Scientific paper

Synthesis, Characterization, and Biological Activity Evaluation of 4,6-Dihydropyrano[3,2-*c*]isochromene-3-carbonitrile as Anticancer Agents

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Abstract

Herein, a series of new 2-amino-4,6-dihydropyrano[3,2-*c*]isochromene-3-carbonitrile derivatives (**4a**–**o**) have been synthesized by one-pot three component reaction of 4-hydroxyisocoumarin, various aromatic aldehydes, and malononitrile in the presence of triethylamine in EtOH at reflux conditions. All the newly synthesized compounds were characterized using standard spectroscopic techniques (FT-IR, ^{1}H and ^{13}C NMR) and elemental analysis. The cytotoxicity of the synthesized compounds was determined by MTT assay on one normal (3T3) and two cancer cell lines (A549 and MCF-7). It was revealed that some of these compounds (**4b**, **4f**, and **4j**) are toxic especially for MCF-7 cell line and can be considered as lead compounds for further investigation. Howevere, the other compounds had low cytotoxicity and can be suitable candidates for other pharmacological effects such as antibacterial, antifungal, antiviral, etc.

Keywords: 4-hydroxyisocoumarin, antiproliferative, three component reaction, cytotoxic, 4,6-dihydropyrano[3,2-*c*] isochromene-3-carbonitrile derivatives.

1. Introduction

During the last decades, isocoumarin scaffolds have been well recognized owing to their diverse presence in natural products, important roles in organic synthesis, and broad spectrum of pharmacological activities.[1] Isocoumarin (the isomer of coumarin) is a class of benzopyrone compounds having fused benzene and α-pyrone ring, so that the carbonyl group is at the 1-position of the molecule. Generally the differences among isocoumarins are in the side group attached to their basic structure.[1,2] Natural isocoumarins have been found in plants and used as valuable leads for the design and synthesis of new medicinally important pharmacophores.[2] Isocoumarin and related moieties display a wide range of biological activities such as antiproliferative (anti-cancer, anti-metastatic),[3,4] antibacterial,[5] antifungal,[6] anti-influenza virus,[7] anti-HIV,[8] anti-diabetic,[9] osteogenic,[10] and carbonic anhydrase inhibitor.[11]

Isocoumarins are used as worthwhile intermediates in the synthesis of heterocyclic compounds such as isoquinolines, isochromenes, isocarbostyrils, and various aromatic compounds.[1] Some compounds with isocoumarin scaffold are displayed in Figure 1.

Despite enormous improvements in treatment, cancer is still a challenge for healthcare systems.[12] Chemotherapy is the most frequently used and suitable strategy in cancer treatment.[13] However, drug resistance especially during long exposure is a matter of concern.[12] This requires more efforts and research in the field of discovery and synthesis of new drugs.

In the present study, 4-hydroxyisocoumarin was used as a framework structure for synthesis of new 2-amino-4,6-dihydropyrano[3,2-*c*]isochromene-3-carbonitrile derivatives **4a**–**o** by one-pot three component reaction with aromatic aldehydes and malononitrile in the presence of triethylamine using ethanol as the reaction solvent, at reflux conditions; then the cyototoxicity of these compounds was evaluated on one normal (3T3) and two cancer cell lines (A549 lung and MCF-7 breast cancer cells).

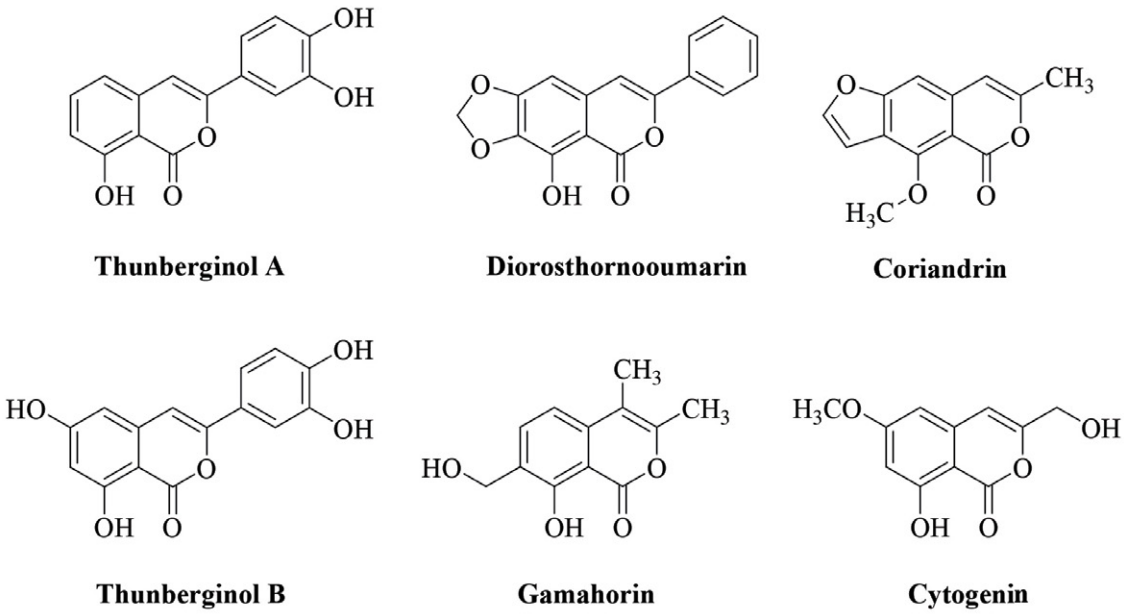


Figure 1. Some compounds with isocoumarin skeletons.

