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Structural Simplification of Bioactive Natural Products with Multicomponent Synthesis. 2. Antiproliferative and Antitubulin Activities of Pyrano[3,2-c]pyridones and Pyrano[3,2-c]quinolones

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Abstract

Pyrano[3,2-c]pyridone and pyrano[3,2-c]quinolone structural motifs are commonly found in alkaloids manifesting diverse biological activities. As part of a program aimed at structural simplification of bioactive natural products utilizing multicomponent synthetic processes, we developed compound libraries based on these privileged heterocyclic scaffolds. The selected library members display low nanomolar antiproliferative activity and induce apoptosis in human cancer cell lines. Mechanistic studies reveal that these compounds induce cell cycle arrest in the G2/M phase and block *in vitro* tubulin polymerization. Because of the successful clinical use of microtubule-targeting agents, these heterocyclic libraries are expected to provide promising new leads in anticancer drug design.

Introduction

Natural products are a rich source of new medicinal leads and, therefore, preparation of natural product-based libraries of compounds is an important area of research in modern drug discovery.[1–6] Such efforts, however, are often complicated by the structural complexity of natural products, which often contain a large number of stereogenic centers and intricate ring systems.[7] We have recently initiated a research program aimed at structural simplification of natural products, specifically by utilizing multicomponent synthetic processes. We showed that the stereochemically complex structure of an important

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Supporting Information Available: X-ray data for compound **15** (CIF and PDF). This material is available free of charge via the internet at http://pubs.acs.org.

anticancer lead podophyllotoxin can be efficiently simplified to a dihydropyridopyrazole scaffold, which is accessible via a one-step multicomponent reaction (MCR).[a] The resulting library of compounds retains a significant portion of podophyllotoxin's cytotoxic potency and apoptosis inducing potential.[8,9]

In continuation of these efforts we have been investigating compound libraries based on pyrano[3,2-c]pyridone and pyrano[3,2-c]quinolone scaffolds. This structural motif is broadly represented by pyranopyridone and pyranoquinolone alkaloids manifesting diverse biological activities. The latter include antibacterial,[10–12] antifungal and antialgal,[13] antiinflammatory[14] and antimalarial[15] properties as well as inhibition of calcium signaling,[16] platelet aggregation[17] and nitric oxide production.[18] Furthermore, many of these alkaloids exhibit cancer cell growth-inhibitory activity and are investigated as potential anticancer agents. Examples include cytotoxic fusaricide (**1**)[19] and melicobisquinolinone B (**2**, Figure 1).[20] In addition, zanthosimuline (**3**) is active against multidrug resistant KB-VI cancer cells, while huajiaosimuline (**4**) exhibits a selective cytotoxicity profile showing the greatest activity with estrogen receptor-positive ZR-75-1 breast cancer cells.[21]

To develop compound libraries based on this fused heterocyclic scaffold we considered a reaction of 4-hydroxypyridones as well as corresponding quinolones with aldehydes and malononitrile (Figure 2a). Although we found only two literature examples pointing to the feasibility of such an MCR for pyranopyridone and pyranoquinolone library preparations,[22] the stepwise version of this process involving the synthesis of the intermediate Knoevenagel adducts and their subsequent reactions with 4-hydroxypyridones and quinolones has been well studied.[23] Inspection of this literature reveals that the biology of compounds prepared using this process has not been investigated with the exception of antibacterial properties reported for some of these heterocycles.[24] Importantly, a number of recent publications and patents have described promising anticancer activity associated with a structurally related chromene scaffold exemplified by compounds **5** and **6** (Figure 2b).[25] These compounds, shown to inhibit tubulin polymerization and induce apoptosis in cancer cells, exhibit high potency against taxoland vinblastine-resistant P-glycoprotein overexpressing cell types.[25d] Furthermore, chromenes **5** and **6** disrupt tumor vasculature in a number of human solid tumor xenografts and they are currently in development as anticancer agents.[25b.c]

Results and Discussion

Prompted by the encouraging literature precedent we embarked on the synthesis and anticancer evaluation of the proposed pyrano[3,2-c]pyridone and pyrano[3,2-c]quinolone libraries.[26] The synthesis of the pyranopyridone library is shown in Figure 3. Pyridone **7** was selected on the basis of its ready availability by a one-step synthesis involving treatment of the corresponding commercially available pyrone with aqueous $MeNH_2$ following a literature procedure.[27] A three-component reaction of pyridone **7** with malononitrile and various aromatic aldehydes in a 1:1:1 ratio proceeds smoothly in refluxing ethanol containing a small quantity of Et_3N. Pyranopyridones **8–18** precipitate directly from the refluxing reaction mixtures and require no further purification. The product yields are given in Table 1.

The structures of the pyranopyridones were established by ^1H and ^{13}C NMR, MS techniques as well as an X-ray structure of the library member **15** (Figure 4).[28]

[a]Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDTA, diaminoethanetetraacetic acid; EtOH, ethanol; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FOG, Ficoll Orange G; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; HHB, Heinz-HEPES buffer; HRMS, high resolution mass spectrometry; MCR, multicomponent reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SAR, structure-activity relationship; TLC, thin layer chromatography; SD, standard deviation.

