Ultrasound assisted synthesis of pyrazolo[1,5-a]pyrimidine-antipyrine hybrids and their anti-inflammatory and anti-cancer activities

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ABSTRACT

A series of antipyrylpyrazolo[1,5-a]pyrimidines have been synthesized by reactions of aminopyrazole (4) with various formylated active proton compounds in the presence of KHSO4 (aqueous media), under ultrasound irradiation. The structures of the compounds have been established with the help of spectral and analytical data. N-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H- pyrazol-4-yl)-7-phenylpyrazolo[1,5-a]pyrimidine-3-carboxamide (6a) was further subjected to X-ray crystallographic studies to avoid any ambiguity of the derived structures. Crystal data for compound 6a, C35H34N12O5 (M = 907.00 g/mol): triclinic, space group P-1 (no. 2), a = 9.955(3) Å, b = 14.087(4) Å, c = 17.4572(4) Å, α = 79.676(2)°, β = 85.283(2)°, γ = 72.647(2)°, V = 2297.97(11) Å³, Z = 2, T = 296.15 K, μ(MoKα) = 0.088 mm⁻¹, Dcalc = 1.311 g/cm³, 29732 reflections measured (4.174° ≤ 2θ ≤ 57.068°), 10681 unique (I > 2σ(I)) and Rint = 0.0400, Rsigma = 0.0533 which were used in all calculations. The final R was 0.0566 (1 > 2σ(I)) and wR2 was 0.1663 (all data). The novel compounds were also screened for their biological activities.

1. Introduction

Pyrazolo[1,5-a]pyrimidine, an important pharmacophore and an adjective structure in medicinal chemistry, is well known for its innumerable biological and pharmacological properties [1,2]: like antitumor [3-5], antileukemic [3], antineoplastic [6], CNS stimulant [7], antihypertensive [8], adenosine receptors [9], tuberculostatic [10], antibacterial and antifungal [11], anti-inflammatory [12], anti-atherosclerotic [12,13] antischistosomal [14], and antileishmanial activities [15]. A well-known drug Zaleplon [16] which has found a positive role as a sedative for the treatment of insomnia without the risk of dependence consists of the core pyrazolo[1,5-a]pyrimidine ring. Also, 4-aminopyrazine, containing the functional parent antipyrene has been reported to show myriads of biological activities such as anti-inflammatory [17], analgesic [18], antipyretic [19], antimicrobial [20], and anti-cancer activities [21]. 4-Aminopyrazine has been used for the protection against oxidative stress as well as prophylactic of some diseases including cancer, and these are found to be important directions in medical applications [22]. In view of this information and in continuation with our research work [23-25] on synthesis of pharmacologically important pyrazolo[1,5-a]pyrimidines, it was found worthy to synthesize novel pyrazolo[1,5-a]pyrimidines incorporating the antipyrene moiety to study their biological properties. Ultrasound (US) irradiation has helped researchers tremendously in accomplishing the green goals of chemistry by shortening the reaction time with easy work-up, resulting in high yields of the products and use of readily available solvents and catalysts [26-28]. Ultrasound tool has been advantageously employed to achieve the desired synthesis.

2. Experimental

2.1. Chemistry

2.1.1. Material and methods
Melting points were recorded by open capillary method and are uncorrected. The IR spectra were recorded on a Perkin-Elmer 983 spectrometer. High-resolution 1H NMR (400 MHz) and 13C NMR (100 MHz) were measured on a DRX-400 Varian spectrometer and Bruker spectrometer, respectively, and CDCl3 and DMSO-d6 were used as the solvent. The chemical shifts (δ, ppm) and the coupling constants (Hz) are reported in the standard fashion with reference to tetramethylsilane (TMS) as internal reference. In the NMR spectral data, the abbreviations s = singlet, t = triplet, m = multiplet, d = doublet, dd = double-double are used. The X-ray diffraction data were collected at 296 K with MoKα radiation (λ = 0.71073 Å) using a Bruker Nonius SMART APEX II CCD diffractometer equipped with a graphite monochromator. The structure was solved by direct methods (SHELXS97) and refined by full-matrix least squares based on $F^2$. All calculations were carried out using WinGX system version 1.80.05 [29]. All the non-H atoms were refined in the anisotropic approximation: H-atoms were placed at calculated positions. The electron spray mass spectra were recorded on a THERMO Finnigan LC Advantage max ion trap mass spectrometer. Elemental analysis was performed on a Vario-EL III instrument. Ultrason irradiation was achieved by column chromatography using silica gel and 100 % EtOAc.

