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RESEARCH ARTICLE

Synthesis and Cytotoxic Activity of Novel Mono- and Bis-Indole Derivatives: Analogues of Marine Alkaloid Nortopsentin

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ARTICLE HISTORY

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Abstract: ***Background*:** The oceans cover more than 70% of the earth's surface, which represents over 95% of the biosphere. Therefore, oceans provide a wealth of marine invertebrates, especially sponges, ascidians, bryozoans and molluscs that produce structurally unique bioactive metabolites such as alkaloids. The bioactive scaffolds of marine alkaloids exhibit cytotoxic activities against human cancer cell lines.

***Objective*:** To prepare analogues of the marine alkaloid nortopsentin [having 2,4-bis(3'-indolyl)imidazole scaffold] as cytotoxic agents *via* structural modification of the core imidazole ring and one of the side indole rings.

***Method*:** Four series of nortopsentin analogues were synthesized in which the imidazole ring was replaced by pyrazole, pyrido[2,3-*d*]pyrimidinone and pyridine rings. Furthermore, one of the side indole rings was replaced by substituted phenyl moiety. The target compounds were tested for their *in vitro* cytotoxic activity against HCT-116 cell-line and the most potent compound was subjected to further investigation on its effect on HCT-116 cell cycle progression.

***Results*:** The cytotoxic screening of the synthesized compounds revealed that bis-indolylpyridine-dicarbonitriles **8a-d** exhibited the most potent cytotoxic activity with IC_{50}=2.6-8.8 µM. Compound **8c** was further tested by flow cytometry analysis to explore its effect on HCT-116 cell cycle progression that, in turn, indicated its anti-proliferative effect.

***Conclusion*:** Marine-derived bis-indole alkaloids (nortopsentins) have emerged as a new class of indole-based antitumor agents. The design of new analogues involved several modifications in order to obtain more selective and potent cytotoxic agents. Indole derivatives bearing a pyridine core displayed more potent cytotoxic activity than those containing pyrido[2,3-*d*]pyrimidin-4(1*H*)-one moiety.

Keywords: 3-Indolyl pyrazoles, 3-indolyl pyrido[2,3-*d*]pyrimidines, bis-indole derivatives, Nortopsentins, Anticancer activity, Cell cycle analysis.

1. INTRODUCTION

Cancer is the second leading cause of death all over the world. Its incidence and mortality are increasing rapidly worldwide. The extraordinary diversity of cancer is captured by the variations in the magnitude and profile of the disease between and within world regions [1]. Thus there is always a constant need to develop alternative or synergistic anticancer drugs with minimal side effects [2].

Recent clinical and preclinical data indicated that cancer is a polygenic disease that involves the de-regulation of extended networks of proteins [3, 4]. A therapeutic proposal based on multiple target modulation may provide a more efficient treatment strategy producing greater benefits than those obtained with single-target approaches [3]. One of the major contributing factors in cancer is protein kinases, as they play a critical role in cell regulation and de-regulation [5].

Wide-ranging attention had been focused on marine natural products, due to their unique scaffolds resulted from the harsh and competitive conditions in the marine environment [4, 6, 7]. Since 1990's, several marine natural products dragged up from marine invertebrates, especially sponges, ascidians, bryozoans and molluscs [7, 8]. A remarkable number of marine-derived compounds target oncogene kinases of signaling pathways that are related to tumorigenesis and tumor progression [4].

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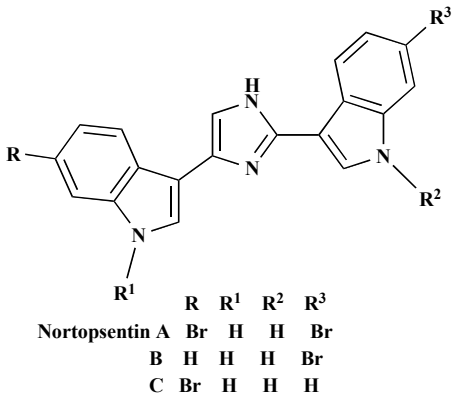


Fig. (1). Nortopsentin A-C.

The alkaloids nortopsentins A–C (Fig. **1**) were isolated from the Caribbean deep-water sponge *Spongosorites ruetzleri* and had a 2,4-bis (3'-indolyl)imidazole structural skeleton. They exhibit *in vitro* anti-tumor activity against P338 cells (IC_{50}, 4.5–20.7 μM) and also antifungal activity against *Candida albicans* [7, 9-11]. Recently, nortopsentin alkaloids A-C were reported to have potent antiviral, anti-phytopathogenic-fungus, and insecticidal activities [12].

The unique structures and biological activities of nortopsentins had attracted many researchers in medicinal chemistry during the last two decades. The structural modification of natural nortopsentins was directed towards the replacement of imidazole ring by other five or six membered rings such as thiazole [11, 13-20], thiophene [21], pyrazole [22], furan, isoxazole [23], oxazole [16], pyrrole [24], oxadiazole [25, 26] and pyridine rings [27]. Alternatively, structural modification of nortopsentins involved replacement of one or both indolyl rings by or both indolyl rings by substituted phenyl rings, thienyl, pyridyl [12, 16], pyrrolo[2,3-*b*]pyridines [18, 20, 26], pyrrolo[3,2-*b*]pyridines [11, 14, 15, 17] and pyrrolo[2,3-*c*]pyridines [19]. Indeed, some of these analogues were reported to act as CDK1 inhibitors [11, 14, 17], exhibited strong inhibitory activity against tumor cancer cell lines and caused cell cycle arrest at G2/M phase [11, 14, 15, 17-19].

Our aim was to prepare multi-target anticancer agents guided with the privileged scaffold of nortopsentins. The design of new indole derivatives was performed as follows (Fig. **2**):

- Analogues of nortopsentins were prepared in which the core scaffold imidazole ring was replaced with a pyrazole ring. One of the side indole rings was replaced with a substituted aromatic ring linked to pyrazole ring with thiourea moiety (compounds **3a-d**).
- Another series of nortopsentin analogues are compounds (**6a-d** and **7a-c**), in which pyrido[2,3-*d*]pyrimidin-4(1*H*)-one replaced the core imidazole ring. Additionally, one of the side indole rings was replaced with a substituted aromatic ring.
- A series of 2,6-di(3'-indolyl)pyridines **8a-d** was synthesized as nortopsentins analogues in which the imidazole ring (core scaffold) was replaced with 4-substituted phenyl-1,4-dihydropyridine ring while the two side indole rings were kept constant.

The synthesized compounds were tested for their *in vitro* anti-proliferative activities towards HCT-116 cell-line. The most potent compound was subjected to further investigation on its effect on HCT-116 cell cycle progression.