The analogue library was tested for antiproliferative activity using the HeLa cell line as a model for human cervical adenocarcinoma. The cells were treated with respective compounds for 48 h and cell viability was assessed through measurements of mitochondrial dehydrogenase activity using the MTT method (Table 1).[29] It is noteworthy that all potent analogues have a 3-bromo substituent on the aromatic ring at position C4 of the pyranopyridone skeleton (compounds **8–14**) and this preference is uniform irrespective of the substitution pattern of this aromatic moiety. The 3-chloro (**15**) and other variously substituted analogues (**16–18**) are significantly less potent or are totally inactive. Further, the substitution of the nitrogen in the pyridone ring by oxygen, as in pyranopyranone **19**, abolishes the activity as well. Moderate potency of the N-(β-arylethyl)pyridone **20**, synthesized in a manner analogous to the rest of the library, warrants further investigation of compounds having a bulky moiety on the pyridone nitrogen. Efforts to prepare a library of such compounds are underway in our laboratories.

Since many clinically used anticancer agents induce apoptosis in cancer cells,[30] we tested the pyranopyridone analogues for their ability to induce apoptosis in Jurkat (model for human T-cell leukemia) cells using the flow cytometric annexin-V/propidium iodide assay (Figure 5).[31] Compounds **8–14**, exhibiting submicromolar or low micromolar potencies for the inhibition of proliferation of HeLa cells, were found to be strong inducers of apoptosis in Jurkat cells at 5 μM concentrations. The magnitude of apoptosis induction is comparable to the known antimitotic agent colchicine used at the same concentration. In contrast, compounds **15**, **17**, **20**, which are much less potent or totally inactive in the HeLa MTT assay, do not induce apoptosis in Jurkat cells at this concentration.

Encouraged by the promising results obtained with pyrano-[3,2-*c*]-pyridone library of compounds, we investigated the corresponding pyrano-[3,2-*c*]-quinolones. The synthesis of analogues **21–45**, utilizing commercially available 4-hydroxy-1-methylquinolin-2(1*H*)-one in lieu of pyridone **7**, turned out to be equally facile and the yields of the library members are given in Table 2.

Analogues **21–45** as well as chromenes **5** and **6** were evaluated for antiproliferative activity in HeLa and MCF-7 (model for breast adenocarcinoma) cell lines. The general SAR trends are very similar to the corresponding pyridone-based library and, with a few exceptions, HeLas are more responsive than MCF-7 cells to both pyranoquinolones and chromenes. Most pleasingly, however, changing the pyranopyridone scaffold to the corresponding pyranoquinolone led to low nanomolar potencies exhibited by a number of library members containing the crucial 3-bromo substituent. Furthermore, the rest of the substitution pattern of this aromatic ring has a profound influence on antiproliferative potency, including the complete reversal of the strength of the effect on the two cell lines. For example, **42** and **44** exhibit low nanomolar potencies in both cell lines, while pairs **40**, **41** and **37**, **45** display respective low nanomolar GI_{50} values only with HeLa or MCF-7 cells, but not both. Synthesis of a larger number of analogues and their evaluation in a broader panel of cell lines will be required to understand these intriguing observations, which may pave the way to compounds with highly selective toxicity profiles.

The induction of apoptosis by the potent library members was investigated using the Jurkat cell line in both time-dependent and dose-dependent manner. To this end, Jurkat cells were treated with analogues **40** and **41**, as well as chromenes **5** and **6**, and the proportion of cells displaying the apoptotic phenotype was determined with the flow cytometric annexin-V/propidium iodide assay every 12 h (Figure 6). For all four compounds we observed a similar, time-dependent increase in the proportion of cells undergoing apoptosis with the maximum occurring after 48 h of treatment.

The time period of 48 h was then selected for the dose-dependent experiments to compare the apoptosis inducing power of the pyranoquinolone library members with chromenes **5** and **6** (Figure 7). While compound **40** is a strong apoptosis inducer at 100 nM, its potency drops as the concentration is reduced to 10 nM. In contrast, similarly to chromenes **5** and **6**, library member **41** retains its potency at this low concentration.

Since endonuclease-mediated cleavage of nuclear DNA resulting in formation of oligonucleosomal DNA fragments (180–200 base pairs long) is a hallmark of apoptosis in many cell types, apoptosis was further investigated with the DNA laddering assay (Figure 8). Jurkat cells were treated with DMSO (lane A), analogues **40** and **41** (lanes B and C), and paclitaxel (lane D) for 36 h. After that the cellular DNA was isolated and electrophoresed in a 1.5% agarose gel. The characteristic ladder pattern was obtained with compounds **40**, **41** and paclitaxel. No laddering was observed when the cells were treated with DMSO control.

Morphological changes of cells treated with potent pyrano-[3,2-*c*]-quinolones can be visually observed with light microscopy (Figure 9). The phenotypic changes of Jurkat cells, such as formation of finger-like extensions and shriveling, become apparent as early as 2 h after their treatment with **40** (C), and **41** (D).

Because of the structural similarity between the pyranopyridone and chromene scaffolds, we suspected that our libraries of compounds exert their antiproliferative properties through inhibition of tubulin dynamics, thereby inducing mitotic arrest and initiating apoptosis in cancer cells. Indeed, the flow cytometric cell cycle analysis, performed with pyranoquinolones **40** and **41** using the Jurkat cell line, shows a pronounced cell cycle arrest in the G2/M phase (Table 3). This effect is characteristic of antimitotic agents disrupting microtubule assembly, and is also observed with chromenes **5** and **6** that bind to or near the colchicine binding site on β-tubulin.[25d]

To obtain further support for the proposed antitubulin mechanism of action for our heterocycles, we assessed the effect of pyranoquinolones on *in vitro* tubulin polymerization.[32] In this assay microtubule formation is monitored by the increase in fluorescence intensity of the reaction mixture. Paclitaxel exhibited potent enhancement of microtubule formation relative to the effect of DMSO control (Figure 10). In contrast, library members **40** and **41** displayed potent microtubule destabilizing effect in a manner similar to the known tubulin polymerization inhibitor podophyllotoxin.