N-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-7-phenylpyrazolo[1,5-a]pyrimidine-3-carboxamide (6a): Color: Yellow solid. Yield: 95 %. M.p.: 191-192 °C. FT-IR (KBr, ν, cm$^{-1}$): 3448 (NH), 1645 (CO), 1637 (CO). 1H NMR (400 MHz, CDCl3, δ, ppm): 2.43 (3H, CH3), 3.17 (3H, NCH3), 7.10 (d, 1H, C6-pyrimidine, J = 4.5 Hz), 7.30-7.34 (m, 1H, ArH), 7.45-7.50 (m, 4H, ArH), 8.03-8.06 (m, 2H, 2H, ArH), 8.72 (d, 1H, C5-pyrimidine, J = 4.5 Hz), 8.75 (s, 1H, C2-pyrazole). 13C NMR (100 MHz, CDCl3, δ, ppm): 23.1, 36.4, 105.4, 108.4, 109.0, 124.4, 127.1, 128.9, 129.3, 129.5, 130.0, 131.8, 134.5, 146.7, 147.5, 148.4, 149.0, 151.1, 160.7, 161.3. MS (EI, m/z): 459 (MH)+. Anal. calcd. for C24H20N6O2: C, 67.91; H, 4.75; N, 19.80. Found: C, 67.98; H, 4.72; N, 19.75 %.

N-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-7-(p-toly1)pyrazolo[1,5-a]pyrimidine-3-carboxamide (6b): Color: Yellow solid. Yield: 94 %. M.p.: 252-253 °C. FT-IR (KBr, ν, cm$^{-1}$): 3448 (NH), 1675 (CO), 1624 (CO). 1H NMR (400 MHz, CDCl3, δ, ppm): 2.42 (3H, CH3), 2.47 (3H, CH3), 3.17 (3H, NCH3), 7.07 (d, 1H, C6-pyrimidine, J = 4.5 Hz), 7.28-7.32 (m, 1H, ArH), 7.39 (d, 2H, ArH, J = 7.8 Hz), 7.44-7.49 (m, 4H, ArH), 7.96 (d, 2H, ArH, J = 7.8 Hz), 8.68 (d, 1H, C5-pyrimidine, J = 4.5 Hz), 8.73 (s, 1H, C2-pyrazole), 9.56 (s, 1H, NH). 13C NMR (100 MHz, CDCl3, δ, ppm): 12.8, 21.7, 36.4, 105.4, 108.4, 109.1, 124.2, 126.9, 127.1, 129.2, 129.5, 134.7, 142.5, 146.6, 147.5, 148.4, 149.1, 151.0, 160.9, 161.3. MS (EI, m/z (%)): 439 (MH)+. Anal. calcd. for C24H22N6O2: C, 68.59; H, 5.04; N, 19.12 %. Found: C, 68.59; H, 5.04; N, 19.12 %.

7-(4-Chlorophenyl)-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)pyrazolo[1,5-a]pyrimidine-3-carboxamide (6c): Color: Yellow solid. Yield: 95 %. M.p.: 261-262 °C. FT-IR (KBr, ν, cm$^{-1}$): 3448 (NH), 1675 (CO), 1624 (CO). 1H NMR (400 MHz, CDCl3, δ, ppm): 2.42 (3H, CH3), 3.16 (3H, NCH3), 7.10 (d, 1H, C6-pyrimidine, J = 4.5 Hz), 7.28-7.32 (m, 1H, ArH), 7.39 (d, 2H, ArH, J = 7.8 Hz), 7.44-7.49 (m, 4H, ArH), 7.96 (d, 2H, ArH, J = 7.8 Hz), 8.63 (d, 1H, C5-pyrimidine, J = 4.5 Hz), 8.73 (s, 1H, C2-pyrazole), 9.56 (s, 1H, NH). 13C NMR (100 MHz, CDCl3, δ, ppm): 12.8, 21.7, 36.4, 105.4, 108.4, 109.1, 124.2, 126.9, 127.1, 129.2, 129.5, 134.7, 142.5, 146.6, 147.5, 148.4, 149.1, 151.0, 160.9, 161.3. MS (EI, m/z (%)): 459 (MH)+. Anal. calcd. for C2H2NO2: C, 68.48; H, 5.06; N, 19.17. Found: C, 68.59; H, 5.04; N, 19.12 %.

2.1.2. Synthesis

2.1.2.1. Synthesis of antipyrryl-7-arylpyrazol[1,5-a]pyrimidines (6a-c)

Aminopyrazole (4) (1 mmol), enamiones (5 (1 mmol), and KHSO$_4$ (2 mmol) were suspended in 5 mL of ethanol:water (1:1, v/v) system and the resulting mixture was irradiated under the influence of ultrasound waves for 3-6 minutes resulting in the formation of a precipitated product (Scheme 2). After the completion of reaction monitored by thin layer chromatography (TLC), the precipitate was collected by filtration, washed repeatedly with water to ensure complete removal of acid and dried to give practically pure pyrazolo

![Scheme 1](image-url)
A mixture of aminopyrazole (4) (1 mmol) and enaminonitriles 7 (1 mmol) in the presence of KHSO4 (2 mmol) in 5 mL of ethanol-water (1:1, v/v) was US irradiated for 3-10 minutes to give a precipitate (Scheme 2). After the completion of reaction (monitored by TLC), the precipitate was collected by filtration, washed repeatedly with ethanol-water (1:1, v/v) ensuring complete removal of acid, and then dried to give practically pure pyrazolopyrimidines (B) in 87-92 % yields. Further, purification was carried out by column chromatography using silica gel and 100 % EtOAc.

