Full Paper

Synthesis and *in-vitro* Cytotoxic Evaluation of Novel Pyridazin-4-one Derivatives

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A new series of *N*-aryl-4-oxo-1,4-dihydro-pyridazine-3-carboxylic acids has been synthesized by condensation of aryldiazonium with 4-hydroxy-6-methyl-2-pyrone. Some of these compounds exhibited *in-vitro* cytotoxic activity with moderate to excellent growth inhibition against the murine P815 mastocytoma cell line. Compound **5b** showed an important cytotoxic activity against cell line P815 (IC_{50} = 0.40 μg/mL).

Keywords: Arylhydrazonopyranedione / Cytotoxic activity / Pyrazolo[4,3-*c*]pyridazinone / Pyridazinone / 2-Pyrone

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Introduction

Pyridazine derivatives, in particular pyridazinones, present various pharmacological properties [1–5]. Introduction of an aryl moiety on the pyridazinone skeleton has resulted in a large number of derivatives exhibiting a plethora of promising pharmacological activities. For example, these molecules have been previously reported to be platelet-aggregation inhibitors [6–8], α-adrenoceptor antagonists [9], anti-hypertensive [10], and antinociceptive agents [11]. Recently, we have reported the synthesis and the cytotoxicity activities of new triazolopyridazinone derivatives. Some of these compounds exhibited significant cytotoxicity against the Hep cell line [12]. In continuation of our studies [12, 13] on the synthesis of new pyridazinone compounds, herein, we report the synthesis of some potent analogs of *N*-substituted-pyridazinone derivatives, which have been tested for their antitumor activity.

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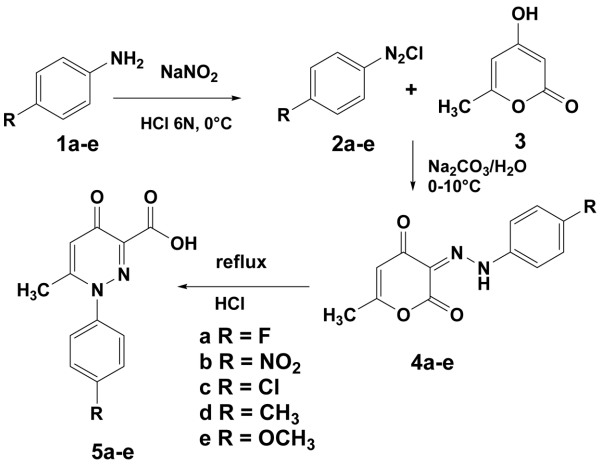
Results and discussion

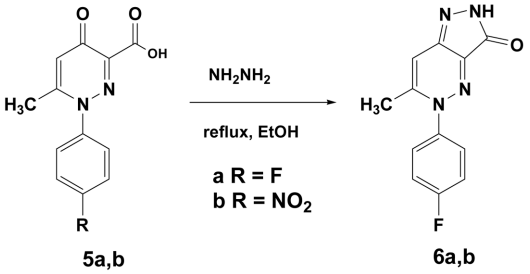
Chemistry

The new series of 1-aryl-1,4-dihydro-4-oxo-6-methyl pyridazine-3-carboxylic acid shown in Scheme 1 were prepared according to the reported methods [14]. The synthetic strategy employed the condensation of a variety of aryldiazonium chloride **2a–e** with commercially available 4-hydroxy-6-methy-2-pyrone **3** followed by an intramolecular cyclization of arylhydrazonopyranediones **4a–e** producing the desired series of 1-aryl-1,4-dihydro-4-oxo-6-methyl pyridazine-3-carboxylic acid **5a–e** (Scheme 1). The structures of compounds **5a–e** were confirmed by ^{1}H-NMR, ^{13}C-NMR, IR, MS spectra and elemental analysis.

Pyridazine-4-ones **5a–e** are suitable intermediates for the preparation of new pyrazolo[4,3-*c*]pyridazinone derivatives. In fact, the reaction between hydrazine hydrate and pyridazinones **5a**, **b** afforded pyrazolo[4,3-*c*]pyridazinones **6a**, **b** in good yield (Scheme 2). The structures of the synthesized compounds were established on the basis of IR, ^{1}H-NMR, and ^{13}C-NMR spectral data. In the IR spectra of compounds **6a**, **b**, the absence of the absorption band at 1741–1745 cm^{-1} for C=O acid and the absorption band at 1630–1645 cm^{-1} for C=O ketone confirms the formation of pyrazolone cycle. The ^{1}H-NMR spectra of **6a** and **6b**



Scheme 1. Synthesis of compounds **5a–e**.