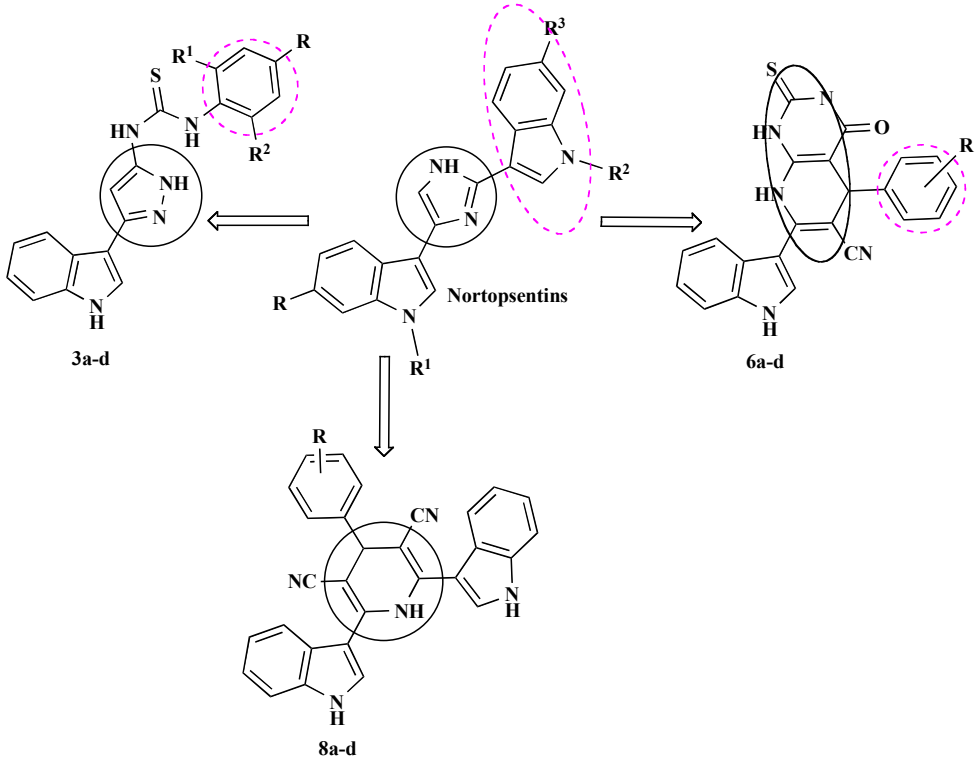


Fig. (2). The design of nortospentin analogues **3**,**6** and **8**.

2. EXPERIMENTAL

2.1. Chemistry

All the melting points were determined on the Stuart apparatus and the values given are uncorrected. The IR spectra were determined using KBr discs on Shimadzu IR 435 spectrophotometer, Microanalytical unit, Faculty of pharmacy, Cairo University and the values are represented in cm^{-1}. The ^1H NMR and ^{13}C NMR spectra were carried out using a 400 MHz Bruker spectrophotometer, Microanalytical unit, Faculty of pharmacy, Cairo University, Egypt, using TMS as an internal standard. Chemical shift values are recorded in ppm on δ scale. The elemental analyses were carried out at the Regional center for Mycology and Biotechnology, Azher University, Egypt. The progress of the reactions was monitored by TLC using TLC sheets pre-coated with UV fluorescent silica gel Merck 60 F 254 and the sheets were visualized using a UV lamp. Reagents purchased commercially were used without further purification. Solvents were dried using standard procedures. The starting compounds 3-cyanoacetylindole (**1**) and aminopyrazole derivative **2** were prepared as reported in the literature [28, 29].

2.1.1. General Procedure for the Synthesis of 1-(3-(1H-indol-3-yl)-1H-pyrazol-5-yl)-3-(substituted phenyl)thioureas 3a-d

A mixture of compound **2** (0.50 g, 0.0025 mol) and selected isothiocyanates (0.0025 mol) in dry dioxane (25 mL) was stirred at room temperature for 48 h in the presence of anhydrous K_2CO_3 (0.35 g, 0.0025 mol) as a catalyst. The mixture was poured onto ice/water mixture (25 mL), the separated solid was filtered, dried and crystallized from methanol.

2.1.2. 1-(3-(1H-Indol-3-yl)-1H-pyrazol-5-yl)-3-(2-chloro-6-methylphenyl)thiourea (3a)

Yield (75%), mp (204-206 °C), IR (KBr) cm^{-1}: 3421-3124 (4 NH), 2962, 2858 (CH-aliphatic); ^1H-NMR (DMSO-d_6, ppm) δ: 2.31 (s, 3H, $-CH_3$), 6.33 (s, 1H, pyrazole Ar-H), 7.15-7.81 (m, 8H, Ar-H), 10.90 (s, 1H, NH, D_2O exchangeable), 11.29 (s, 1H, NH, D_2O exchangeable), 11.54 (s, 1H, NH, D_2O exchangeable), 12.70 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 18.9 (CH_3), 91.2 (pyrazole CH), 104.7, 112.6, 119.2, 120.6, 122.4, 124.4, 124.8, 127.4, 128.8, 129.5, 132.6, 135.7, 136.8, 138.8, 139.6, 150.6 (Ar-C), 178.4 (C=S). Anal. calc for $C_{19}H_{16}ClN_5S$: C, 59.76, H, 4.22, N, 18.34. Found: C, 59.89, H, 4.37, N, 18.21.

2.1.3. 1-(3-(1H-Indol-3-yl)-1H-pyrazol-5-yl)-3-(4-chlorophenyl)thiourea (3b)

Yield (90%), mp (188-190°C), IR (KBr) cm^{-1}: 3437-3124 (4 NH); ^1H-NMR (DMSO-d_6, ppm) δ: 6.35 (s, 1H, pyrazole Ar-H), 7.15-7.82 (m, 9H, Ar-H), 10.85 (s, 1H, NH, D_2O exchangeable), 11.55 (s, 1H, NH, D_2O exchangeable), 11.91 (s, 1H, NH, D_2O exchangeable), 12.80 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 91.4 (pyrazole CH), 104.7, 112.6, 119.2, 120.6, 122.4, 124.5, 124.7, 126.2, 128.8, 129.5, 136.8, 138.4, 138.8, 150.4 (Ar-C), 176.7 (C=S). Anal. calc. for $C_{18}H_{14}ClN_5S$: C, 58.77, H, 3.84, N, 19.04. Found: C, 59.01, H, 3.91, N 19.32.

2.1.4. 1-(3-(1H-Indol-3-yl)-1H-pyrazol-5-yl)-3-(4-methoxyphenyl)thiourea (3c)

Yield (88%), mp (197-199°C), IR (KBr) cm^{-1}: 3390-3120 (4 NH), 2931, 2835 (CH-aliphatic); ^1H-NMR (DMSO-d_6, ppm) δ: 3.78 (s, 3H, OCH_3), 6.33 (s, 1H, pyrazole Ar-H), 6.95-7.81 (m, 9H, Ar-H), 10.67 (s, 1H, NH, D_2O exchangeable), 11.54 (s, 1H, NH, D_2O exchangeable), 11.70 (s, 1H, NH, D_2O exchangeable), 12.74 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 55.7 (OCH_3), 91.2 (pyrazole CH), 104.7, 112.6, 114.1, 119.2, 120.6, 122.4, 124.4, 124.7, 126.5, 132.3, 136.8, 138.8, 150.6, 157.3 (Ar-C), 177.1 (C=S). Anal. calc. for $C_{19}H_{17}N_5OS$: C, 62.79, H, 4.71, N, 19.27. Found: C, 62.63, H, 4.88, N, 19.11.