2. Experimental

2. 1. General Methods

All used reagents and chemicals were prepared from commercial sources and applied with no additional purification. Determination of the melting points was done on the Electrothermal 9100 apparatus without correction. Using KBr pellets, IR spectra were recorded on a Bruker FT-IR spectrophotometer (Alpha model). ^{13}C NMR (75 MHz) and ^{1}H NMR (300 MHz) spectra were recorded on a Bruker AVANCE III 300 MHz spectrometer in dimethyl sulfoxide (DMSO-d_6) and TMS was an internal standard. Coupling constants (J) are given in Hz and chemical shifts (δ) are expressed in parts per million (ppm). Thin layer chromatography (TLC) was used to monitor reactions on the aluminium-backed silica gel sheets (GF254) and were observed under UV light (254 nm). Elemental analyses were obtained utilizing a Heraeus CHN-O-Rapid analyzer.

The cell lines (3T3, A549, and MCF-7) were purchased from the Iranian Biological Resource Center (IBRC, Tehran, Iran). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were supplied by Gibco, USA; and antibiotics (penicillin, streptomycin) were purchased from Biosera, France. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Melford, England.

2. 2. General Procedure for the Preparation of 2-Amino-4,6-dihydropyrano[3,2-*c*] isochromene-3-carbonitrile Derivatives 4a–o

In a 50 mL round bottom flask, a mixture of 2 mmol 4-hydroxyisocoumarin (**1**), 2 mmol aromatic aldehydes **2a–o**, 2.1 mmol malononitrile (**3**), and three drops of triethylamine in 10 mL of ethanol was stirred and refluxed for 90 min. After the reaction was completed (the progress of the reaction was monitored by TLC using hexane/ethyl acetate as an eluent), the mixture was cooled and the precipitated products were collected by filtration, washed with cold ethanol, and then recrystallized from ethanol to give pure solid sample for analysis.

2-Amino-6-oxo-4-phenyl-4,6-dihydropyrano[3,2-*c*]isochromene-3-carbonitrile (4a)

Pale yellow powder; yield: 94%; mp 248.5–249 °C; IR (KBr, cm^{-1}) ν_{max}: 3461, 3318 (NH_2), 3012 (CH, aromatic), 2197 (C≡N), 1721 (C=O), 1683, 1633 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.13 (d, J = 6 Hz, 1H, ArH), 8.00 (t, J = 6 Hz, 1H, ArH), 7.78 (d, J = 6 Hz, 1H, ArH), 7.68 (t, J = 6 Hz, 1H, ArH), 7.44–7.31 (m, 5H, ArH), 7.25 (s, 2H, NH_2), 4.72 (s, 1H, CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.16 (C=O), 159.57 (C-2), 141.77, 137.28, 136.07, 130.64, 130.04, 129.97, 129.29, 129.25, 128.49, 128.17, 120.37, 120.09, 119.96 (C≡N), 56.95 (C-3), 40.76 (C-4). Anal. calcd. for $C_{19}H_{12}N_2O_3$: C, 72.15; H, 3.82; N, 8.86%. Found: C, 71.91; H, 3.65; N, 8.68%.

2-Amino-6-oxo-4-(*ortho*-tolyl)-4,6-dihydropyrano [3,2-*c*]isochromene-3-carbonitrile (4b)

Cream powder; yield: 93%; mp 269–269.5 °C; IR (KBr, cm^{-1}) ν_{max}: 3456, 3350 (NH_2), 3015 (CH, aromatic), 2979 (CH, aliphatic), 2196 (C≡N), 1729 (C=O), 1683, 1633 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.14 (d, J = 6 Hz, 1H, ArH), 8.04–7.99 (m, 1H, ArH), 7.80 (d, J = 6 Hz, 1H, ArH), 7.72–7.66 (m, 1H, ArH), 7.22 (s, 6H, ArH, NH_2), 5.01 (s, 1H, CH), 2.46 (s, 3H, CH_3); ^{13}C NMR (75

MHz, DMSO-d_6) δ 160.14 (C=O), 159.62 (C-2), 139.82, 137.67, 136.41, 136.11, 131.18, 130.61, 130.06, 129.94, 129.36, 129.12, 127.91, 127.22, 120.31, 120.03, 119.96 (C≡N), 56.67 (C-3), 37.25 (C-4), 19.44 (CH_3). Anal. calcd. for $C_{20}H_{14}N_2O_3$: C, 72.72; H, 4.27; N, 8.48%. Found: C, 72.75; H, 4.07; N, 8.29%.

2-Amino-4-(2-chlorophenyl)-6-oxo-4,6-dihydropyrano [3,2-*c*]isochromene-3-carbonitrile (4c)

White powder; yield: 91%; mp 234.5–235 °C; IR (KBr, cm^{-1}) ν_{max}: 3463, 3304 (NH_2), 3009 (CH, aromatic), 2925 (CH, aliphatic), 2195 (C≡N), 1727 (C=O), 1686, 1634 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.14 (d, J = 6 Hz, 1H, ArH), 8.03 (t, J = 6 Hz, 1H, ArH), 7.80 (d, J = 6 Hz, 1H, ArH), 7.71 (t, J = 6 Hz, 1H, ArH), 7.52–7.36 (m, 4H, ArH), 7.30 (s, 2H, NH_2), 5.20 (s, 1H, CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.07 (C=O), 159.85 (C-2), 138.38, 136.22, 136.15, 133.20, 131.55, 130.54, 130.40, 130.10, 130.06, 129.89, 128.50, 120.40, 120.19, 119.58 (C≡N), 55.75 (C-4), 38.33 (C-3). Anal. calcd. for $C_{19}H_{11}ClN_2O_3$: C, 65.06; H, 3.16; N, 7.99%. Found: C, 64.88; H, 3.07; N, 7.90%.