Scheme 2. Synthesis of compounds **6a** and **6b**.

exhibited broad signals at δ = 11.90 and 12.50 ppm, respectively, due to the NH proton.

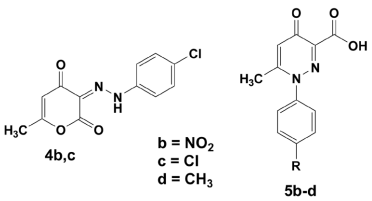
Cytotoxic activity

The preliminary cytotoxic activities of some compounds against the murine P815 mastocytoma cell line were evaluated *in vitro* (under scattered light) as shown in Table 1. The IC_{50} represents the drug concentration (μg/mL) required to inhibit cell growth by 50%.

The arylhydrazonopyranediones **4b**, **c** have shown slight cytotoxic activity against cell line P815. The cytotoxic activity of the 1-aryl-1,4-dihydro-4-oxo-6-methyl pyridazine-3-carboxylic acids **5b–d** is interesting, and among them, compound **5b** (R = NO_2) showed good activity against cell line P815 (IC_{50} = 0.40 μg/mL). It should be noted that the substituent at the *para*-position of benzene in compounds **5b–d** may also play an important role in determining relative activities.

In conclusion, we have synthesized novel pyridazin-4-one derivatives and tested them for their cytotoxic activity on the P815 cell line. Compound **5b** (R = NO_2) was identified as the most potent having an IC_{50} value of 0.40 μg/mL. Our results indicate that these new cytotoxic compounds may be useful as leads for the development of novel anticancer agents.

Table 1. Cytotoxicity of compounds **4b**, **c** and **5b–d** against the murine mastocytoma cell line P815.



Compound	R	IC_{50} (μg/mL)
4b	NO_2	11.73
4c	Cl	3.14
5b	NO_2	0.40
5c	Cl	1.01
5d	CH_3	0.72

Experimental

General

Melting points were determined using a Büchi-Tottoli apparatus (Büchi, Switzerland) and are uncorrected. IR spectra were recorded on a Perkin-Elmer 577 spectrometer (Perkin-Elmer, USA) using KBr disks; only noteworthy IR absorptions are listed (cm^{-1}). ^1H- and ^{13}C-NMR spectra were recorded in $CDCl_3$, DMSO-d_6 and solution (unless otherwise specified) with TMS as an internal reference using Bruker AC 300 MHz (^1H) or 75 MHz (^{13}C) instruments (Bruker Bioscience, USA). Chemical shifts are given in *d* parts per million (ppm) downfield from TMS. Multiplicities of ^{13}C-NMR resources were assigned by distortionless enhancement by polarization transfer (DEPT) experiments. Low-resolution mass spectra (MS) were recorded on a Perkin-Elmer Sciex API 3000 spectrometer (Perkin-Elmer). Column chromatography was carried out on SiO_2 (silica gel 60 Merck 0.063-0.200 mm; Merck, Germany). Thin-layer chromatography (TLC) was carried out on SiO_2 (silica gel 60, F 254 Merck 0.063–0.200 mm; Merck), and the spots were located with UV light. Commercial reagents were used without further purification unless stated.

Chemistry

*General procedure for synthesis of 1-(aryl substituted)-1,4-dihydro-4-oxo-6-methyl pyridazine-3-carboxylic acid **5a–e***

4-Hydroxy-6-methyl-2-pyrone **3** (0.5 g, 3.96 mmol) was dissolved in 20 mL water and sodium carbonate (2.24 g, 21.03 mmol) was added to the suspension to make the solution alkaline.

In a separate flask, 4.20 mmol of required aniline derivative **1a–e** was dissolved in 10 mL 6 N HCl and cooled to 0°C. A solution of sodium nitrite (0.35 g, 5.04 mmol) in 5 mL water was added at 0°C and the resulting diazonium chloride solution was added dropwise to a stirred pyrone solution while maintaining the temperature at 5–10°C and pH at 10–12. The resulting hydrazone was refluxed for 3 h at 110°C and then neutralized with acetic acid up to pH = 7. A small amount of charcoal was added and again

refluxed for 30 min. The solid was filtered while being hot, the filtrate then cooled to 0–5°C and treated with conc. HCl. The precipitated solid was collected by filtration, washed well with water, and dried completely to yield the desired compound.