2.1.5. 1-(3-(1H-Indol-3-yl)-1H-pyrazol-5-yl)-3-(p-tolyl)thiourea (3d)

Yield (92%), mp (198-200°C), IR (KBr) cm^{-1}: 3406-3124 (4 NH), 2962, 2862 (CH-aliphatic); ^1H-NMR (DMSO-d_6, ppm) δ: 2.32 (s, 3H, CH_3), 6.35 (s, 1H, pyrazole Ar-H), 7.15-7.83 (m, 9H, Ar-H), 10.72 (s, 1H, NH, D_2O exchangeable), 11.55 (s, 1H, NH, D_2O exchangeable), 11.83 (s, 1H, NH, D_2O exchangeable), 12.77 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 21.0 (CH_3), 91.3 (pyrazole CH), 104.7, 112.6, 119.2, 120.6, 122.4, 124.5, 124.6, 124.8, 129.4, 134.9, 136.8, 136.9, 138.8, 150.6 (Ar-C), 176.7 (C=S). Anal. calc. for $C_{19}H_{17}N_5S$: C, 65.68, H, 4.93, N, 20.16. Found: C, 65.41, H, 5.14, N, 20.32.

2.1.6. General Procedure for the Synthesis of 2-(1H-indole-3-carbonyl)-3-(substituted phenyl)acrylonitriles 4a-d

A mixture of compound **1** (0.5 g, 0.0027 mol), substituted benzaldehyde (0.0027 mol), and 4-5 drops of piperidine in absolute ethanol (5 mL) was heated under reflux for 5 h. The mixture was allowed to cool, the separated solid was filtered, dried and crystallized from methanol except for compound **4d** which was crystallized from glacial acetic acid.

2.1.7. 3-(3-Fluorophenyl)-2-(1H-indole-3-carbonyl)acrylonitrile (4a)

Yield (89%), mp (246-248°C), IR (KBr) cm^{-1}: 3224 (NH), 2222 (CN), 1597 (C=O); ^1H-NMR (DMSO-d_6, ppm) δ: 7.26-8.25 (m, 9H, Ar-H), 8.48 (s, 1H, =CH), 12.33 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 113.0, 113.4, 113.9, 116.8, 117.1, 117.7, 119.3, 121.7, 123.0, 124.1, 126.4, 131.7, 135.2, 136.9, 150.8, 161.2, 163.6 (Ar-C and CN), 181.5 (C=O). Anal. calc. for $C_{18}H_{11}FN_2O$: C, 74.47, H, 3.82, N, 9.65. Found: C, 74.79, H, 3.96, N, 9.89.

2.1.8. 2-(1H-Indole-3-carbonyl)-3-(2-methoxyphenyl)acrylonitrile (4b)

Yield (94%), mp (239-241°C), IR (KBr) cm^{-1}: 3217 (NH), 2924 (CH aliphatic), 2218 (CN), 1597 (C=O); ^1H-NMR (DMSO-d_6, ppm) δ: 3.89 (s, 3H, OCH_3), 7.15-8.41 (m, 9H, Ar-H), 8.45 (s, 1H, =CH), 12.29 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 56.4 (OCH_3), 111.9, 112.4, 113.0, 114.1, 118.2, 121.1, 121.3, 121.8, 122.9, 124.1, 126.5, 128.9, 134.8, 136.0, 137.1, 147.5, 158.9 (Ar-C and CN), 181.6 (C=O). Anal. calc. for $C_{19}H_{14}N_2O_2$: C, 75.48, H, 4.67, N, 9.27. Found: C, 75.79, H, 4.85, N, 9.38.

2.1.9. 2-(1H-Indole-3-carbonyl)-3-(m-tolyl)acrylonitrile (4c)

Yield (69%), mp (188-190°C), IR (KBr) cm^{-1}: 3224 (NH), 2924 (CH aliphatic), 2218 (CN), 1600 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.39 (s, 3H, CH_3), 7.26-8.20 (m, 9H, Ar-H), 8.46 (s, 1H, =CH), 12.29 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 21.3 ($\underline{C}H_3$), 111.7, 112.9, 114.0, 118.1, 121.8, 122.9, 124.0, 126.5, 127.7, 129.5, 131.4, 132.8, 133.4, 136.4, 137.1, 138.9, 152.6 (Ar-C and CN), 181.8 (C=O). Anal. calc. for $C_{19}H_{14}N_2O$: C, 79.07, H, 4.93, N, 9.78. Found: C, 79.43, H, 5.11, N, 9.94.

2.1.10. 2-(1H-Indole-3-carbonyl)-3-(2-nitrophenyl)acrylonitrile (4d)

Yield (58%), mp (250-252°C), IR (KBr) cm^{-1}: 3224 (NH), 2233 (CN), 1600 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 7.30-8.45 (m, 9H, Ar-H), 8.64 (s, 1H, =CH), 12.41 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 113.0, 113.7, 116.2, 116.5, 121.7, 123.2, 124.3, 125.6, 126.3, 129.4, 131.2, 132.4, 135.2, 136.8, 137.2, 147.6, 151.3 (Ar-C and CN), 181.1 (C=O). Anal. calc. for $C_{18}H_{11}N_3O_3$: C, 68.14, H, 3.49, N, 13.24. Found: C, 68.01, H, 3.71, N, 13.12.

2.1.11. General Procedure for the Synthesis of 7-(1H-indol-3-yl)-4-oxo-2-thioxo-5-(substituted phenyl)-1,2,3,4,5,8-hexahydropyrido[2,3-d]pyrimidine-6-carbonitriles 6a-d

A mixture of 6-aminothiouracil (**5**) (0.15 g, 0.001 mol) and acrylonitriles **4a-d** (0.001 mol) in glacial acetic acid (20 mL) was heated under reflux for 17 h (except for compound **6d** which was refluxed for 22 h). A solid was precipitated on hot. The mixture was concentrated under reduced pressure, the separated solid was filtered, dried and crystallized from ethanol.

2.1.12. 5-(3-Fluorophenyl)-7-(1H-indol-3-yl)-4-oxo-2-thioxo-1,2,3,4,5,8-hexahydropyrido[2,3-d]pyrimidine-6-carbonitrile (6a)

Yield (61%), mp (>300°C), IR (KBr) cm^{-1}: 3545-3251 (4 NH), 2194 (CN), 1631 (C=O), 1246 (C=S); ^{1}H-NMR (DMSO-d_6, ppm) δ: 4.68 (s, 1H, $\underline{H}$C), 7.09-7.91 (m, 9H, Ar-H), 8.92 (s, 1H, NH, D_2O exchangeable), 11.76 (s, 1H, NH, D_2O exchangeable), 11.89 (s, 1H, NH, D_2O exchangeable), 12.30 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 39.0 (H$\underline{C}$), 83.1, 91.1, 107.1, 112.9, 114.3, 114.6, 114.9, 120.4, 120.8, 122.9, 124.1, 124.6, 128.6, 130.9, 136.6, 143.0, 144.2, 147.9, 160.5 (Ar-C and CN), 161.5 (C=O), 174.1 (C=S). Anal. calc. for $C_{22}H_{14}FN_5OS$: C, 63.60, H, 3.40, N, 16.86. Found: C, 63.87, H, 3.23, N, 16.71.