2.1.2.2. Synthesis of 7-amino-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-6-arylpyrazolo[1,5-a]pyrimidine-3-carboxamide (8a,b)

A mixture of aminopyrazole (4) (1 mmol) and enaminopyrazole (1 mmol) in the presence of KHSO4 (2 mmol) in 5 mL of ethanol-water (1:1, v/v) was US irradiated for 3-10 minutes to give a precipitate (Scheme 2). After the completion of reaction (monitored by TLC), the precipitate was collected by filtration, washed repeatedly with ethanol-water (1:1, v/v) ensuring complete removal of acid, and then dried to give practically pure pyrazolopyrimidines (B) in 87-92 % yields. Further, purification was carried out by column chromatography using silica gel and 100 % EtOAc.

2.1.2.3. Synthesis of antipyrinyl-7-hetarylpyrazolo[1,5-a]pyrimidines (9,10)

Aminopyrazole (4) (1 mmol), enamino nitriles 7 (1 mmol) in the presence of KHSO4 (2 mmol) were suspended in 5 mL of ethanol-water (1:1, v/v) and the mixture was irradiated under the influence of US waves for 5-6 minutes giving a precipitated product (Scheme 3). After the completion of reaction monitored by TLC, the precipitate was collected by filtration, washed repeatedly with water ensuring complete removal of acid and dried to produce practically pure pyrazolopyrimidines (9, 10) in 90-94 % yields. Further, purification was achieved by column chromatography using silica gel and 100 % EtOAc.

2.1.2.4. Synthesis of 6-acetyl/carboalkoxy-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-7-(4-nitrophenyl)pyrazolo[1,5-a]pyrimidine-3-carboxamide (6a-e)

An = CO, Ar = C6H5, 4-CH3C6H4, 4-ClC6H4, 4-MeOC6H4, 4-NO2C6H4; Ar1 = C6H5, 4-CH3C6H4, 4-ClC6H4, 4-MeOC6H4, 4-NO2C6H4.
In order to synthesize the target pyrazolo[1,5-α]pyrimidine (13), formylated active proton compounds of type 12 were required. This was accomplished by the microwave irradiation of acyclic active proton compounds (11) (1 mmol) with DMF-DMA in a microwave digester for 5 minutes. The reaction mixture (monitored by TLC) was evaporated to dryness under reduced pressure. To the resulting residue, aminopyrazole (4) (1 mmol) was added, and the content was dissolved in 5 mL of ethanol:water mixture (1:1, v/v). KHSO₄ (2 mmol) was then added, and the solution was subjected to ultrasound (US) irradiation for 4-6 min giving a precipitate (Scheme 4). After the completion of reaction (monitored by TLC), the precipitate was collected by filtration, washed repeatedly with ethanol: water (1:1, v/v), and dried over anhydrous CaCl₂ to give practically pure products (13a, b) in 92-96 % yields. Further, purification was achieved by column chromatography (silica gel, 100 % EtOAc).

6-Acetyl-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-7-methylpyrazolo[1,5-d]pyrimidine-3-carboxamide (13a): Color: Light yellow solid. Yield: 92 %. M.p.: 211-212 °C. FT-IR (KBr, cm⁻¹): 3328 (NH), 1692 (CO), 1680 (CO).

1H NMR (400 MHz, CDCl₃, δ, ppm): 2.41 (s, 3H, CH₃), 2.72 (s, 3H, CH₃), 3.12 (s, 3H, NCH₃), 3.21 (s, 3H, CH₃), 7.27-7.29 (m, 1H, ArH), 7.45-7.46 (m, 4H, ArH), 8.79 (s, 1H, C5-CH ₃), 9.10 (s, 1H, C2-pyrazole), 9.25 (s, 1H, NH). 13C NMR (100 MHz, CDCl₃, δ, ppm): 2.41, 22.2, 30.0, 36.6, 106.6, 109.2, 119.0, 123.9, 126.8, 130.5, 145.0, 152.2. Anal. calcd. for C₂₁H₂₀N₆O₄: C, 59.99; H, 4.79; N, 19.99. Found: C, 59.81; H, 4.81; N, 19.94 %.

2.2. Biology

2.2.1. Material and methods

2.2.1.1. Anti-inflammatory test

Griess reagent system (naphthylethylenediamine dihydrochloride, sulphanilamide and nitrite standard) from Promega (USA), Freund’s Complete Adjuvant (FCA) from GeNei (India), Wright stain, sodium chloride, disodium hydrogen phosphate dihydrate, potassium dihydrogen orthophosphate, dimethyl sulfoxide (DMSO) were procured from HiMEDA (India), methanol was purchased from MERCK (India). Swiss Albino mice of both sexes were purchased from Pasteur institute, Shillong. The animals were kept in a temperature controlled room with a 12 hours light and dark cycle. Cell lines CHO K1 (Chinese Hamster Ovary) were procured from National Centre for Cell Science (NCCS), Pune.

