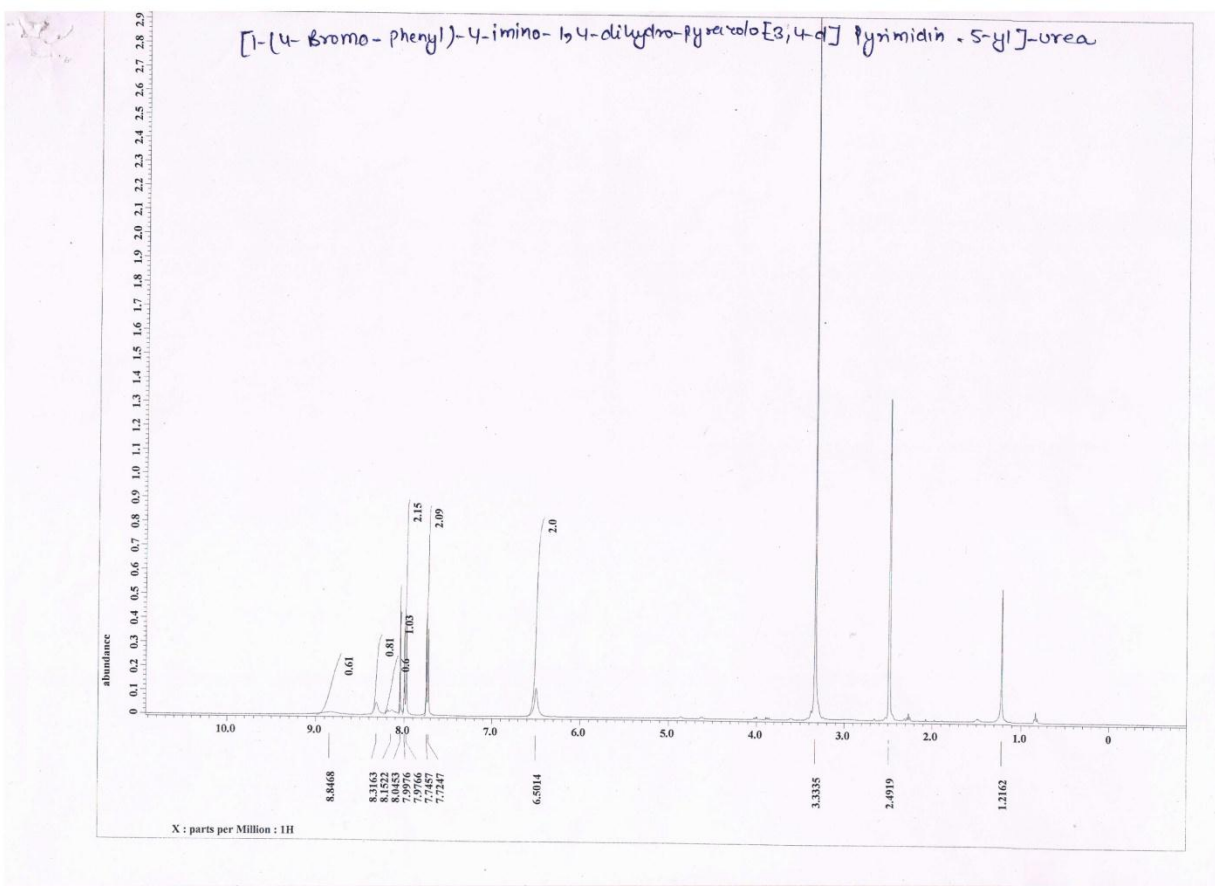
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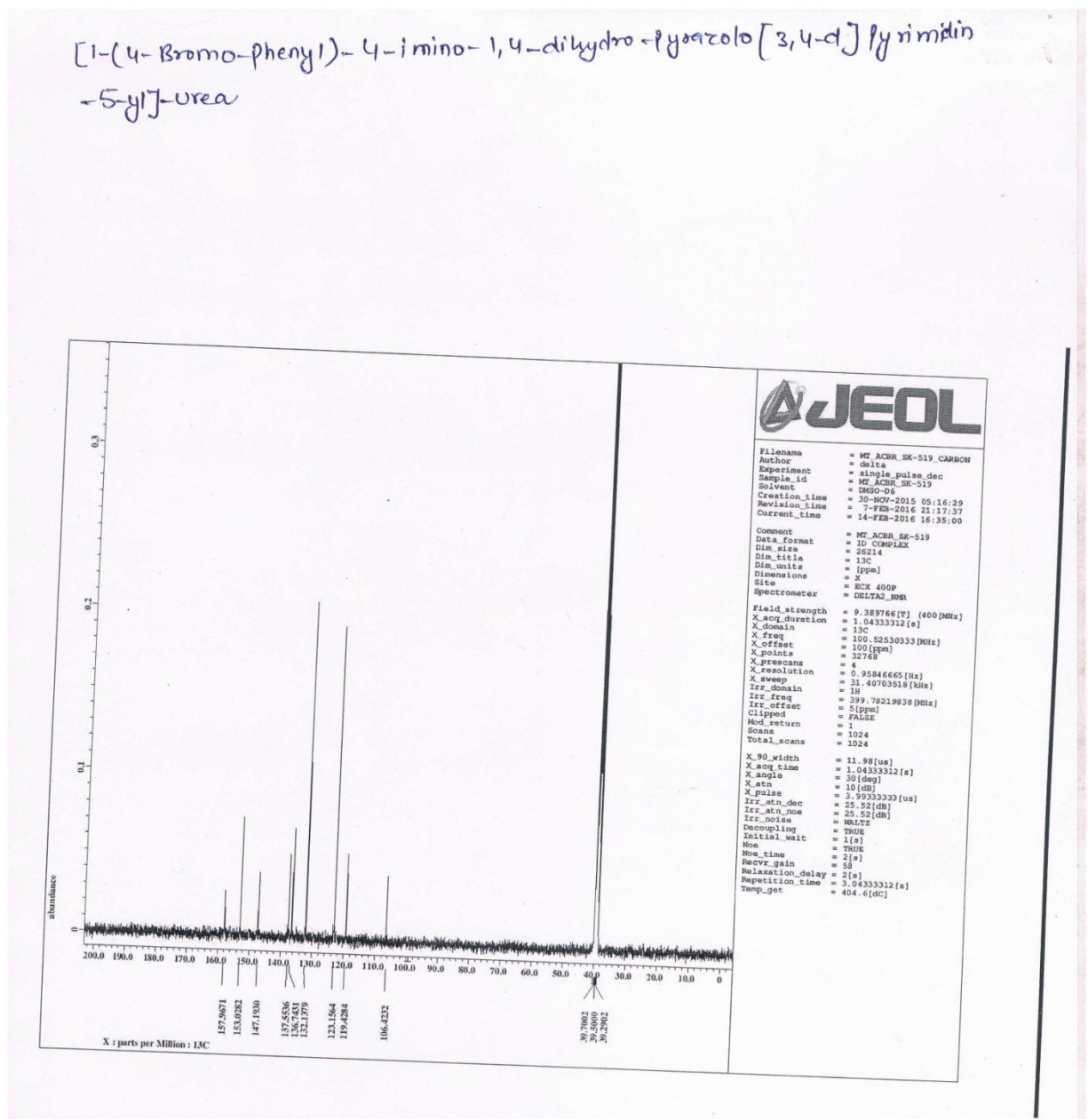


