Pyrazolo[3,4-*d*]pyrimidine-based neplanocin analogs an identified possible *de-novo* pharmacophore as dual-target HBV inhibitor

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1. Materials and Methods

All chemicals and anhydrous solvents were procured from commercial sources and used without further purification. Moisture-sensitive reactions are performed under N_2-atmosphere. Reactions were monitored by thin-layer chromatography (TLC) on pre-coated plates (silica gel 60 F254) purchased from Merck. The TLC plates were visualized with UV light and staining with 5% H_2SO_4 in MeOH. UV spectra were recorded on Thermo Scientific Evolution 201 and 220 UV-visible spectrophotometers. Melting points were taken on BUCHI (B-540) apparatus. Column chromatography was generally performed on a silica gel (100-200 mesh). NMR (Nuclear magnetic resonance) all samples were recorded on a Varian Mercury spectrometer at 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR or Brucker Ascend at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C

NMR using $CDCl_3$, DMSO-d_6 or CD_3OD as a solvent. HRMS were recorded on a Thermo Q Exactive (resolution = 1,40,000 FWHM) with electrospray ionization (ESI-Orbitrap) in positive mode and are given to four decimal places.

2. The anti-HBV assay and cytotoxicity assay

HepG2.2.15.7 cells were seeded in a 96-microtiter plate at a count of 1×10^4 cells/well. After overnight incubation, the cells were treated with different concentrations (0-100 μM) of test compounds. The culture medium was changed every three days to maintain the appropriate compound concentrations. Following a nine-day incubation period, culture supernatants were collected and analyzed for HBV DNA and surface antigen (HBsAg) levels using real-time PCR and ELISA, respectively. Additionally, cell viability was evaluated using a tetrazolium dye method.

EC_{50} (HBV DNA): 50% Effective concentration based on the inhibition of HBV DNA levels in culture supernatants.

EC_{50} (HBsAg): 50% Effective concentration based on the inhibition of HBs antigen levels in culture supernatants.

CC_{50}: 50% Cytotoxic concentration based on the reduction of viable cell number.

3. General Information:

All reactions were carried out in oven-dried glassware under nitrogen atmosphere. Unless stated otherwise, reagents and solvents were purchased from commercial suppliers and used without further purification. Analytical thin-layer chromatography (TLC) was performed on pre-coated plates (silica gel 60 F254) purchased from Merck. The TLC plates were visualized with UV light and by staining with 5% H_2SO_4 in MeOH and heated as developing agents. UV spectra were recorded on a Thermo Scientific Evolution 201 and 220 UV-Visible Spectrophotometers. Melting points were taken on BUCHI (B-540) apparatus and uncorrected column chromatography was generally performed on a silica gel (100-200 mesh). NMR (Nuclear magnetic resonance) was

recorded on a Varian Mercury spectrometer at 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR or Brucker Ascend at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR and using $CDCl_3$, DMSO-d_6 or CD_3OD as solvent and, in some cases, tetramethylsilane (TMS) as internal standard ($\delta = 0$) and calibrated using residual undertreated solvent as an internal reference ($CHCl_3$ @ δ 7.26 ppm ^{1}H NMR, δ 77.16 ppm ^{13}C NMR; DMSO @ δ 2.50 ppm ^{1}H NMR, δ 39.52 ppm ^{13}C NMR; MeOH @ δ 3.30 ppm ^{1}H NMR, δ 48.36 ppm ^{13}C NMR). Chemical shifts of ^{1}H and ^{13}C NMR spectra are reported in parts per million (δ) and multiplicities are quoted as s (singlet), d (doublet), dd (doublet of doublet), t (triplet), splitting patterns that could not be interpreted or easily visualized were designated as multiple (m). All coupling constants (J values) were noted in Hertz (Hz). High Resolution Mass Spectra (HRMS) were recorded on a Thermo Q Exactive (resolution = 1, 40, 000 FWHM) with electrospray ionization (ESI-Orbitrap) in positive mode and are given to four decimal places.

4. Experimental procedure & Product characterization:

General procedure for preparation of 4-cycloalkyl amino pyrazolo pyrimidine:

A mixture of cyclopropyl/cyclohexyl amine (12.96 mmol), 4-chloro-1*H*-pyrazolo[3,4-*d*] pyrimidine (6.48 mmol), and 1,4-dioxane were combined and allowed to stir at 80 ^{o}C for 4 h. The reaction progress is monitored by LCMS/TLC. After completion of the reaction, remove the volatiles under reduced pressure. The crude residue was purified on a silica gel (100–200 mesh) column chromatography, elution gradient 0–1% MeOH in CH_2Cl_2. Appropriate fractions are combined and concentrated under vacuum.

***N*-Cyclopropyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (2a):** Yield: 77%; off white solid, (TLC: R_f 0.2, 5% MeOH in CH_2Cl_2); UV (MeOH) λ_{max}: 265 nm; mp: 212–213 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.76–0.78 (m, 2H), 0.89–0.92 (m, 2H), 2.96–2.98 (m, 1H), 8.07 (bs, 1H), 8.11 (s, 3H), 8.29 (s, 1H), 13.468 (bs, 1H); MS-ESI (m/z): 176.2.0 [M^{+}+1].

***N*-Cyclohexyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (2d):** Yield: 81%; pale yellow solid; (TLC: R_f0.3, 50% EtOAc in hexane); UV (MeOH) λ_{max}: 262 nm; mp: 209–210 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.12–1.88 (m, 10H), 2.98–2.99 (m, 1H), 7.91 (bs, 1H, NH), 8.10 (s, 1H), 8.20 (s, 1H), 13.91 (bs, 1H, NH); MS-ESI (m/z): 218.0 [M^{+}+1].

General procedure for synthesis of 3-halo-*N*-cycloalkyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine: *N*-halo succinimide (5.64 mmol) was added to a solution of 4-cyclopropyl/cyclohexyl amino pyrazolo pyrimidines (**2a, 2d**) (5.13 mmol) in dry DMF under argon at rt. The reaction mixture was stirred at rt for 1 h. The reaction was monitored with LCMS/TLC. After completion of the reaction, the reaction mixture was poured into crushed ice and extracted with EtOAc (x3). The combined organic layer was washed with brine, dried over anhydrous sodium sulfate, and filtered. The solvent was evaporated in *vacuo,* and the residue was purified by silica gel (100–200 mesh) column chromatography, elution gradient 0–60% EtOAc in hexane. Collected the pure fractions and concentrated under vacuum.

3-Chloro-*N*-cyclopropyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (2b): Yield: 82%; off white solid, (TLC: R_f 0.3, 50% EtOAc in hexane); UV (MeOH) λ_{max}: 267 nm; mp: 215–217 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.78–0.80 (m, 2H), 0.88–0.91 (d, 2H), 2.98–3.00 (m, 1H), 7.26 (bs, 1H, NH), 8.39 (s, 1H), 13.70 (bs, 1H, NH); MS-ESI (m/z): 209.9 [M^{+}+1], 211.9 [M^{+}+2].

3-Bromo-*N*-cyclopropyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (2c): Yield: 85%; off white solid, (TLC: R_f0.3, 50% EtOAc in hexane); UV (MeOH) λ_{max}: 268 nm; mp: 219–224 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.78–0.80 (m, 2H), 0.88–0.91 (m, 2H), 2.98–2.99 (m, 1H), 6.91 (bs, 1H, NH), 8.39 (s, 1H), 13.91 (bs, 1H, NH); MS-ESI (m/z): 253.8 [M^{+}+1], 255.9 [M^{+}+2].

3-Bromo-*N*-cyclohexyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (2e): Yield: 75%; yellow solid, (TLC: R_f0.3, 50% EtOAc in hexane); UV (MeOH) λ_{max}: 264 nm; mp: 219–220 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.12–1.88 (m, 10H), 2.88–2.29 (m, 1H), 7.91 (bs, 1H, NH), 8.20 (s, 1H), 14.21 (bs, 1H, NH); MS-ESI (m/z): 295.9 [M^{+}+1], 297.9 [M^{+}+2].

4-Methyl-1*H*-pyrazolo[3,4-*d*]pyrimidine (2f) was synthesized according to the literature procedure[5]. Yield: 65%; yellow solid, (TLC: R_f 0.1, 60% EtOAc in hexane); UV (MeOH) λ_{max}: 246 nm; mp: 152–153 ^{o}C; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.76 (s, 3H), 8.43 (s, 3H), 8.81 (s, 1H), 13.9 (bs, 1H, NH); MS-ESI (m/z): 135.07 [M^{+}+1].

3-Iodo-4-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidine (2g): *N*-halo succinimide (5.64 mmol) was added to a solution of 4-methyl pyrazolo pyrimidines (**2f**) (5.13 mmol) in dry DMF under argon

at rt. The reaction mixture was stirred at rt for 1 h. The reaction was monitored with LCMS/TLC. After completion of the reaction, the reaction mixture was poured into crushed ice and extracted with EtOAc (x3). The combined organic layer was washed with brine, dried over anhydrous sodium sulfate, and filtered. The solvent was evaporated in *vacuo,* and the residue was purified by silica gel (100–200 mesh) column chromatography, elution gradient 0–60% EtOAc in hexane. Collected the pure fractions and concentrated under vacuum.

Yield: 69%; off white solid, (TLC: R_f 0.1, 60% EtOAc in hexane); UV (MeOH) λ_{max}: 249 nm; mp: 159–162 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.92 (s, 3H), 8.87 (s, 1H), 14.41 (bs, 1H, NH); MS-ESI (m/z): 260.8 [M^{+}+1].

General procedure for the preparation of compound 3a-g

To a mixture of suitable base **2a-g** (1.5 mmoles), carbocyclic sugar **1** (1.57 mmoles) and Ph_3P (3.75 mmoles) in THF was added DIAD (3.75 mmoles) dropwise at 0°C. The reaction mixture was brought up to rt with continued stirring. Completion of the reaction was analyzed by TLC, solvent evaporated under reduced pressure and crude was purified on silica gel column chromatography (ethyl acetate in hexane: 0-20%) to give the couple products **3a-g** in 79-88% yield (TLC Rf: 0.5-0.7, 20% EtOAc in hexane).

***N*-Cyclopropyl-1-(2,2-dimethyl-6-(trityloxymethyl)-4,6a-dihydro-3a*H*-cyclopenta[*d*][1,3]dioxol-4-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (3a)**

Yield: 80%; mp: 145°C; MS (m/z): 585.9 [M^{+}]; UV (MeOH): λ_{max} 270 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.69–0.73 (s, 2H, cyclopropyl-CH_2), 0.98–1.03 (s, 2H, cyclopropyl-CH_2), 1.25 (s, 3H, CH_3), 1.37 (s, 3H, CH_3), 3.01–3.03 (m, 1H, cyclopropyl-CH), 3.71–3.75 (d, 1H, J^2= 15.2 Hz, 5'H), 3.86–3.90 (d, 1H, J^2= 15.2 Hz, 5'H), 4.82–4.83 (d, 1H, J^3= 5.6 Hz, 2'H), 5.26–5.27 (d, 1H, J^3= 5.6 Hz, 3'H), 5.96–5.99 (m, 2H, 1' & 6'H), 6.22 (s, 1H, NH), 7.14–7.38 (m, 15H, Tr-H), 7.9 (s, 1H, 3-CH), 8.3 (s, 1H, 6-CH).

3-Chloro-*N*-cyclopropyl-1-(2,2-dimethyl-6-(trityloxymethyl)-4,6a-dihydro-3a*H*-cyclopenta[*d*][1,3]dioxol-4-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (3b)

Yield: 83%; mp: 157°C; MS (m/z): 620.1 [M^{+}], 622.1 [M^{+}+2]; UV (MeOH): λ_{max} 264 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.69–0.73 (s, 2H, cyclopropyl-CH_2), 0.98–1.03 (s, 2H, cyclopropyl-CH_2), 1.33 (s, 3H, CH_3), 1.45 (s, 3H, CH_3), 3.01–3.03 (m, 1H, cyclopropyl-CH), 3.76–3.80 (d, 1H, J^2= 15.8 Hz, 5'H), 3.93–3.97 (d, 1H, J^2= 14.9 Hz, 5'H), 4.85–4.86 (d, 1H, J^3= 5.7 Hz, 2'H), 5.30–

5.31 (d, 1H, J^3= 5.7 Hz, 3'H), 5.98–6.00 (s, 2H, 1' & 6'H), 6.22 (s, 1H, NH), 7.22–7.47 (m, 15H, Tr-H), 8.53 (s, 1H, 6-CH).

