Electronic Supplementary Material (ESI) for RSC Advances.


# Supplemental File

# For

## *In vitro* biological studies and computational prediction-based analyzes of pyrazolo[1,5-*a*]pyrimidine derivatives

**Abdulrahman A. Almehizia [a], Wael M. Aboulthana [b], Ahmed M. Naglah [a], Ashraf S. Hassan [c,*]**

[a] Drug Exploration & Development Chair (DEDC), Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia

[b] Biochemistry Department, Biotechnology Research Institute, National Research Centre, Dokki 12622, Cairo, Egypt

[c] Organometallic and Organometalloid Chemistry Department, National Research Centre, Dokki 12622, Cairo, Egypt

**Correspondence**
**Ashraf S. Hassan**, Organometallic and Organometalloid Chemistry Department, National Research Centre, Dokki 12622, Cairo, Egypt.
Email: ashraf_salmoon@yahoo.com and as.el-salmoon@nrc.sci.eg

**Table S1.** Native electrophoretic α-amylase isoenzymes pattern showing the anti-diabetic activity of 12a and 12b derivatives compared to Acarbose (standard) on the physiological state of α-amylase enzyme.

| | Enzyme | | | Enzyme treated with 12a at Conc. | | | | | | Enzyme treated with 12b at Conc. | | | | | | Enzyme treated with STD at Conc. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 mg/ mL | | | $IC_{50}$ | | | 1 mg/ mL | | | $IC_{50}$ | | | 1 mg/ mL | | | $IC_{50}$ | | |
| | Rf | Qty | B% | Rf | Qty | B% | Rf | Qty | B% | Rf | Qty | B% | Rf | Qty | B% | Rf | Qty | B% | Rf | Qty | B% |
| α-amy1 | 0.38 | 10.33 | 58.54 | 0.39 | 11.72 | 31.47 | - | - | - | 0.37 | 9.09 | 49.50 | 0.37 | 9.62 | 100.00 | - | - | - | - | - | - |
| | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.53 | 19.25 | 100.00 |
| | - | - | - | 0.68 | 25.52 | 68.53 | - | - | - | 0.70 | 9.27 | 50.50 | - | - | - | 0.69 | 9.94 | 100.00 | - | - | - |
| α-amy2 | 0.83 | 7.32 | 41.46 | - | - | - | 0.84 | 23.87 | 100.00 | - | - | - | - | - | - | - | - | - | - | - | - |
| SI% | - | | | 50.00 | | | 66.67 | | | 50.00 | | | 66.67 | | | 0.00 | | | 0.00 | | |
| Diff% | - | | | 50.00 | | | 33.33 | | | 50.00 | | | 33.33 | | | 100.00 | | | 100.00 | | |

**B4 = 12a and B9= 12b**

**Rf.:** Relative Mobility, **Qty:** Band Quantity, **B%:** Band Percent, **SI%:** Similarity Percent, **Diff%:** Difference Percent.

**Table S2.** The electrophoretic α-glucosidase enzyme checked by SDS-PAGE showing the anti-diabetic activity of 12a and 12b derivatives compared to Acarbose (standard) on the physiological state of α-glucosidase enzyme.

| Marker (KDa) | Enzyme | | | Enzyme treated with 12a at Conc. | | | | | | Enzyme treated with 12b at Conc. | | | | | | Enzyme treated with STD at Conc. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 mg/ mL | | | $IC_{50}$ | | | 1 mg/ mL | | | $IC_{50}$ | | | 1 mg/ mL | | | $IC_{50}$ | | |
| | Rf | Int. | Qty | Rf | Int. | Qty | Rf | Int. | Qty | Rf | Int. | Qty | Rf | Int. | Qty | Rf | Int. | Qty | Rf | Int. | Qty |
| 66 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 45 | 0.42 | 115.00 | 1.48 | 0.43 | 100.80 | 0.88 | 0.44 | 74.85 | 0.65 | 0.44 | 87.25 | 0.76 | 0.43 | 52.89 | 0.46 | 0.43 | 25.73 | 0.22 | - | - | - |
| 36 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 25 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Diff% | - | | | 40.54 | | | 56.08 | | | 48.65 | | | 68.92 | | | 84.14 | | | - | | |

**Rf.:** Relative Mobility, **Int.:** Band Intensity, **Qty:** Band Quantity, **Diff%:** Difference Percent.

**Table S3:** The in vitro cytotoxic activity of 12a, 12b, and Doxorubicin against the human lung cancer (A549) cell line

| **12a** | | | | | | |
|---|---|---|---|---|---|---|
| **Conc. (μg/mL)** | **0.00** | **6.25** | **12.50** | **25.00** | **50.00** | **100.00** |
| OD | 0.35 | 0.30 | 0.26 | 0.19 | 0.12 | 0.10 |
| | 0.36 | 0.29 | 0.27 | 0.2 | 0.12 | 0.08 |
| | 0.34 | 0.31 | 0.29 | 0.19 | 0.13 | 0.08 |
| Mean | 0.35 | 0.30 | 0.27 | 0.19 | 0.12 | 0.09 |
| Viability % | 100.00 | 98.71 | 88.56 | 62.16 | 47.73 | 21.45 |
| **$IC_{50}$ %** | **47.83** μg/mL | | | | | |

| **12b** | | | | | | |
|---|---|---|---|---|---|---|
| **Conc. (μg/mL)** | **0.00** | **6.25** | **12.50** | **25.00** | **50.00** | **100.00** |
| OD | 0.47 | 0.41 | 0.37 | 0.28 | 0.22 | 0.09 |
| | 0.49 | 0.41 | 0.37 | 0.26 | 0.22 | 0.10 |
| | 0.47 | 0.42 | 0.36 | 0.28 | 0.21 | 0.10 |
| Mean | 0.48 | 0.41 | 0.37 | 0.27 | 0.22 | 0.10 |
| Viability % | 100.00 | 92.21 | 81.16 | 49.76 | 38.33 | 19.85 |
| **$IC_{50}$ %** | **40.54** μg/mL | | | | | |

| **Doxorubicin** | | | | | | |
|---|---|---|---|---|---|---|
| **Conc. (μg/mL)** | **0.00** | **6.25** | **12.50** | **25.00** | **50.00** | **100.00** |
| OD | 0.27 | 0.22 | 0.18 | 0.14 | 0.11 | 0.78 |
| | 0.28 | 0.23 | 0.17 | 0.14 | 0.095 | 0.75 |
| | 0.26 | 0.23 | 0.18 | 0.13 | 0.10 | 0.75 |
| Mean | 0.27 | 0.23 | 0.18 | 0.14 | 0.10 | 0.76 |
| Viability % | 100.00 | 82.11 | 70.56 | 38.16 | 20.17 | 12.45 |
| **$IC_{50}$ %** | **31.32** μg/mL | | | | | |