2.2.1.1. Measurement of paw edema

This experiment was performed according to Lai et al. [34] with certain modifications. Swiss Albino male and female mice aged between 10-12 weeks (3 per group) were injected with about 50 µL of FCA into left hind paw of the mice to induce inflammation. The test compounds (50 mg/kg body weight in DMSO) were then administered 1 hour after FCA injection and the diameter of paw edema was then measured at different time intervals such as 0, 1, 2, 3 and 24 hours using a caliper. After 24 hours, blood was collected by retro-orbital bleeding and stored for nitric oxide assay and differential WBC count. The paws were then excised, weighed and kept in ice-cold normal saline. These were then homogenized in 10 % ice-cold normal saline, centrifuged at 12,000 rpm and the supernatant was collected and stored at -20 °C for nitric oxide assay.

2.2.1.2. Nitric oxide assay

The amount of nitric oxide produced was calculated using Griess reaction. The sulphanilamide and naphthylethylenediamine dihydrochloride (NED) solutions were made to equilibrate to room temperature for 15 to 30 minutes.
To a 96 well microtiter plate, 50 µL of the sample was added in triplicate. To each of these wells, 50 µL of the sulfanilamide solution was then added and incubated for 5 to 10 minutes at room temperature protected from light. After the incubation period, 50 µL of the NED solution was added to each of the wells and incubated again in dark at room temperature. A purple colour developed which was further quantified at 520 nm.

Three columns in the 96 wells plate were used for the nitrite standard reference curve in which a six serial fold dilution of 100 µM nitrite solution (50 µL/well) in triplicate was performed to generate the nitrite standard reference curve.

Differential WBCs count. Differential WBCs counts were performed according to the method described by Houwen [35]. In brief, blood film was prepared on glass slides till it dried. The film was fixed in absolute methanol for 30 seconds. The slides were then stained with Wright’s stain for 2 minutes in a horizontal position after which Sorensen’s buffer (KH2PO4, Na2HPO4, pH = 6.4) was added and mixed. This was allowed to stand for 3 minutes and then rinsed with distilled water and dried. The slides were then observed under a microscope and differential WBC counting was done.

2.2.1.2. Anti-cancer assay

CHO K1 cell lines were procured from NCCS (Pune). Dulbecco’s modified Eagle medium/Nutrient mixture F-12, dimethyl sulfoxide, fetal bovine serum (FBS), sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, trypsin phosphate versene glucose (TPVG) solution, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from HIMEDIA. Antibiotic antymyotic solution was purchased from Sigma. The cytotoxicity of the test compounds was assessed using MTT assay, according to Freshney [36] with certain modifications.

CHO K1 cells at a concentration of 0.5 × 10 to 103 in 100 µL growth medium (Dulbecco’s modified Eagle medium/ Nutrient mixture F-12 with 10 % FBS) per well were seeded in a 96 well-microtiter plate. The cells were then incubated in the CO2 incubator at 37 °C. After 24 hours, the medium was removed and 80 µL of the fresh medium was added. To six wells, 20 µL (10 mg/mL in 10 % DMSO) of test compounds (in triplicate) was added, while three wells were loaded with 20 µL of 10 % DMSO to serve as control. The plate was again incubated for 24 hours and at the end of incubation period, the spent medium was replaced with 100 µL of the fresh medium and 20 µL of MTT (5 mg/mL) reagent prepared in phosphate buffer saline (PBS) was added to all the wells. The plates were then wrapped in aluminium foil and returned to the incubator for 2 hours until an insoluble purple formazan product was formed. After 2 hours, the medium was removed and 200 µL of 10 % DMSO was added to all the wells to dissolve the formazan product. Contents were mixed and the absorbance was recorded at 570 nm.

3. Results and discussion

3.1. Chemistry

To start with, 4-aminoantipyrine was fused with ethyl cyanoacetate at 150 °C for the formation of compound 2 [32]. This was then formylated with DMP-DMA using microwave (850 W, 5 min). The reaction mixture was subsequently treated with hydrazine hydrate in refluxing ethanol [33], yielding practically pure aminopyrazole 4 (Scheme 1). The aminopyrazole 4 thus obtained was used as a synthon for subsequent reactions without further purification.

In order to optimize the conditions for the reaction between pyrazole 4 and enamines, the reaction of compounds 4 and 5a was taken as a model experiment and a series of experiments was conducted under various conditions as presented in Table 1. Sonication at 60 °C in water-ethanol gave the desired product 6a within 4 minutes in 94 % yield. The most remarkable aspect of this condition is that the product precipitated out and could be isolated by simple filtration in practically pure form. Therefore, it was decided to carry out the rest of the reactions of the series of experiments was conducted under various conditions as presented in Table 1. Sonication at 60 °C in water-ethanol gave the desired product 6a within 4 minutes in 94 % yield. The most remarkable aspect of this condition is that the product precipitated out and could be isolated by simple filtration in practically pure form. Therefore, it was decided to carry out the rest of the reactions of the series in water-ethanol at 60 °C under US irradiation.