2-Amino-4-(2-methoxyphenyl)-6-oxo-4,6-dihydropyrano[3,2-*c*]isochromene-3-carbonitrile (4d)

Pale yellow powder; yield: 92%; mp 260–260.8 °C; IR (KBr, cm^{-1}) ν_{max}: 3454, 3325 (NH_2), 3016 (CH, aromatic), 2966 (CH, aliphatic), 2197 (C≡N), 1722 (C=O), 1686, 1636 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.14 (d, J = 6 Hz, 1H, ArH), 8.02 (t, J = 6 Hz, 1H, ArH), 7.80 (d, J = 6 Hz, 1H, ArH), 7.69 (t, J = 6 Hz, 1H, ArH), 7.31 (t, J = 6 Hz, 1H, ArH), 7.21 (d, J = 6 Hz, 1H, ArH), 7.13 (s, 2H, NH_2), 7.07 (d, J = 6 Hz, 1H, ArH), 6.97 (t, J = 6 Hz, 1H, ArH), 5.02 (s, 1H, CH), 3.79 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.30 (C=O), 159.95 (C-2), 157.66, 137.50, 136.08, 130.79, 130.05, 129.81, 129.66, 129.49, 129.46, 121.40, 120.21, 120.02 (C≡N), 112.31, 56.57 (C-3), 56.34 (OCH_3), 34.93 (C-4). Anal. calcd. for $C_{20}H_{14}N_2O_4$: C, 69.36; H, 4.07; N, 8.09%. Found: C, 69.09; H, 4.08; N, 7.97%.

2-Amino-4-(2-bromophenyl)-6-oxo-4,6-dihydropyrano [3,2-*c*]isochromene-3-carbonitrile (4e)

White powder; yield: 90%; mp 243–244 °C; IR (KBr, cm^{-1}) ν_{max}: 3453, 3300 (NH_2), 3003 (CH, aromatic), 2922 (CH, aliphatic), 2194 (C≡N), 1727 (C=O), 1686, 1637 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.16 (d, J = 6 Hz, 1H, ArH), 8.04 (t, J = 6 Hz, 1H, ArH), 7.81 (d, J = 6 Hz, 1H, ArH), 7.75–7.67 (m, 2H, ArH), 7.46–7.41 (m, 2H, ArH), 7.32–7.26 (m, 3H, ArH, NH_2), 5.22 (s, 1H, CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.07 (C=O), 159.77 (C-2), 140.05, 136.29, 136.15, 133.64, 131.70, 130.55, 130.34, 130.12, 129.90, 129.10, 123.51, 120.43, 120.22, 119.51 (C≡N), 55.95 (C-3), 39.16 (C-4). Anal. calcd. for $C_{19}H_{11}BrN_2O_3$: C, 57.74; H, 2.81; N, 7.09%. Found: C, 57.51; H, 2.69; N, 6.91%.

2-Amino-4-(2-nitrophenyl)-6-oxo-4,6-dihydropyrano [3,2-*c*]isochromene-3-carbonitrile (4f)

Brown yellow powder; yield: 90%; mp 234–235 °C; IR (KBr, cm^{-1}) ν_{max}: 3437, 3307 (NH_2), 3009 (CH, aromatic), 2980 (CH, aliphatic), 2201 (C≡N), 1731 (C=O), 1691, 1638 (C=C), 1527, 1325 (NO_2); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.15 (d, J = 6 Hz, 1H, ArH), 8.06–7.98 (m, 2H, ArH), 7.82–7.58 (m, 5H, ArH), 7.40 (s, 2H, NH_2), 5.41 (s, 1H, CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.02 (C=O), 159.90 (C-2), 149.89, 136.13, 135.78, 134.69, 134.28, 131.89, 130.47, 130.21, 130.11, 129.91, 129.79, 124.97, 120.46, 120.28, 119.46 (C≡N), 55.24 (C-3), 36.32 (C-4). Anal. calcd. for $C_{19}H_{11}N_3O_5$: C, 63.16; H, 3.07; N, 11.63%. Found: C, 63.01; H, 2.98; N, 11.59%.

2-Amino-4-(3-fluorophenyl)-6-oxo-4,6-dihydropyrano [3,2-*c*]isochromene-3-carbonitrile (4g)

Yellow powder; yield: 91%; mp 245–246.3 °C; IR (KBr, cm^{-1}) ν_{max}: 3463, 3314 (NH_2), 3016 (CH, aromatic), 2924 (CH, aliphatic), 2197 (C≡N), 1723 (C=O), 1684, 1633 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.14 (d, J = 6 Hz, 1H, ArH), 8.01 (t, J = 6 Hz, 1H, ArH), 7.79 (d, J = 6 Hz, 1H, ArH), 7.70 (t, J = 6 Hz, 1H, ArH), 7.58 (s, 1H, ArH), 7.56–7.51 (m, 1H, ArH), 7.41–7.37 (m, 2H, ArH), 7.32 (s, 2H, NH_2), 4.80 (s, 1H, CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.47 (C=O), 161.24 (C-2), 160.14, 159.64, 144.63, 144.55, 136.57, 136.04, 131.30, 131.19, 130.63, 130.04, 129.52, 124.70, 124.67, 120.44, 120.26, 119.82 (C≡N), 115.50, 115.22, 114.96, 56.47 (C-3), 40.32 (C-4). Anal. calcd. for $C_{19}H_{11}FN_2O_3$: C, 68.26; H, 3.32; N, 8.38%. Found: C, 67.94; H, 3.07; N, 8.09%.