**Table S4:** The in vitro cytotoxic activity of 12a, 12b, and Doxorubicin against the human colon cancer (CaCo-2) cell line

| **12a** | | | | | | |
|---|---|---|---|---|---|---|
| **Conc. (μg/mL)** | **0.00** | **6.25** | **12.50** | **25.00** | **50.00** | **100.00** |
| OD | 0.31 | 0.24 | 0.19 | 0.12 | 0.09 | 0.09 |
| | 0.32 | 0.25 | 0.19 | 0.12 | 0.10 | 0.09 |
| | 0.31 | 0.23 | 0.20 | 0.13 | 0.08 | 0.10 |
| Mean | 0.31 | 0.24 | 0.19 | 0.12 | 0.09 | 0.09 |
| Viability % | 100.00 | 84.18 | 75.66 | 56.63 | 34.47 | 27.09 |
| **$IC_{50}$ %** | **38.15** | | | | | |

| **12b** | | | | | | |
|---|---|---|---|---|---|---|
| **Conc. (μg/mL)** | **0.00** | **6.25** | **12.50** | **25.00** | **50.00** | **100.00** |
| OD | 0.41 | 0.28 | 0.21 | 0.12 | 0.08 | 0.07 |
| | 0.39 | 0.27 | 0.21 | 0.11 | 0.09 | 0.07 |
| | 0.40 | 0.28 | 0.22 | 0.12 | 0.09 | 0.06 |
| Mean | 0.40 | 0.28 | 0.21 | 0.12 | 0.09 | 0.07 |
| Viability % | 100.00 | 59.96 | 40.40 | 29.79 | 16.02 | 12.68 |
| **$IC_{50}$ %** | **29.77** μg/mL | | | | | |

| **Doxorubicin** | | | | | | |
|---|---|---|---|---|---|---|
| **Conc. (μg/mL)** | **0.00** | **6.25** | **12.50** | **25.00** | **50.00** | **100.00** |
| OD | 0.44 | 0.39 | 0.31 | 0.22 | 0.24 | 0.11 |
| | 0.45 | 0.38 | 0.29 | 0.21 | 0.23 | 0.09 |
| | 0.42 | 0.38 | 0.28 | 0.21 | 0.24 | 0.12 |
| Mean | 0.44 | 0.38 | 0.29 | 0.21 | 0.24 | 0.11 |
| Viability % | 100.00 | 62.07 | 40.52 | 31.61 | 12.77 | 9.80 |
| **$IC_{50}$ %** | **28.45** μg/mL | | | | | |

**Table S5:** The in vitro cytotoxic activity of 12a, 12b, and Doxorubicin against the normal lung (WI-38) cell line

| **12a** | | | | | | |
|---|---|---|---|---|---|---|
| **Conc. (μg/mL)** | **0.00** | **31.13** | **62.50** | **125.00** | **250.00** | **500.00** |
| OD | 0.42 | 0.39 | 0.35 | 0.32 | 0.21 | 0.19 |
| | 0.41 | 0.4 | 0.39 | 0.28 | 0.21 | 0.17 |
| | 0.43 | 0.4 | 0.38 | 0.30 | 0.23 | 0.17 |
| Mean | 0.42 | 0.40 | 0.37 | 0.30 | 0.22 | 0.18 |
| Viability % | 100.00 | 82.65 | 76.72 | 70.79 | 55.44 | 39.53 |
| **$IC_{50}$%** | **134.24** μg/mL | | | | | |

| **12b** | | | | | | |
|---|---|---|---|---|---|---|
| **Conc. (μg/mL)** | **0.00** | **31.13** | **62.50** | **125.00** | **250.00** | **500.00** |
| OD | 0.37 | 0.35 | 0.35 | 0.33 | 0.30 | 0.22 |
| | 0.36 | 0.36 | 0.34 | 0.33 | 0.29 | 0.26 |
| | 0.38 | 0.34 | 0.34 | 0.32 | 0.27 | 0.27 |
| Mean | 0.37 | 0.35 | 0.34 | 0.32 | 0.28 | 0.25 |
| Viability % | 100.00 | 97.71 | 89.75 | 81.78 | 75.8 | 61.74 |
| **$IC_{50}$%** | **304.88** μg/mL | | | | | |

| **Doxorubicin** | | | | | | |
|---|---|---|---|---|---|---|
| **Conc. (μg/mL)** | **0.00** | **31.13** | **62.50** | **125.00** | **250.00** | **500.00** |
| OD | 0.44 | 0.41 | 0.40 | 0.38 | 0.26 | 0.26 |
| | 0.43 | 0.42 | 0.39 | 0.35 | 0.27 | 0.26 |
| | 0.44 | 0.43 | 0.38 | 0.34 | 0.28 | 0.25 |
| Mean | 0.43 | 0.42 | 0.39 | 0.35 | 0.27 | 0.25 |
| Viability % | 100.00 | 67.2 | 58.87 | 52.04 | 42.44 | 28.24 |
| **$IC_{50}$%** | **75.98** μg/mL | | | | | |

## Pyrazolo[1,5-a]pyrimidines derivatives 12a, b

**Procedure for Synthesis of Pyrazolo[1,5-a]pyrimidines derivatives 12a, b.**

A mixture of compounds **10a, b** (0.01 mol) with 3-(dimethylamino)-1-phenylprop-2-en-1-one (**11**) (0.01 mol) in glacial acetic acid (25 mL), the reaction mixture was refluxed for 1 h and then left to cool. The solid product was filtered off, washed with ethanol, dried, and finally recrystallized from DMF/$H_2O$.