3-Bromo-*N*-cyclopropyl-1-(2,2-dimethyl-6-(trityloxymethyl)-4,6a-dihydro-3a*H*-cyclopenta[*d*][1,3]dioxol-4-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (3c)

Yield: 85%; mp: 173°C; MS (m/z): 664.0 [M^+], 666.0 [M^++2]; UV (MeOH): λ_{max} 265 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.69–0.71 (d, 2H, cyclopropyl-CH_2), 0.96–0.99 (d, 2H, cyclopropyl-CH_2), 1.31 (s, 3H, CH_3), 1.43 (s, 3H, CH_3), 2.99-3.01 (m, 1H, cyclopropyl-CH), 3.75–3.79 (d, 1H, J^2= 15.3 Hz, 5'H), 3.91–3.95 (d, 1H, J^2= 15.3 Hz, 5'H), 4.84–4.86 (d, 1H, J^3= 6.1 Hz, 2'H), 5.28–5.30 (d, 1H, J^3 = 5.6 Hz, 3'H), 5.95–6.02 (m, 2H, 1' & 6'H), 6.22 (s, 1H, NH), 7.21–7.45 (m, 15H, Tr-H), 8.5 (s, 1H, 6-CH).

***N*-Cyclohexyl-1-(2,2-dimethyl-6-(trityloxymethyl)-4,6a-dihydro-3a*H*-cyclopenta[*d*][1,3]dioxol-4-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (3d)**

Yield: 80%; mp: 135°C; MS-ESI (m/z): 628.2 [M^+]; UV (MeOH): λ_{max} 270 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.25 (s, 3H, CH_3), 1.37 (s, 3H, CH_3), 1.44–2.20 (m, 11H, cyclohexyl-CH), 3.75–3.79 (d, 1H, J^2= 15.2 Hz, 5'H), 3.94–3.98 (d, 1H, J^2= 15.2 Hz, 5'H), 4.84–4.86 (d, 1H, J^3= 5.6 Hz, 2'H), 5.30–5.32 (d, 1H, J^3= 5.2 Hz, 3'H), 6.02–6.05 (m, 2H, 1' & 6'H), 6.42–6.38 (s, 1H, NH), 7.14–7.38 (m, 15H, Tr-H), 7.4 (s, 1H, 3-CH), 8.3 (s, 1H, 6-CH).

3-Bromo-*N*-cyclohexyl-1-(2,2-dimethyl-6-(trityloxymethyl)-4,6a-dihydro-3a*H*-cyclopenta[*d*][1,3]dioxol-4-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (3e)

Yield: 79%; mp: 147°C; MS-ESI (m/z): 706.1 [M^+], 708.1 [M^++2]; UV (MeOH): λ_{max} 269 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.28 (s, 3H, CH_3), 1.28 (s, 3H, CH_3), 1.38 (s, 3H, CH_3), 1.44–2.20 (m, 11H, cyclohexyl-CH), 3.75–3.79 (d, 1H, J^2= 15.6 Hz, 5'H), 3.91–3.95 (d, 1H, J^2= 15.6 Hz, 5'H), 4.85–4.86 (d, 1H, J^3= 5.6 Hz, 2'H), 5.28–5.30 (d, 1H, J^3= 5.6 Hz, 3'H), 5.94 (m, 1H, 6'H), 6.02–6.05 (m, 1H, 1'H), 7.21–7.46 (m, 15H, Tr-H), 8.4 (s, 1H, 6-CH).

1-(2,2-Dimethyl-6-(trityloxymethyl)-4,6a-dihydro-3a*H*-cyclopenta[*d*][1,3]dioxol-4-yl)-4-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidine (3f)

Yield: 86%; mp: 146°C; MS-ESI (m/z): 544.7 [M^+]; UV (MeOH) λ_{max}: 289 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.24 (s, 3H, CH_3), 1.33 (s, 3H, CH_3), 2.83 (s, 3H, 6-CH_3), 3.72–3.76 (d, 1H, J^2= 15.2 Hz, 5'H), 3.82–3.86 (d, 1H, J^2= 15.2 Hz, 5'H), 4.79–4.80 (d, 1H, J^3= 5.6 Hz, 6'H), 5.26–5.27

(d, 1H, J^3= 5.6 Hz, 3'H), 5.95–5.98 (m, 2H, 1' & 6'H), 7.11–7.32 (m, 15H, Tr-H), 8.3 (s, 1H, 3-CH), 8.8 (s, 1H, 6-CH).

1-(2,2-Dimethyl-6-(trityloxymethyl)-4,6a-dihydro-3a*H*-cyclopenta[*d*][1,3]dioxol-4-yl)-3-iodo-4-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidine (3g)

Yield: 88%; mp: 157°C; MS (m/z): 670.9 [M^+]; UV (MeOH) λ_{max}: 292 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.25 (s, 3H, CH_3), 1.37 (s, 3H, CH_3), 2.93 (s, 3H, 6-CH_3), 3.71–3.75 (d, 1H, J^2= 15.2 Hz, 5'H), 3.86–3.90 (d, 1H, J^2= 15.2 Hz, 5'H), 4.82–4.83 (d, 1H, J^3= 5.6 Hz, 2'H), 5.26–5.27 (d, 1H, J^3= 5.6 Hz, 3'H), 5.99–5.96 (m, 2H, 1' & 6'H), 7.38–7.14 (m, 15H, Tr-H), 8.8 (s, 1H, 6-CH).

General procedure for the synthesis of 4a-h:

The deprotection of trityl and acetonide groups of **3a-g,** was carried out by stirring at rt in methanolic HCl. After completion (monitored by TLC), the reaction mixture was concentrated under reduced pressure, and the crude solid was dissolved in methanol, neutralized with $NaHCO_3$ and the crude was purified by silica gel column chromatography using 5–10% MeOH in CH_2Cl_2 to afford pure carbocyclic nucleoside **4a-g** in 85–94% yield. The compound **4h** was synthesized from **4g** by dissolving it in dry DMF, and to it, tributylvinyltin and $Pd(PPh_3)_4$ were added subsequently and stirred at 100 °C for 4 h to obtain **4h** with an 84% yield.