2-Amino-6-oxo-4-(*meta*-tolyl)-4,6-dihydropyrano[3,2-*c*] isochromene-3-carbonitrile (4h)

Pale yellow powder; yield: 93%; mp 245–246.2 °C; IR (KBr, cm^{-1}) ν_{max}: 3426, 3315 (NH_2), 3011 (CH, aromatic), 2917 (CH, aliphatic), 2194 (C≡N), 1738 (C=O), 1688, 1640 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.14 (d, J = 6 Hz, 1H, ArH), 8.04–7.99 (m, 1H, ArH), 7.80 (d, J = 6 Hz, 1H, ArH), 7.72–7.67 (m, 1H, ArH), 7.31–7.24 (m, 3H, ArH), 7.14 (d, J = 6 Hz, 3H, ArH, NH_2), 4.67 (s, 1H, CH), 2.32 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.19 (C=O), 159.52 (C-2), 141.77, 138.52, 137.39, 136.08, 130.68, 130.05, 129.95, 129.19, 129.16, 128.90, 125.71, 120.37, 120.10, 119.99 (C≡N), 57.05 (C-3), 40.76 (C-4), 21.48 (CH_3). Anal. calcd. for $C_{20}H_{14}N_2O_3$: C, 72.72; H, 4.27; N, 8.48%. Found: C, 72.53; H, 4.11; N, 8.26%.

2-Amino-4-(3-chlorophenyl)-6-oxo-4,6-dihydropyrano [3,2-*c*]isochromene-3-carbonitrile (4i)

White powder; yield: 92%; mp 241–242 °C; IR (KBr, cm^{-1}) ν_{max}: 3429, 3315 (NH_2), 3010 (CH, aromatic), 2986 (CH, aliphatic), 2198 (C≡N), 1735 (C=O), 1687, 1642 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.13 (d, J = 6 Hz, 1H, ArH), 8.03–7.98 (m, 1H, ArH), 7.79 (d, J = 6 Hz, 1H, ArH), 7.72–7.67 (m, 1H, ArH), 7.47–7.32 (m, 6H, ArH,

NH_2), 4.81 (s, 1H, CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.11 (C=O), 159.68 (C-2), 144.17, 136.48, 136.02, 133.90, 131.18, 130.60, 130.03, 129.53, 128.39, 128.27, 127.38, 120.44, 120.26, 119.81 (C≡N), 56.36 (C-3), 39.16 (C-4). Anal. calcd. for $C_{19}H_{11}ClN_2O_3$: C, 65.06; H, 3.16; N, 7.99%. Found: C, 64.81; H, 2.99; N, 7.91%.

2-Amino-4-(3-methoxyphenyl)-6-oxo-4,6-dihydropyrano[3,2-*c*]isochromene-3-carbonitrile (4j)

Yellow powder; yield: 90%; mp 255–255.5 °C; IR (KBr, cm^{-1}) ν_{max}: 3392, 3311 (NH_2), 3060 (CH, aromatic), 2985 (CH, aliphatic), 2203 (C≡N), 1735 (C=O), 1689, 1646 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.14 (d, J = 6 Hz, 1H, ArH), 8.03–7.98 (m, 1H, ArH), 7.79 (d, J = 6 Hz, 1H, ArH), 7.71–7.66 (m, 1H, ArH), 7.35–7.30 (m, 1H, ArH), 7.24 (s, 2H, NH_2), 6.93–6.90 (m, 3H, ArH), 4.70 (s, 1H, CH), 3.76 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.17 (C=O), 159.98 (C-2), 159.58, 143.35, 137.19, 136.05, 130.65, 130.42, 130.04, 129.95, 129.25, 120.60, 120.38, 120.12, 119.94 (C≡N), 114.57, 113.17, 56.88 (C-3), 55.55 (OCH_3), 40.70 (C-4). Anal. calcd. for $C_{20}H_{14}N_2O_4$: C, 69.36; H, 4.07; N, 8.09%. Found: C, 69.23; H, 3.99; N, 7.89%.

2-Amino-4-(3-bromophenyl)-6-oxo-4,6-dihydropyrano[3,2-*c*]isochromene-3-carbonitrile (4k)

Yellow powder; yield: 92%; mp 260–261.1 °C; IR (KBr, cm^{-1}) ν_{max}: 3431, 3316 (NH_2), 3013 (CH, aromatic), 2986 (CH, aliphatic), 2199 (C≡N), 1737 (C=O), 1690, 1640 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.14 (d, J = 6 Hz, 1H, ArH), 8.04–7.98 (m, 1H, ArH), 7.79 (d, J = 6 Hz, 1H, ArH), 7.73–7.67 (m, 1H, ArH), 7.58–7.50 (m, 2H, ArH), 7.40–7.37 (m, 2H, ArH), 7.32 (s, 2H, NH_2), 4.80 (s, 1H, CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.12 (C=O), 159.68 (C-2), 144.42, 136.50, 136.03, 131.50, 131.24, 131.18, 130.60, 130.04, 129.51, 127.78, 122.53, 120.45, 120.26, 119.82 (C≡N), 56.36 (C-3), 40.26 (C-4). Anal. calcd. for $C_{19}H_{11}BrN_2O_3$: C, 57.74; H, 2.81; N, 7.09%. Found: C, 57.51; H, 2.57; N, 6.76%.