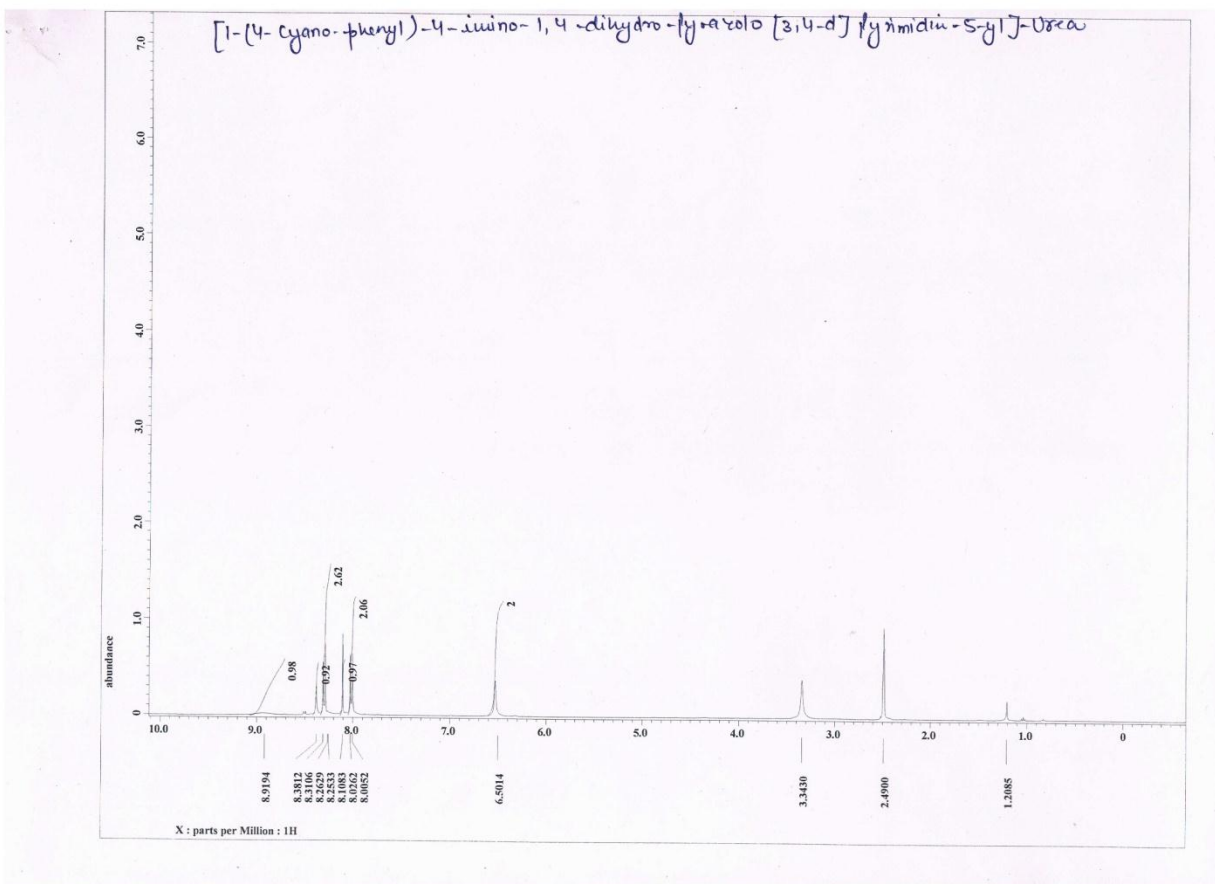
$^{13}\text{C}$  of Compound 32

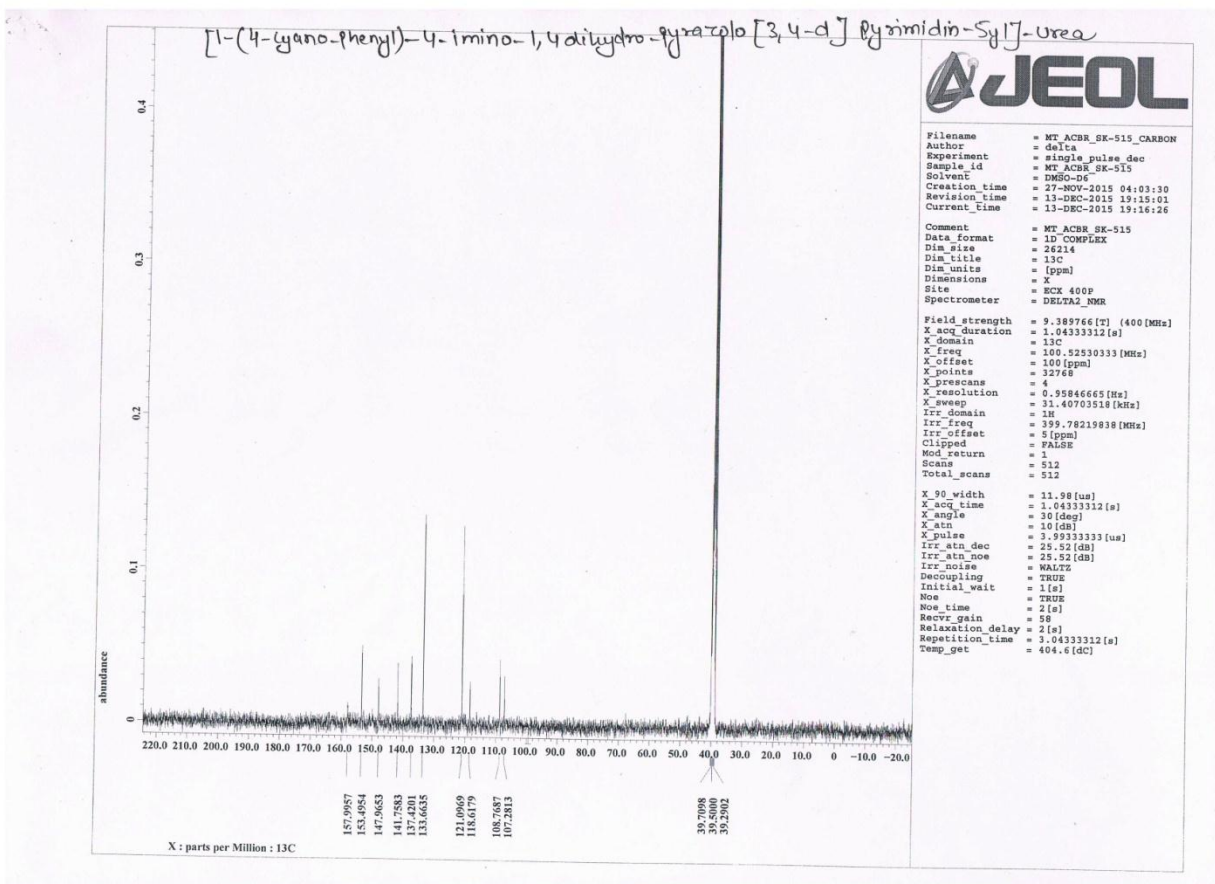
**$^1\text{H}$  of Compound 