5. ^{1}H, ^{13}C, and HRMS Spectra of few representative compounds

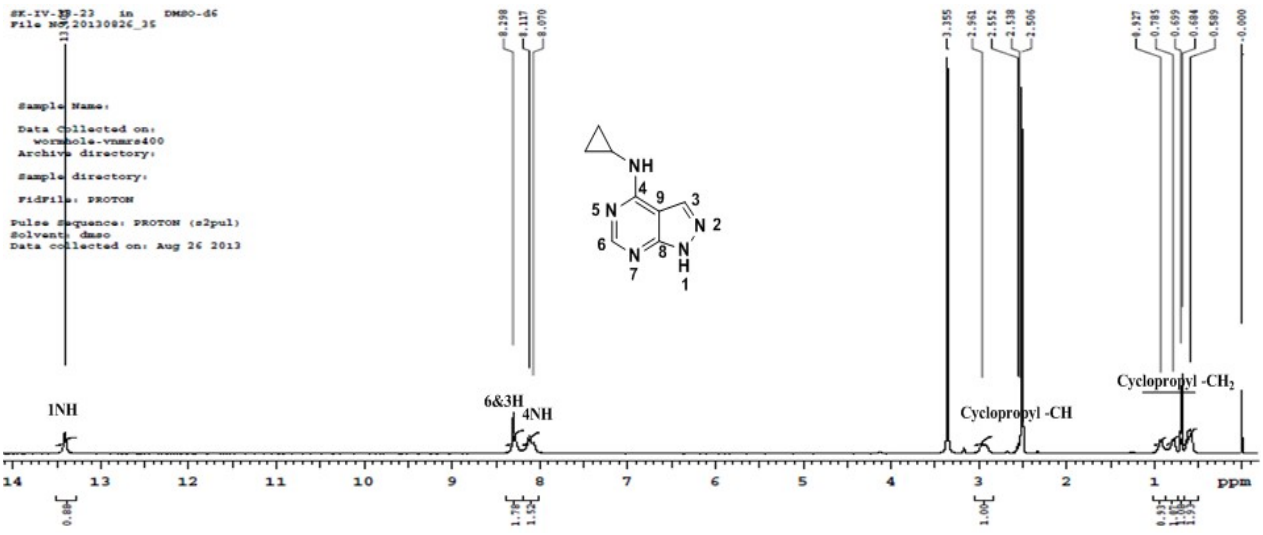

Figure 1: ^{1}H-NMR (400 MHz, DMSO-d_6) of compound **2a**

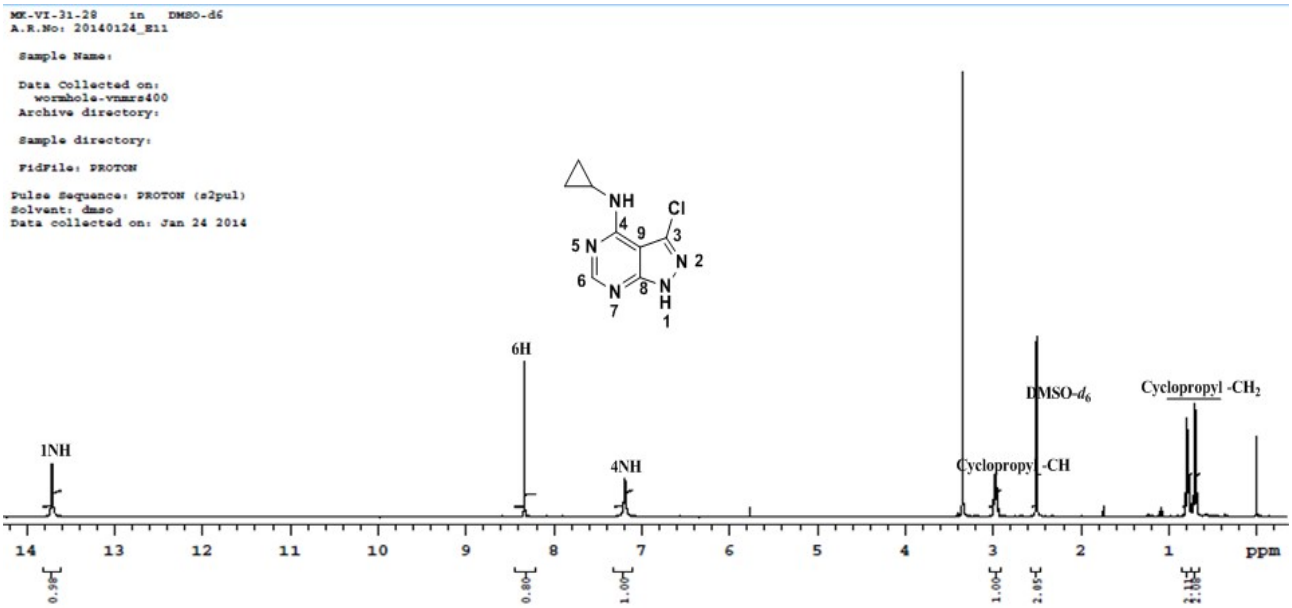

Figure 2: ^{1}H-NMR (400 MHz, DMSO-d_6) of compound **2b**

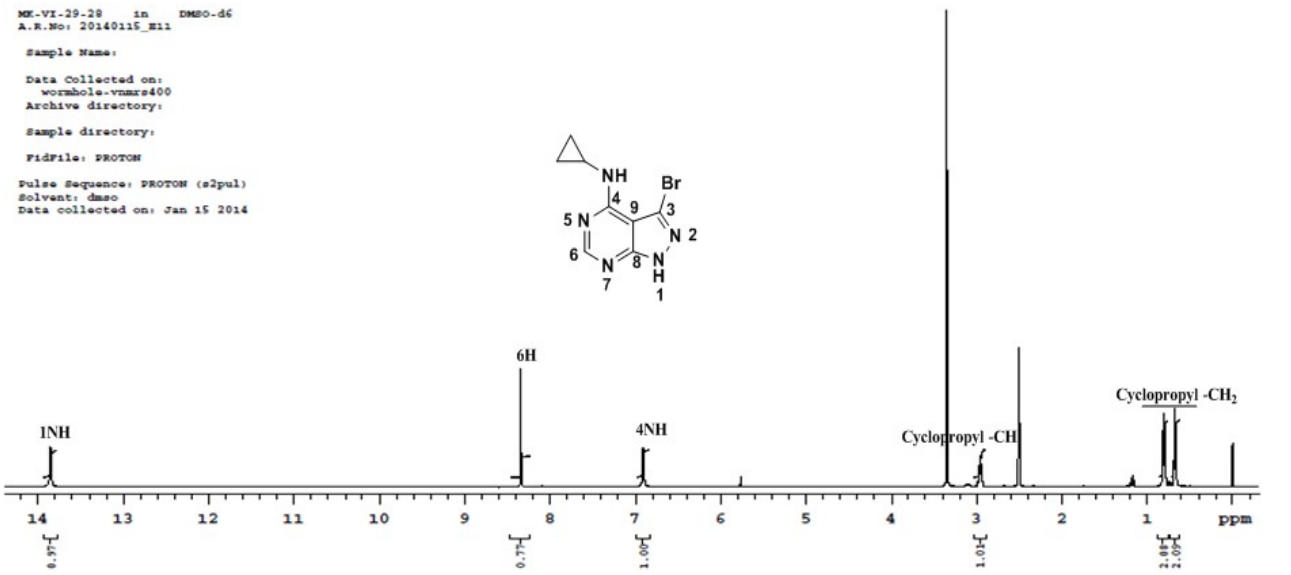

Figure 3: ^{1}H-NMR (400 MHz, DMSO-d_6) of compound **2c**

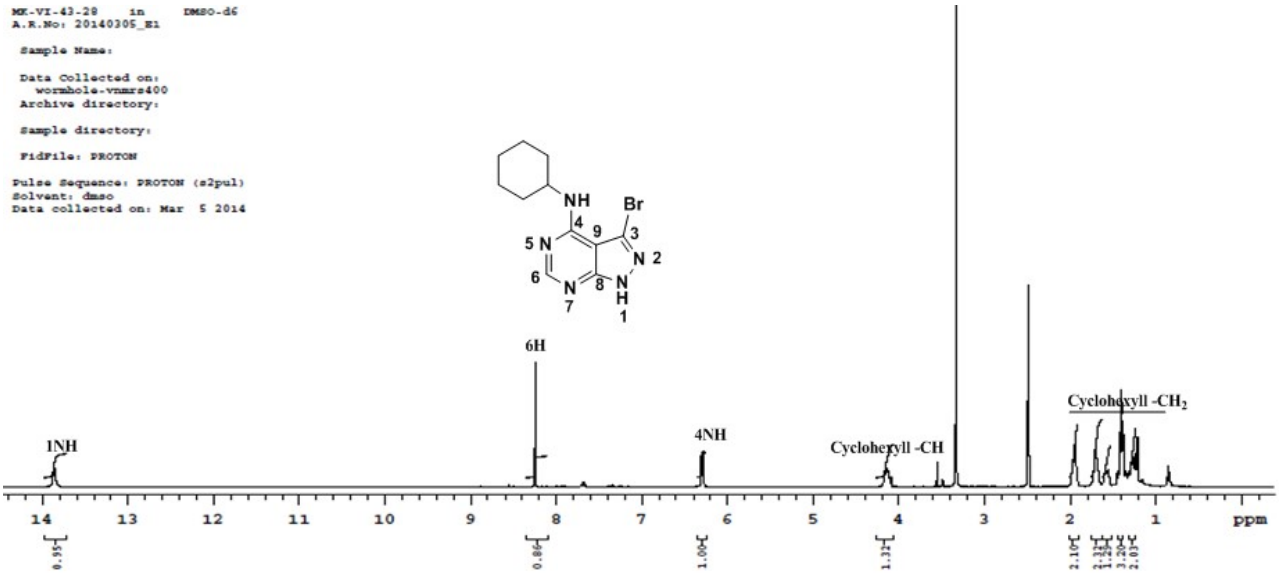

Figure 4: [1]H-NMR (400 MHz, DMSO-d_6) of compound **2e**