Conclusions

The utilization of multicomponent synthesis led to the development of compound libraries based on pyrano[3,2-c]pyridone and pyrano[3,2-c]quinolone scaffolds, which are commonly found in structurally complex alkaloids manifesting diverse biological activities. Many of the synthesized analogues exhibit low nanomolar (down to 3 nM) antiproliferative properties in HeLa and MCF-7 human cancer cell lines. Furthermore, the antiproliferative effect results from the potent apoptosis inducing ability of these heterocycles, as confirmed by the flow cytometric annexin-V staining and DNA laddering assays. The potent apoptosis inducing power of a selected library member is pronounced at concentrations as low as 10 nM. The structural similarity of these heterocycles with compounds based on a related chromene scaffold prompted investigations of a possible mode of action based on inhibition of tubulin dynamics and cell cycle arrest in the G2/M phase. Indeed, the flow cytometric detection of large populations of cells with the 4N DNA content and potent inhibition of the *in vitro* tubulin polymerization by library analogues in a manner similar to the well-established antitubulin agents, such as colchicine and podophyllotoxin, support this hypothesis. Because the antitubulin agents are some of the most effective drugs in cancer chemotherapy[33] and

many of them are now investigated as possible inhibitors of angiogenesis in cancer tissues,[34] we believe that further investigation of compound libraries based on pyrano[3,2-c]pyridone and pyrano[3,2-c]quinolone scaffolds will result in important new leads in anticancer drug design.

Experimental Section

All aldehydes, malononitrile, and 4-hydroxy-1-methylquinolin-2(1*H*)-one were purchased from commercial sources and used without purification. 4-Hydroxy-1,6-dimethylpyridin-2(1*H*)-one was prepared following a literature procedure.[27] Triethylamine (Et_3N) was distilled from CaH_2. All reactions were performed in a reaction vessel open to the atmosphere and monitored by thin layer chromatography (TLC) on pre-coated (250 μm) silica gel $60F_{254}$ glass-backed plates. Visualization was accomplished with UV light and aqueous ceric ammonium molybdate solution or potassium permanganate stain followed by charring on a hot-plate. ^1H and ^{13}C NMR spectra were recorded on JEOL 300 MHz spectrometer. MS analyses were performed at the Mass Spectrometry Facility, University of New Mexico. All compounds **8**–**45** decompose at temperatures exceeding 260 °C without melting.

General procedure for the synthesis of pyrano-[3,2-c]-pyridones 8–16 and pyrano-[3,2-c]-quinolones 21–45

A mixture of 4-hydroxy-1,6-dimethylpyridin-2(1*H*)-one (0.8 mmol) or 4-hydroxy-1-methylquinolin-2(1*H*)-one, malononitrile (0.8 mmol), triethylamine (0.05 mL) and a corresponding aldehyde (0.8 mmol) in EtOH (96% aqueous solution, 3 mL) was refluxed for 50 minutes. The reaction mixture was allowed to cool to room temperature, the precipitated product was collected by filtration and washed with EtOH (5 mL). In most cases the product was > 98% pure as judged by ^1H NMR analysis. When necessary the products were recrystallized from DMF.

2-Amino-4-[3-bromo-4-(dimethylamino)phenyl]-6,7-dimethyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]pyridine-3-carbonitrile (8)

83%; ^1H NMR (DMSO-d_6) δ 7.27 – 7.08 (m, 5H), 6.06 (s, 1H), 4.28 (s, 1H), 3.33 (s, 3H), 2.66 (s, 6H), 2.33 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 161.7, 159.8, 155.4, 153.7, 148.8, 145.0, 143.1, 122.9, 112.7, 105.5, 97.4, 60.5, 56.6, 39.3, 37.2, 31.2, 20.8; HRMS *m/z* (ESI) calcd for $C_{19}H_{19}BrN_4O_2$ ($M+Na^+$) 437.0589, found 437.0580.

2-Amino-4-(3-bromo-4,5-dimethoxyphenyl)-6,7-dimethyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]pyridine-3-carbonitrile (9)

87%; ^1H NMR (DMSO-d_6) δ 6.93 (d, *J* = 6.9 Hz, 2H), 6.87–6.85 (m, 2H), 6.03 (s, 1H), 4.37 (s, 1H), 3.80 (s, 3H), 3.73 (s, 3H), 3.35 (s, 3H), 2.35 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 161.7, 159.8, 155.4, 153.6, 148.5, 145.2, 142.7, 123.2, 120.1, 116.8, 113.1, 105.6, 97.4, 60.6, 58.2, 56.8, 37.3, 31.2, 20.6; HRMS *m/z* (ESI) calcd for $C_{19}H_{19}BrN_3O_4$ ($M+Na^+$) 454.0378, found 454.0371.