33**

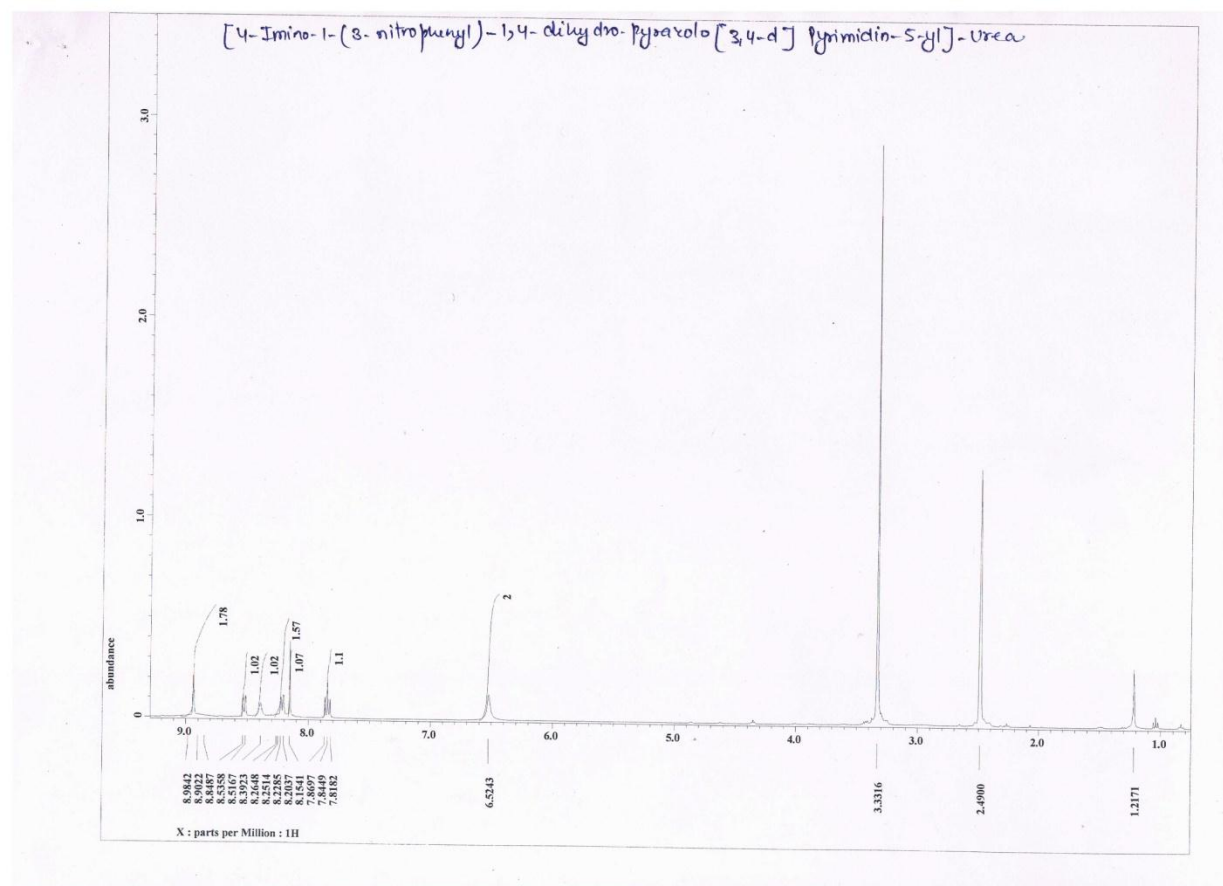
**<sup>13</sup>C of Compound 33**

**$^1\text{H}$  of Compound 34**

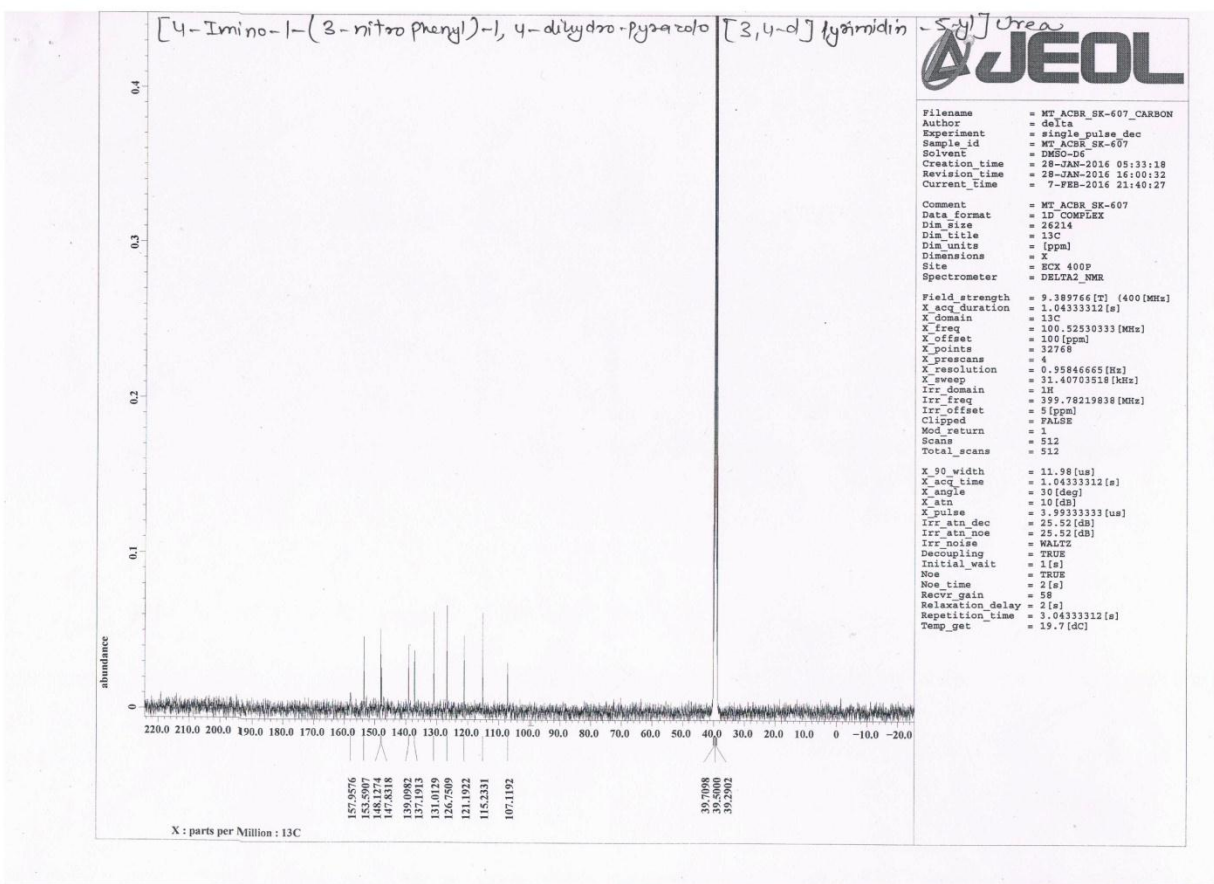