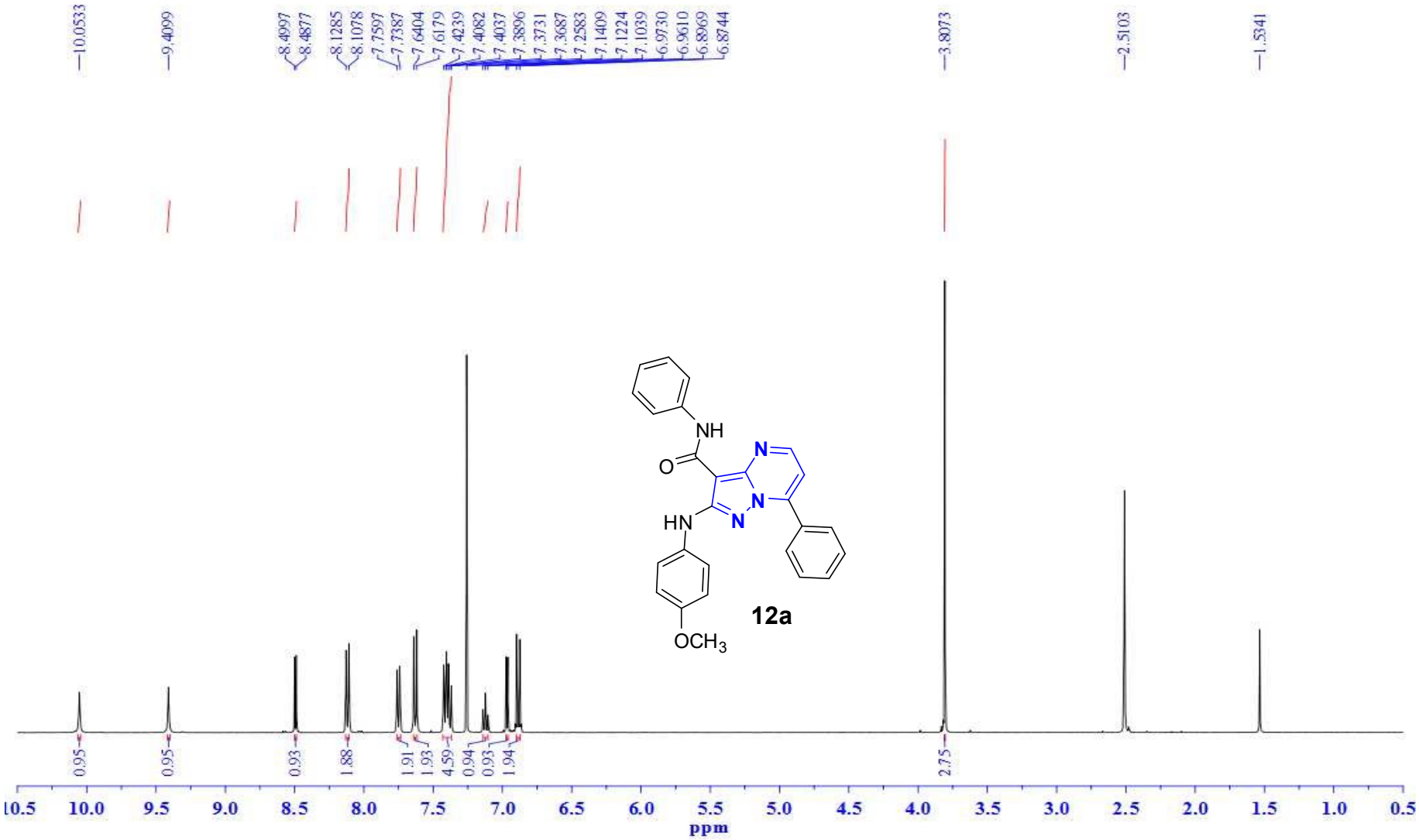
### 2-(4-Methoxyphenylamino)-*N*,7-diphenylpyrazolo[1,5-*a*]pyrimidine-3-carboxamide (12a)

Yellow crystals, m.p. 218-220 °C, yield (72%). IR (KBr) $\nu_{max}$/cm$^{-1}$ 3346 (NH), 1658 (C=O). $^1$H-NMR ($CDCl_3$, 400 MHz, $\delta$ ppm): 3.80 (s, 3H, $OCH_3$), 6.88 (d, 2H, $J$ = 9.0 Hz,ArH), 6.96 (d, 1H, $J$ = 4.8 Hz, pyrimidine), 7.12 (t, 1H, ArH), 7.36–7.42 (m, 5H, ArH), 7.62 (d, 2H, $J$ = 9.0 Hz,ArH), 7.74 (d, 2H, $J$ = 8.4 Hz,ArH), 8.11 (d, 2H, $J$ = 8.3 Hz,ArH), 8.49 (d, 1H, $J$ = 4.8 Hz, pyrimidine), 9.40 (s, 1H, NH), 10.05 (s, 1H, NH). $^{13}$C-NMR ($CDCl_3$, 100 MHz, $\delta$ ppm): 55.7 (C, $OCH_3$), 87.8 (C, $C_3$-pyrazolopyrimidine), 107.0 (C, $C_6$-pyrazolopyrimidine), 114.4, 119.2, 120.2, 123.7, 127.7, 129.1, 129.5, 129.6 (14C, Ar), 134.1 (C, $C_{3a}$-pyrazolopyrimidine), 138.8, 142.4, 146.7 (3C, Ar), 147.9 (C, $C_7$-pyrazolopyrimidine), 149.6 (C, Ar), 154.5 (C, $C_2$-pyrazolopyrimidine), 157.8 (C, $C_5$-pyrazolopyrimidine), 163.3 (C=O). MS (*m*/*z*, %): 435 ($M^+$, 73.86). Anal. Calcd. (%) for $C_{26}H_{21}N_5O_2$ (435.48): C, 71.71; H, 4.86; N, 16.08. Found: C, 71.80; H, 4.81; N, 16.00%.