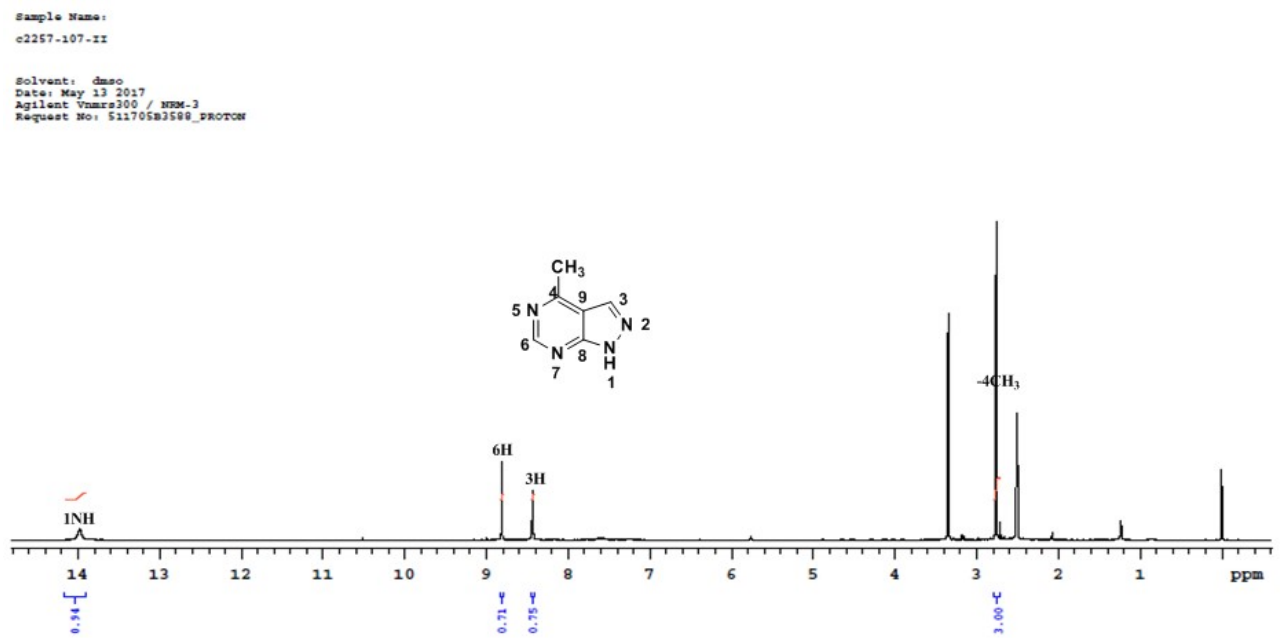

Figure 5: [1]H-NMR (300 MHz, DMSO-d_6) of compound **2f**

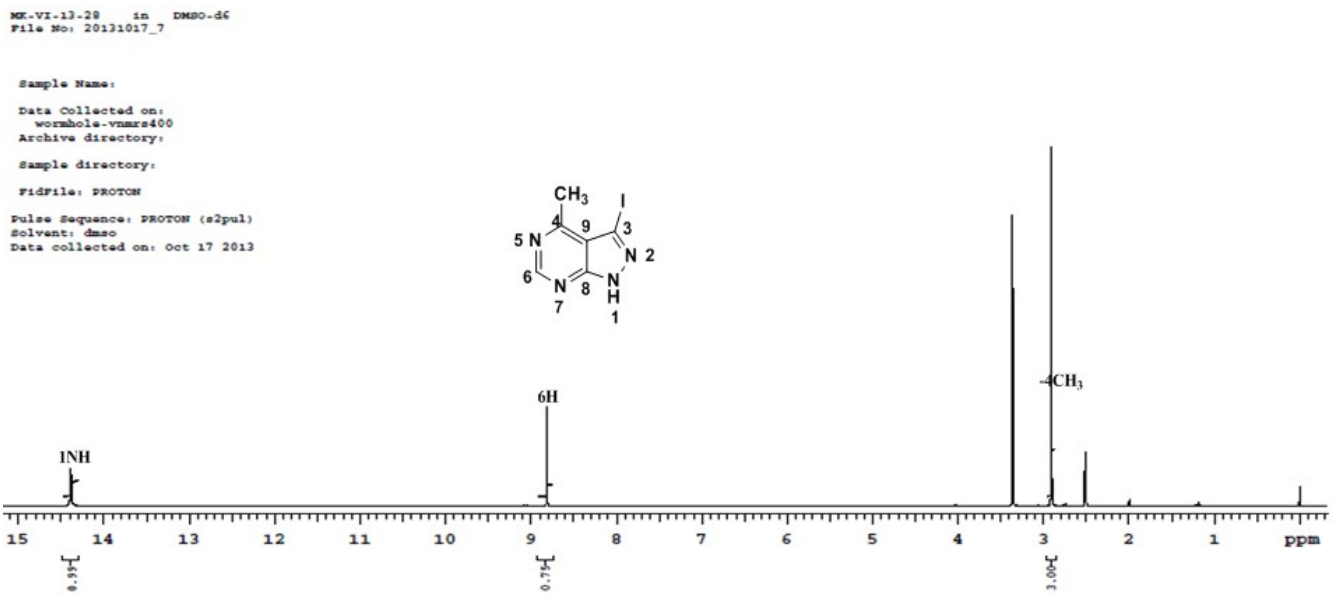

Figure 6: ^{1}H-NMR (400 MHz, DMSO-d_6) of compound **2g**

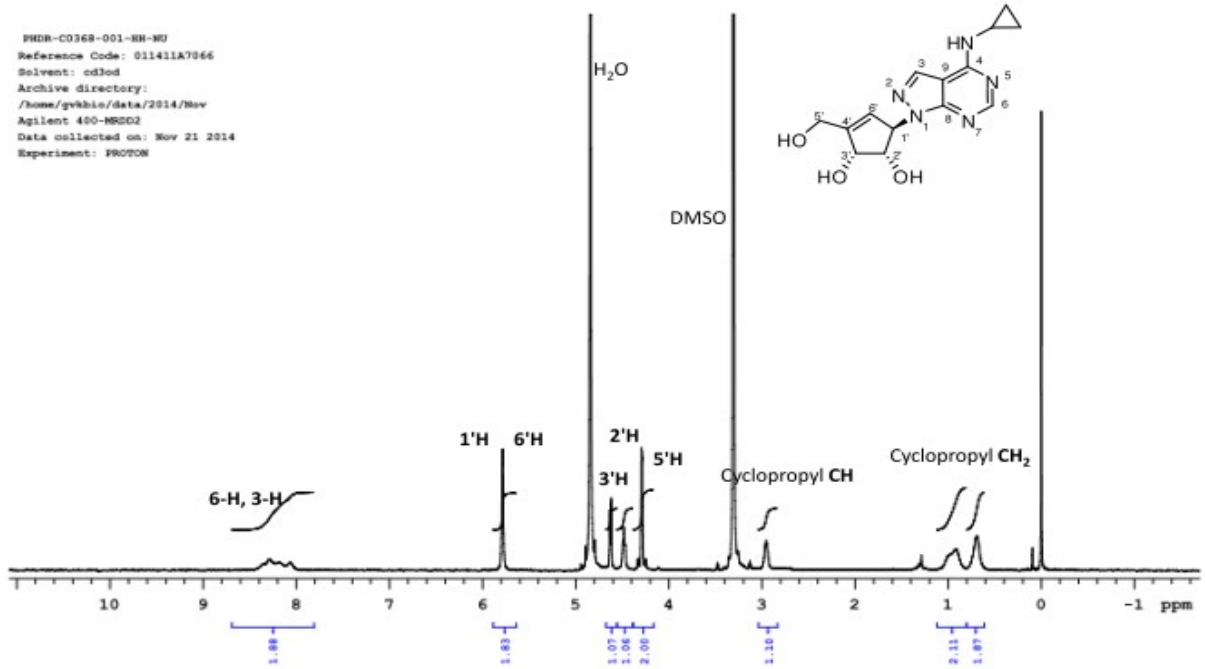

Figure 7: ^{1}H-NMR (400 MHz, CD_3OD) of compound **4a**

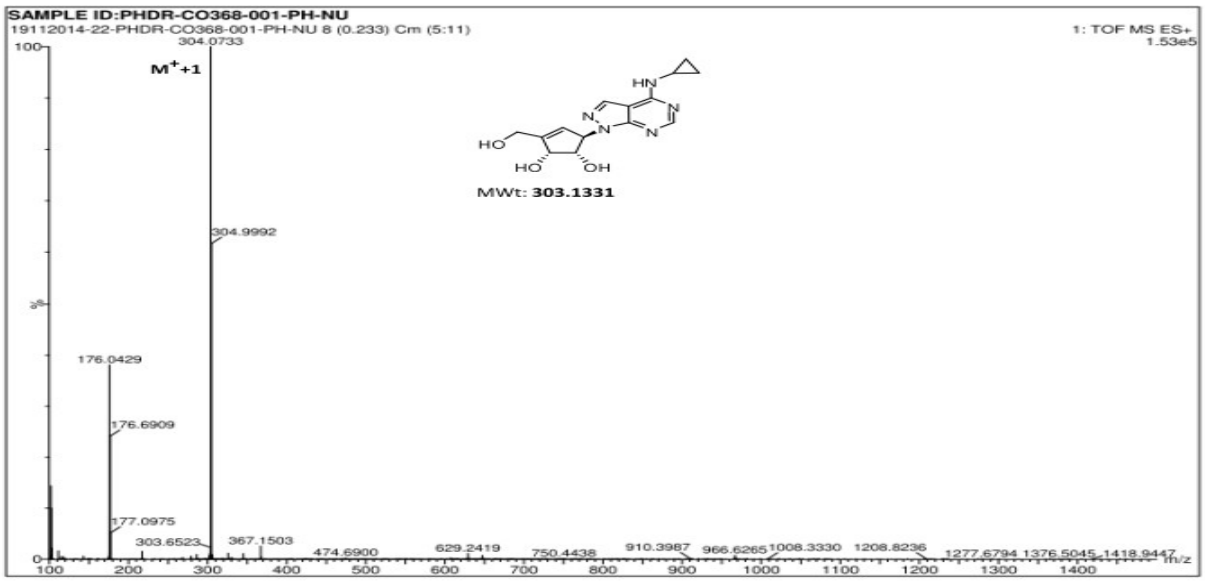

Figure 8: HRMS spectra of compound 4**a**

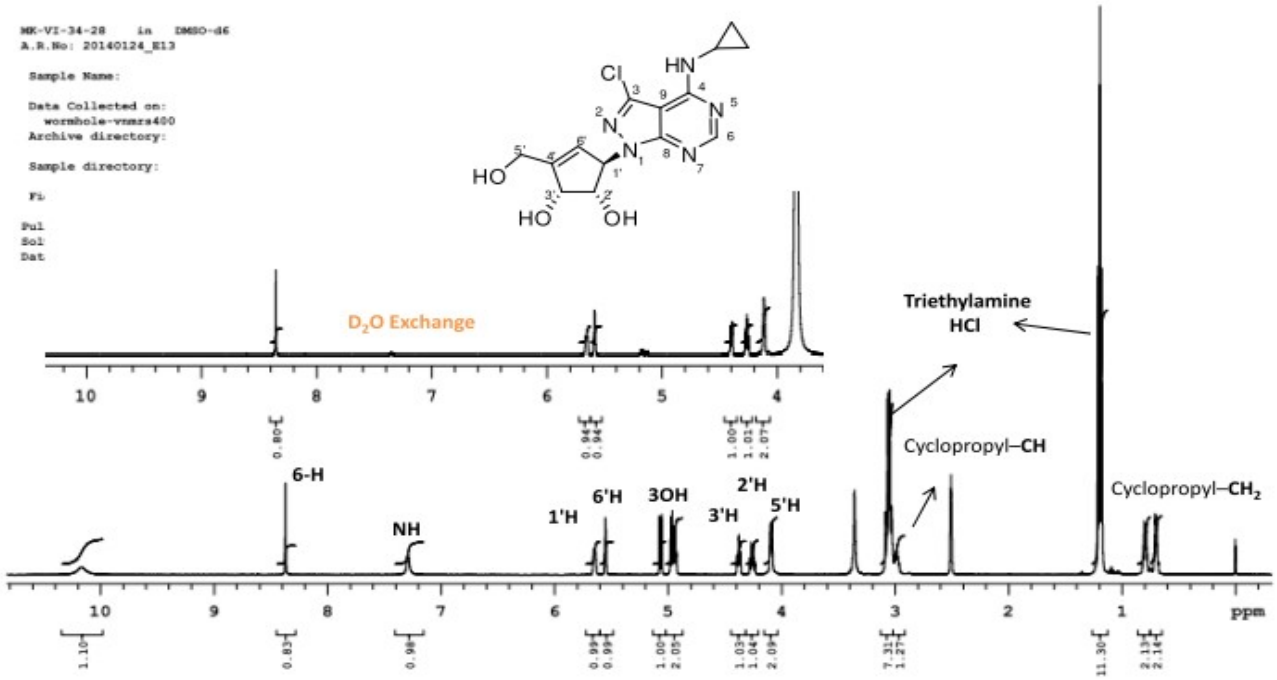

Figure 9: ^{1}H-NMR (400 MHz, DMSO-d_6) of compound **4b**