2.1.13. 7-(1H-Indol-3-yl)-5-(2-methoxyphenyl)-4-oxo-2-thioxo-1,2,3,4,5,8-hexahydropyrido[2,3-d]pyrimidine-6-carbonitrile (6b)

Yield (66%), mp (>300°C), IR (KBr) cm^{-1}: 3549-3228 (4 NH), 2954, 2835 (CH aliphatic), 2194 (CN), 1631 (C=O), 1253 (C=S); ^{1}H-NMR (DMSO-d_6, ppm) δ: 3.80 (s, 3H, O$\underline{C}H_3$), 4.92 (s, 1H, $\underline{H}$C), 6.91-7.82 (m, 9H, Ar-H), 8.84 (s, 1H, NH, D_2O exchangeable), 11.69 (s, 1H, NH, D_2O exchangeable), 11.81 (s, 1H, NH, D_2O exchangeable), 12.20 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 34.2 (H$\underline{C}$), 56.2 (O$\underline{C}H_3$), 83.2, 90.8, 107.4, 112.2, 112.8, 120.3, 120.7, 120.8, 121.0, 122.8, 124.7, 128.2, 128.9, 129.8, 132.8, 136.5, 142.8, 144.7, 157.5 (Ar-C and CN), 160.3 (C=O), 173.9 (C=S). Anal. calc. for $C_{23}H_{17}N_5O_2S$: C, 64.60, H, 4.01, N, 16.38. Found: C, 64.49, H, 4.19, N, 16.54.

2.1.14. 7-(1H-Indol-3-yl)-4-oxo-2-thioxo-5-(m-tolyl)-1,2,3,4,5,8-hexahydropyrido[2,3-d]pyrimidine-6-carbonitrile (6c)

Yield (64%), mp (>300°C), IR (KBr) cm^{-1}: 3545-3251 (4 NH), 2970, 2908 (CH aliphatic), 2194 (CN), 1635 (C=O), 1246 (C=S); ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.32 (s, 3H, $\underline{C}H_3$), 4.57 (s, 1H, $\underline{H}$C), 7.08-7.89 (m, 9H, Ar-H), 8.88 (s, 1H, NH, D_2O exchangeable), 11.76 (s, 1H, NH, D_2O exchangeable), 11.87 (s, 1H, NH, D_2O exchangeable), 12.27 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 21.6 ($\underline{C}H_3$), 39.1 (H$\underline{C}$), 83.8, 91.7, 107.2, 112.9, 120.4, 120.7, 120.9, 122.9, 124.6, 125.1, 128.3, 128.50, 128.54, 128.9, 136.6, 138.0, 142.6, 144.0, 145.1 (Ar-C and CN), 160.5 (C=O), 174.0 (C=S). Anal. calc. for $C_{23}H_{17}N_5OS$: C, 67.13, H, 4.16, N, 17.02. Found: C, 67.40, H, 4.18, N, 16.89

2.1.15. 7-(1H-Indol-3-yl)-5-(2-nitrophenyl)-4-oxo-2-thioxo-1,2,3,4,5,8-hexahydropyrido[2,3-d]pyrimidine-6-carbonitrile (6d)

Yield (60%), mp (>300°C), IR (KBr) cm^{-1}: 3549-3194 (4 NH), 2202 (CN), 1658 (C=O), 1242 (C=S); ^{1}H-NMR (DMSO-d_6, ppm) δ: 5.48 (s, 1H, $\underline{H}$C), 7.18-7.95 (m, 9H, Ar-H), 8.93 (s, 1H, NH, D_2O exchangeable), 11.91 (s, 1H, NH, D_2O exchangeable), 12.13 (s, 1H, NH, D_2O exchangeable), 12.27 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 33.7 (H$\underline{C}$), 82.1, 84.4, 91.6, 107.0, 112.9, 120.4, 120.8, 123.0, 124.1, 124.6, 128.7, 131.9, 134.2, 136.6, 139.2, 143.9, 144.1, 148.9, 157.7 (Ar-C and CN), 160.4 (C=O), 174.0 (C=S). Anal. calc. for $C_{22}H_{14}N_6O_3S$: C, 59.72, H, 3.19, N, 18.99. Found: C, 59.89, H, 3.40, N, 19.25.

2.1.16. General Procedure for the Synthesis of 7-(1H-indol-3-yl)-5-(substituted phenyl)-2-(methylthio)-4-oxo-1,4,5,8-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitriles 7a-c

The selected pyridopyrimidine **6a-c** (0.0025 mol) was dissolved in 10% aqueous KOH (2.5 g of KOH in 25 mL of water), then dimethyl sulphate (0.0025 mol) was added. The mixture was stirred overnight, acidified with glacial acetic acid (5 mL) then left to settle in the fridge overnight. The obtained solid was filtered, dried and crystallized from ethyl acetate.

2.1.17. 5-(3-Fluorophenyl)-7-(1H-indol-3-yl)-2-(methylthio)-4-oxo-1,4,5,8-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (7a)

Yield (65%), mp (233-235°C), IR (KBr) cm^{-1}: 3410-3360 (3 NH), 2198 (CN), 1651 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.52 (s, 3H, S$\underline{C}H_3$), 4.77 (s, 1H, $\underline{H}$C), 7.08-7.84 (m, 9H, Ar-H), 10.02 (s, 1H, NH, D_2O exchangeable), 11.77 (s, 1H, NH, D_2O exchangeable), 12.47 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 13.1 (S$\underline{C}H_3$), 39.0 (H$\underline{C}$), 82.1, 95.1, 107.6, 112.6, 114.2, 114.4, 120.3, 121.3, 122.4, 123.8, 125.4, 128.4, 131.0, 136.4, 145.5, 148.72, 148.78, 152.5, 161.5, 163.9 (Ar-C and CN), 170.8 (C=O). Anal. calc. for $C_{23}H_{16}FN_5OS$: C, 64.32, H, 3.76, N, 16.31. Found: C, 64.53, H, 3.95, N, 16.48.

2.1.18. 7-(1H-Indol-3-yl)-5-(2-methoxyphenyl)-2-(methylthio)-4-oxo-1,4,5,8-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (7b)

Yield (75%), mp (275-277°C), IR (KBr) cm^{-1}: 3664-3271 (3 NH), 2954 (CH aliphatic), 2191 (CN), 1643 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.53 (s, 3H, S$\underline{CH_3}$), 3.80 (s, 3H, O$\underline{CH_3}$), 5.04 (s, 1H, $\underline{H}$C), 6.89-7.71 (m, 9H, Ar-H), 9.84 (s, 1H, NH, D_2O exchangeable), 11.65 (s, 1H, NH, D_2O exchangeable), 12.34 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 13.1 (S$\underline{CH_3}$), 34.2 (H$\underline{C}$), 56.1 (O$\underline{CH_3}$), 82.7, 108.0, 112.1, 112.5, 117.1, 120.2, 121.0, 121.4, 122.3, 125.4, 127.8, 128.6, 129.1, 129.4, 132.3, 133.9, 136.2, 144.9, 157.2, 160.5 (Ar-C and CN), 175.0 (C=O). Anal. calc. for $C_{24}H_{19}N_5O_2S$: C, 65.29, H, 4.34, N, 15.86. Found: C, 64.96, H, 4.50, N, 16.04.