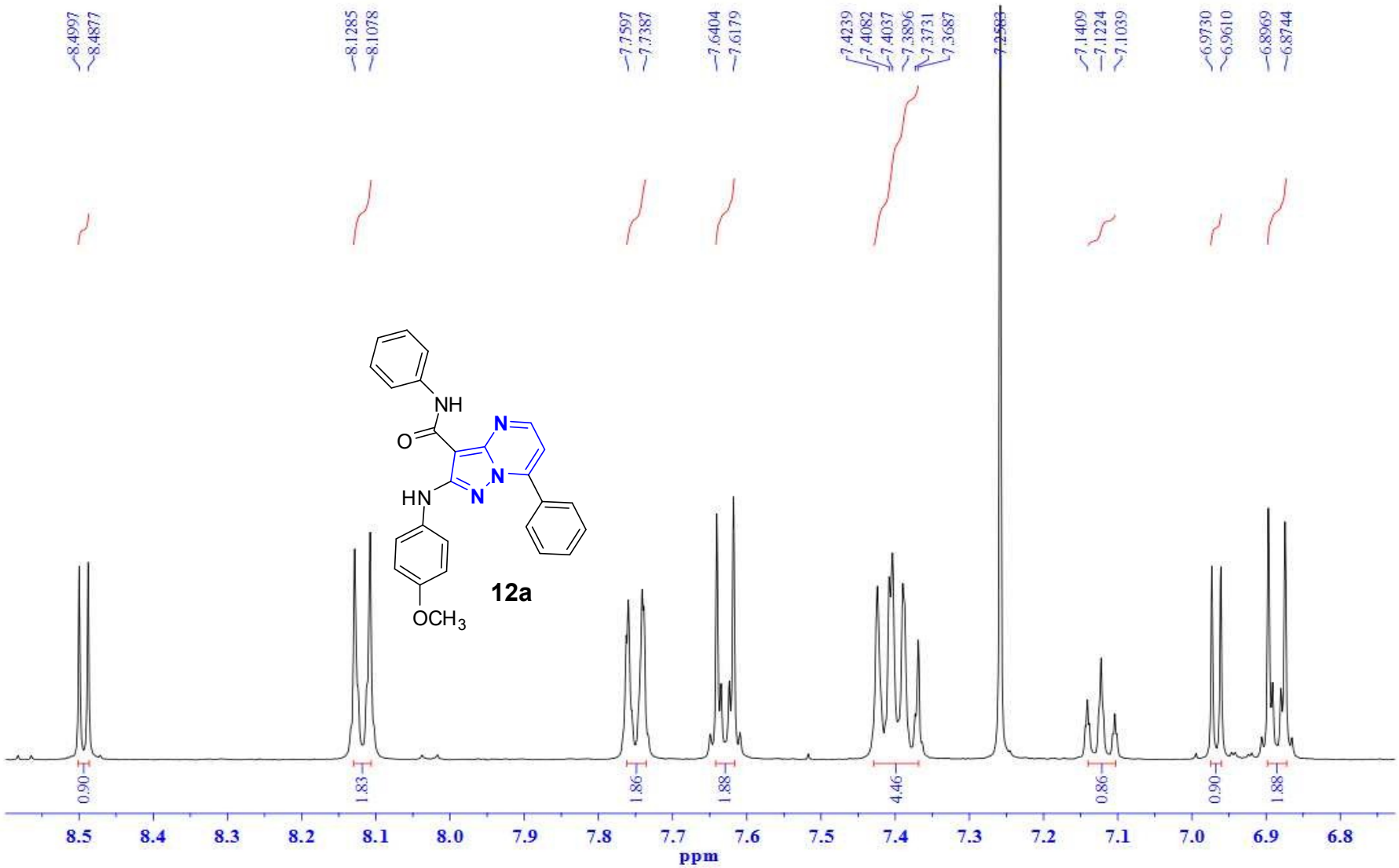
### *N*-(4-Chlorophenyl)-2-(4-methoxyphenylamino)-7-phenylpyrazolo[1,5-*a*]pyrimidine-3-carboxamide (12b)

Yellow crystals, m.p. 252–254 °C, yield (73%). IR (KBr) $\nu_{max}$/cm$^{-1}$ 3336 (NH), 1650 (C=O). $^1$H-NMR (DMSO-$d_6$, 400 MHz, $\delta$ ppm): 3.72 (s, 3H, $OCH_3$), 6.90 (d, 2H, $J$ = 9.0 Hz, ArH), 7.40 (d, 1H, $J$ = 4.8 Hz, pyrimidine), 7.44 (d, 2H, $J$ = 8.8 Hz, ArH), 7.61 (d, 2H, $J$ = 9.0 Hz, ArH), 7.68–7.70 (m, 3H, ArH), 7.78 (d, 2H, $J$ = 8.9 Hz, ArH), 8.23 (d, 2H, $J$ = 7.2 Hz, ArH), 8.75 (d, 1H, $J$ = 4.8 Hz, pyrimidine), 9.20 (s, 1H, NH), 10.11 (s, 1H, NH). $^{13}$C-NMR (DMSO-$d_6$, 100 MHz, $\delta$ ppm): 55.7 (C, $OCH_3$), 87.9 (C, $C_3$-pyrazolopyrimidine), 107.0 (C, $C_6$-pyrazolopyrimidine), 114.5, 119.1, 120.2, 124.0, 128.4, 129.1, 130.9, 131.8 (13C, Ar), 134.0 (C, $C_{3a}$-pyrazolopyrimidine), 134.6, 135.9, 138.0, 138.7 (4C, Ar), 145.6 (C, $C_7$-pyrazolopyrimidine), 149.7 (C, Ar), 154.8 (C, $C_2$-pyrazolopyrimidine), 158.0 (C, $C_5$-