2-Amino-4-(3-bromo-4-ethoxy-5-methoxyphenyl)-6,7-dimethyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]pyridine-3-carbonitrile (10)

88%; ^1H NMR (DMSO-d_6) δ 7.09 (s, 2H), 6.90 (s, 1H), 6.79 (s, 1H), 6.07 (d, *J* = 2.7 Hz, 1H), 4.33 (d, *J* = 3.0 Hz, 1H), 3.92 (q, *J* = 3.0 Hz, 2H), 3.76 (s, 3H), 3.33 (s, 3H), 2.33 (s, 3H), 1.27 (t, *J* = 3.0 Hz. 3H); ^{13}C NMR (DMSO-d_6) δ 161.6, 159.8, 155.4, 153.8, 148.6, 144.3, 142.7, 122.9, 117.5, 112.6, 105.5, 97.42, 68.9, 57.8, 56.6, 37.1, 31.1, 20.8, 16.1; HRMS *m/z* (ESI) calcd for $C_{20}H_{20}BrN_{3O}O_4$ ($M+Na^+$) 468.0535, found 468.0540.

2-Amino-4-(3-bromo-4-hydroxy-5-methoxyphenyl)-6,7-dimethyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]pyridine-3-carbonitrile (11)

75%; ^{1}H NMR (DMSO-d_6) δ 9.32 (s, 1H), 7.04 (s, 2H), 6.82 – 6.71 (m, 2H), 6.06 (s, 1H), 4.28 (s, 1H), 3.77 (s, 3H), 3.32 (s, 3H), 2.34 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 161.6, 159.6, 155.1, 148.5, 148.4, 143.0, 137.5, 123.1, 111.5, 109.6, 105.9, 97.4, 58.0, 56.7, 37.0, 31.1, 20.8; HRMS *m/z* (ESI) calcd for $C_{18}H_{16}BrN_3O_4$ (M+Na^+) 440.0222, found 440.0223.

4-(2-Amino-3-cyano-6,7-dimethyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]pyridin-4-yl)-2-bromo-6-methoxyphenyl acetate (12)

83%; ^{1}H NMR (DMSO-d_6) δ 7.02 (s, 1H), 6.97 (s, 2H), 6.92 (s, 1H) 6.05 (s, 1H), 4.44 (s, 1H), 3.77 (s, 3H), 3.34 (s, 3H), 2.34 (s, 3H), 2.30 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 168.2, 161.9, 159.9, 155.6, 152.5, 148.9, 145.4, 136.5.0, 122.9, 120.2, 116.6, 112.5, 105.4, 97.4, 57.6, 56.7, 37.2, 31.1, 20.5; HRMS *m/z* (ESI) calcd for $C_{20}H_{18}BrN_3O_5$ (M+Na^+) 482.0328, found 482.0322.

2-Amino-4-(3-bromo-4-fluorophenyl)-6,7-dimethyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]pyridine-3-carbonitrile (13)

84%; ^{1}H NMR (DMSO-d_6) δ 7.42 (m, 1H), 7.29–7.15 (m, 2H), 7.07 (s, 2H) 6.05 (s, 1H), 4.37 (s, 1H), 2.33 (s, 3H), 2.30 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 161.6, 159.8, 155.35, 148.7, 143.5, 132.9, 129.3, 120.2, 117.2, 116.8, 108.1, 105.3, 97.5, 57.9, 36.8, 30.9, 20.7; HRMS *m/z* (ESI) calcd for $C_{17}H_{13}BrFN_3O_2$ (M+Na^+) 412.0073, found 412.0073.

2-Amino-4-(3-bromophenyl)-6,7-dimethyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]pyridine-3-carbonitrile (14)

97%; ^{1}H NMR (DMSO-d_6) δ 7.39–7.11 (m, 6H), 6.07 (s, 1H), 4.35 (s, 1H), 3.30 (s, 3H), 2.34 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 161.5, 159.6, 155.3, 148.7, 148.1, 131.0, 130.7, 129.9, 127.2, 121.9, 120.3, 105.3, 97.4, 57.6, 37.4, 31.1, 20.5; HRMS *m/z* (ESI) calcd for $C_{17}H_{14}BrN_3O_2$ (M+Na^+) 394.0167, found 394.0159.

2-Amino-4-(3,4-dichlorophenyl)-6,7-dimethyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]pyridine-3-carbonitrile (15)

98%; ^{1}H NMR (DMSO-d_6) δ 7.54–7.49 (m, 1H), 7.38 (s, 1H), 7.18–7.16 (m, 1H), 6.83 (s, 2H), 6.03 (s, 1H), 4.43 (s, 1H) 3.35 (s, 3H), 2.35 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 161.6, 159.8, 155.5, 148.7, 146.4, 131.3, 130.9, 130.0, 129.8, 128.3, 119.8, 105.1, 97.3, 58.0, 36.9, 31.1, 20.4; HRMS *m/z* (ESI) calcd for $C_{17}H_{13}Cl_2N_3O_2$ (M+Na^+) 384.0283, found 384.0282.

2-Amino-6,7-dimethyl-5-oxo-4-(3,4,5-trimethoxyphenyl)-5,6-dihydro-4*H*-pyrano[3,2-*c*]pyridine-3-carbonitrile (16)

97%; ^{1}H NMR (DMSO-d_6) δ 7.01 (s, 2H), 6.42 (s, 2H), 6.06 (s, 1H), 4.35 (s, 1H), 3.69 (s, 6H), 3.60 (s, 3H), 3.32 (s, 3H), 2.34 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 161.6, 159.8, 155.4, 153.3, 148.2, 140.9, 137.3, 120.2, 106.2, 105.6, 97.3, 60.6, 58.5, 56.4, 37.5, 31.0, 20.7; HRMS *m/z* (ESI) calcd for $C_{20}H_{21}BrN_3O_5$ (M+Na^+) 406.1379, found 406.1379.