Thus, 3-Aminopyrazole 4 was irradiated for 3-4 minutes with enamines 5 and KHSO4 in an aqueous medium in an ultrasonic bath at 60 °C (Scheme 2) to give products 6a-e in 93-96 % yields. The structures of these compounds were well established on the basis of their spectral and analytical data. These reaction conditions could well be applied for the reactions of 4 with enaminothiones (7) giving the 7-amino-pyrazolopyrimidines 8a, b in 87-92 % overall yields (Scheme 2).

The synthetic protocol was applied for the synthesis of antipyrinyl-pyrazolo[1,5-a]pyrimidines with hetaryl group at C-7 position of the pyrimidine ring. This was achieved by ultrasound irradiation of 3-aminopyrazole 4 with hetarylen aminonines I or II and KHSO4 in water-ethanol mixture 60 °C (Scheme 3). The products were obtained in 90-96 % yields in 5-6 minutes. Further, the reaction of aminopyrazole 4 with formylated active proton compounds 12 (Scheme 4) derived from 1,3-diketones and prepared in situ was carried out under similar reaction conditions. The reaction proceeded smoothly with the product 13a, b precipitating out in 92-96 % yields within 4-6 minutes.
The infrared spectra of compounds 6a-d showed NH stretching peak in the region 3199-3449 cm⁻¹ and the NH₂ stretches at about 3394-3640 ppm. The carbonyl groups gave characteristic bands at around 1645-1678 and 1624-1662 cm⁻¹. In the ¹H NMR spectra of all the compounds, the aromatic protons resonated as singlet at around δ 7.09 and 7.11 ppm. The C5-H and C2-H protons signals overlapped and appeared as multiplet in the range δ 8.71-8.75 ppm. In compound 6d the C6-H proton signal was obscured by the aromatic protons while the C5-H and C2-H protons signals overlapped and appeared as multiplet in the range δ 8.71-8.75 ppm. In compound 6d the C6-H proton signal was at δ 8.75 ppm and for compound 6b and 6d it resonated as singlet at δ 8.73 ppm. The carbonyl groups gave characteristic bands at around δ 160.4-162.0 ppm indicating the presence of carbonyl groups. Mass spectrometry results were in full support of the assigned structures. The synthesized antipyrinyl-pyrazolo[1,5-a]pyrimidines are mentioned in Table 2.

### 3.2. X-ray crystallography

The structure of N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-7-phenylpyrazolo[1,5-a]pyrimidine-3-carboxamide (6a) was further supported by using X-ray crystallography. The crystal data and structure refinement [37] values of 6a are mentioned in Table 3. The molecular structure of compound 6a is depicted in Figure 1. The details of the structure of compound 6a have been deposited with the Cambridge Crystallographic Data Centre No. CCDC-1401935. The bond length and angles are within the normal ranges [38]. Selected bond lengths and bond angles are given in Tables 4 and 5.

The compound exists as a dimer in an asymmetric unit arranged in an opposite manner, with a molecule of acetone trapped. This could be probably due to the existence of short contacts between N5 and H21A at a distance of 2.744 Å, C42 and H20 at a distance of 2.828 Å and C34 and C11 at a distance of 3.391 Å which are definitely shorter than the sum of the corresponding van der Waal radii of N (1.55 Å) and H (1.2 Å); C (1.7 Å) and H (1.2 Å); and C (1.7 Å) and C (1.7 Å) respectively.
Table 3. Crystal data and structure refinement for N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-7-phenylpyrazolo[1,5-a]pyrimidine-3-carboxamide (6a).

| Parameter                  | Value                                      |
|---------------------------|--------------------------------------------|
| Empirical formula         | C14H14N6O5                                |
| Formula weight            | 907.00                                     |
| Temperature (K)           | 296.15                                     |
| Crystal system            | triclinic                                  |
| Space group               | p₁                                          |
| a (Å)                     | 9.955(1)                                   |
| b (Å)                     | 14.087(4)                                  |
| c (Å)                     | 17.457(4)                                  |
| α (°)                     | 79.476(2)                                  |
| β (°)                     | 85.283(2)                                  |
| γ (°)                     | 72.647(2)                                  |
| Volume (Å³)               | 2297.97(11)                                |
| μ (mm⁻¹)                  | 0.088                                      |
| F(000)                    | 952.0                                      |
| Crystal size (mm³)        | 0.30 ± 0.14 × 0.08                         |
| Radiation                 | MoKα (λ = 0.71073)                         |
| 2θ range for data collection (°) | 4.174 to 57.068                     |
| Index ranges              | -13 ≤ h ≤ 13, -18 ≤ k ≤ 18, -23 ≤ l ≤ 22 |
| Reflections collected    | 29732                                      |
| Independent reflections   | 10681 (Rint = 0.0400, Rsme = 0.0533)        |
| Data/restraints/parameters| 10607/0/627                                |
| Goodness-of-fit on F²     | 1.058                                      |
| Final R indexes (I≥ 2σ(I))| R₁ = 0.0566, wR₂ = 0.1433                 |
| Final R indexes [all data]| R₁ = 0.1032, wR₂ = 0.1663                 |
| Largest diff. peak/ hole (e Å⁻³)| 0.47/0.30                                  |