**13C of Compound 34**

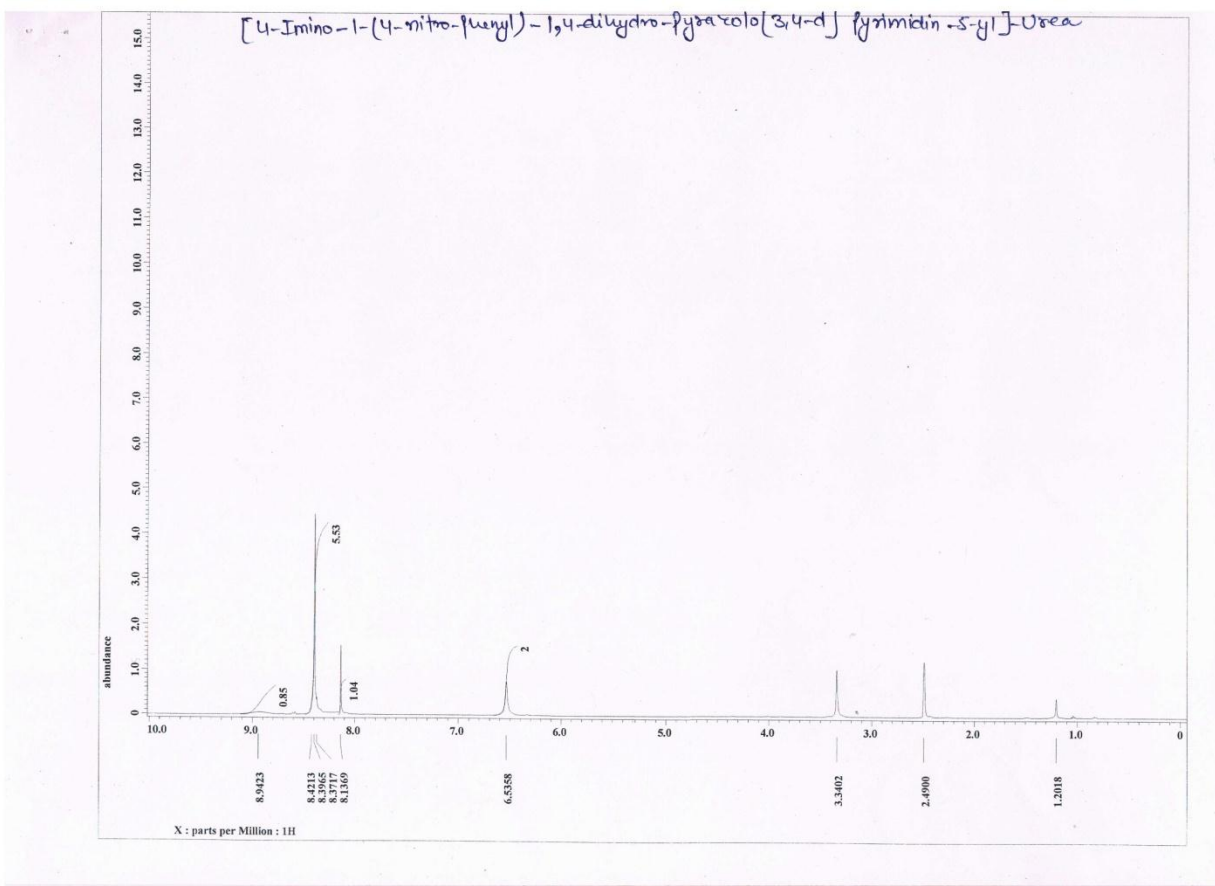
**$^1\text{H}$  of Compound 35**

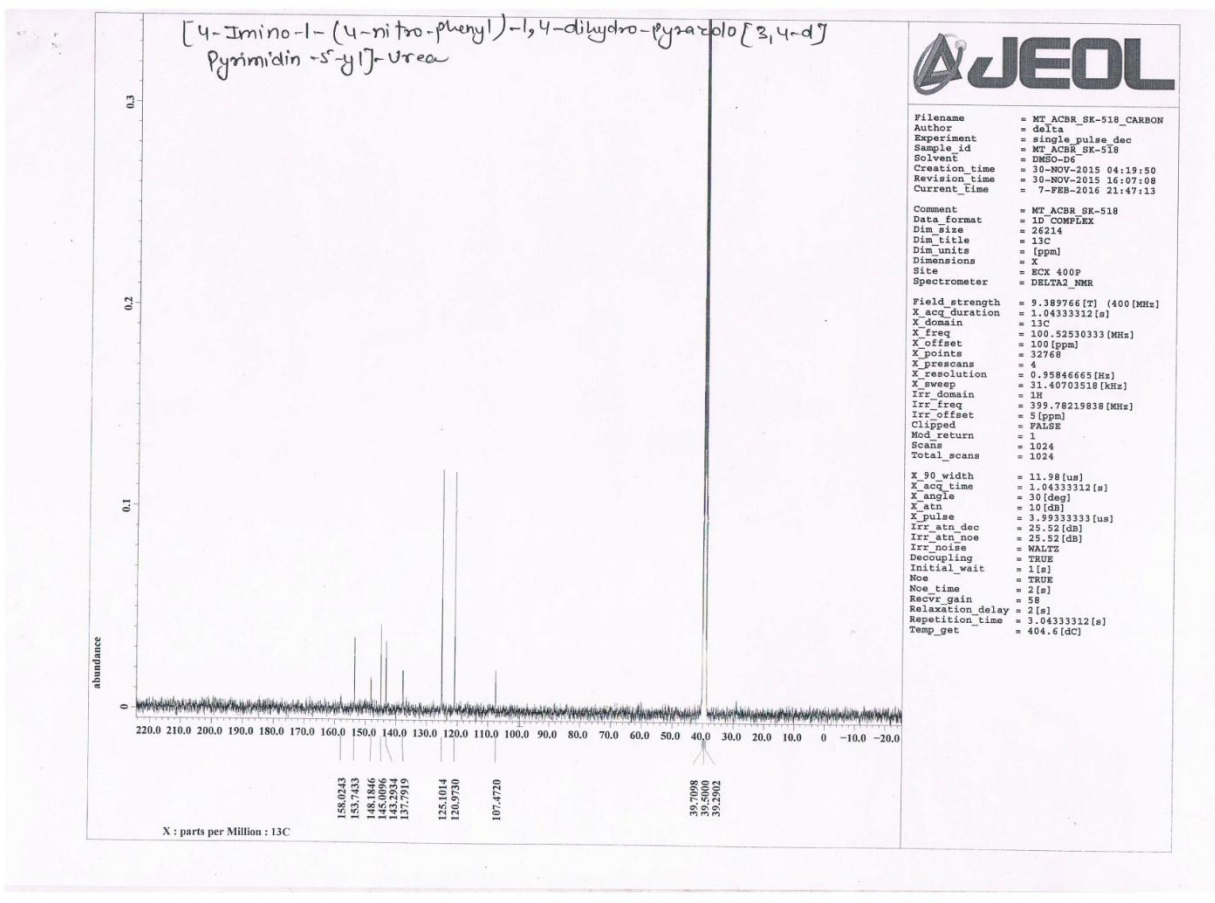