2-Amino-4-(4-isopropylphenyl)-6,7-dimethyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]pyridine-3-carbonitrile (17)

81%; ^{1}H NMR (DMSO-d_6) δ7.15–7.08 (m, 4H), 6.75 (s, 2H), 6.02 (s, 1H), 4.34 (s, 1H), 3.32 (s, 3H), 2.88–2.79 (m, 1H), 2.34 (s, 3H), 1.18 (d, J = 6.9 Hz, 6H); ^{13}C NMR (DMSO-d_6) δ 161.7, 159.8, 155.4, 148.0, 147.0, 142.7, 127.8, 126.5, 120.2, 106.6, 97.4, 59.3, 37.2, 33.5, 31.1, 24.3, 20.6; HRMS *m/z* (ESI) calcd for $C_{20}H_{21}N_3O_2$ (M+Na^+) 358.1531, found 358.1519.

2-Amino-6,7-dimethyl-4-(3-nitrophenyl)-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]pyridine-3-carbonitrile (18)

97%; ^{1}H NMR (DMSO-d_6) δ8.04 (d, *J* = 7.7 Hz, 1H), 7.95 (s, 1H), 7.65–7.53 (m, 2H) 6.15 (s, 2H), 6.06 (s, 1H), 4.52 (s, 1H), 3.27 (s, 3H), 2.32 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 161.8, 159.9, 155.6, 149.1, 148.4, 147.6, 134.9, 130.3, 122.6, 122.1, 120.1, 105.0, 97.5, 56.6, 37.4, 31.1, 20.5; HRMS *m/z* (ESI) calcd for $C_{17}H_{14}N_4O_4$ (M+Na$^+$) 361.0913, found 361.0909.

2-Amino-4-(3-bromophenyl)-7-methyl-5-oxo-4*H*,5*H*-pyrano[4,3-*b*]pyran-3-carbonitrile (19)

87%; ^{1}H NMR (DMSO-d_6) δ7.43 (d, *J* = 8.0 Hz, 1H), 7.38 (s, 1H), 7.32–7.27 (m, 1H), 7.21 (d, *J* = 8.0 Hz, 1H), 7.15 (s, 2H), 6.26 (s, 1H), 4.34 (s, 1H), 2.22 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 163.7, 161.9, 159.1, 158.8, 146.8, 131.3, 130.8, 130.5, 127.3, 122.1, 119.5, 100.6, 98.5, 58.1, 36.6, 19.7; HRMS *m/z* (ESI) calcd for $C_{16}H_{11}BrN_2O_3$ (M+Na$^+$) 380.9845, found 380.9839.

2-Amino-6-(3,4-dimethoxyphenethyl)-7-methyl-5-oxo-4-(3,4,5-trimethoxyphenyl)-5,6-dihydro-4*H*-pyrano[3,2-*c*]pyridine-3-carbonitrile (20)

80%; ^{1}H NMR (DMSO-d_6) δ 6.90 (s, 2H), 6.83 (d, *J* = 8.5 Hz, 1H), 6.69–6.63 (m, 2H), 6.52 (s, 2H),5.89 (s, 1H), 4.41 (s, 1H), 4.13–3.98 (m, 2H), 3.74 (s, 6H), 3.71 (s, 3H), 3.67 (s, 3H), 3.65 (s, 3H), 2.86–2.70 (m, 2H), 2.19 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 161.6, 160.0, 155.6, 153.4, 149.5, 148.4, 147.6, 141.0, 137.4, 131.4, 121.3, 120.5, 113.6, 112.9, 106.6, 105.6, 97.6, 60.5, 58.4, 56.5, 56.3, 56.1, 40.1, 37.2, 33.3, 20.0; HRMS *m/z* (ESI) calcd for $C_{29}H_{31}N_3O_7$ (M+Na$^+$) 556.2060, found 556.2057.

2-Amino-6-methyl-5-oxo-4-phenyl-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (21)

78%; ^{1}H NMR (DMSO-d_6) δ8.06 (d, *J* = 7.6 Hz, 1H), 7.70–7.66 (m, 1H), 7.52 (d, *J* = 7.6 Hz, 1H), 7.40–7.18 (m, 6H), 6.98 (s, 2H), 4.56 (s, 1H), 3.55 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.5, 159.6, 150.9, 144.9, 139.4, 132.1, 128.8, 128.0, 127.2, 122.9, 122.8, 122.6, 119.9, 115.3, 113.4, 109.8, 59.2, 37.8, 29.8; HRMS *m/z* (ESI) calcd for $C_{20}H_{15}N_3O_2$ (M+Na$^+$) 352.1062, found 352.1058.

2-Amino-6-methyl-5-oxo-4-(3,4,5-trimethoxyphenyl)-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (22)

86%; ^{1}H NMR (DMSO-d_6) δ8.03 (d, *J* = 8.0 Hz, 1H), 7.72–7.66 (m, 1H), 7.55 (d, *J* = 7.6 Hz, 1H), 7.40–7.35 (m, 1H), 7.14 (s, 2H), 6.51 (s, 2H), 4.53 (s, 1H), 3.71 (s, 6H), 3.64 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.6, 159.6, 153.4, 150.8, 140.4, 139.3, 137.5, 132.2, 122.9, 122.7, 120.1, 115.4, 113.3, 109.4, 105.9, 60.5, 58.6, 56.6, 38.2, 29.9; HRMS *m/z* (ESI) calcd for $C_{23}H_{21}N_3O_5$ (M+Na$^+$) 442.1379, found 442.1389.