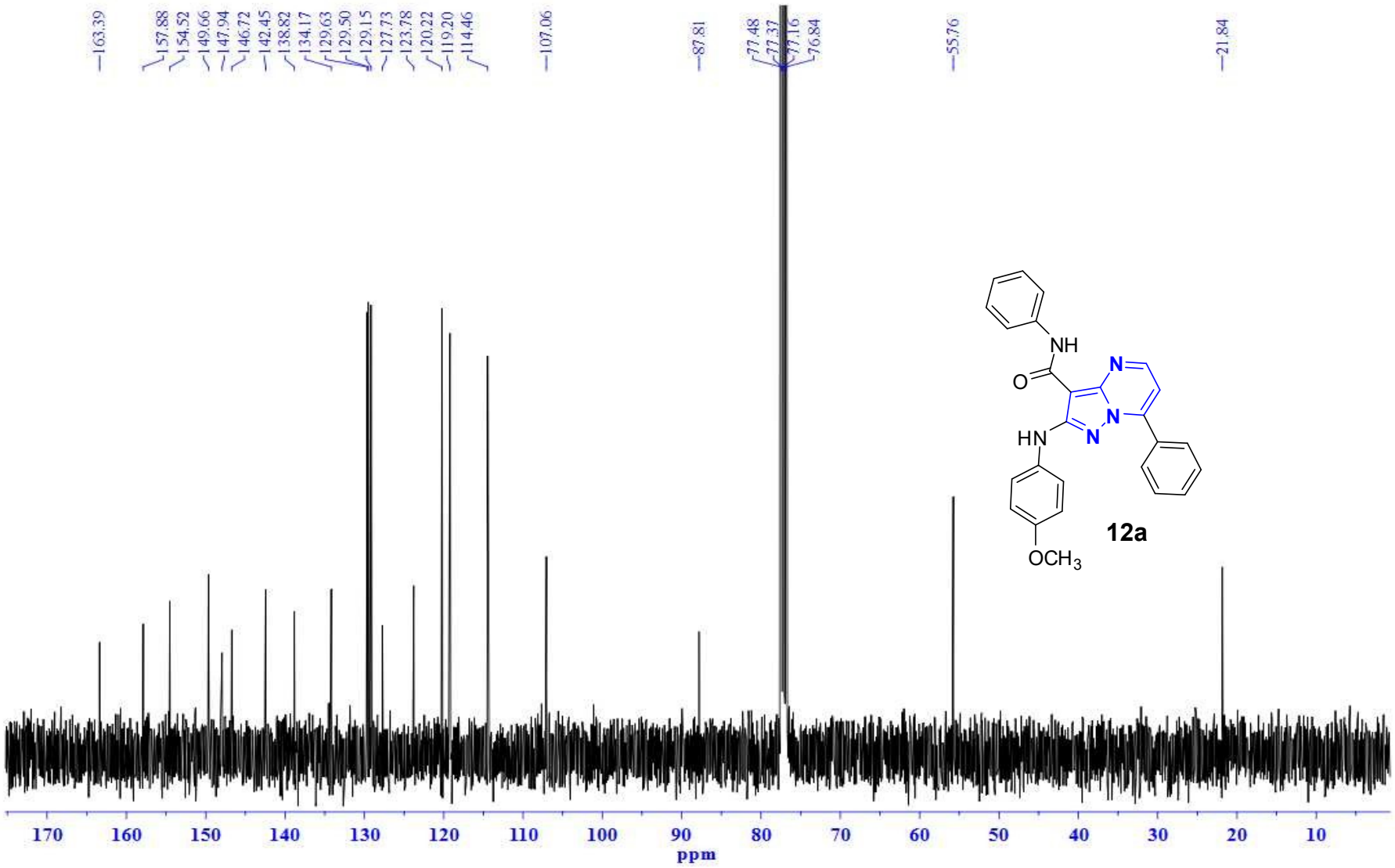
pyrazolopyrimidine), 163.8 (C=O). MS (*m*/*z*, %): 469 ($M^+$, 29.83). Anal. Calcd. (%) for $C_{26}H_{20}ClN_5O_2$ (469.92): C, 66.45; H, 4.29; N, 14.90. Found: C, 66.40; H, 4.35; N, 14.85%.