2-Amino-4-(4-fluorophenyl)-6-oxo-4,6-dihydropyrano[3,2-*c*]isochromene-3-carbonitrile (4l)

White powder; yield: 92%; mp 239–240 °C; IR (KBr, cm^{-1}) ν_{max}: 3502, 3402 (NH_2), 3008 (CH, aromatic), 2969 (CH, aliphatic), 2196 (C≡N), 1723 (C=O), 1686, 1633 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.13 (d, J = 6 Hz, 1H, ArH), 8.03–7.97 (m, 1H, ArH), 7.79 (d, J = 6 Hz, 1H, ArH), 7.71–7.66 (m, 1H, ArH), 7.44–7.39 (m, 2H, ArH), 7.27–7.20 (m, 4H, ArH, NH_2), 4.77 (s, 1H, CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.70 (C=O), 160.48 (C-2), 160.13, 159.55, 137.93, 137.89, 136.99, 136.03, 130.62, 130.57, 130.45, 130.02, 129.98, 129.27, 120.39, 120.14, 119.89 (C≡N), 116.20, 115.92, 56.80 (C-3), 40.53 (C-4). Anal. calcd. for $C_{19}H_{11}FN_2O_3$: C, 68.26; H, 3.32; N, 8.38%. Found: C, 68.19; H, 3.10; N, 8.19%.

2-Amino-4-(4-chlorophenyl)-6-oxo-4,6-dihydropyrano[3,2-*c*]isochromene-3-carbonitrile (4m)

Yellow powder; yield: 94%; mp 223–224 °C; IR (KBr, cm^{-1}) ν_{max}: 3384, 3312 (NH_2), 3008 (CH, aromatic), 2966 (CH, aliphatic), 2192 (C≡N), 1742 (C=O), 1687, 1641 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.14 (d, J = 6 Hz, 1H, ArH), 8.04–7.98 (m, 1H, ArH), 7.79 (d, J = 6 Hz, 1H, ArH), 7.72–7.67 (m, 1H, ArH), 7.48–7.38 (m, 4H, ArH), 7.30 (s, 2H, NH_2), 4.79 (s, 1H, CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.10 (C=O), 159.61 (C-2), 140.71, 136.75, 136.07, 132.85, 130.59, 130.44, 130.05, 129.39, 129.26, 120.41, 120.18, 119.83 (C≡N), 56.51 (C-3), 40.07 (C-4). Anal. calcd. for $C_{19}H_{11}ClN_2O_3$: C, 65.06; H, 3.16; N, 7.99%. Found: C, 64.77; H, 3.13; N, 7.80%.

2-Amino-4-(4-methoxyphenyl)-6-oxo-4,6-dihydropyrano[3,2-*c*]isochromene-3-carbonitrile (4n*)*

Yellow powder; yield: 91%; mp 220.5–221.3 °C; IR (KBr, cm^{-1}) ν_{max}: 3420, 3313 (NH_2), 3038 (CH, aromatic), 2967 (CH, aliphatic), 2196 (C≡N), 1725 (C=O), 1688, 1646 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.13 (d, J = 6 Hz, 1H, ArH), 8.03–7.97 (m, 1H, ArH), 7.78 (d, J = 6 Hz, 1H, ArH), 7.71–7.66 (m, 1H, ArH), 7.29–7.24 (m, 2H, ArH), 7.21 (s, 2H, NH_2), 6.98–6.93 (m, 2H, ArH), 4.66 (s, 1H, CH), 3.76 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.21 (C=O), 159.45 (C-2), 159.23, 137.55, 136.06, 134.10, 133.73, 130.69, 130.03, 129.91, 129.59, 128.99, 120.35, 120.04 (C≡N), 114.64, 57.17 (C-3), 55.56 (OCH_3), 40.52 (C-4). Anal. calcd. for $C_{20}H_{14}N_2O_4$: C, 69.36; H, 4.07; N, 8.09%. Found: C, 69.15; H, 3.85; N, 7.77%.

2-Amino-4-(4-bromophenyl)-6-oxo-4,6-dihydropyrano[3,2-*c*]isochromene-3-carbonitrile (4o)

Yellow powder; yield: 92%; mp 222–223 °C; IR (KBr, cm^{-1}) ν_{max}: 3381, 3310 (NH_2), 3008 (CH, aromatic), 2967 (CH, aliphatic), 2189 (C≡N), 1741 (C=O), 1686, 1641 (C=C); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.14 (d, J = 6 Hz, 1H, ArH), 8.04–7.99 (m, 1H, ArH), 7.79 (d, J = 6 Hz, 1H, ArH), 7.73–7.67 (m, 1H, ArH), 7.61–7.58 (m, 2H, ArH), 7.35–7.30 (m, 4H, ArH, NH_2), 4.77 (s, 1H, CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.10 (C=O), 159.60 (C-2), 141.13, 136.69, 136.08, 133.67, 132.19, 130.79, 130.58, 130.06, 129.39, 121.41, 120.41, 120.18, 119.83 (C≡N), 56.43 (C-3), 40.14 (C-4). Anal. calcd. for $C_{19}H_{11}BrN_2O_3$: C, 57.74; H, 2.81; N, 7.09%. Found: C, 57.72; H, 2.63; N, 6.89%.

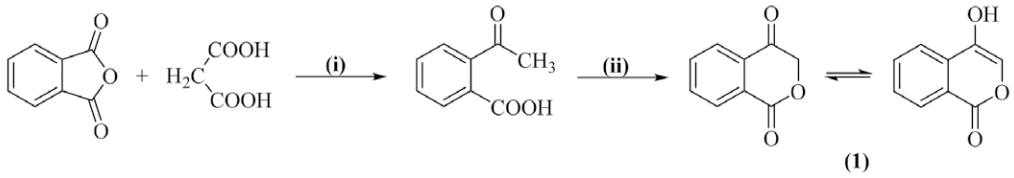
2. 3. Cytotoxicity Assay

The three cell lines were cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and incubated at 37 °C, 5% CO_2, and 95% relative humidity up to at least 80% confluent. Then, the cells were trypsinized, cultured in 96-well microplates ($1{\cdot}10^4$ cells in each well), and incubated in the mentioned conditions for 24 h. The next day, different