2-Amino-4-(3-hydroxy-4-methoxyphenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (23)

82%; ^{1}H NMR (DMSO-d_6) δ8.90 (s, 1H), 8.00 (d, *J* = 8.0 Hz, 1H), 7.72–7.67 (m, 1H), 7.55 (d, *J* = 7.6 Hz, 1H), 7.41–7.36 (m, 1H), 7.23 (s, 2H), 6.79 (d, *J* = 6.8 Hz, 1H), 6.61 (s, 2H), 4.37 (s, 1H), 3.71 (s, 3H), 3.55 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.3, 159.3, 150.4, 147.1, 146.8, 139.1, 137.6, 132.0, 122.8, 120.4, 118.7, 115.5, 115.1, 113.2, 112.7, 110.0, 58.8, 56.4, 37.2, 29.8; HRMS *m/z* (ESI) calcd for $C_{21}H_{17}N_3O_4$ (M+Na$^+$) 398.1117, found 398.1123.

2-Amino-4-(4-hydroxy-3-methoxyphenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (24)

84%; ^{1}H NMR (DMSO-d_6) δ8.70 (s, 1H), 8.03 (d, *J* = 7.5 Hz, 1H), 7.70–7.65 (m, 1H), 7.53 (d, *J* = 7.6 Hz, 1H), 7.39–7.34 (m, 1H), 7.08 (s, 2H), 6.84 (s, 1H), 6.79 (d, *J* = 8.2 Hz, 1H), 6.60–6.57 (m, 1H), 4.47 (s, 1H), 3.73 (s, 3H), 3.55 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.5, 159.5, 150.4, 147.7, 146.3, 139.3, 136.0, 132.3, 122.7, 120.4, 116.2, 115.2, 113.3, 113.1, 110.0, 59.0, 56.4, 37.1, 29.8; HRMS *m/z* (ESI) calcd for $C_{21}H_{17}N_3O_4$ ($M+Na^+$) 398.1117, found 398.1119.

2-Amino-4-(4-hydroxy-3-methoxy-5-nitrophenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (25)

89%; ^{1}H NMR (DMSO-d_6) δ10.20 (s, 1H), 8.03 (d, *J* = 8.0 Hz, 1H), 7.73–7.67 (m, 1H), 7.55 (d, *J* = 7.6 Hz, 1H), 7.41–7.36 (m, 1H), 7.23 (s, 4H), 4.61 (s, 1H), 3.85 (s, 3H), 3.54 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.5, 159.7, 150.9, 149.9, 142.3, 139.3, 137.4, 135.8, 132.3, 122.7, 120.0, 117.2, 115.4, 114.8, 113.2, 108.7, 57.9, 57.4, 37.4, 29.9; HRMS *m/z* (ESI) calcd for $C_{21}H_{16}N_4O_6$ ($M+Na^+$) 443.0968, found 443.0966.

2-Amino-6-methyl-4-(3-nitrophenyl)-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (26)

77%; ^{1}H NMR (DMSO-d_6) δ8.11–8.04 (m, 3H), 7.75–7.54 (m, 4H), 7.41–7.36 (m, 1H), 7.00 (s, 2H), 4.78 (s, 1H), 3.56 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.4, 160.0, 150.7, 149.9, 139.3, 134.0, 133.8, 132.4, 131.3, 128.4, 124.3, 122.9, 122.7, 119.4, 115.5, 113.1, 108.8, 56.9, 32.6, 29.8; HRMS *m/z* (ESI) calcd for $C_{20}H_{14}N_4O_4$ ($M+Na^+$) 397.0913, found 397.0917.

2-Amino-6-methyl-4-(2-nitrophenyl)-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (27)

73%; ^{1}H NMR (DMSO-d_6) δ8.06–8.03 (m, 1H), 7.86–7.84 (m, 1H), 7.73–7.66 (m, 1H), 7.61–7.51 (m, 2H), 7.45–7.36 (m, 3H), 7.29 (s, 2H), 5.34 (s, 1H), 3.48 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.4, 160.1, 150.8, 150.0, 139.4, 134.0, 133.8, 132.4, 131.3, 128.5, 124.4, 122.8, 119.5, 115.3, 113.2, 108.8, 57.0, 32.6, 29.8; HRMS *m/z* (ESI) calcd for $C_{20}H_{14}N_4O_4$ ($M+Na^+$) 397.0913, found 397.0910.

2-Amino-6-methyl-5-oxo-4-(4-pyridinyl)-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (28)

79%; ^{1}H NMR (DMSO-d_6) δ8.45 (d, *J* = 8.5 Hz, 2H), 8.03 (d, *J* = 8.0 Hz, 1H), 7.73–7.67 (m, 1H), 7.54 (d, *J* = 7.5 Hz, 1H), 7.41–7.22 (m, 5H), 4.55 (s, 1H), 3.52 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.3, 159.7, 153.1, 151.2, 150.4, 150.2, 139.3, 132.3, 123.4, 122.9, 119.8, 115.3, 113.1, 107.9, 57.0, 37.6, 29.8; HRMS *m/z* (ESI) calcd for $C_{19}H_{14}N_4O_2$ ($M+Na^+$) 353.1014, found 353.1017.