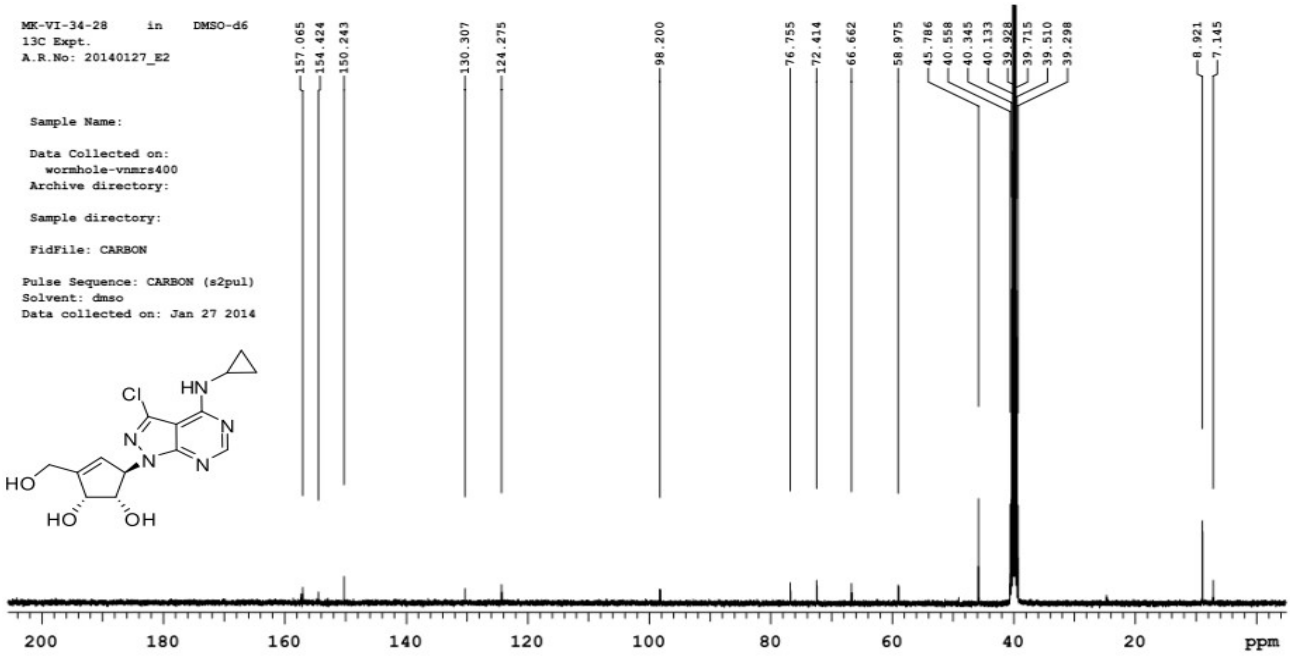

Figure 10: ^{13}C-NMR (100 MHz, DMSO-d_6) of compound **4b**

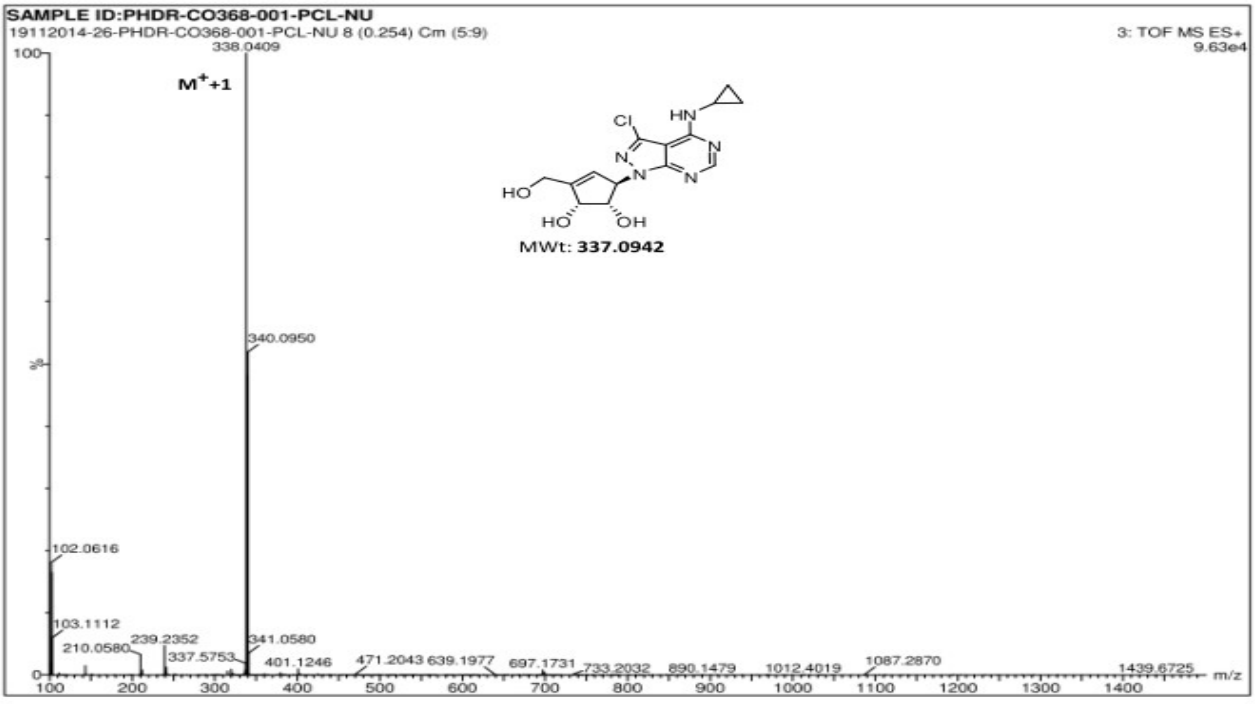

Figure 11: HRMS spectra of compound 4**b**

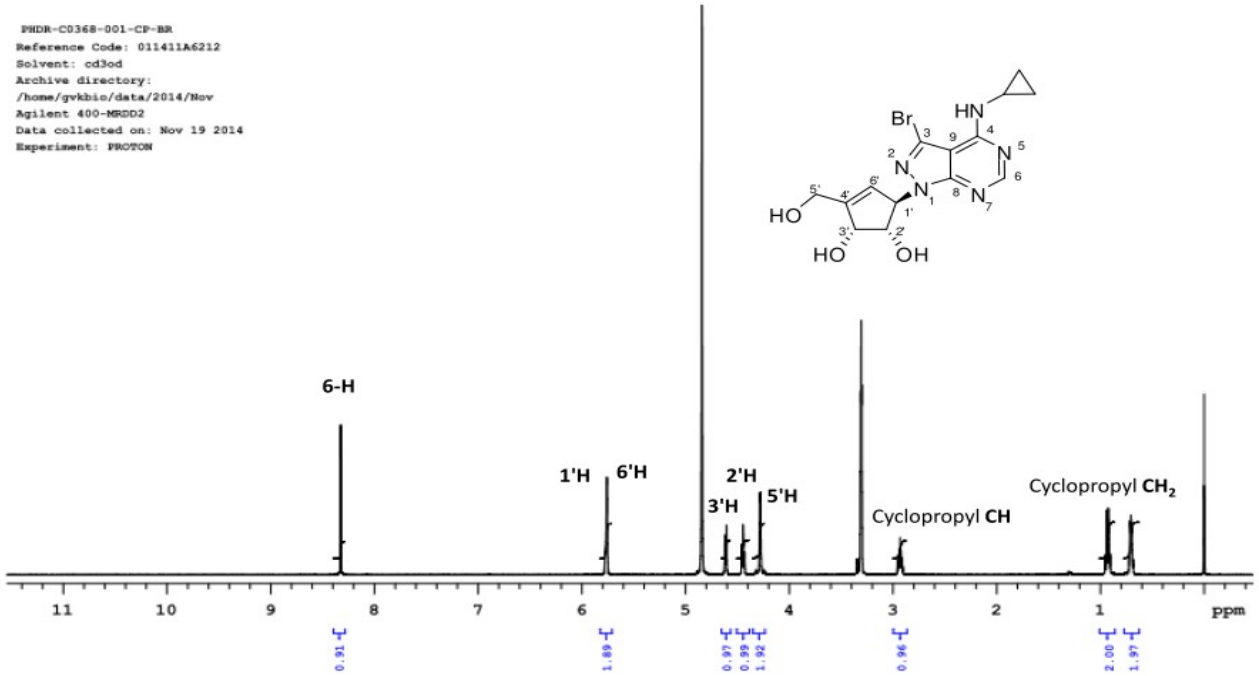

Figure 12: ^{1}H-NMR (400 MHz, CD_3OD) of compound **4c**

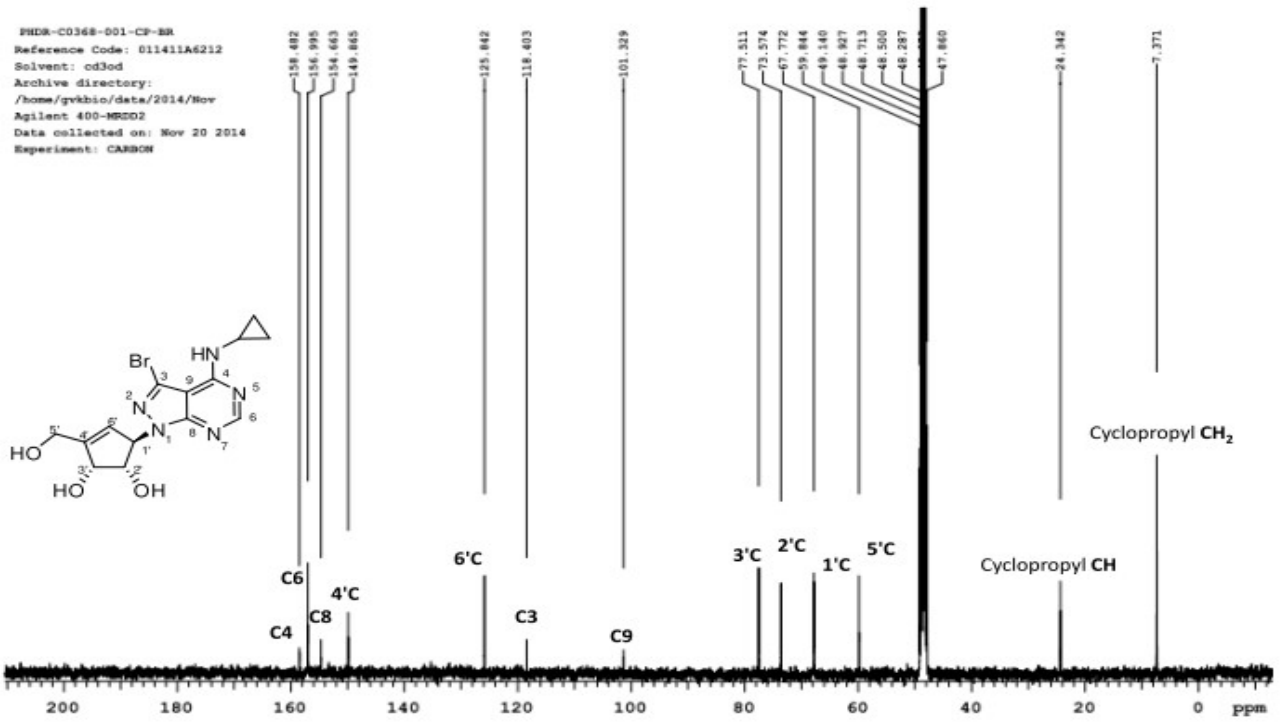

Figure 13: ^{13}C-NMR (100 MHz, CD_3OD) of compound **4c**

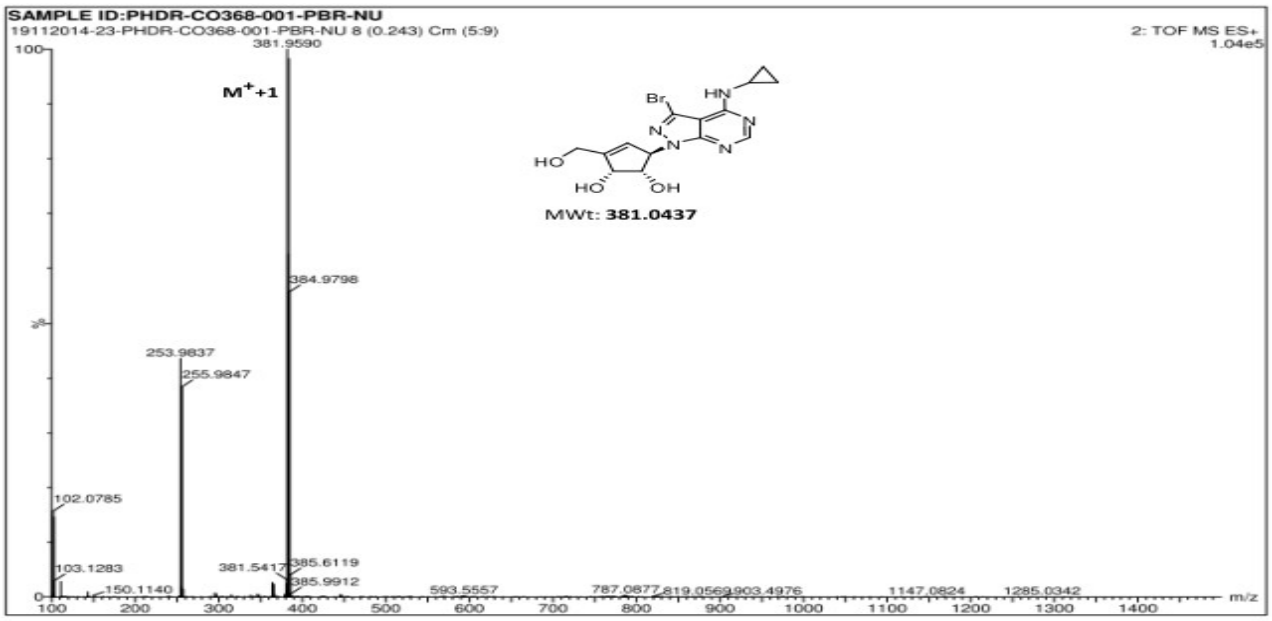

Figure 14: HRMS spectra of compound **4c**