**13C of Compound 35**

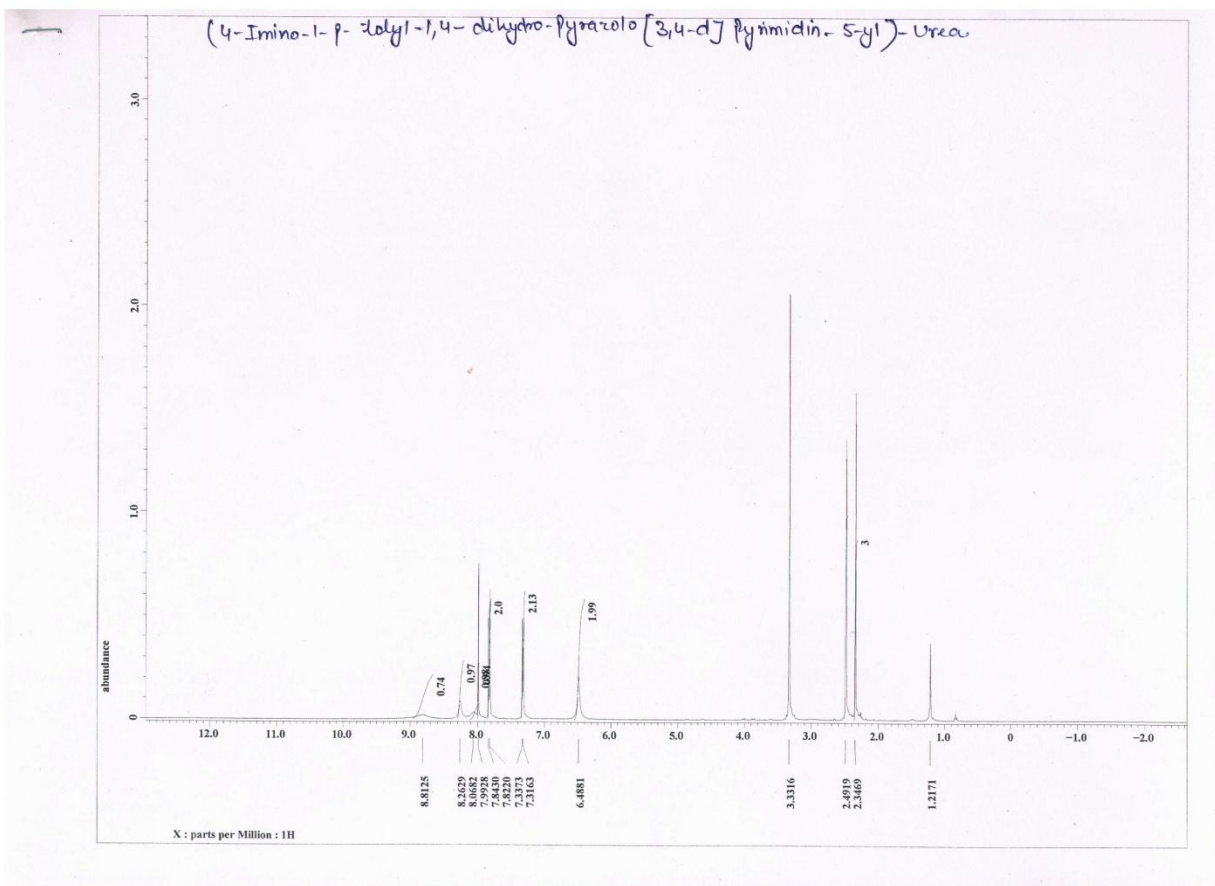
**$^1\text{H}$  of Compound 36**

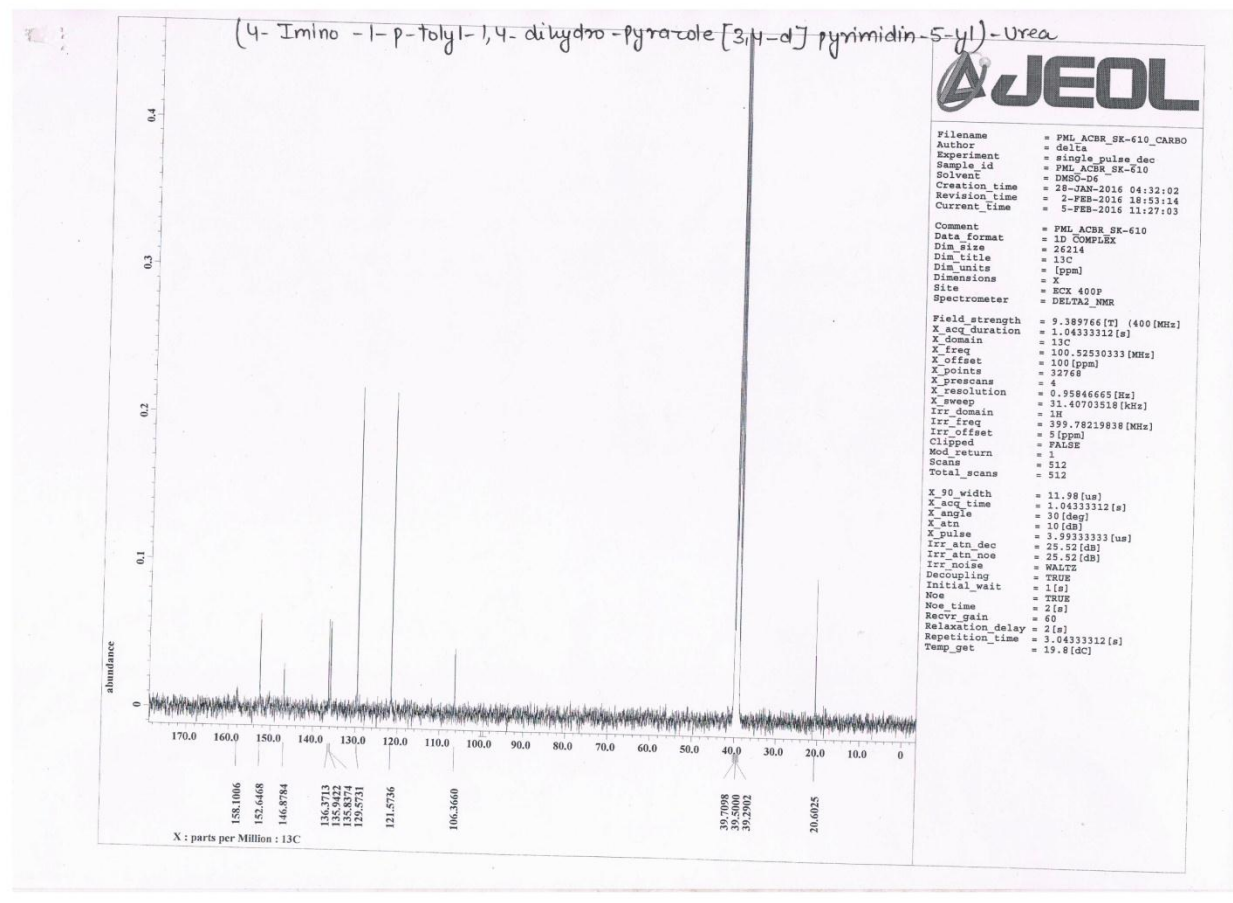