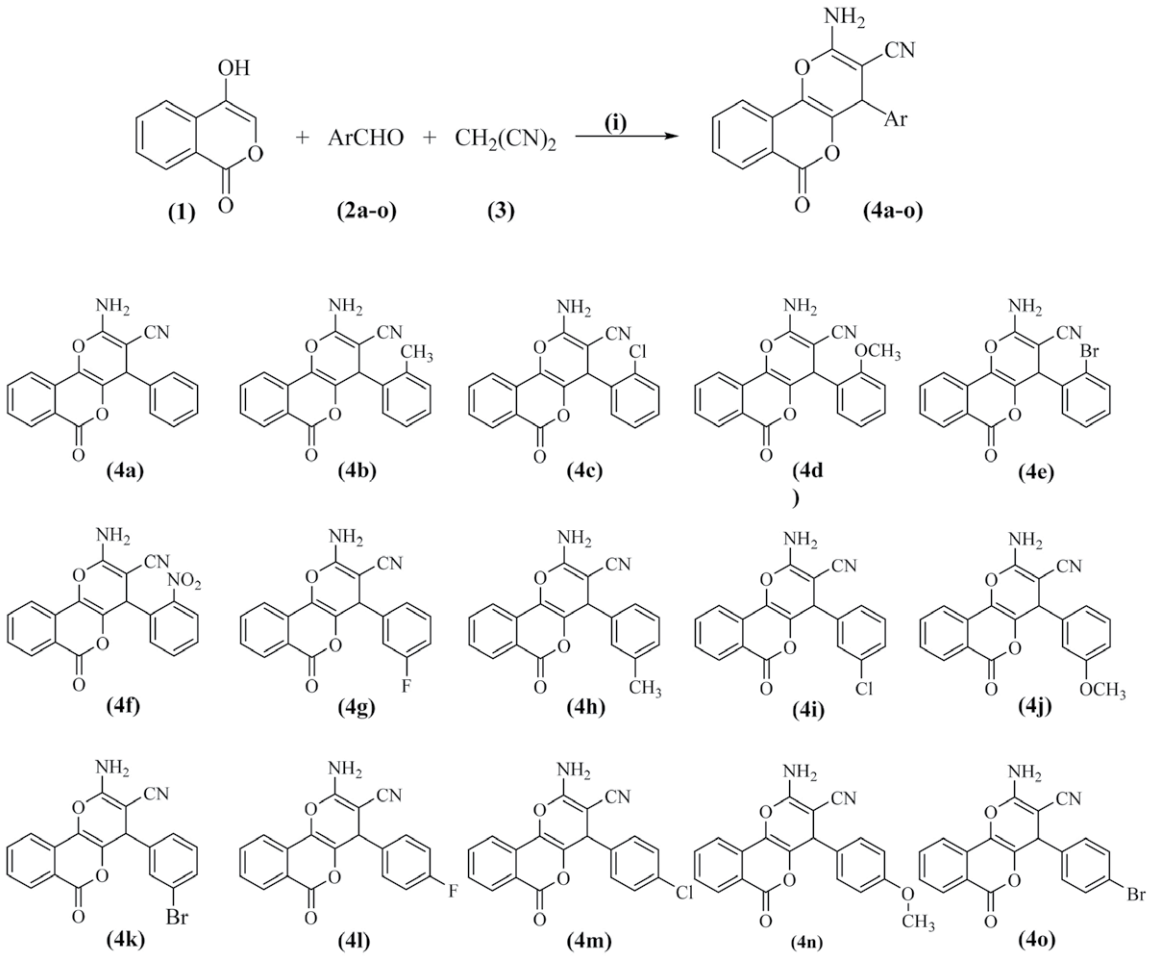
concentrations of the compounds were added to the wells (at least three wells for each concentration), and the cells were incubated for a further 24 h. Finally, 10 μL of 5 mg/mL MTT solution was added to the wells, and the microplates were incubated for 3 h protected from light. The formed formazan crystals were solubilized in 100 μL DMSO. The absorbance was measured at 570 nm with a reference of 620 nm. The experiment was repeated 3 times. Doxorubicin was used as the reference drug. The IC_{50} values were calculated using a non-linear curve of dose-response in GraphPad® Prism from the percent of viable cells *vs.* logarithm of concentrations. One way ANOVA with Tukey's post-hoc was used for investigating the statistical significance of the differences.

3. Results and Discussions

To begin our research, we have synthesized 4-hydroxyisocoumarin (**1**) by the reported procedure as substrate to prepare **4a**–**o** derivatives.[14] The reaction of phthalic anhydride with malonic acid in the presence of triethylamine as the solvent/base at 80 °C for 10 h, and then neutralized with aqueous HCl resulted in the formation of 2-acetylbenzoic acid. Treatment of 2-acetylbenzoic acid with Br_2/HBr in chlorobenzene at 30 °C for 3 h, then addition of H_2O and reflux for 3 h as the next step, afforded the formation of 4-hydroxyisocoumarin (**1**) in good yield (Scheme 1).



Scheme 1. Synthesis of 4-hydroxyisocoumarin. Reagents and conditions: (i): 1) Et_3N, heat; 2) HCl (aq); (ii): 1) Br_2/HBr, chlorobenzene; 2) H_2O, reflux.



Scheme 2. Synthesis of 2-amino-4,6-dihydropyrano[3,2-*c*]isochromene-3-carbonitrile derivatives **4a**–**o**. Reagents and conditions: (i): Et_3N, EtOH, reflux.