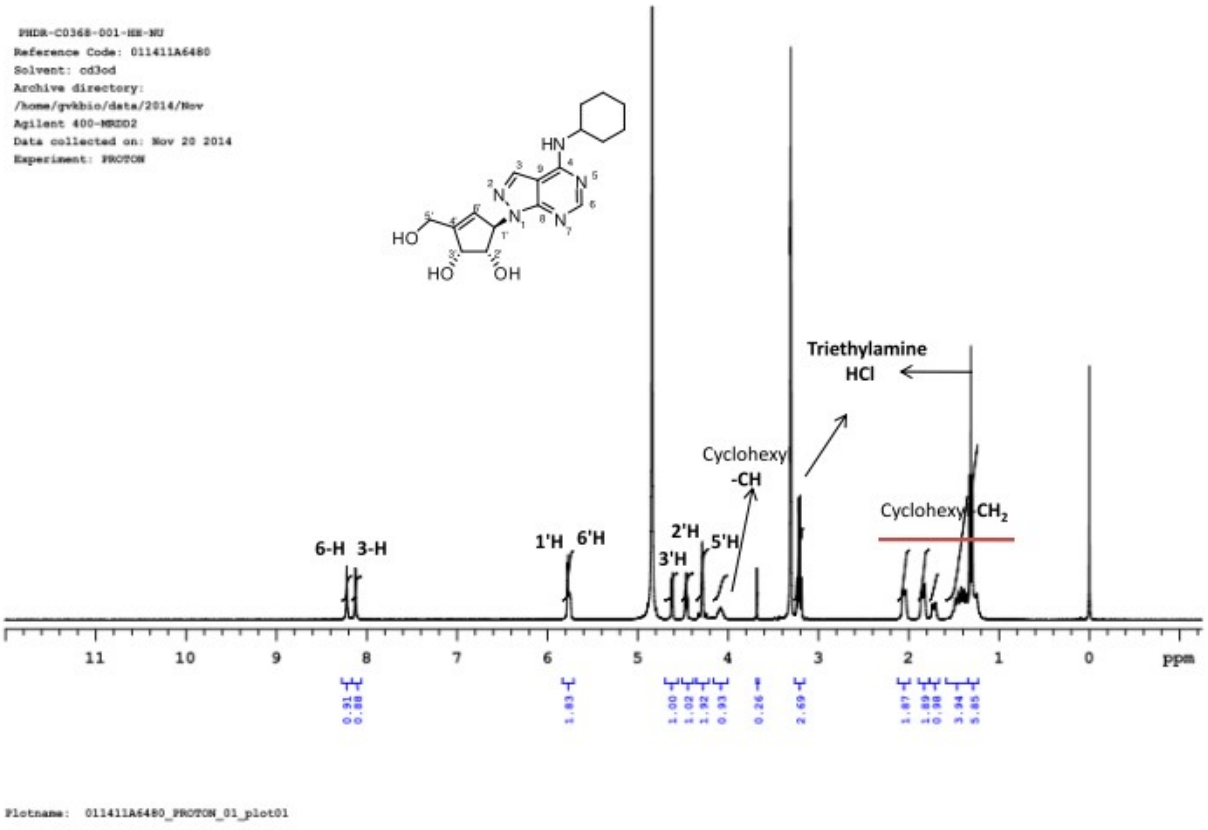

Figure 15: ^{1}H-NMR (400 MHz, CD_3OD) of compound **4d**

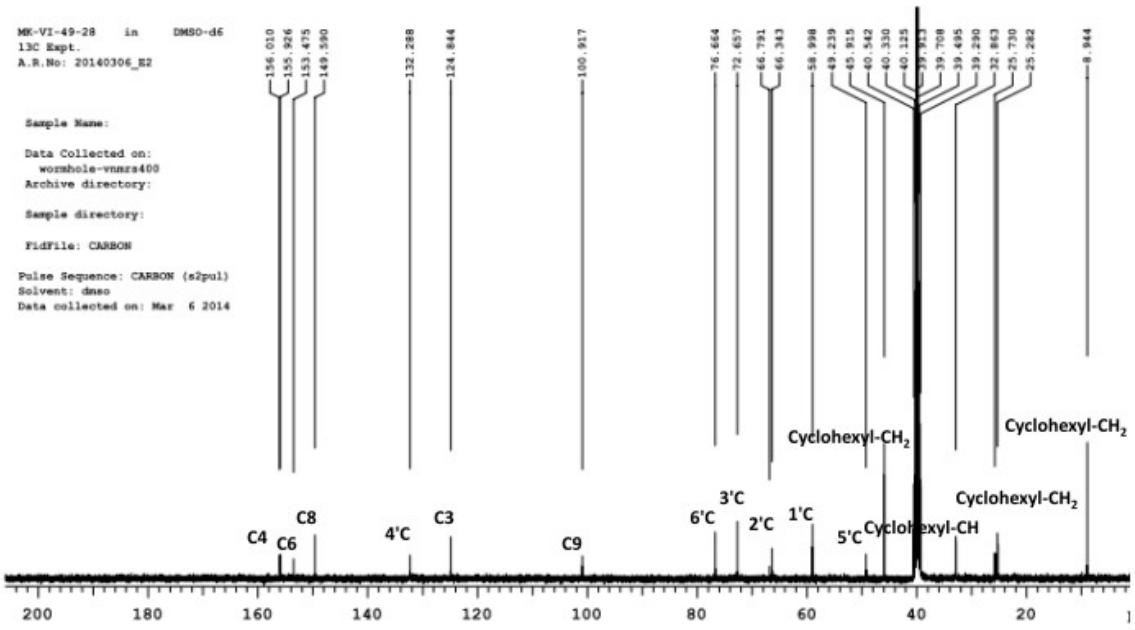

Figure 16: ^{13}C-NMR (100 MHz, DMSO-d_6) of compound **4d**

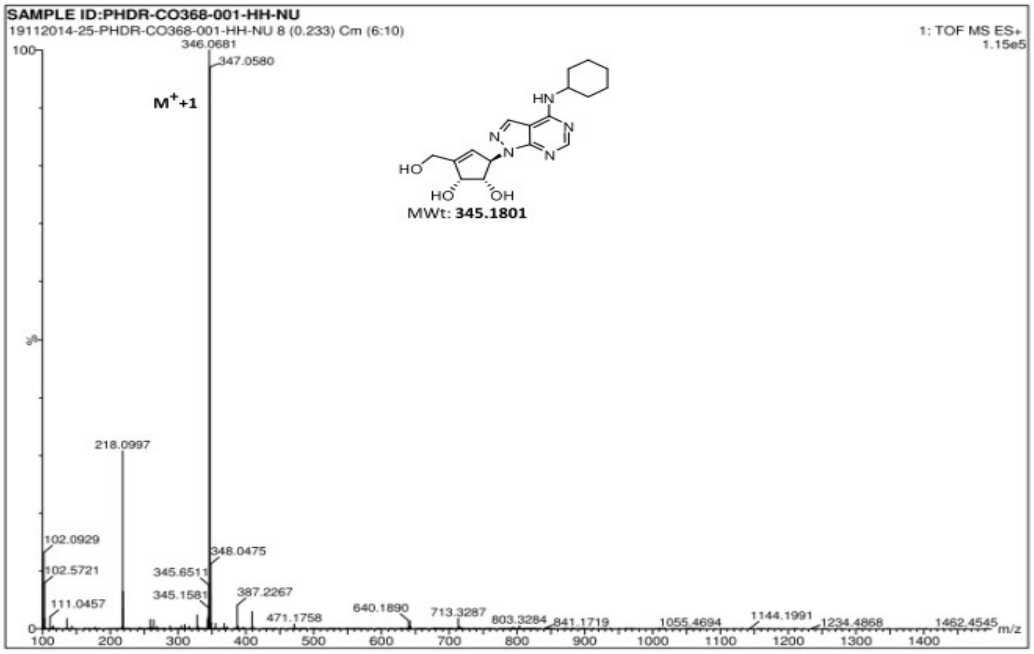

Figure 17: HRMS spectra of compound **4d**

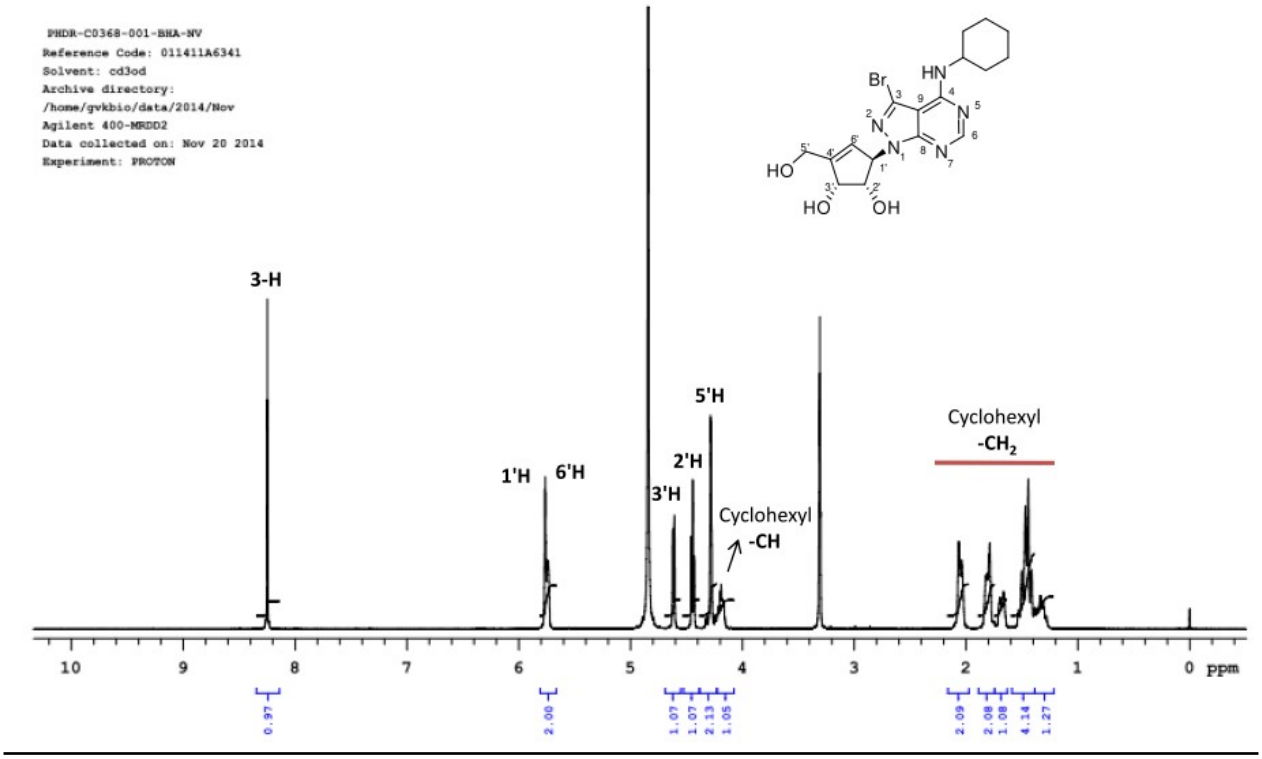

Figure 18: ^{1}H-NMR (400 MHz, CD_3OD) of compound **4e**

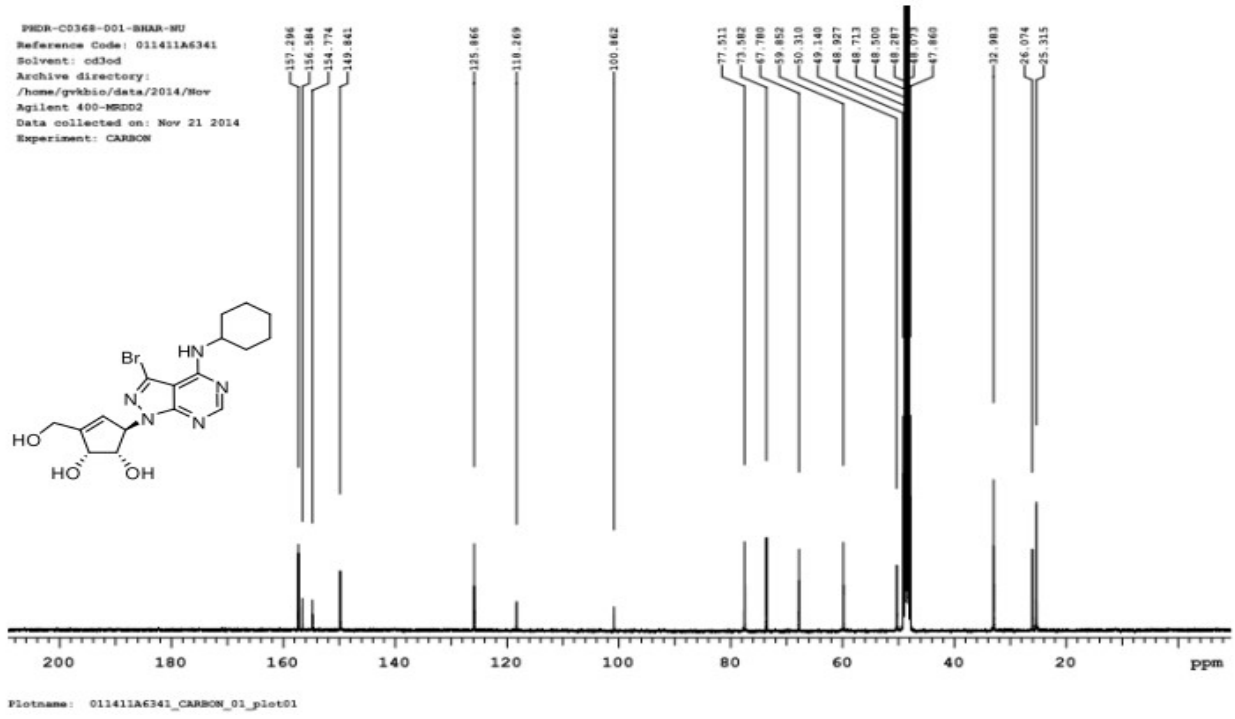

Figure 19: ^{13}C-NMR (100 MHz, CD_3OD) of compound **4e**