Table 4. Selected bond lengths for compound 6a (Å).

| Atom | Atom | Length |
|------|------|--------|
| N3   | C14  | 1.408(2) |
| N3   | C13  | 1.366(2) |
| N1   | C10  | 1.353(2) |
| N1   | C9   | 1.320(2) |
| N2   | N0   | 1.371(19)|
| N2   | C12  | 1.329(2) |
| N5   | C34  | 1.351(2) |
| N5   | C33  | 1.321(2) |
| N6   | N7   | 1.374(2) |
| N6   | C36  | 1.327(2) |
| N4   | C37  | 1.362(2) |
| N4   | C38  | 1.407(2) |
| N7   | C34  | 1.392(2) |
| N7   | C31  | 1.373(2) |
| N8   | C10  | 1.391(2) |
| N8   | C11  | 1.374(2) |
| C10  | C11  | 1.393(2) |
| C11  | C12  | 1.397(2) |
| C11  | C13  | 1.467(2) |
| C14  | C24  | 1.448(3) |
| C14  | C23  | 1.392(2) |
| C14  | C21  | 1.373(2) |
| C11  | C16  | 1.374(3) |
| C10  | C20  | 1.384(3) |
| C10  | C22  | 1.384(3) |
| C12  | C21  | 1.387(3) |
| C12  | C22  | 1.387(3) |
| C12  | C23  | 1.387(3) |
| C13  | C18  | 1.351(3) |
| C13  | C19  | 1.372(4) |
| C14  | C34  | 1.399(3) |
| C14  | C35  | 1.399(3) |
| C14  | C36  | 1.399(3) |
| C14  | C37  | 1.399(3) |
| C14  | C38  | 1.399(3) |
| C14  | C39  | 1.399(3) |
| C14  | C40  | 1.399(3) |
| C14  | C41  | 1.399(3) |
| C14  | C42  | 1.399(3) |
| C14  | C43  | 1.399(3) |
| C14  | C44  | 1.399(3) |
| C14  | C45  | 1.399(3) |
| C14  | C46  | 1.399(3) |
| C14  | C47  | 1.399(3) |
| C14  | C48  | 1.399(3) |
| C14  | C49  | 1.399(3) |
| C14  | C50  | 1.399(3) |

Also, the π-π stacking of the pyrazole rings could be the contributing factor. In both molecules, the pyrazolo[1,5-a]pyrimidine rings are planar. The bond lengths and angles are expected for C-C single bonds. Also the bond lengths of C34-C36, C33-C32, C9-C8 and C11-C12 are 1.388, 1.390, 1.402 and 1.397 Å, respectively, which are much shorter than that expected for C=C single bonds. Also the bond lengths of C34-C36, C33-C32, C10-C11 and C7-C8 are 1.399, 1.377, 1.393 and 1.368 Å, respectively, which are longer than C=C bond and close to that of C-C single bond. This indicates a considerable degree of delocalization of the aromatic 10-pi-electrons around the heterocyclic pyrazolopyrimidine ring system [39].

3.3. Biological activities

3.3.1. Anti-inflammatory assay

3.3.1.1. Percentage Inhibition of Paw Diameter

Untreated paw edema bearing mice served as control group. The mice (except the control) were then treated with the compounds (50 mg/kg body weight) via intraperitoneal (i.p) injection. The ability of the test compound to reduce the edema caused by Freund's Complete Adjuvant (FCA) was taken as a parameter. When induced with injection of FCA into the plantar side of left hind paw of the mice, all the groups of mice showed an increase in the paw diameter. In our study, we found that when these mice were treated with the test compounds (except the control which is untreated) some of the compounds showed a decrease in the paw diameter, which indirectly means that the edema has reduced. The ability of these test compounds to reduce the paw edema was calculated as percentage inhibition of paw diameter by using the formula [(a-b)/a]×100 [39] where 'a' and 'b' denote the mean increase of paw diameter of the control and drug treated mice respectively. The decrease/increase in the paw diameter was monitored at different intervals of zero, one, two, three and 24 h, respectively. Values are expressed as ±SEM.
From the Table 6, it shows that compound 8b led to a percentage inhibition in the paw diameter of the mice.

Table 6 reveals the percentage inhibition of paw edema of the different test groups. In this study, it was found that the standard drug ibuprofen could produce the highest percentage inhibition of up to 66.67 % at four hours but this inhibition was found to be absent at 24 hours. The highest percentage inhibition of the edema at 24 hours after intraperitoneal (i.p) drug administration was seen in case of test compound 8b (25 %) and 13a (16.57 %), respectively, while at four hours, this effect was seen to be highest in case of test compound 8b, followed by test compounds 9 and 13a, 13b, 6a, 6c, 6e and 10, respectively.