2.1.19. 7-(1H-Indol-3-yl)-2-(methylthio)-4-oxo-5-(m-tolyl)-1,4,5,8-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (7c)

Yield (53%), mp (216-218°C), IR (KBr) cm^{-1}: 3387-3116 (3 NH), 2954 (CH aliphatic), 2198 (CN), 1647 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.31 (s, 3H, $\underline{CH_3}$), 2.52 (s, 3H, S$\underline{CH_3}$), 4.66 (s, 1H, $\underline{H}$C), 7.07-7.83 (m, 9H, Ar-H), 9.98 (s, 1H, NH, D_2O exchangeable), 11.73 (s, 1H, NH, D_2O exchangeable), 12.40 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 13.1 (S$\underline{CH_3}$), 21.6 ($\underline{CH_3}$), 39.1 (H$\underline{C}$), 83.0, 95.7, 107.8, 112.5, 112.7, 120.2, 120.3, 120.4, 120.5, 121.5, 122.3, 124.9, 125.5, 128.0, 128.2, 128.9, 136.3, 137.9, 145.0, 145.9 (Ar-C and CN), 174.5 (C=O). Anal. calc. for $C_{24}H_{19}N_5OS$: C, 67.74, H 4.50, N 16.46. Found: C, 67.88, H, 4.73, N, 16.59.

2.1.20. General Procedure for the Synthesis of 4-(substituted phenyl)-2,6-di(1H-indol-3-yl)-1,4-dihydropyridine-3,5-dicarbonitriles 8a-d

A mixture of compound **1** (0.5 g, 0.0027 mol), the appropriate aromatic aldehyde (0.00135 mol) and ammonium acetate salt (6.8 g, 0.088 mol) was fused for 27-35 h. The mixture was poured onto ice/water mixture (25 mL), the separated solid was filtered, dried and crystallized from ethanol.

2.1.21. 4-(4-Bromophenyl)-2,6-di(1H-indol-3-yl)-1,4-dihydropyridine-3,5-dicarbonitrile (8a)

Reaction time 27 h; yield (70%), mp (>300°C), IR (KBr) cm^{-1}: 3444-3321 (3 NH), 2920 (CH aliphatic), 2191 (CN); ^{1}H-NMR (DMSO-d_6, ppm) δ: 4.69 (s, 1H, pyridine $\underline{H}$C-4), 7.14-7.94 (m, 14H, Ar-H), 9.90 (s, 1H, NH, D_2O exchangeable), 11.81 (s, 2H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 42.6 (pyridine H$\underline{C}$-4), 81.1, 107.7, 112.6, 120.4, 120.5, 121.0, 121.3, 122.4, 125.4, 128.9, 129.8, 132.5, 136.5, 144.7, 144.8 (Ar-C and CN). Anal. calc. for $C_{29}H_{18}BrN_5$: C, 67.45, H, 3.51, N, 13.56. Found: C, 67.63, H, 3.78, N, 13.80.

2.1.22. 4-(4-Chlorophenyl)-2,6-di(1H-indol-3-yl)-1,4-dihydropyridine-3,5-dicarbonitrile (8b)

Reaction time 28 h; yield (90%), mp (>300°C), IR (KBr) cm^{-1}: 3444-3356 (3 NH), 2924 (CH aliphatic), 2191 (CN); ^{1}H-NMR (DMSO-d_6, ppm) δ: 4.71 (s, 1H, Pyridine $\underline{H}$C-4), 7.14-7.95 (m, 14H, Ar-H), 9.90 (s, 1H, NH, D_2O exchangeable), 11.81 (s, 2H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 42.5 (pyridine H$\underline{C}$-4), 81.2, 107.7, 112.6, 120.4, 120.5, 121.0, 121.3, 122.5, 125.4, 128.8, 129.4, 132.8, 136.5, 144.3, 144.7 (Ar-C and CN). Anal. calc. for $C_{29}H_{18}ClN_5$: C, 73.80, H, 3.84, N, 14.84. Found: C, 74.06, H, 3.89, N, 14.69.

2.1.23. 2,6-Di(1H-indol-3-yl)-4-(2-methoxyphenyl)-1,4-dihydropyridine-3,5-dicarbonitrile (8c)

Reaction time 34 h; yield (80%), mp (257-259°C), IR (KBr) cm^{-1}: 3387-3309 (3 NH), 2920 (CH aliphatic), 2191 (CN); ^{1}H-NMR (DMSO-d_6, ppm) δ: 3.89 (s, 3H, O$\underline{CH_3}$), 4.92 (s, 1H, pyridine $\underline{H}$C-4), 7.04-7.92 (m, 14H, Ar-H), 9.80 (s, 1H, NH, D_2O exchangeable), 11.78 (s, 2H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 37.2 (pyridine H$\underline{C}$-4), 56.1 (O$\underline{CH_3}$), 80.6, 108.0, 112.1, 112.6, 120.4, 121.2, 121.4, 122.4, 125.5, 128.6, 128.8, 129.0, 129.4, 132.9, 136.5, 145.0, 157.0 (Ar-C and CN). Anal. calc. for $C_{30}H_{21}N_5O$: C, 77.07, H, 4.53, N, 14.98. Found: C, 77.28, H, 4.70, N, 15.12.

2.1.24. 2,6-Di(1H-indol-3-yl)-4-(m-tolyl)-1,4-dihydropyridine-3,5-dicarbonitrile (8d)

Reaction time 35 h; yield (65%), mp (>300°C), IR (KBr) cm^{-1}: 3410-3290 (3 NH), 2924 (CH aliphatic), 2187 (CN); ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.39 (s, 3H, $\underline{CH_3}$), 4.58 (s, 1H, pyridine $\underline{H}$C-4), 7.15-7.93 (m, 14H, Ar-H), 9.83 (s, 1H, NH, D_2O exchangeable), 11.80 (s, 2H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6, ppm): 21.6 ($\underline{CH_3}$), 43.2 (pyridine H$\underline{C}$-4), 81.7, 107.8, 112.6, 120.3, 120.4, 121.2, 122.4, 124.8, 125.5, 128.1, 128.7, 128.8, 129.5, 136.5, 138.5, 144.4, 145.5 (Ar-C and CN). Anal. calc. for $C_{30}H_{21}N_5$: C, 79.80, H, 4.69, N, 15.51. Found: C, 79.64, H, 4.85, N, 15.68.

2.2. *In vitro* Anticancer Assay

The synthesized compounds (**3a-d**, **4a-d**, **6a-d**, **7a-c** and **8a-d**) were evaluated for their cytotoxic activity on HCT-116 (colon carcinoma) cell line using SRB cytotoxicity assay [30,31].

2.2.1. Cell Culture Protocol

HCT-116 cell line was obtained from Nawah Scientific Inc., (Mokatam, Cairo, Egypt). The cells were maintained in DMEM media supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin, and 10% of heat-inactivated fetal bovine serum in humidified, 5% (v/v) CO_2 atmosphere at 37°C.