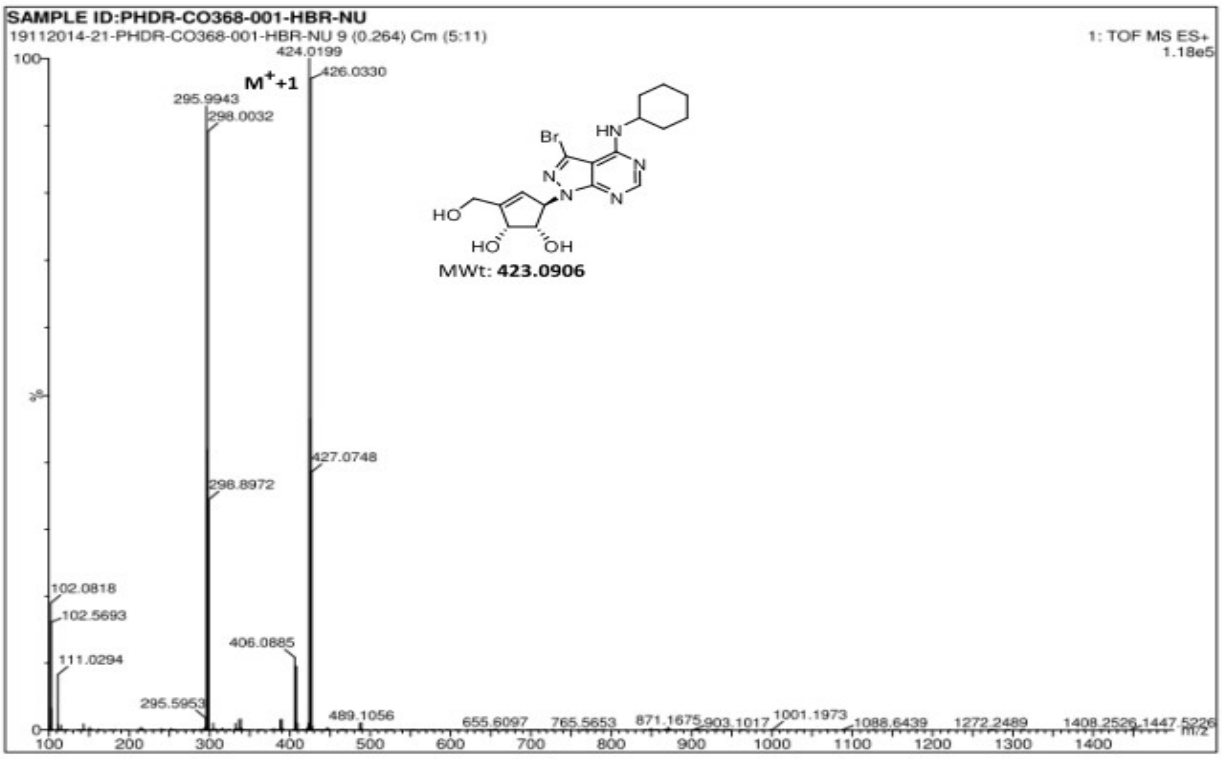

Figure 20: HRMS spectra of compound **4e**

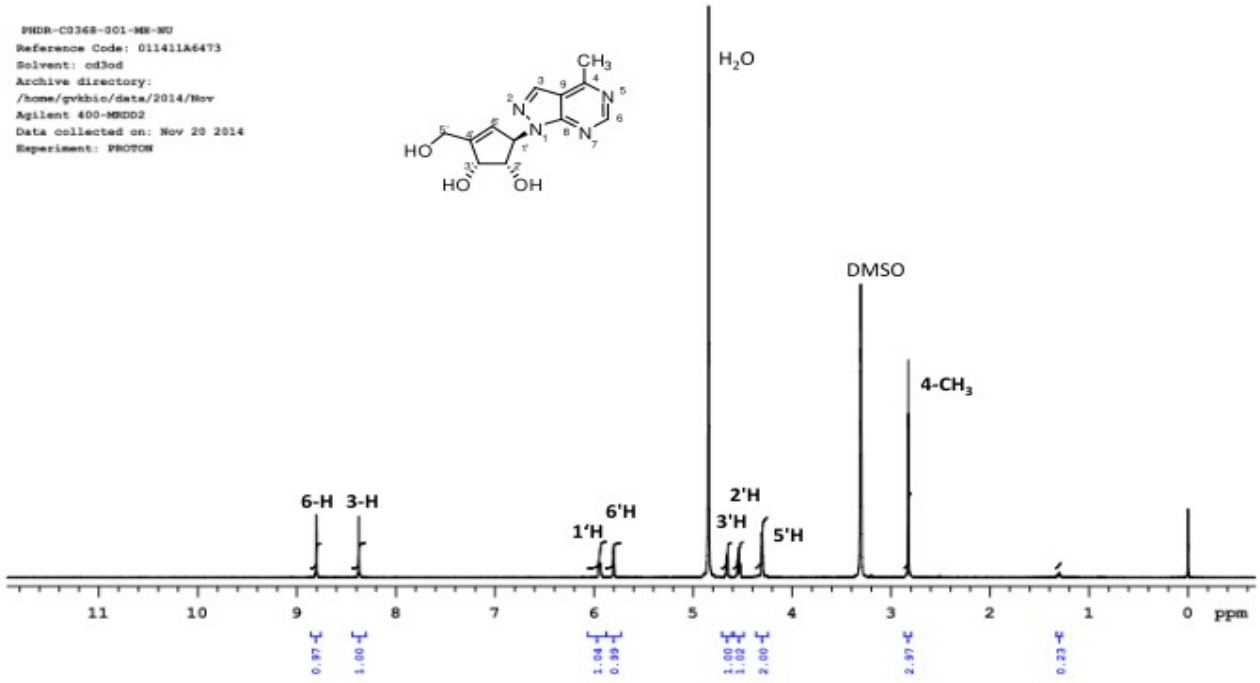

Figure 21: ^{1}H-NMR (400 MHz, CD_3OD) of compound **4f**

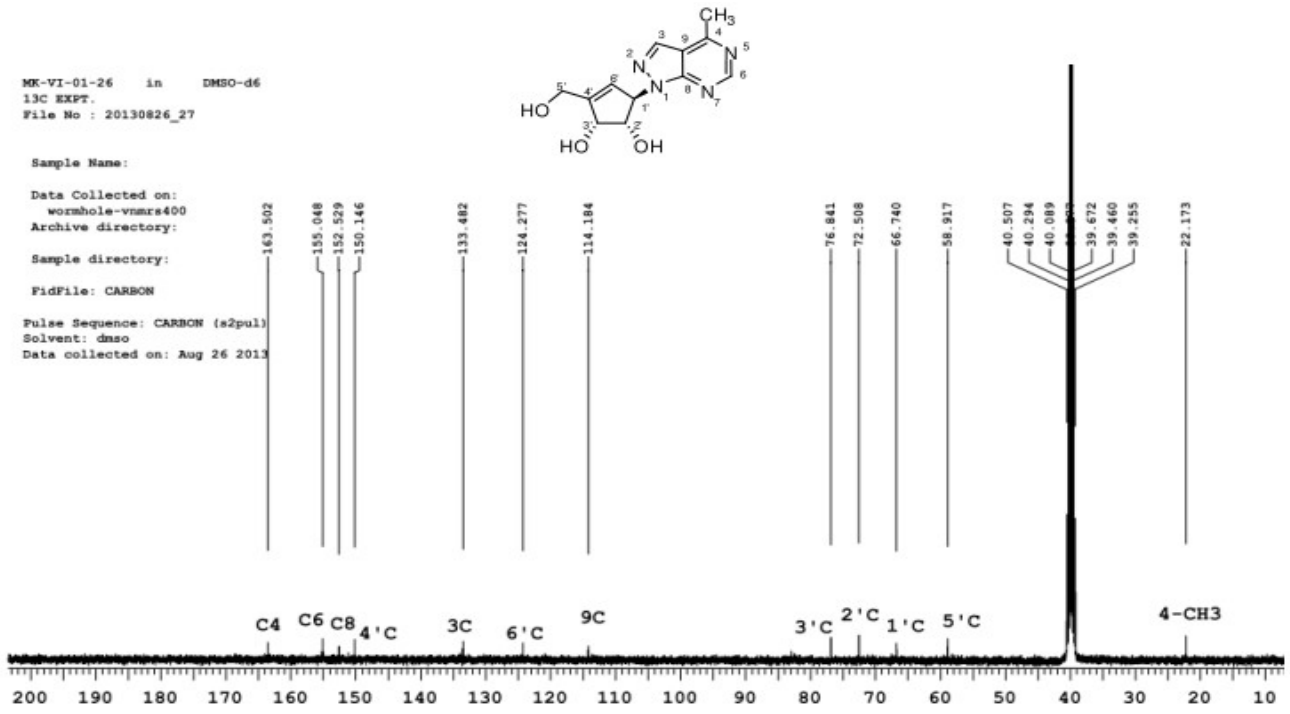

Figure 22: ^{13}C-NMR (100 MHz, DMSO-d_6) of compound **4f**

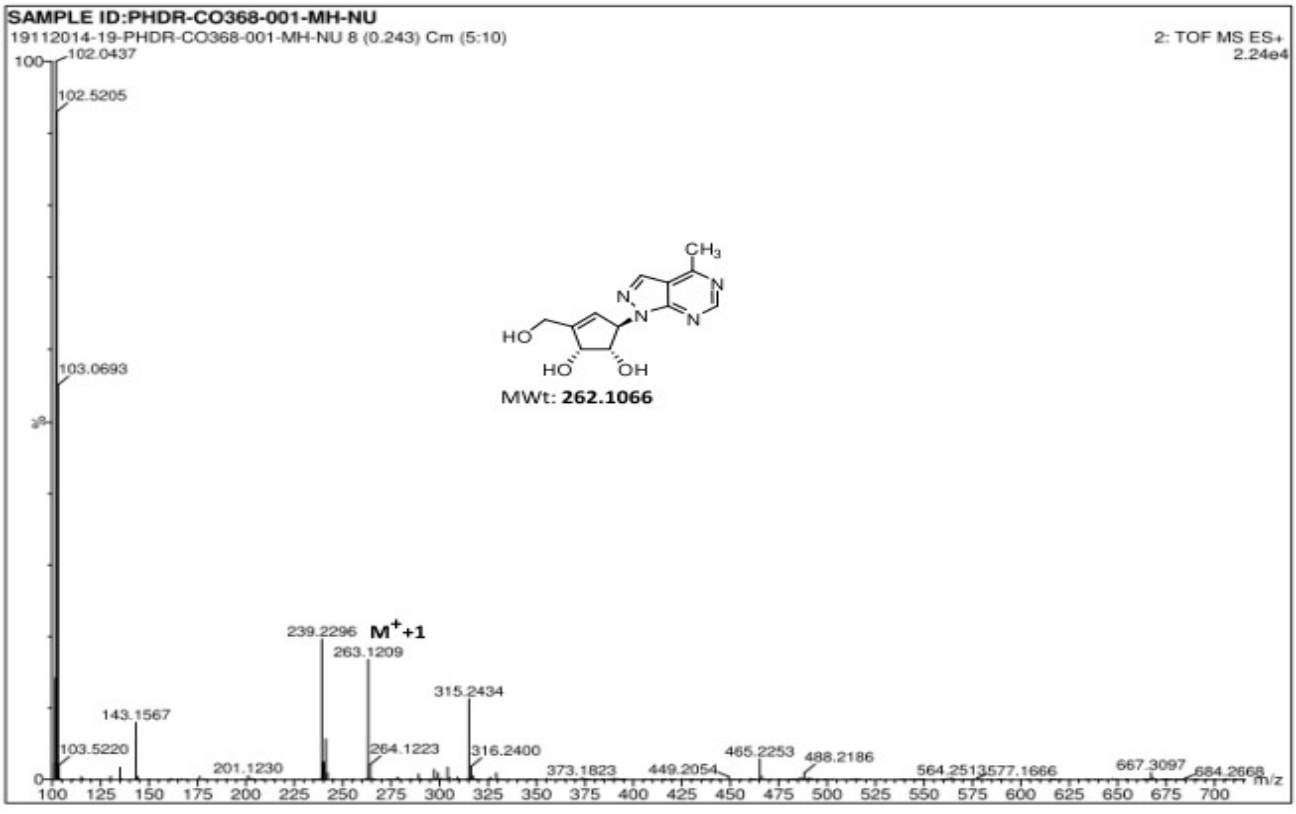

Figure 23: HRMS spectra of compound **6f**

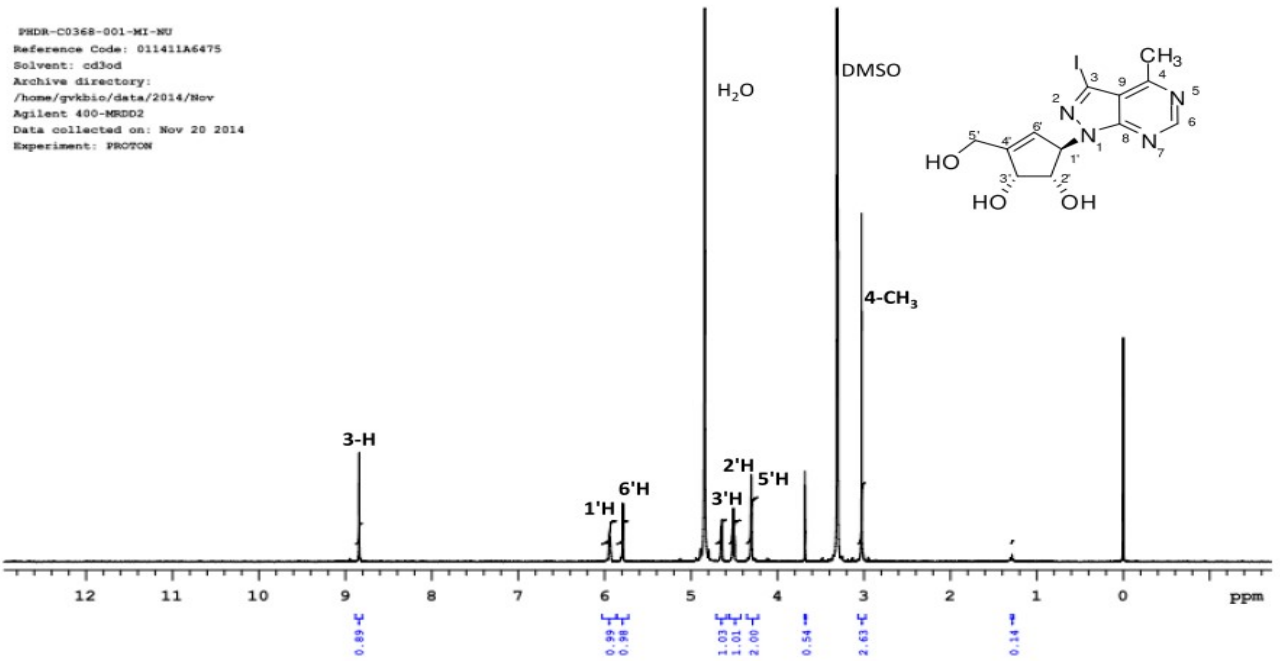

Figure 24: ^{1}H-NMR (400 MHz, CD_3OD) of compound **4g**