1-(4-Fluoro-phenyl)-1,4-dihydro-4-oxo-6-methyl pyridazine-3-carboxylic acid ***5a***

Yield: 68%; m.p.: 158–160°C; IR (cm^{-1}): 3400 (OH), 1745 (C=O), 1645 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 2.40 (s, 3H, CH_3) ,6.95 (s, 1H, CH), 7.35 (d, 2H, *J* = 6.8 Hz, H-Ar), 7.50 (d, 2H, *J* = 6.8 Hz, H-Ar), 15.40 (s, 1H, OH); ^{13}C-NMR (DMSO-d_6) δ: 171.10 (C=O), 162.10 (C=O), 155.57 (C=N), 144.72, 142.65, 141.01 (3C), 128.15 (2 CHAr), 121.67 (2CHAr), 120.12 (=CH), 20.48 (CH_3); MS (SI) *m/z*: 249 $[M + 1]^+$. Anal. calcd. for $C_{12}H_9FN_2O_3$: C, 58.07; H, 3.65; N, 11.29. Found: C, 57.90; H, 3.74; N, 11.10.

1-(4-Nitro-phenyl)-1,4-dihydro-4-oxo-6-methyl pyridazine-3-carboxylic acid ***5b***

Yield: 61%; m.p.: 255–257°C; IR (cm^{-1}): 3414 (OH), 1741 (C=O), 1630 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 2.28 (s, 3H, CH_3), 7.09 (s, 1H, =CH), 7.95 (d, 2H, *J* = 9.0 Hz, H-Ar), 8.45 (d, 2H, *J* = 9.0 Hz, H-Ar); ^{13}C-NMR (DMSO-d_6) δ: 170.60 (C=O), 162.60 (C=O), 155.07 (C=N), 147.97, 146.28, 143.18 (3 C), 128.19 (2 CHAr), 125.00 (2 CHAr), 120.56 (=CH), 20.32 (CH_3); MS (SI) *m/z*: 276 $[M + 1]^+$. Anal. calcd. for $C_{12}H_9N_3O_5$: C, 52.37; H, 3.30; N, 15.27. Found: C, 51.98; H, 3.50; N, 15.15.

1-(4-Chloro-phenyl)-1,4-dihydro-4-oxo-6-methyl pyridazine-3-carboxylic acid ***5c***

Yield: 57%; m.p.: 227–229°C; IR (cm^{-1}): 3416 (OH), 1739 (C=O acide), 1620 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 2.25 (s, 3H, CH_3), 7.10 (s, 1H, =CH), 7.29 (d, 2H, *J* = 6.8 Hz, H-Ar), 7.46 (d, 2H, *J* = 6.8 Hz, H-Ar); ^{13}C-NMR (DMSO-d_6) δ: 170.80 (C=O), 162.61 (C=O), 155.57 (C=N), 142.42, 140.57, 134.88 (3 C), 129.67 (2 CHAr), 128.30 (2 CHAr), 120.17 (=CH), 20.39 (CH_3); MS (SI) *m/z*: 265 (^{35}Cl) $[M + 1]^+$, 267 (^{37}Cl) $[M + 3]^+$. Anal. calcd. for $C_{12}H_9ClN_2O_3$: C, 54.46; H, 3.43; N, 10.58. Found: C, 54.28; H, 3.54; N, 10.74.

1-(4-Methyl-phenyl)-1,4-dihydro-4-oxo-6-methyl pyridazine-3-carboxylic acid ***5d***

Yield: 47%; m.p.: 166–168°C; IR (cm^{-1}): 3410 (OH), 1728 (C=O), 1630 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 2.32 (s, 3H, CH_3), 2.44 (s, 3H, CH_3Ar), 6.90 (s, 1H, =CH), 7.28 (d, 2H, *J* = 9.00 Hz, H-Ar), 7.33 (d, 2H, *J* = 9.0 Hz, H-Ar), 15.56 (s, 1H, OH); ^{13}C-NMR (DMSO-d_6) δ: 171.87 (C=O), 162.77 (C=O), 155.13 (C=N), 141.61, 141.07, 139.14 (3 C), 130.48 (2 CHAr), 125.68 (2 CHAr), 121.12 (=CH), 21.26 (CH_3); MS (SI) *m/z*: 245 $[M + 1]^+$. Anal. calcd. for $C_{13}H_{12}N_2O_3$: C, 63.93; H, 4.95; N, 11.47. Found: C, 63.74; H, 4.78; N, 11.65.