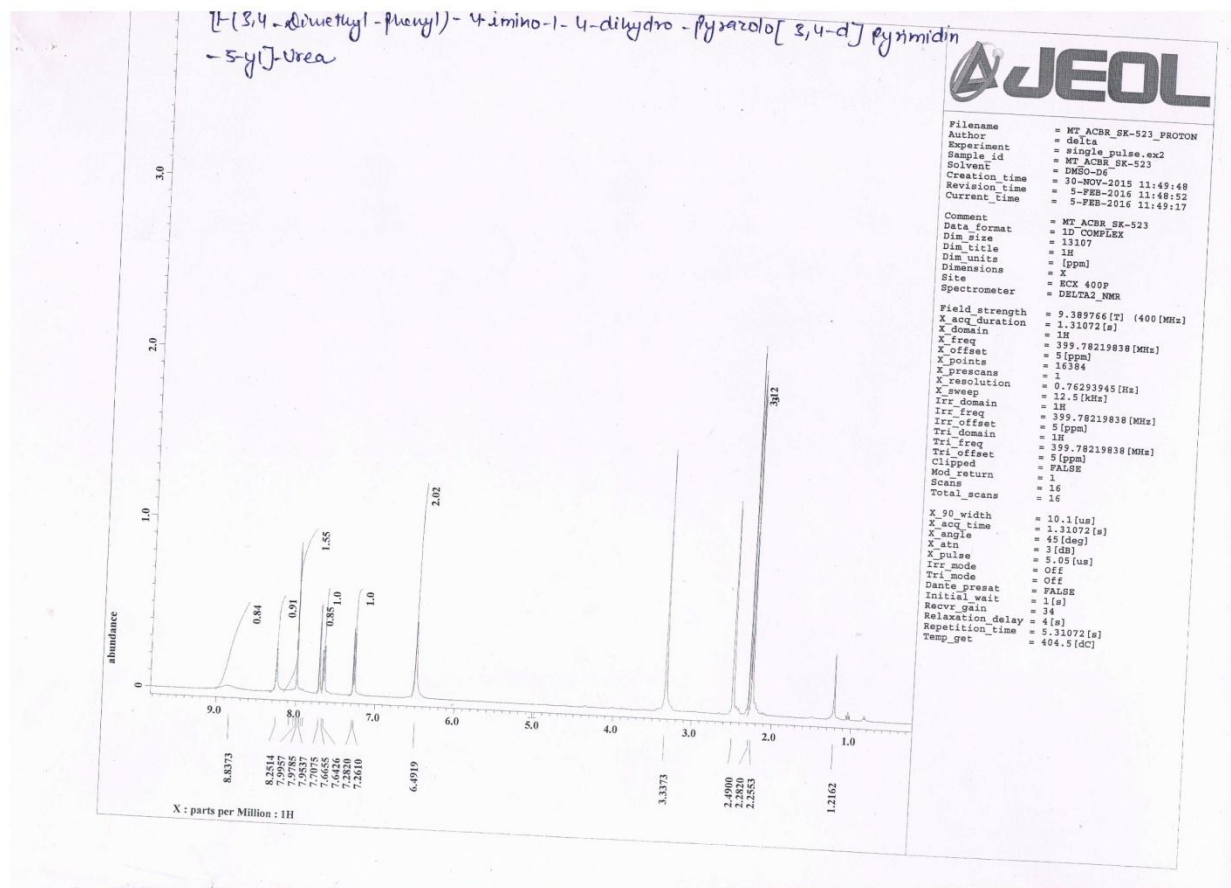
**<sup>13</sup>C of Compound 36**

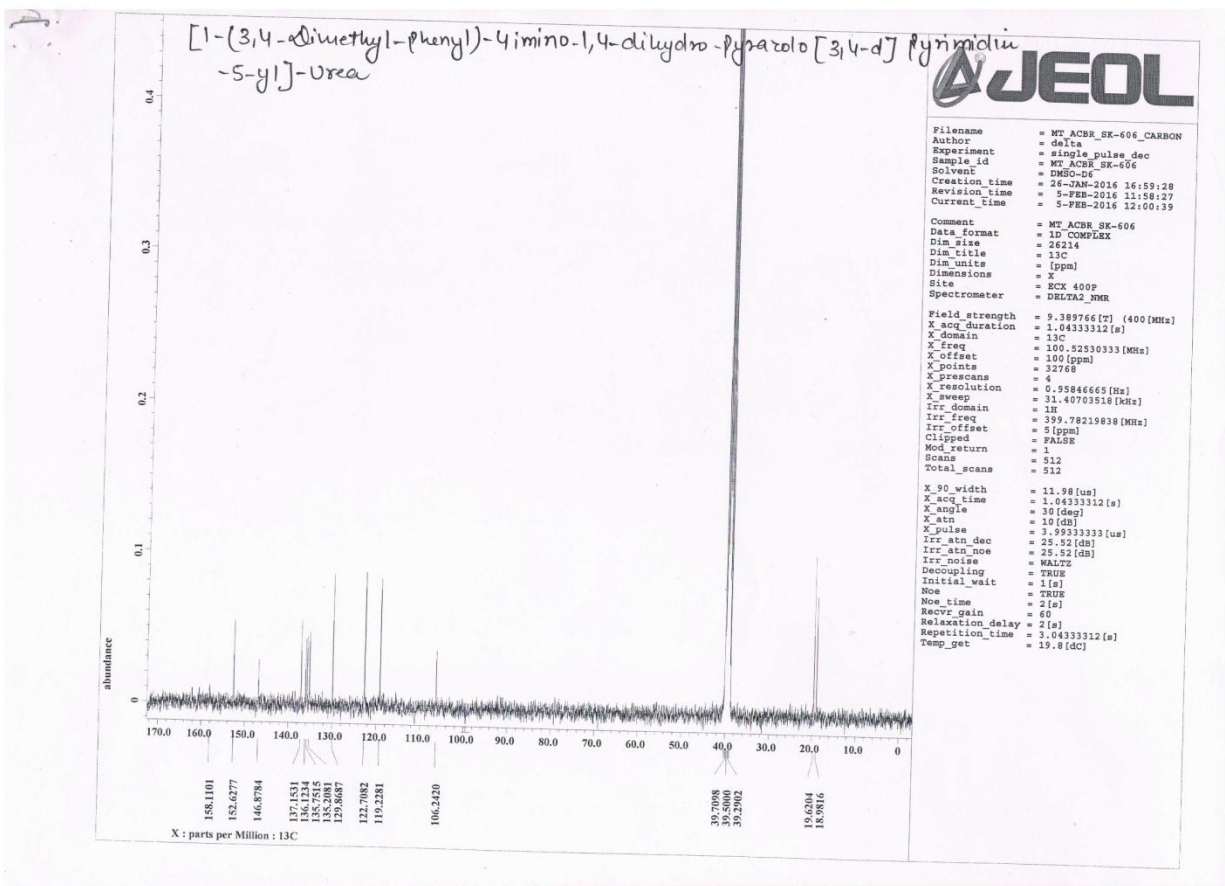