3.3.1.2. Concentration of nitric oxide (NO)

This assay was used to assess the potentiality of the test compounds as anti-inflammatory agents. As nitric oxide is produced by inducible nitric oxide synthase (iNOS) in activated macrophages during inflammation, this compound therefore can serve as a good biological marker for inflammation. This means that the agents that can inhibit the over production of nitric oxide may have anti-inflammatory activities [36,40].

The concentration of nitric oxide was assessed in paw exudates and whole blood. Of the different compounds tested, compound 13b was found to exert marked reduction in the concentration of NO in paw exudates (Figure 2) while in blood, compound 6a showed some magnitude of reduction (Figure 3).

3.3.1.3. Differential WBC count in the blood

During inflammation, a variety of cells such as neutrophils, eosinophils and basophils mediate an effective immune response [41]. Differential leucocyte count in blood smear was performed for the test compounds (Table 7) to study its anti-inflammatory properties. When compared with the untreated blood sample count, the blood samples that showed a reduction in the percentage count of the neutrophils, eosinophils and basophils might have anti-inflammatory effect. Most of the compounds tested resulted in lower counts of both neutrophils and eosinophils as compared to control mice. The percentage count of neutrophils was found to be significantly reduced in case of 8b and 13b (p < 0.0005 **), the effects of which are higher than that of ibuprofen (p < 0.001 *) treated mice. The test compound 13a showed comparable effects to ibuprofen in the reduction of neutrophils (p < 0.001 **). In the reduction of eosinophils, test compounds 10 and 8a (p < 0.001 *) showed comparable activities to ibuprofen (p < 0.001 *), while test compound 8b showed lower effect (p < 0.005 *). It is clearly understood that the compounds with the highest ability to reduce the marked indicators of inflammation, neutrophils and eosinophils are test compounds 8b and 13b.

3.3.2. Anti-cancer assay

3.3.2.1. MTB-based cytotoxic assay

The anti-cancer screening of the synthesized compounds were performed in cultured Chinese hamster ovary K1 (CHO K1) cell lines. The ability of the enzyme succinate tetrazolium reductase to metabolize 3-[4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide to purple formazan product after treatment with the test compounds in comparison to the untreated (control) cells was assessed. It was found that the test compounds 8a, 6b, 8a, 13a, and 13b, resulted in a decrease in metabolism of MTB by CHO K1 cells (Table 8). In particular, test compound 6a produced the lowest metabolism of MTB by these cells (Figure 4), followed by test compounds 8b, 13a. This assay is commonly used to monitor cell viability and proliferation and hence can also be used for assessing cytotoxicity [36,42].
Table 6. Percentage inhibition of paw edema in two different test groups, one administered with test compounds and the other with ibuprofen.

| Test compounds | Mean increase in paw diameter (mm) | 1 h | 2 h | 3 h | 4 h | 24 h |
|----------------|-------------------------------------|-----|-----|-----|-----|-----|
| Control        |                                     | 1.5 | 1.7 | 1.3 | 1.5 | 1.2 |
| Ibuprofen      |                                     | 2.0 | 1.2 | 1.0 | 0.5 | 1.8 |
| 6a             |                                     | 2.3 | 1.9 | 1.3 | 1.2 | 1.8 |
| 6b             |                                     | 2.0 | 2.0 | 1.7 | 1.8 | 2.3 |
| 6c             |                                     | 1.7 | 1.1 | 1.2 | 1.3 | 1.5 |
| 6d             |                                     | 2.0 | 1.7 | 1.5 | 1.5 | 1.9 |
| 6e             |                                     | 2.0 | 1.9 | 1.5 | 1.3 | 1.2 |
| 6a             |                                     | 1.8 | 1.9 | 1.6 | 0.9 | 0.9 |
| 6b             |                                     | 2.0 | 2.0 | 1.6 | 1.3 | 1.2 |
| 6c             |                                     | 1.0 | 1.5 | 1.3 | 0.9 | 1.2 |
| 6d             |                                     | 2.0 | 1.5 | 1.1 | 1.0 | 1.2 |

Table 7. Differential WBC count in three different test groups: untreated paw edema bearing mice (control), ibuprofen treated paw edema bearing mice and test compounds treated paw edema bearing mice. The values are SEM (* denote significance against paw edema bearing control mice at p < 0.0005**, p < 0.001***, p < 0.0005***).