1-(4-Methoxy-phenyl)-1,4-dihydro-4-oxo-6-methyl pyridazine-3-carboxylic acid ***5e***

Yield: 45%; m.p.: 170–172°C; IR (cm^{-1}): 3400 (OH), 1720 (C=O), 1625 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 2.32 (s, 3H, CH_3), 3.87 (s, 3H, OCH_3), 6.89 (s, 1H, =CH), 7.01 (d, 2H, *J* = 6.9 Hz, H-Ar), 7.30 (d, 2H, *J* = 6.9 Hz, H-Ar); ^{13}C-NMR (DMSO-d_6) δ: 171.86 (C=O), 162.78 (C=O), 155.29 (C=N), 160.91, 141.65, 134.47 (3 C), 127.23 (2 CHAr), 121.10 (=CH), 114.96 (2 CHAr), 55.77 (OCH_3), 21.33 (CH_3); MS (SI) *m/z*: 261 $[M + 1]^+$. Anal. calcd. for $C_{13}H_{12}N_2O_4$: C, 60.00; H, 4.65; N, 10.76. Found: C, 59.74; H, 4.75; N, 11.05.

General procedure for synthesis of pyrazolo[4,3-c] pyridazinones ***6a, b***

A mixture of **5a**, **b** (1.2×10^{-3} mol) and hydrazine hydrate (0.6 g, 1.2×10^{-3} mol) was refluxed for 4 h in ethanol. After cooling, the reaction mixture was poured onto ice. The solid obtained was filtered and washed with ether.

5-(4-Fluorophenyl)-6-methyl-2H-pyrazolo[4,3-c] pyridazin-3(5H)-one ***6a***

Yield: 73%; m.p.: 276–278°C; IR (cm^{-1}): 3380 (NH), 1665 (C=O), 1654 (C=N); ^{1}H-NMR (DMSO-d_6) δ: 2.17 (s, 3H, CH_3), 7.01 (s, 1H, CH), 7.31 (d, 2H, *J* = 9.1 Hz, H-Ar), 7.70 (d, 2H, *J* = 9.1 Hz, H-Ar), 11.90 (s, 1H, NH); ^{13}C-NMR (DMSO-d_6) δ: 159.94, 142.42, 142.38, 139.76, 138.45, 134.76 (5 C), 128.76 (2 CHAr), 115.97 (2 CHAr), 109.60 (=CH), 20.02 (CH_3); MS (SI) *m/z*: 245 $[M + 1]^+$.

5-(4-Nitrophenyl)-6-methyl-2H-pyrazolo[4,3-c]pyridazin-3(5H)-one ***6b***

Yield: 80%; m.p.: 288–290°C; IR (cm^{-1}): 3355 (NH), 1670 (C=O), 1645 (C=N); ^{1}H-NMR (DMSO-d_6) δ: 2.18 (s, 3H, CH_3), 6.52 (s, 1H, CH), 7.31 (d, 2H, *J* = 11.5 Hz, H-Ar), 8.10 (d, 2H, *J* = 11.5 Hz, H-Ar), 12.50 (s, 1H, NH); ^{13}C-NMR (DMSO-d_6) δ: 165.38 (CO), 149.73, 149.70, 138.76, 138.70, 133.50 (5 C), 125.75 (2 CHAr), 112.13 (2 CHAr), 104.07 (=CH), 34.75 (CH_3). MS (SI) *m/z*: 272 $[M + 1]^+$.

Cytotoxic activity

The cytotoxic activity was studied against P815 (murine mastocytoma cell line) using the colorimetric MTT assay as described and modified by Tim Mossman [15]. Cells were washed by centrifugation in PBS (phosphate buffered saline), and incubated in 96-well microtiter plates (Bioster, Italy) at a density of 1.5×10^5 cells/mL in 100 µL per well of culture medium (D-MEM) supplemented with 5% of fetal calf serum, and 100 UI/mL of penicillin and 100 µg/mL streptomycin, 0.2% sodium bicarbonate). Then, 100 µL of fresh culture medium containing appropriate serial concentrations of the test compounds were added to each well. After incubation for 48 h at 37°C and 5% CO_2, 100 µL of medium were carefully aspirated from each well and replaced by 20 µL of MTT solution (5 mg/mL PBS). After incubation during 4 h at the same conditions, the plates were treated with a mixture of HCl/isopropanol (24:1) to dissolve the blue intracellular formazan product. One hour later, the optical density in the wells was read on a MicroElisa reader (Dynatech Laboratories, USA) using dual-wavelength mode (540–630 nm). DMSO and Adriamycine were used as negative and positive control, respectively. The cytotoxicity in (%) = $100 \times (1\text{-}OD_t/OD_o)$, where OD_o and OD_t are the optical density of control and treated wells, respectively. Three independent sets of experiments performed in duplicate were evaluated.

The authors have declared no conflict of interest.

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