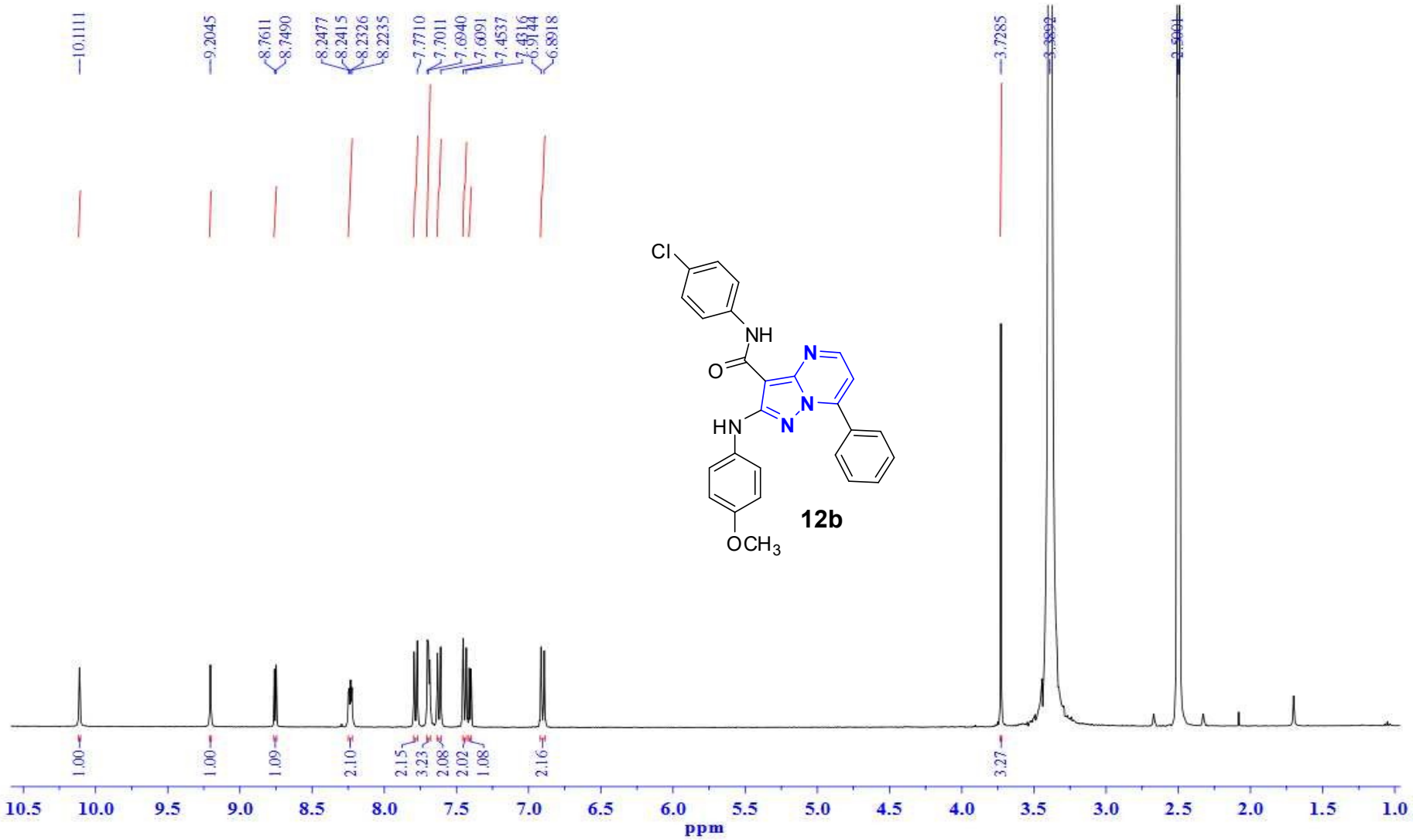

The $^{1}$H NMR (400 MHz) spectrum of compound **12a**



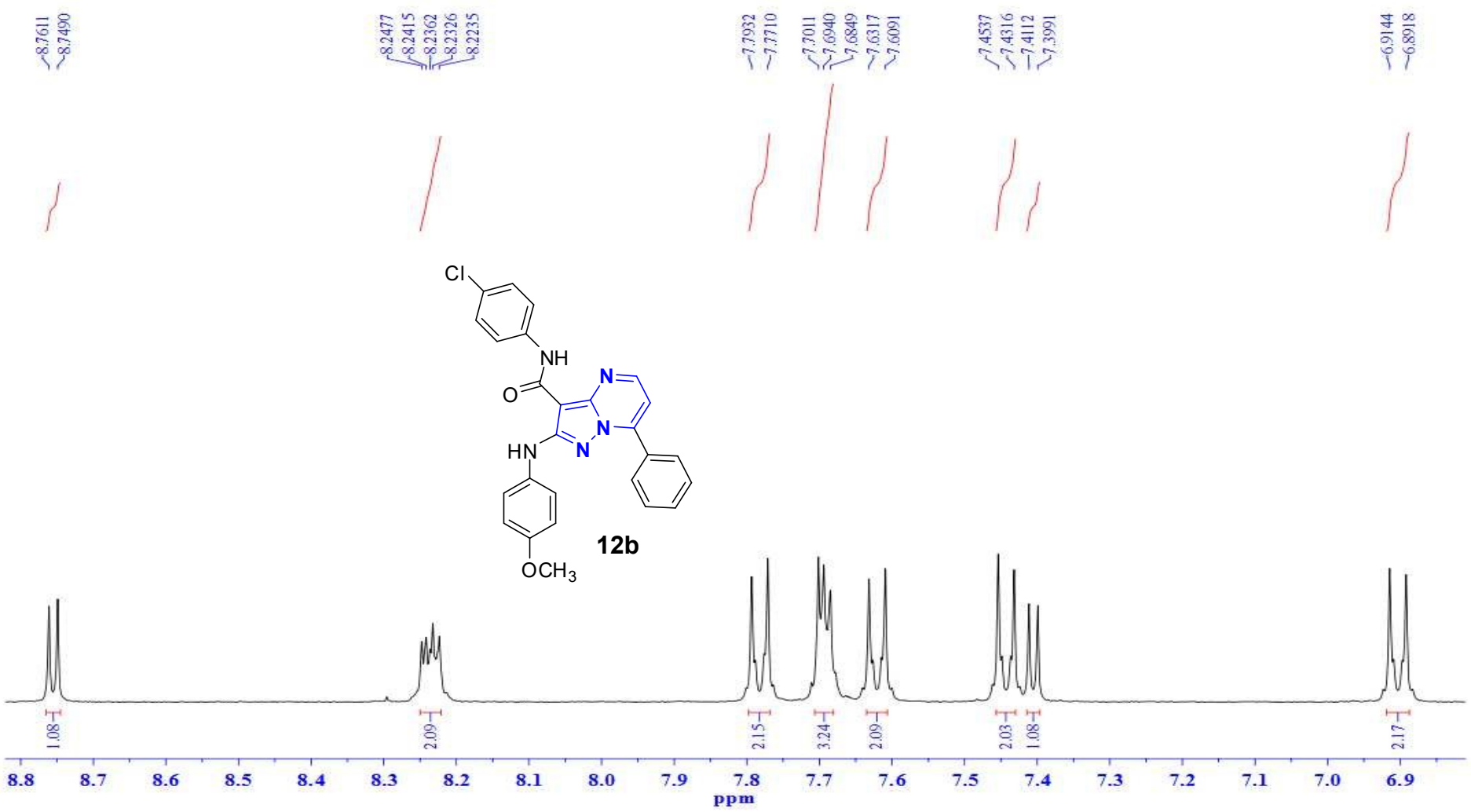

The $^{1}$H NMR (400 MHz) spectrum (aromatic region) of compound **12a**




The $^{13}C$ NMR (100 MHz) spectrum of compound **12a**




The $^{1}$H NMR (400 MHz) spectrum of compound **12b**




The $^{1}$H NMR (400 MHz) spectrum (aromatic region) of compound **12b**

### 4.2. *In vitro* biological activities

#### 4.2.1 Antioxidant activity

The total antioxidant capacity (TAC) was measured in mg gallic acid/g by analyzing the green phosphate/$Mo^{5+}$ complex at a wavelength ($\lambda$) of 695 nm, following the procedure described by Prieto *et al*. Samples were mixed with a reagent solution containing 0.3 N sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. Methanol (80%) was used in place of the sample for the blank. The tubes were sealed and incubated in a boiling water bath for 90 minutes. After cooling to room temperature, the absorbance was measured at 695 nm against the blank. The antioxidant capacity was expressed as mg gallic acid equivalent per gram dry weight. All samples were analyzed in triplicate.