| Test compounds | Lymphocytes | Neutrophils | Eosinophils | Basophils | Monocytes |
|----------------|-------------|-------------|-------------|-----------|-----------|
| Control        | 62.67       | 14.00       | 6.00        | 8.67      | 8.67      |
| Ibuprofen      | 86.67       | 4.00        | 2.00        | 6.67      | 0.33      |
| 6a             | 68.00       | 16.00       | 6.00        | 7.30      | 2.70      |
| 6b             | 77.33       | 8.67        | 6.00        | 6.00      | 2.00      |
| 6c             | 93.33       | 19.33       | 7.33        | 3.33      | 0.67      |
| 6d             | 64.67       | 19.33       | 9.33        | 4.67      | 2.00      |
| 6e             | 78.67       | 10.00       | 8.00        | 2.67      | 0.67      |
| 8a             | 82.67       | 6.33        | 2.00        | 9.00      | 0.00      |
| 8b             | 93.00       | 1.67        | 3.00        | 3.00      | 0.00      |
| 9              | 76.00       | 10.00       | 8.67        | 4.67      | 0.67      |
| 10             | 71.33       | 27.33       | 5.06        | 0.67      | 0.00      |
| 13a            | 79.67       | 6.67        | 5.00        | 7.00      | 1.67      |
| 13b            | 80.00       | 3.00        | 4.00        | 3.00      | 2.00      |

Table 8. MTT assay in CHO K1 cell lines after exposure to 20 µL of 10 mg/mL test compounds (in 10 % DMSO). Absorbance was recorded at 570 nm. Cells exposed to 10 % DMSO served as control. Values are mean ± SD of three readings.

| Test compounds | Absorbance at 570 nm | Standard deviation |
|----------------|----------------------|--------------------|
| Control        | 0.30                 | ±0.0015            |
| 6a             | 0.02                 | ±0.0048            |
| 6c             | 0.07                 | ±0.0025            |
| 6d             | 0.08                 | ±0.0012            |
| 8a             | 0.39                 | ±0.0012            |
| 8b             | 0.19                 | ±0.0060            |
| 9              | 0.05                 | ±0.0077            |
| 10             | 0.64                 | ±0.0068            |
| 13a            | 0.20                 | ±0.0050            |
| 13b            | 0.37                 | ±0.0091            |
**Figure 2.** Concentration of nitric oxide in paw exudates of different test groups (treated with test compounds and ibuprofen) in comparison to the control (untreated) paw edema bearing mice. The edema was induced by injection of FCA into the plantar side of left hind paw of the mice. The mice (except the control) were then treated with the compounds (50 mg/kg body weight) via i.p injection. After 24 h, the paws were excised, weighed, homogenized and centrifuged at 12,000 rpm in 10 per cent ice-cold normal saline. The supernatant was collected and used for NO assay. Absorbance was recorded at 520 nm and the concentration of NO was calculated using the standard nitrite curve. Values are expressed as mean ±SEM (*denotes significance against paw edema bearing control mice at p < 0.0005***, p < 0.001**, p < *0.005).

**Figure 3.** Concentration of nitric oxide (NO) in blood groups of treated and untreated paw edema bearing mice. Untreated groups served as control. The edema was induced by injection of FCA into the plantar side of the left hind paw of the mice. These mice (except the control) were then treated with the compounds (50 mg/kg body weight) via i.p injection. After 24 h, blood was collected by retro-orbital bleeding and used for nitric oxide assay. Absorbance was recorded at 520 nm and the concentration of nitric oxide was quantified using the standard nitrite curve. Values are expressed as mean ±SEM (*denotes significance against paw edema bearing control mice at p < 0.0005***, p < 0.001**, p < *0.005).

**Figure 4.** The cytotoxic effects of the test compounds on CHO K1 cell lines were compared against the control using the MTT based assay. CHO K1 cells were grown in microtitre plates and treated with 20 µL of 10 mg/mL test compounds (in 10 % DMSO). Their ability to metabolise MTT was measured by taking the absorbance at 570 nm. Cells exposed to 10 % DMSO were used as control. This means that the lower the production of purple formazan in cells treated with the test compounds, the lower is the number of viable cells, the higher is their cytotoxic effects and hence the greater is their potential as anticancer compounds. Hence, these compounds 6a, 6b, 6c, 6b, 8a, 13a, 13b may be concluded to have cytotoxic effect on CHO K1 cell lines.

### 4. Conclusion

We were able to successfully combine the bio-labile rings together in a molecular framework. The synthetic protocol features a new route that utilizes ultrasound irradiation for the diversification of pyrazolopyrimidine derivatives. The
starting materials used were developed by methods reported from our group from the commercially available active proton compounds suggesting that further modification of this component may be a viable strategy for future development of bioactive compound libraries. From the results obtained, the test compound 13b can be selected as a good candidate for anti-inflammatory effects. This test compound showed an anti-inflammatory and anti-cancer drugs.

On the basis of the anti-cancer assay, test compound 6a was found to show the maximum cytotoxic effect, followed by compounds 8b, 13a, 13b, 8a and 6b, respectively. The percentage inhibition of these test compounds was found to be 6a (97.10 %), 8b (72.96 %), 13a (72.46 %), 13b (49.24 %) and 8a (45.75 %).

Further investigation on these compounds are therefore crucial in order to elucidate the exact mechanism of action on target cells and hence to therefore add them in the list of potent anti-inflammatory and anti-cancer drugs.

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