Afterwards, in continuation of our previous work on environmental friendly multi-component reactions,[15–17] we continued with the synthesis as a one-pot three component reaction of 4-hydroxyisocoumarin (**1**), aromatic aldehydes **2a**–**o**, and malononitrile (**3**) in the presence of three drops of triethylamine in ethanol as the solvent and at reflux conditions. After completion of the reactions, the crude products were purified by recrystallization and a series of 2-amino-4,6-dihydropyrano[3,2-*c*]isochromene-3-carbonitrile derivatives **4a**–**o** was prepared in 90–94% yields (Scheme 2).

The reaction first leads to Knoevenagel condensation between aromatic aldehydes **2a**–**o** and malononitrile (**3**) in the presence of Et_3N, followed by dehydration, affording various arylidene malononitriles. Thereafter, Michael addition reaction between 4-hydroxyisocoumarin (**1**) and various arylidene malononitriles led to completion of the reaction and to the synthesis of **4a**–**o** derivatives in good yields without any byproducts.

For benzaldehyde derivatives, the presence of electron-donating and electron-withdrawing substituents in the *ortho*- (**4b**–**f**), *meta*- (**4g**–**k**), and *para*- (**4l**–**o**) positions were synthesized. These compounds were fully characterized by standard spectroscopic techniques (IR, ^1H and ^{13}C NMR) and elemental analyses.

The IR spectrum of **4a** showed the presence of NH_2 at 3461 and 3318 cm^{-1}, CN at region 2197 cm^{-1}, and one sharp band at 1721 cm^{-1} due to the vibration of the carbonyl (C=O) group. The ^1H NMR spectrum of **4a** indicated one kind of aliphatic proton at 4.42 ppm. On the other hand, the aryl protons displayed at 8.13–7.31 ppm (9H, m), and NH_2 group appeared at 7.25 ppm (2H, s). The ^{13}C NMR of compound **4a** showed two signals at 160.16 and 119.96 ppm, which occur due to the carbonyl and CN carbons, respectively. The aromatic carbons appeared at 141.77–120.09 ppm. Two signals reflected the shift of carbons at 56.95 and 40.76 ppm, which are due to the C3 and C4, respectively.

The MTT assay, which is one of the simplest methods for screening of the effects of compounds on viability of cultured cells, was used in this study. It relies on converting the yellow tetrazolium dye into the violet formazan crystals by the mitochondrial enzymes of live cells.[18] Based on the previous definitions, IC_{50} > 500 μg/mL was considered as non-toxic, IC_{50} values of 201 to 500 μg/mL as weakly toxic, and IC_{50} values of 21 to 200 μg/mL as moderately toxic.[19] The data obtained by this assay showed that most of the compounds are non-toxic for A549 cell line and only five (**4a**, **4b**, **4f**, **4j**, **4o**) compounds were weakly toxic on these cells. However, in case of MCF-7 cells, compounds **4b**, **4d**, **4f**, **4g**, **4h**, **4i**, **4k**, **4l**, and **4o** had weak cytotoxicity and five compounds were non-toxic. Additionally, compound **4j** was moderately toxic for MCF-7 cell line. In contrast, four of the compounds were non-toxic for 3T3 (the embryonic mouse fibroblasts considered as normal cells), while the rest of the compounds showed weak (6 compounds) and moderate cytotoxicity (**4a**, **4b**, **4d**, **4f**, and **4j**). The IC_{50} values are presented in Table 1. The data indicate that some of these compounds inhibit the proliferation of cancer cells in a dose-dependent manner, as shown in Figure 2 for three of the compounds that were more toxic on MCF-7 cells.

Overall, addition of a CH_3 or NO_2 group at *ortho* position of the aromatic ring can improve the antiproliferative effects of the compounds (**4b** and **4f** are more toxic than **4a** on MCF-7 cell line). However, in *meta* position, the CH_3 and especially OCH_3 groups work better (**4h** and **4j** are more potent compared to **4a**). In *para* position, only Br and somewhat F substituents could slightly enhance the cytotoxicity of the compounds (**4o** and **4l**). Compounds with these structures were not previously synthesized and evaluated in term of biological effects. However, Cholayil Palapetta *et al.* evaluated the anticancer activity of some dihydropyrano[3,2-*c*]chromene derivatives and observed that some of the compounds had moderate cytotoxicity on HT-29 cell line.[20]

Table 1. IC_{50} (μg/mL) values of the synthesized compounds for A549, MCF-7, and 3T3 cell lines presented as mean ± S.E.M.

Compd. No.	A549	MCF-7	3T3
4a	485.33 ± 12.93	>500	169.01 ± 8.14
4b	459.95 ± 19.65	204.41 ± 6.90	200.84 ± 7.28
4c	>500	>500	>500
4d	>500	497.51 ± 16.21	185.25 ± 7.80
4e	>500	>500	>500
4f	473.02 ± 17.74	247.45 ± 9.18	175.83 ± 8.99
4g	>500	492.95 ± 12.50	313.48 ± 15.03
4h	>500	375.71 ± 15.36	265.13 ± 7.78
4i	>500	443.02 ± 10.28	336.65 ± 10.86
4j	375.50 ± 15.81	112.67 ± 7.39	68.24 ± 6.99
4k	>500	486.51 ± 14.72	414.67 ± 13.05
4l	>500	454.31 ± 21.06	208.40 ± 6.73
4m	>500	>500	>500
4n	>500	>500	>500
4o	377.29 ± 12.58	332.10 ± 15.62	382.04 ± 9.11
Doxorubicin	7.15 ± 0.57	4.21 ± 0.36	4.37 ± 0.62

4. Conclusion

In summary, we have reported an efficient procedure for the one-pot three component reaction of 4-hydroxyisocoumarin, various aromatic aldehydes, and malononitrile, which leads to the synthesis of new 2-amino-4,6-dihydropyrano[3,2-*c*]isochromene-3-carbonitrile derivatives. These reactions were performed in the presence of triethylamine in ethanol as the solvent at reflux conditions. Simple operation procedure, clean reaction conditions, easy to obtain products, and good yields are advantages of this procedure. The synthesized compounds showed no or