The iron reducing power was determined in μg/mL using the method proposed by Oyaizu, with ascorbic acid as the standard. In summary, 1ml of the tested sample (1mg/mL) was combined with 1mL of 200mM sodium phosphate buffer (pH 6.6) and 1mL of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 20 minutes, followed by the addition of 1mL of trichloroacetic acid (10%). After centrifugation at 2000 rpm for 10 minutes, the upper layer solution (2.5 mL) was mixed with 2.5 mL of double deionized water and 1mL of fresh ferric chloride (0.1%). The absorbance was measured at 700 nm against a blank prepared without the extract. Ascorbic acid at various concentrations was used as a standard. A high absorbance at 700nm indicates a higher reducing power in the reaction mixture.

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activities were assessed using the method described by Rahman *et al*. An antioxidant substance capable of donating a hydrogen atom to a solution containing DPPH- can reduce the stable free radical, causing the solution to change color from violet to pale yellow. The remaining DPPH- radical was quantified by measuring the intensity of a light-purple colored DPPH methanol solution in the visible range at 518 nm using a

spectroscopic method. Various concentrations ranging from 0.78 to 100 μg/mL were prepared from different samples. Two milliliters of a 100 μM DPPH solution in ethanol were mixed with 2 mL of the sample (100 μg/mL). The effective test concentrations of DPPH and the sample were 50 μM and 50 μg/mL, respectively. The reaction mixture for each concentration was thoroughly vortexed and then incubated in the dark at room temperature for 30 minutes. The absorbance was then measured spectrophotometrically at 518 nm against a blank (ethanol). For the control, 2 mL of ethanol was added instead of the sample and run simultaneously with the test. Ascorbic acid was used as a positive control. Percent inhibition of the DPPH free radical was calculated. Then, the inhibition % was plotted against concentration, and from the graph the median inhibitory concentration ($IC_{50}$) was calculated.

For the 2, 2'-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, the procedure followed the method suggested by Arnao *et al*. with some modifications. The stock solutions consisted of ABTS solution (7 mM) and potassium persulfate solution (2.4 mM). The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react at room temperature in a dark place for 14 hours. The solution was then diluted by mixing 1 mL of ABTS solution with 60 mL of methanol to obtain an absorbance of 0.706 ± 0.01 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. The tested compounds (1 mL) were allowed to react with 1 mL of the ABTS solution and the absorbance was taken at 734 nm after 7 minutes using a spectrophotometer. The ABTS scavenging capacity of the compound was compared with that of ascorbic acid.

**4.2.2. Anti-diabetic activity**

**4.2.2.1. Enzymes assay**

This assay involved calculating the inhibition percentage (%) of $\alpha$-amylase enzyme using method based on the technique demonstrated by Wickramaratne *et al*.

with Acarbose as the standard drug. During the assay, 0.5 ml of the test solution was combined with 0.5 ml of $\alpha$-amylase solution (0.5 mg/ml) and buffer ($Na_2HPO_4/NaH_2PO_4$ (0.02 M), NaCl (0.006 M) at pH 6.9) to create concentrations ranging from 25 to 800 μg/mL. The mixture was then left at room temperature for 10 minutes before adding 200 μL of starch solution (1% in water (w/v) buffer ($Na_2HPO_4/NaH_2PO_4$ (0.02 M), NaCl (0.006 M) at pH 6.9)). The reaction was stopped by adding 200 μL of DNSA (coloring) reagent (12 g of sodium potassium tartrate tetrahydrate in 8.0 mL of 2 M NaOH and 20 mL of 96 mM of DNSA solution). The test tubes were then placed in a boiling water bath (100 °C) for 10 minutes and the mixture was cooled to room temperature and diluted with 5 mL of distilled water. The absorbance was measured at 540 nm using a UV-Visible spectrophotometer. The $IC_{50}$ of each tested sample was calculated by plotting a curve using a series of sample concentrations against the percent of $\alpha$-amylase inhibition.

The inhibition percentage (%) of the $\alpha$-glucosidase enzyme was determined using the method proposed by Pistia-Brueggeman and Hollingsworth with Acarbose as the standard drug. Five μL of the $\alpha$-glucosidase solution (10 units $mL^{-1}$, 0.1$moll^{-1}$ potassium phosphate buffer, pH 6.8) was pre-mixed with 10 μL of the sample solution at different concentrations (in 10% DMSO) in 620 μL of 0.1 $moll^{-1}$ potassium phosphate buffer (pH 6.8). After incubation at 37.5 °C for 20 minutes, 10 μL of p-nitro phenyl glucopyranoside (pNPG, 10 $mmoll^{-1}$) as a substrate was added to the mixture to start the reaction. The reaction mixture was then incubated at 37.5°C for 30 minutes, followed by the addition of 650 μL of 1 $moll^{-1}$ $Na_2CO_3$ solution to terminate the reaction. The amount of released product (p-nitro phenol) was measured at 410 nm using a UV spectrometer (UV-2550, Shimadzu, Japan) to estimate the enzymatic activity. The inhibition assay was performed in triplicate for all tests. The $IC_{50}$ of each tested sample was calculated by plotting a curve using a series of sample concentrations against the percent of $\alpha$-glucosidase inhibition.