2.2.2. SRB-Cytotoxicity Assay Protocol

The cell viability was assessed by SRB assay. Aliquots of 100 µL cell suspension ($5x10^3$ cells) were introduced into 96-well plates and incubated in complete media for 24 h. The cells were treated with another aliquot of 100 µL media containing the test compounds at two concentrations (10 and 100 µM). After 72 h of compound exposure, the cells were fixed by replacing media with 150 µL of 10% trichloroacetic acid (TCA) and incubated at 4 °C for 1 h. The TCA solution was removed, and the cells were washed 5 times with distilled water. Aliquots of 70 µL SRB solution (0.4% w/v) were added and incubated in a dark place at room temperature for 10 min. The plates were washed 3 times with 1% acetic acid and allowed to air-dry overnight, then 150 µL of Tris(hydroxymethyl)aminomethane (TRIS) (10 mM) was

added to dissolve protein-bound SRB stain. The absorbance was measured at 540 nm using a BMG Labtech®- FLUOstar Omega microplate reader (Ortenberg, Germany). The IC_{50} of the most potent series (the bis-indolyl pyridine dicarbonitriles **8a-d**) was determined following the same procedure using five concentrations (0.01, 0.1, 1, 10 and 100 µM).

2.2.3. Analysis of Cell Cycle Distribution (Flow Cytometry) [32–37]

Compound **8**c, the most potent compound, was subjected for further investigation on its effect on HCT-116 cell cycle progression.

2.2.4. Methodology

After treatment with the test compound **8c** at IC_{50} 2.6 µM for 24 or 48 h and cells (10^5 cells) were collected by trypsinization and washed twice with ice-cold PBS (pH 7.4). The cells were re-suspended in 2 mL of 60% ice-cold ethanol and incubated at 4ºC for 1 h for fixation. Fixed cells were washed twice again with PBS (pH 7.4) and re-suspended in 1 mL of PBS containing 50 µg/mL RNAase A and 10 µg/mL propidium iodide (PI). After 20 min of incubation in the dark at 37ºC, the cells were analyzed for DNA contents using flow cytometry analysis using FL2 (λex/em 535/617 nm) signal detector (ACEA Novocyte™ flow cytometer, ACEA Biosciences Inc., San Diego, CA, USA). For each sample, 12,000 events are acquired. The cell cycle distribution was calculated using ACEA NovoExpress™ software (ACEA Biosciences Inc., San Diego, CA, USA).

3. RESULTS AND DISCUSSION

3.1. Chemistry

Schemes **1-3** outline the synthesis of the target compounds. The key precursor, aminopyrazole **2** was obtained in good yield (71%) by the fusion of 3-cyanoacetylindole (**1**) [28] with 5 molar equivalents of hydrazine hydrate as reported earlier by our group [29].

Previous work published by Ahmad *et al.* [38] indicated that refluxing a mixture of the aminopyrazole **2** and phenyl isothiocyanate in acetonitrile for 16 h afforded 3-phenylthiourea derivative in 62% yield. In this work, a milder and more efficient procedure was applied, which afforded the target compounds in high yields (75-92%). Thus, stirring the equimolar amount of compound **2** and substituted phenyl isothiocyanates at room temperature for 48 h in dry dioxane in the presence of anhydrous potassium carbonate gave the thiourea derivatives **3a-d**. The use of basic catalysts (potassium carbonate) enabled the reaction to be carried out at room temperature and afforded a high yield of the products. The ^{1}H-NMR spectra of compounds **3a-d** showed four exchangeable singlet signals at δ 10.67-12.80 ppm corresponding to NH protons. Furthermore, the ^{13}C-NMR spectra of compounds **3a-d** revealed the appearance of C=S carbon at δ 176.7-178.4 ppm (Scheme **1**).

The acrylonitrile derivatives **4a-d** were synthesized *via* Knoevenagel condensation reaction of 3-cyanoacetylindole (**1**) with the appropriately substituted benzaldehyde using a catalytic amount of piperidine. The IR spectra of compounds **4a-d** indicated the shifting of the C=O group to lower frequency due to conjugation (from 1635 cm^{-1} in compound **I** to1597-1600 cm^{-1} in compounds **4a-d**). Moreover, the ^{1}H-NMR spectra of compounds **4a-d** revealed the disappearance of the singlet signal corresponding to CH_2 at δ 4.50 ppm (from the starting compound **I**) and the appearance of singlet signal corresponding to =CH proton at δ 8.45-8.64 ppm. The ^{13}C-NMR spectra of the compounds revealed the absence of the aliphatic CH_2 signal and the appearance of extra aromatic signals corresponding to the introduced aromatic rings (Scheme **2**).

The synthesis of 7-indolylpyrido[2,3-*d*]pyrimidine derivatives was previously reported *via* a one-pot reaction of compound **1**, aromatic aldehyde and 6-aminouracils either in acetic acid [39] or in ethanol and $InCl_3$ as a catalyst [40].

In the present work, trials to prepare compounds **6a-d** *via* multicomponent one-pot reaction of compound **1**, aromatic aldehyde and 6-aminothiouracil (**5**) in refluxing glacial acetic acid for 14-22 h failed to give the desired products. The products were 5,5'-(phenylmethylene)bis(6-amino-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one) derivatives as confirmed by spectral data (data not shown).

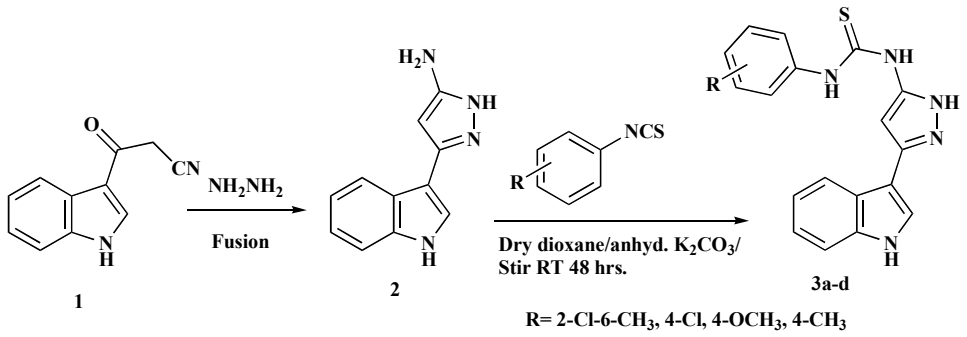
Therefore, a two-step reaction was adopted to synthesize the target compounds **6a-d**. Thus, compound **1** was allowed to react with the aromatic aldehyde to give acrylonitriles **4a-d,** which were subsequently reacted with 6-aminothiouracil **(5)** in glacial acetic acid to give the target compounds **6a-d**.

The IR spectra of compounds **6a-d** indicated the appearance of one CN group absorption band at 2194-2202 cm^{-1}, amino group at 3549-3194 cm^{-1} in addition to C=O group at 1658-1631 cm^{-1} and C=S group at 1242-1253 cm^{-1}. The ^{1}H-NMR spectra showed singlet signal corresponding to the chiral CH proton at δ 4.57-5.48 ppm and the presence of four exchangeable singlet signals corresponding to NH protons at δ 8.84-12.30 ppm. Meanwhile, the ^{13}C-NMR spectra of compounds **6a-d** indicated the presence of (C=O) and (C=S) carbons at δ 160.3-161.5 ppm and δ 173.9-174.1 ppm, respectively.