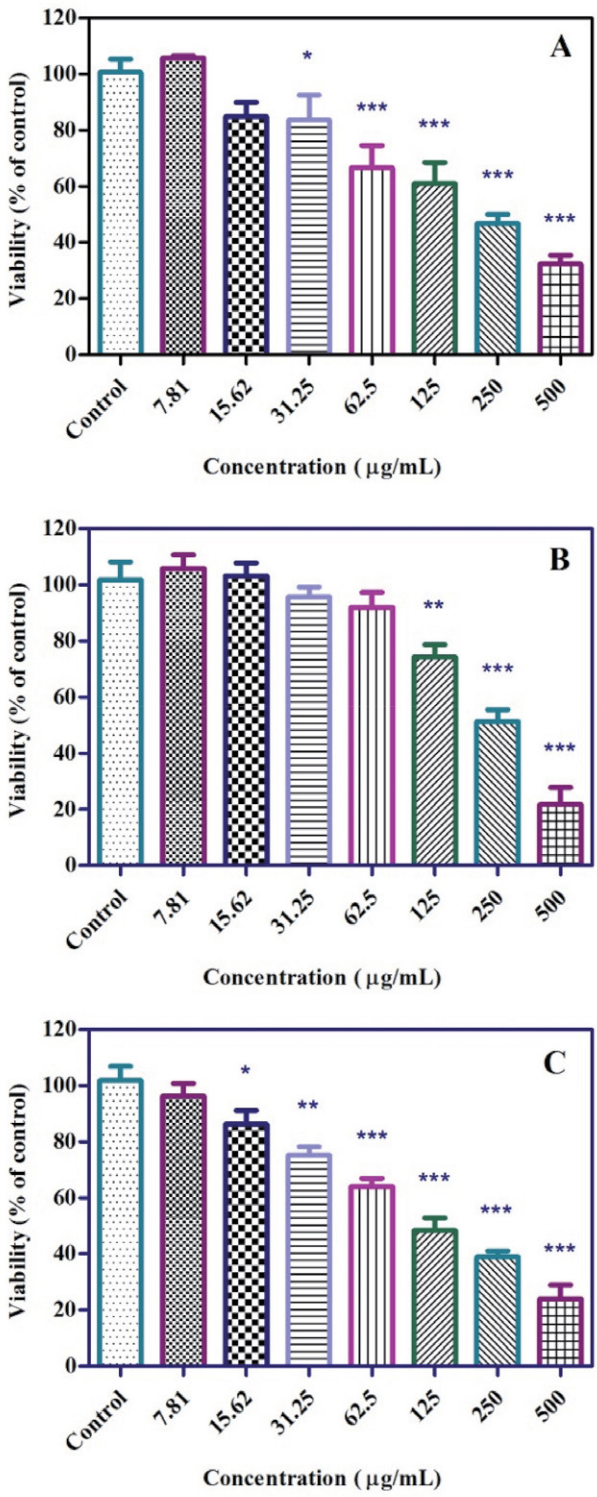


Figure 2. The viability percents of MCF-7 cells treated with different concentrations of compound A: **4b**; B: **4f**; and C: **4j** for 24 hours. *: *p-value* < 0.05; ** *p-value* < 0.01; ***: *p-value* < 0.001 compared to the control group.

low toxicity on A549 and MCF-7 cell lines. However, compounds **4b**, **4f**, and **4j** can be considered as lead compounds for further research. Most of the compounds showed moderate toxicity on 3T3 cells. Nevertheless, the compounds that are non-toxic or weakly toxic on this cell line, might be good candidates for investigating other pharmacological activities such as antibacterial, antifungal, antiviral, etc.

Abbreviations

Et_3N: triethylamine; MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HUVEC: Human Umbilical Vein Endothelial Cells; IC50: 50% Inhibition concentration; KBr: Potassium bromide; ppm: parts per million; TLC: thin layer chromatography.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.17344/acsi.2024.9050

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Authors' contributions

Mehdi Abaszadeh, designed, synthesized and performed experiments, analysed data and wrote the paper. Salehe Sabouri and Fatemeh Haghani, designed and performed the biologic assay and data analysis and contributed in writing the manuscript. All authors were involved in revising the content, agree to take accountability for the integrity and accuracy of the work, and have read and approved the final manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Povzetek

S pomočjo enolončne trokomponentne sinteze med 4-hidroksiizokumarinom, različnimi aromatskimi aldehidi in malononitrilom v prisotnosti trietilamina v EtOH pod pogoji refluksa smo pripravili serijo novih 2-amino-4,6-dihidropirano[3,2-*c*]izokromen-3-karbonitrilnih derivatov (**4a**–**o**). Vse nove spojine smo karakterizirali s standardnimi spektroskpskimi tehnikami (FT-IR, ^{1}H in ^{13}C NMR) in z elementno analizo. S pomočjo MTT testa na eni normalni (3T3) in dveh rakastih celičnih linijah (A549 in MCF-7) smo preučili citotoksičnost pripravljenih spojin. Ugotovili smo, da so nekatere izmed teh spojin (**4b**, **4f** in **4j**) citotoksične predvsem za MCF-7 celično linijo in bi zato lahko bile spojine vodnice za nadaljnje raziskave. Po drugi strani pa so ostale spojine izkazale nizko citotoksičnost in bi zato lahko bile primerni kandidati z drugačnimi farmakološkimi učinki, npr. antibakterijskimi, antiglivičnimi, antivirusnimi itd.