The β-glucocidase enzyme inhibition percentage (%) was measured using the *p*NPG method suggested by Han *et al*. with Acarbose as the standard drug. To determine enzyme activity, 100 µl of enzyme was mixed with 900 µl of substrate (1 mM pNPG in 10 ml of phosphate citrate buffer pH 5) and incubated at 35°C for 30 min. The reaction was stopped by adding 100 µL of 1M $Na_2CO_3$. As a blank, 100 µL of aquadest and 900 µl of substrate treated the same as the sample condition were used. The liberation of *p*-nitrophenol was read with a spectrophotometer at λ 405 nm and then compared to a *p*-nitrophenol standard curve. One unit of enzyme activity was equivalent to the amount of enzyme required to produce 1 µmol *p*-nitrophenol/min. The inhibition assay was performed in triplicate for all tests. The $IC_{50}$ of each tested sample was calculated by plotting a curve using a series of sample concentrations against the percent of β-glucocidase inhibition.

#### 4.2.2.2. Native Electrophoretic Patterns

##### 4.2.2.2.1. Electrophoretic *α*-amylase isoenzyme pattern

This assay used polyacrylamide gel electrophoresis (PAGE) following the method suggested by Rammesmayer and Praznik. After the electrophoresis run, the native gel was dissected and washed with Tris-HCl buffer (pH 7.1), then incubated with a working buffer containing 50 mL of Tris-HCl (pH 7.5) (6 g /1L), 110 mg $CaCl_2$, and soluble starch (0.5 g). The electrophoretically separated *α*-amylase types were visualized as yellow bands by incubation in a staining solution consisting of 300 mg Pot. Iodide and 130 mg iodine dissolved in 100 mL of distilled water.

##### 4.2.2.2.2. Electrophoretic *α*-glucosidase pattern

The vertical slab polyacrylamide gel electrophoresis (PAGE) was conducted following the method suggested by Laemmli using Mini-gel electrophoresis (BioRad, USA) to determine the activity of the *α*-glucosidase enzyme. The gel was run in a buffer containing Tris (24 mM) and glycine (194 mM) at room temperature. After completing the electrophoretic run, protein bands were visualized by staining with

Coomassie Brilliant Blue G-250 and destained overnight with 7% (v/v) glacial acetic acid after documentation. A protein ladder was designed using chmotrypsinogene (25 KDa), glyceraldehyde 3-phosphate dehydrogenase (36 KDa), ovalbumin (45 KDa), and bovine serum albumin (66 KDa) as reported by **Warangkar and Khobragade** to determine the molecular weights. The marker (5µL) was loaded in the first well with the samples.

**4.2.3 Anti-Alzheimer's and anti-arthritic activities**

In the anti-Alzheimer's activity study, we assessed the inhibition percentage of the acetylcholinesterase (AChE) enzyme using Ellman's method with donepezil as the standard drug. The tested sample was dissolved in a 0.1 M phosphate buffer at pH 8. For each run, 5 µL of Acetylthiocholine (ATCh) at a concentration of 0.5 mM, 5 µL of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) at a concentration of 0.03 mM, and 5 µL of the tested sample solution at various concentrations were added to a flat bottom 96-well plate. The mixture was then incubated for 10 minutes at 30°C. After incubation, 5 µL of AChE at a concentration of 0.3 U/mL was added to start the reaction, and the absorbance was measured at 412nm. A control run was also performed, which included all the components except for the test extract. All experiments were conducted in triplicate. The median inhibitory concentration ($IC_{50}$) of each tested sample was calculated by plotting a curve using a series of sample concentrations against the percent of AChE inhibition.

In the anti-arthritic activity study, this assay involved determining the percentage of protein denaturation and proteinase inhibition using diclofenac sodium as the standard non-steroidal anti-inflammatory drug, as prepared according to **Meera *et al*.** The protein denaturation percentage was measured by mixing 0.5mL of the test control solution, prepared by combining 0.45 mL of bovine serum albumin (BSA) (5% w/v aqueous solution) with 0.05 mL of distilled water. Then, 0.05 mL of the test solution was added to 0.45 mL of distilled water to form the product control (0.5 mL). The different samples (test solution) and diclofenac sodium

(standard) were used. The pH value in all prepared solutions was adjusted to 6.3 using HCl (1N). All the samples were incubated at 37 °C for 20 min, and the temperature was then increased to 57 °C, maintaining the samples at that degree for 3 min. After cooling, 2.5 mL of phosphate buffer was added to the prepared solutions. The absorbance was determined at 416 nm using a UV-Visible spectrophotometer. The percentage of protein denaturation inhibition can be calculated. Proteinase inhibitory activity was assessed by combining the test sample (1 mL) with a reaction mixture containing 0.06 mg trypsin dissolved in 1 mL of 20 mM Tris HCl buffer (pH 7.4). The mixture was then incubated for 5 minutes at 37°C, followed by the addition of 1 mL of casein (0.8% w/v). After an additional 20 minutes of incubation, 2 mL of perchloric acid (70%) was added to stop the reaction. The cloudy suspension was then centrifuged, and the absorbance of the supernatant was measured at 210 nm against buffer as the blank. The percentage of proteinase inhibitory activity was then calculated.

**4.2.4. Cytotoxicity**

The effectiveness of the compounds tested against human lung (A549) and colon cancer (Caco-2) cell lines, as well as the normal lung (WI-38) cell line, was determined by measuring the optical density (OD) at a wavelength of 590 using the 3-[4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay, which is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells. Cells were dispensed in a 96 well sterile microplate ($5 \times 10^4$ cells/well), and incubated at 37 °C with series of different concentrations, in DMSO, of each tested compound or Doxorubicin® (positive control) for 48 h in a serum free medium prior to the MTT assay. After incubation, media were carefully removed, 40 µL of MTT (2.5 mg/mL) were added to each well and then incubated for an additional 4 h. The purple formazan dye crystals were solubilized by the addition of 200 µL of DMSO. The absorbance was measured at 590 nm using a SpectraMax® Paradigm® Multi-Mode microplate reader. The relative cell viability was expressed

as the mean percentage of viable cells compared to the untreated control cells. The median inhibitory concentration ($IC_{50}$) calculation software was used to calculate the $IC_{50}$ and percentage of cell growth inhibition (%).