2-Amino-6-methyl-5-oxo-4-(3-pyridinyl)-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (29)

81%; ^{1}H NMR (DMSO-d_6) δ8.51 (d, *J* = 3.6 Hz, 1H), 8.40 (m, 1H) 8.05 (m, 1H), 7.72–7.52 (m, 3H), 7.38–7.27 (m, 2H), 7.10 (s, 2H), 4.62 (s, 1H), 3.53 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.5, 159.8, 151.2, 149.8, 148.6, 140.1, 139.5, 135.5, 132.2, 124.4, 123.0, 122.6, 119.7, 115.3, 113.3, 108.7, 58.2, 35.7, 29.7; HRMS *m/z* (ESI) calcd for $C_{19}H_{14}N_4O_2$ ($M+Na^+$) 353.1014, found 453.1018.

2-Amino-4-(2-furyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (30)

75%; ^{1}H NMR (DMSO-d_6) δ8.04 (d, *J* = 8.0 Hz, 1H), 7.73–7.67 (m, 1H), 7.56 (d, *J* = 7.6 Hz, 1H), 7.41–7.34 (m, 2H), 6.93 (s, 2H), 6.32 (s, 1H), 6.14 (d, *J* = 6.1 Hz, 1H), 4.72 (s, 1H), 3.60 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.4, 155.9, 151.5, 142.3, 142.1, 139.4, 132.1, 122.9, 122.5, 119.5, 115.2, 113.4, 110.9, 107.2, 106.2, 56.7, 31.8, 29.9; HRMS *m/z* (ESI) calcd for $C_{18}H_{13}N_3O_3$ ($M+Na^+$) 342.0855, found 342.0851.

2-Amino-6-methyl-4-(5-methyl-2-furyl)-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (31)

78%; ^{1}H NMR (DMSO-d_6) δ8.03 (d, *J* = 8.0 Hz, 1H), 7.71–7.57 (m, 2H), 7.40–7.35 (m, 1H), 7.01 (s, 2H), 5.99–5.91 (m, 2H), 4.64 (s, 1H), 3.60 (s, 3H), 2.17 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.4, 159.7, 151.5, 151.5, 145.0, 139.5, 132.5, 131.2, 129.3, 128.8, 123.1, 122.8, 119.6, 115.4, 113.1, 108.3, 56.9, 36.4, 29.9; HRMS *m/z* (ESI) calcd for $C_{19}H_{15}N_3O_3$ ($M+Na^+$) 356.1011, found 356.1022.

2-Amino-4-(2,3-dichlorophenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (32)

95%; ^{1}H NMR (DMSO-d_6) δ8.05 (d, *J* = 8.0 Hz, 1H), 7.75–7.70 (m, 1H), 7.55 (d, *J* = 8.8 Hz, 1H), 7.48–7.38 (m, 2H), 7.27–7.14 (m, 4H), 5.15 (s, 1H), 3.52 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.4, 159.6, 151.5, 145.0, 139.5, 132.5, 131.2, 129.3, 128.8, 123.1, 122.8, 122.7, 119.6, 115.6, 115.4, 113.1, 108.3, 56.9, 36.4, 29.9; HRMS *m/z* (ESI) calcd for $C_{20}H_{13}Cl_2N_3O_2$ ($M+Na^+$) 420.0283, found 420.0284.

2-Amino-4-(2,6-dichlorophenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (33)

88%; ^{1}H NMR (DMSO-d_6) δ8.05 (d, *J* = 8.0 Hz, 1H), 7.72–7.67 (m, 1H), 7.55–7.21 (m, 5H), 6.91 (bs, 2H), 5.62 (s, 1H), 3.52 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.1, 139.6, 137.0, 132.4, 132.2, 129.6, 129.4, 122.8, 122.6, 122.4, 119.1, 115.1, 113.1, 106.7, 55.2, 34.6, 29.5; HRMS *m/z* (ESI) calcd for $C_{20}H_{13}Cl_2N_3O_2$ ($M+Na^+$) 420.0283, found 420.0280.

2-Amino-4-(3,4-dichlorophenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (34)

97%; ^{1}H NMR (DMSO-d_6) δ8.03 (d, *J* = 8.0 Hz, 1H), 7.72–7.67 (m, 1H), 7.55–7.19 (m, 7H), 4.58 (s, 1H), 3.52 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.4, 159.5, 150.9, 145.9, 139.4, 132.5, 131.4, 131.1, 130.2, 129.8, 128.5, 123.0, 122.7, 119.7, 115.5, 113.2, 108.4, 57.7, 37.3, 29.8; HRMS *m/z* (ESI) calcd for $C_{20}H_{13}Cl_2N_3O_2$ ($M+Na^+$) 420.0283, found 420.0274.

2-Amino-4-(3-chlorophenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (35)

91%; ^{1}H NMR (DMSO-d_6) δ8.02 (d, *J* = 8.0 Hz, 1H), 7.73–7.68 (m, 1H), 7.58–7.16 (m, 8H), 4.55 (s, 1H), 3.53 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.4, 159.5, 150.8, 147.4, 139.3, 133.4, 131.0, 128.0, 126.8, 122.8, 122.6, 120.1, 113.1, 108.7, 57.8, 37.3, 29.8; HRMS *m/z* (ESI) calcd for $C_{20}H_{14}ClN_3O_2$ ($M+Na^+$) 386.0673, found 386.0677.