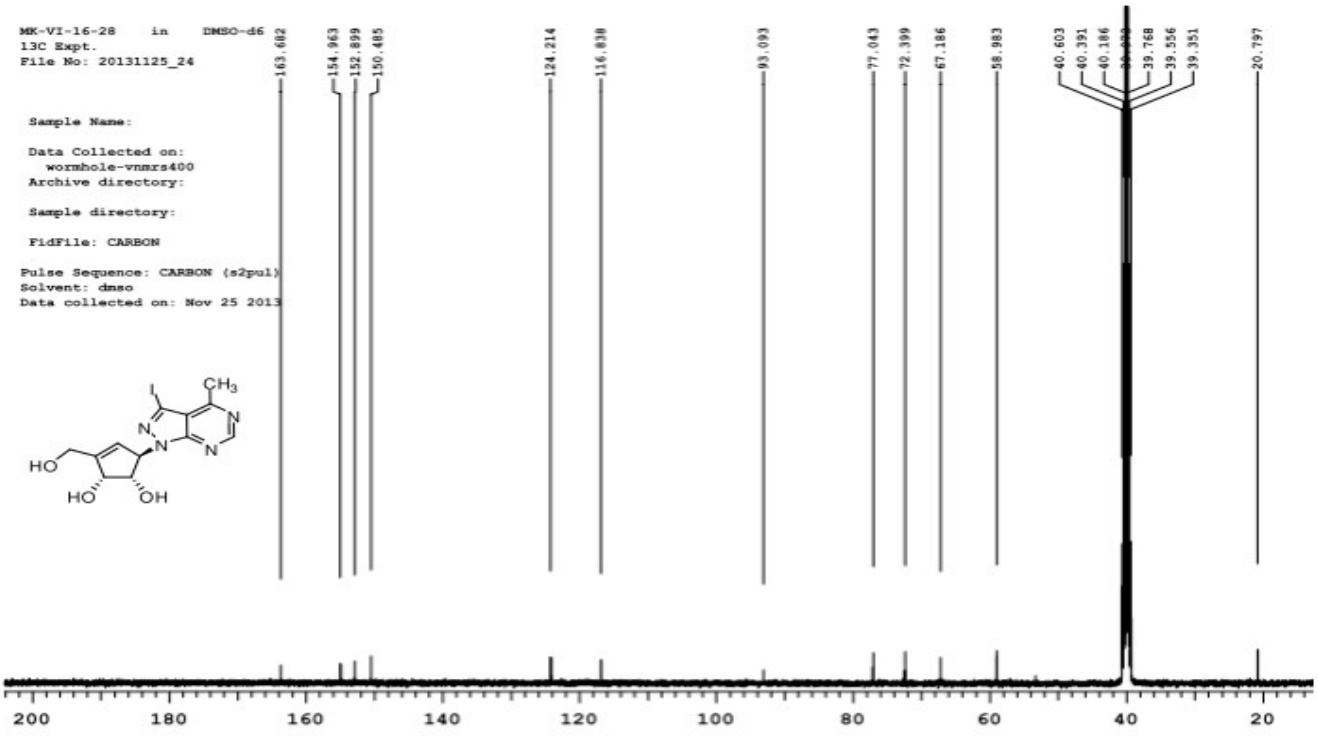

Figure 25: ^{13}C-NMR (100 MHz, DMSO-d_6) of compound **4g**

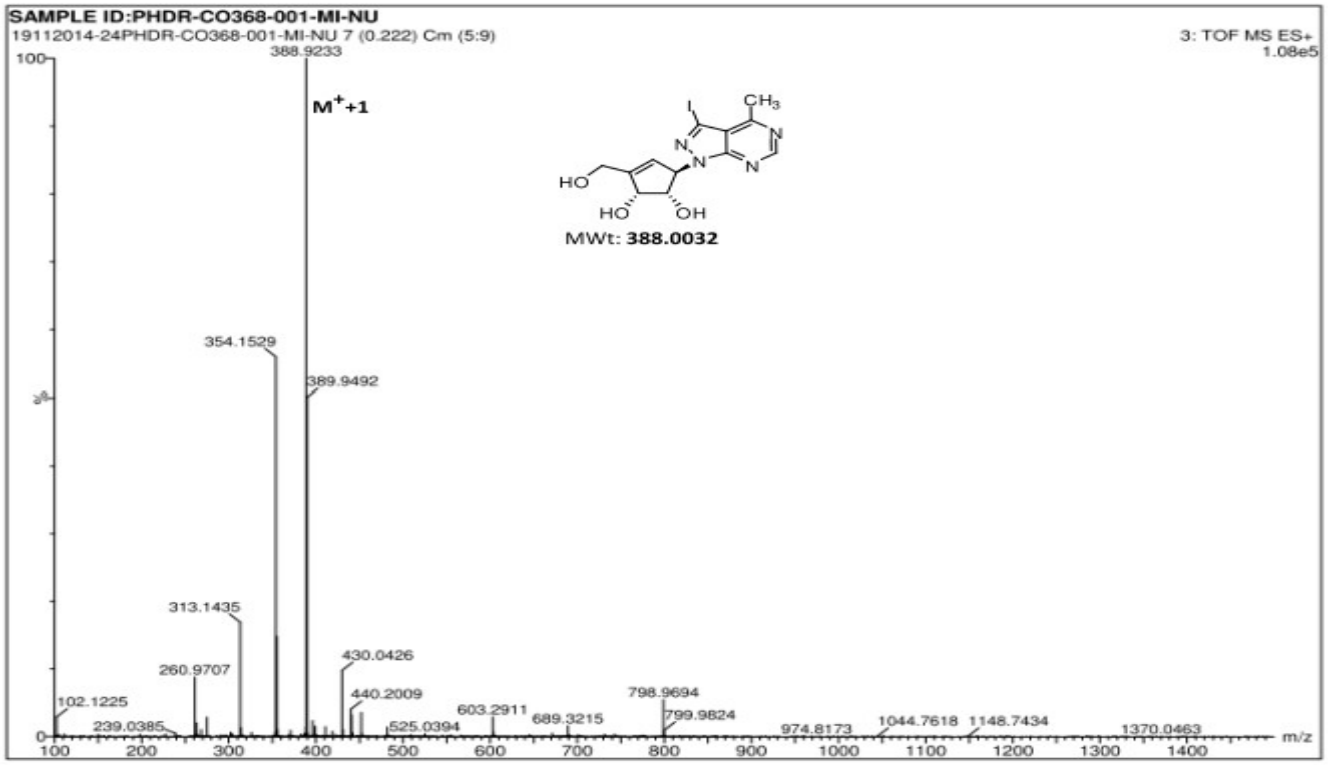

Figure 26: HRMS spectra of compound **6g**

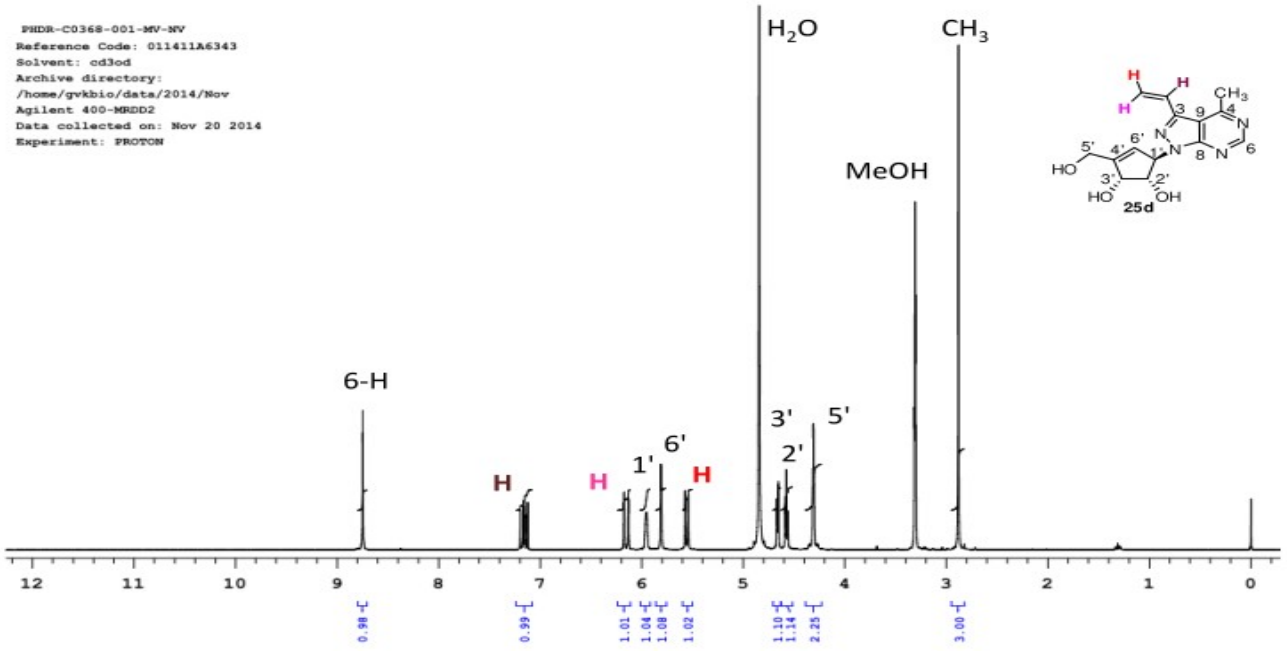

Figure 27: ^{1}H-NMR (400 MHz, CD_3OD) of compound **4h**

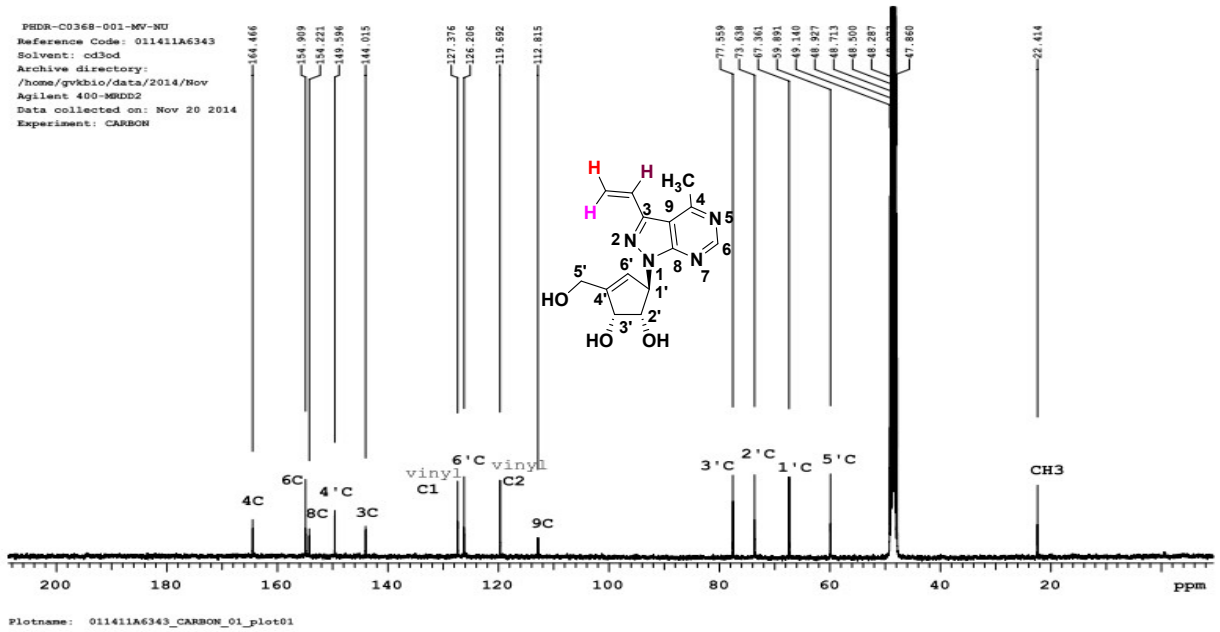

Figure 28: ^{13}C-NMR (100 MHz, CD_3OD) of compound **4h**

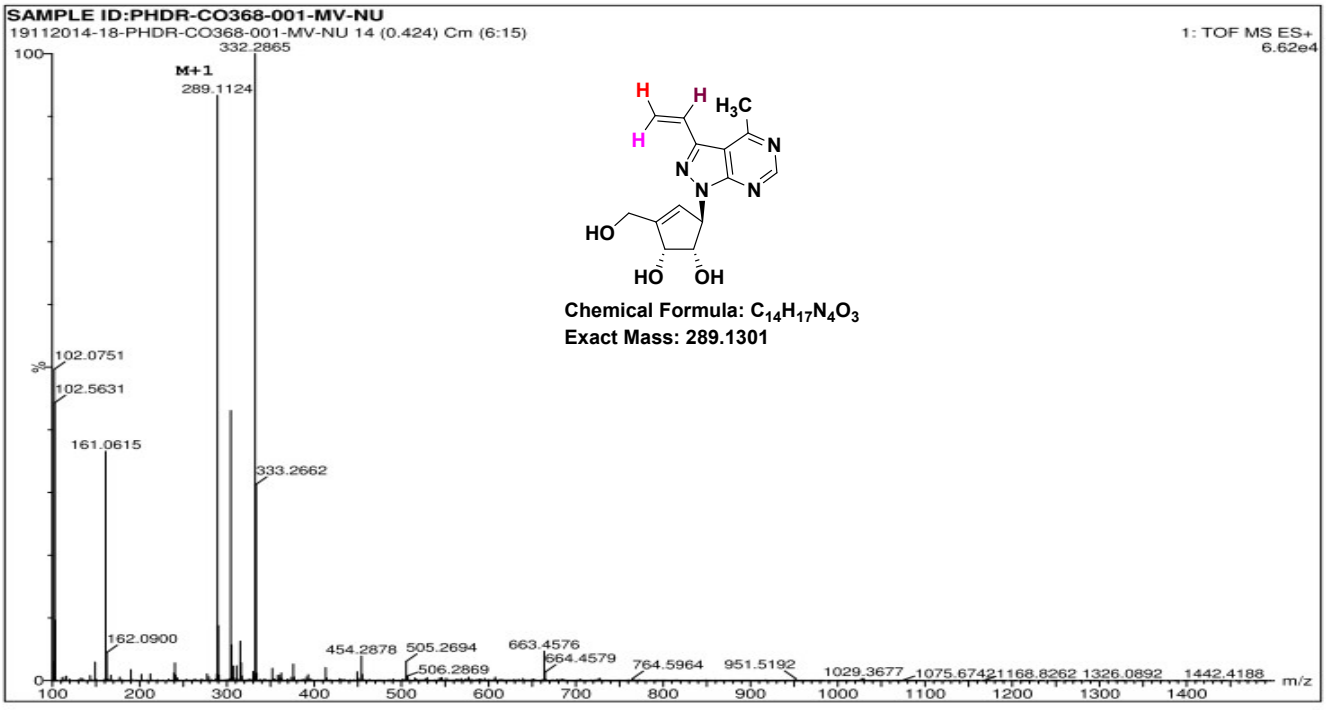

Figure 29: HRMS spectra of compound **4h**