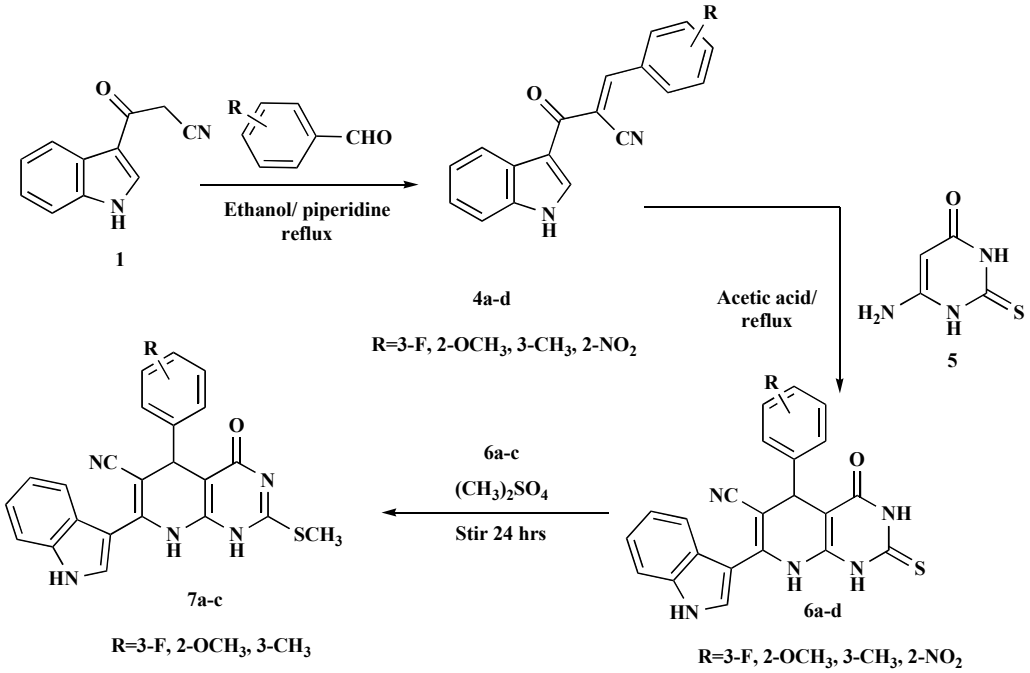
S-methylation of compounds **6a-c** was achieved using the equimolar amount of dimethyl sulphate in 10% aqueous solution of potassium hydroxide. The structure of compounds **7a-c** was confirmed by ^{1}H-NMR spectra that indicated the appearance of singlet signal corresponding to *S*-CH_3 at δ 2.52-2.53 ppm. Meanwhile, the ^{13}C-NMR spectra of compounds **7a-c** revealed the appearance of *S*-CH_3 at δ 13.1 ppm (Scheme **2**).

In 2008, Zhu *et al*. [42], reported the synthesis of bis(3-indolyl)pyridine derivatives through a multicomponent reaction of aldehydes, 3-cyanoacetylindole (**1**) and ammonium acetate under microwave irradiation conditions. Thirumurugan *et al*. [43,44], slightly modified this method by reacting indolylpropanonitrile **1** with ketones and aldehydes in the presence of ammonium acetate under either reflux or MW irradiation to obtain a wide range of asymmetrical pyridines.

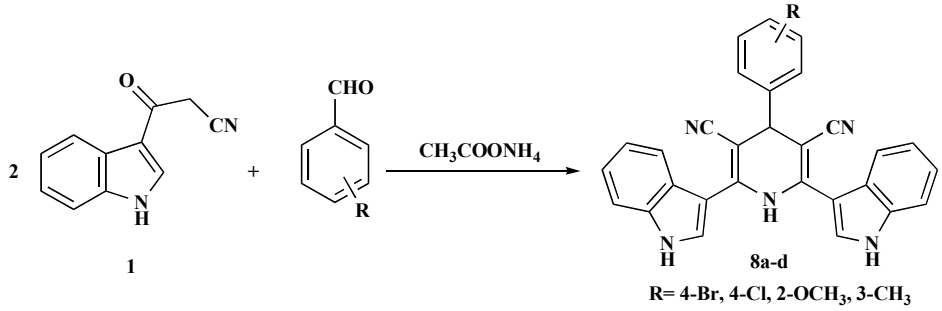
In this work, the synthesis of compounds **8a-d** was achieved *via* the fusion reaction of two molar equivalents of compound **1**, the appropriate aldehyde and ammonium acetate. The IR spectra of compounds **8a-d** indicated the ap-



Scheme 1. Synthesis of indolyl pyrazoles **3a-d**.



Scheme 2. Synthesis of pyrido[2,3-*d*]pyrimidines **6a-d** and **7a-c**.



Scheme 3. Synthesis of 2,6-di-indolyl pyridines **8a-d**.

pearance of NH absorption bands at 3444-3290 cm^{-1}, in addition to the CH aliphatic absorption bands at 2924-2920 and CN absorption band at 2191-2187 cm^{-1}. Moreover, the ^{1}H-NMR spectra of compounds **8a-d** revealed the appearance of a new exchangeable singlet signal at δ 9.80-9.90 ppm corresponding to NH proton of pyridine ring and the appearance of singlet signal at δ 4.58-4.92 ppm assigned to the proton of C-4 of pyridine ring that indicated the 1,4-dihydro structure. On the other hand, the ^{13}C-NMR spectra of compounds **8a-d** revealed the appearance of C-4 of pyridine ring at δ 37-43 ppm (Scheme **3**).

3.2. *In vitro* Cytotoxic Activity

3.2.1. In vitro Two-dose Anticancer Assay

The synthesized compounds were evaluated for their cytotoxic activity on HCT-116 (colon carcinoma) cell line using SRB cytotoxicity assay [30, 31]. The results expressed as cell viability % are summarized in Table **1** and graphically represented in Fig. (**3**). Most of the prepared compounds exhibited moderate to strong cytotoxic activity against HCT-116 cell line.

Table 1. Results of *in vitro* cytotoxicity assay against HCT-116 cell-line.

Comp. No.	Cell Viability %		IC_{50} (µM)	Comp. No.	Cell Viability %		IC_{50} (µM)
	10 µM	100 µM			10 µM	100 µM	
3a	78.0	1.6	ND*	**6c**	71.0	6.6	ND
3b	56.1	1.8	ND	**6d**	60.9	5.9	ND
3c	86.0	6.5	ND	**7a**	53.0	5.2	ND
3d	57.0	1.1	ND	**7b**	94.7	96.3	ND
4a	55.3	13.3	ND	**7c**	82.3	2.6	ND
4b	57.4	35.0	ND	**8a**	14.0	1.6	3.2
4c	57.3	20.3	ND	**8b**	16.2	2.2	5.3
4d	63.9	11.8	ND	**8c**	25.0	1.3	2.6
6a	40.9	1.9	ND	**8d**	36.1	2.9	8.8
6b	126.3	38.7	ND				