**$^1\text{H}$  of Compound 37**

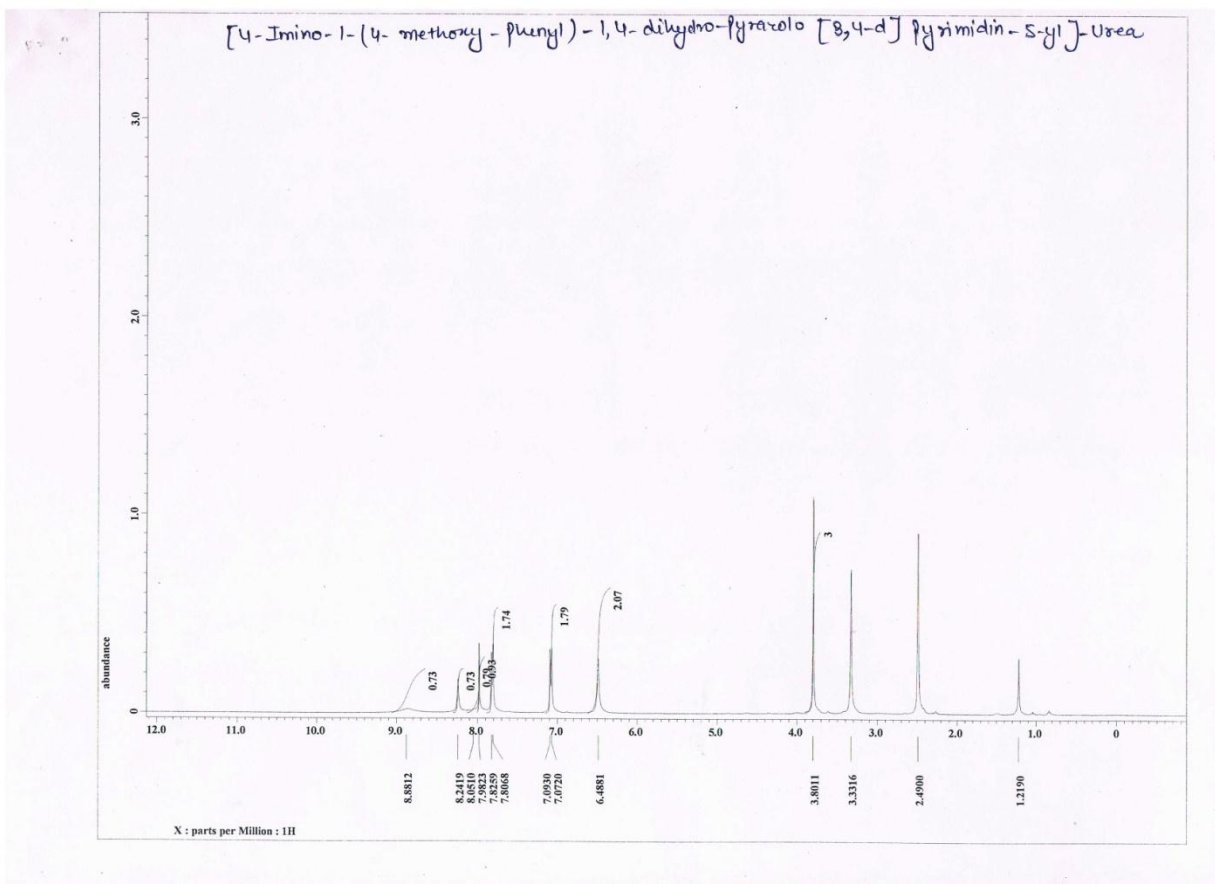
**<sup>13</sup>C of Compound 37**

**<sup>1</sup>H of Compound 38**

**<sup>13</sup>C of Compound 38**

$^1\text{H}$  of Compound 39

**<sup>13</sup>C of Compound 39**

$^1\text{H}$  of Compound 40



**<sup>13</sup>C of Compound 40**