*ND: not determined

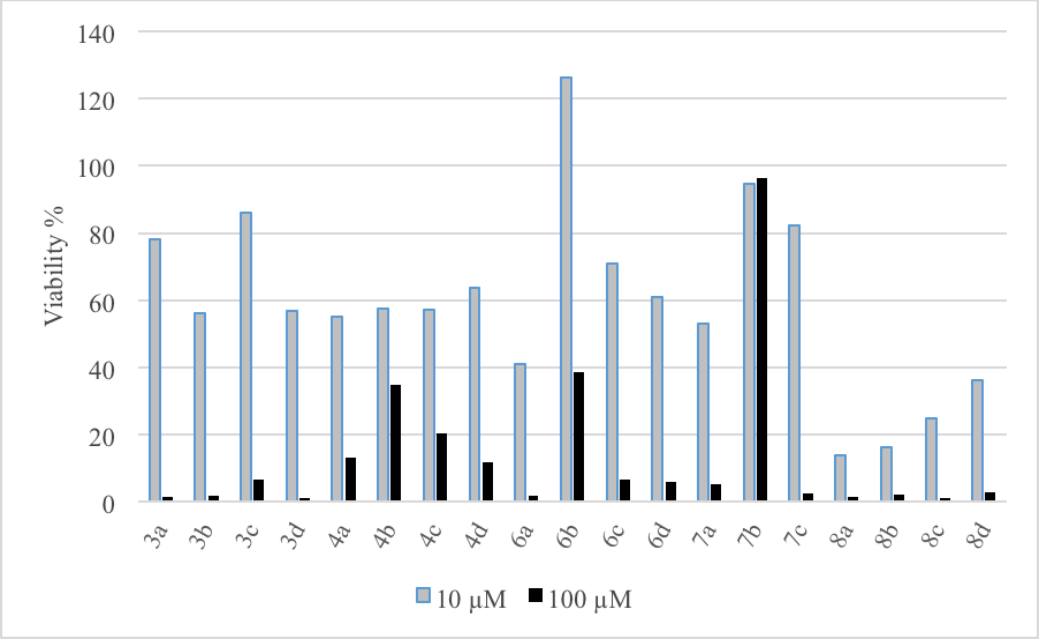


Fig. (3). Results of *in vitro* anticancer assay against the HCT-116 cell-line.

Revising the data in Table **1** indicated the following observations:

- The thiourea derivatives **3a-d** showed moderately potent cytotoxic activity. The substitution of the phenyl ring with the methoxy group showed the least anti-proliferative effect among this class.
- The acrylonitriles **4a-d** showed poor cytotoxic activity. Cyclization into pyrido[2,3-*d*]pyrimidines **6a-d** enhanced the cytotoxic activity dramatically compared to the acrylonitrile derivatives.
- *S*-methylation of pyrido[2,3-*d*]pyrimidines led to a noticeable decrease in the anti-proliferative activity (compounds **7a-c**).
- The bis-indolylpyridine-dicarbonitriles **8a-d** were the most potent compounds among the tested compounds.

Based on the results of the two doses cytotoxic screening, the bis-indolylpyridine-dicarbonitriles **8a-d** were subjected to five-dose anti-proliferative assay to determine their IC_{50} and the results were shown in Table **1**. The results indicated that compound **8c** bearing 2-methoxyphenyl group showed the highest anti-proliferative effect on HCT-116 cell line with IC_{50}= 2.6 µM. Whilst, the substitution of the phenyl ring with halogens (Br, Cl) gave comparable results [compounds **8a** and **8b** (IC_{50}= 3.2 and 5.3 µM, respectively)]. Finally, compound **8d**, carrying 3-methylphenyl group, was the least potent with IC_{50}= 8.8 µM.

Table 2. Results of cell cycle inhibition activity of compound 8c.

Compound	%G_0/G_1	%S	%G_2/M	Pre-G_1
8c/ HCT-116	53.06	22.77	16.62	1.87
Control/ HCT-116	41.26	26.63	22.38	1.86

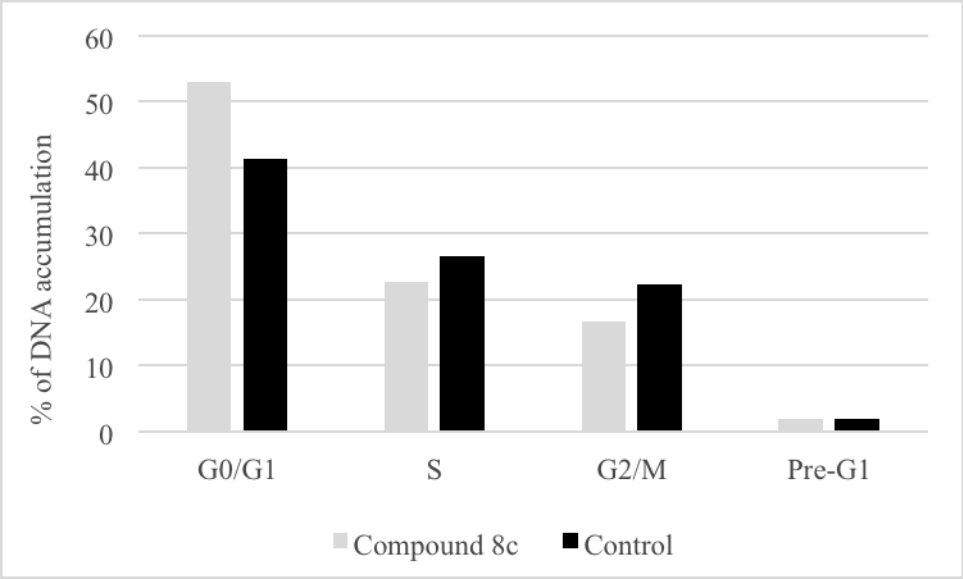


Fig. (4). Flow cytometric analysis of compound **8c** on HCT-116 cell line.

3.2.2. Analysis of Cell Cycle Distribution (Flow Cytometry) [32–37]

Compound **8c**, the most potent compound in this study, was subjected to further investigation to determine its effect on HCT-116 cell cycle progression. The results in terms of the percentage of DNA content were displayed in Table **2** and represented graphically in Fig. (**4**).

Exposure of HCT-116 cells to compound **8c** at its IC_{50} (2.6 µM) caused interference with the normal distribution of the cell cycle of this cell line. It could be seen that 41.26 % of the untreated HCT-116 cells (control) synchronized themselves at the G_0/G_1 phase, 26.63 % at S phase, 22.38 % at G_2/M phase and only 1.87% in sub G_0 phase that usually contains the apoptotic fraction. Compound **8c** induced an increase in the cell population in G_1 phase concomitant with depletion of cells from the S and G_2 compartments. This phase denotes the non-proliferating cells. The accumulation of cells in this phase indicated that the compound **8c** had an anti-proliferative effect.

CONCLUSION

The synthesis and anti-tumor activity of novel nortopsentin analogues were achieved. The analouges included thiourea derivatives **3a-d**, pyrido[2,3-*d*]pyrimidine derivatives **6a-d**, their *S*-methylated analogues **7a-c** and bis-indolyl pyridine-dicarbonitriles **8a-d**. The latter series **8a-d** exhibited pronounced antitumor activity against colon cancer cell line (HCT-116) among the tested series. Compound **8c** [2,6-di(1*H*-indol-3-yl)-4-(2-methoxyphenyl)-1,4-dihydropyridine-3,5-dicarbonitrile] was considered the most potent compound among this series with IC_{50} value of 2.6 µM against HCT-116 cell line. Moreover, **8c** revealed an accumulation of cells in the G_1 phase during flow cytometry assay, which in turn indicated its anti-proliferative effect. Thus, bis-indolylpyridine dicarbonitrile represents a lead structure and further study is still needed to study the SAR and to improve the anticancer activity of this class of compounds. Besides, a kinase profile and ADME study will be conducted on the prepared compounds.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, [Hala B. El-Nassan], upon reasonable request.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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