2-Amino-4-(3-fluorophenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (36)

90%; ^{1}H NMR (DMSO-d_6) δ 8.05–8.02 (m, 1H), 7.72–7.67 (m, 1H), 7.54 (d, *J* = 7.5 Hz, 1H), 7.40–7.00 (m, 7H), 4.58 (s, 1H), 3.54 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.4, 159.6, 150.9, 147.8, 139.4, 132.2, 130.8, 124.0, 122.7, 119.9, 115.5, 114.9, 114.6, 114.2, 113.9,

133.2, 108.9, 58.1, 37.7, 29.9; HRMS *m/z* (ESI) calcd for $C_{20}H_{14}FN_3O_2$ ($M+Na^+$) 307.0968, found 370.0963.

2-Amino-4-(3-bromophenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (37): 93%

^1H NMR (DMSO-d_6) δ 8.07 (d, *J* = 8.0 Hz, 1H), 7.73–7.68 (m, 1H), 7.57–7.24 (m, 7H), 6.98 (s,1H), 4.59 (s, 1H), 3.57 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.5, 159.0, 151.8, 147.9, 139.3, 133.8, 131.0, 128.4, 122.8, 120.0, 113.0, 108.7, 105.4, 57.87 37.5, 29.7; HRMS *m/z* (ESI) calcd for $C_{20}H_{14}BrN_3O_2$ ($M+Na^+$) 430.0167, found 430.0160.

2-Amino-4-(3-bromo-4-fluorophenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (38)

95%; ^1H NMR (DMSO-d_6) δ 8.03 (d, *J* = 8.0 Hz, 1H), 7.72–7.23 (m, 8H), 4.59 (s, 1H), 3.54 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.4, 159.5, 150.9, 143.0, 139.3, 133.0, 132.8, 132.3, 129.5, 123.0, 122.8, 119.9, 117.2, 115.5, 113.2, 108.6, 58.1, 36.7, 29.7; HRMS *m/z* (ESI) calcd for $C_{20}H_{13}BrFN_3O_2$ ($M+Na^+$)448.0073, found 448.0056.

2-Amino-4-(3,5-dibromo-4-hydroxyphenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (39)

92%; ^1H NMR (DMSO-d_6) δ 8.03 (d, *J* = 8.0 Hz, 1H), 7.72–7.23 (m, 8H), 4.50 (s, 1H), 3.55 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.4, 159.5, 150.9, 150.2, 139.5, 139.3, 132.2, 131.8, 123.0, 122.8, 119.9, 115.6, 113.4, 112.3, 108.7, 58.0, 37.5, 29.9; HRMS *m/z* (ESI) calcd for $C_{20}H_{14}Br_2N_3O_3$ ($M+Na^+$) 523.9221, found 523.9210.

2-Amino-4-(3-bromo-4-hydroxy-5-methoxyphenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (40)

94%; ^1H NMR (DMSO-d_6) δ 9.16 (s, 1H), 8.02 (d, *J* = 8.0 Hz, 1H), 7.71–7.53 (m, 2H), 7.39–7.34 (m, 1H), 7.15 (s, 2H), 6.86 (s, 1H), 6.82 (s, 1H), 4.47 (s, 1H), 3.77 (s, 3H), 3.54 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.4, 159.5, 150.6, 148.8, 143.3, 139.2, 136.9, 132.2, 123.5, 123.0, 122.7, 120.1, 115.5, 113.3, 111.8, 109.8, 109.3, 58.5, 57.0, 37.2, 29.9; HRMS *m/z* (ESI) calcd for $C_{21}H_{16}BrN_3O_4$ ($M+Na^+$) 476.0222, found 476.0226.

2-Amino-4-(3-bromo-4,5-dimethoxyphenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (41)

95%; ^1H NMR (DMSO-d_6) δ 8.03 (d, *J* = 8.0 Hz, 1H), 7.72–7.67 (m, 1H), 7.55 (d, *J* = 8.0 Hz, 1H), 7.40–7.35 (m, 1H), 7.21 (s, 2H), 6.98 (d, *J* = 1.7 Hz, 1H), 6.92 (d, *J* = 1.7 Hz, 1H) 4.56 (s, 1H), 3.79 (s, 3H), 3.71 (s, 3H), 3.56 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.4, 159.6, 153.8, 150.9, 145.3, 142.2, 139.3, 132.3, 123.3, 123.0, 122.5, 120.1, 117.0, 115.5, 113.3, 113.0, 108.9, 60.5, 58.2, 56.5, 37.4, 29.8; HRMS *m/z* (ESI) calcd for $C_{22}H_{18}BrN_3O_4$ ($M+Na^+$) 490.0379, found 490.0371.

2-Amino-4-(3,5-dibromophenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (42)

82%; ^1H NMR (DMSO-d_6) δ 8.05 (d, *J* = 8.3 Hz, 1H), 7.65–7.57 (m, 2H), 7.48–7.38 (m, 4H), 7.29 (s, 2H), 4.60 (s, 1H), 3.56 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.4, 159.7, 151.2, 149.6, 132.6, 130.1, 122.8, 115.5, 113.2, 107.8, 57.5, 37.6, 29.7; HRMS *m/z* (ESI) calcd for $C_{20}H_{13}Br_2N_3O_2$ ($M+Na^+$) 507.9272, found 507.9257.

2-Amino-4-(3-bromo-4-methoxyphenyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (43)

64%; ^{1}H NMR (DMSO-d_6) δ 8.02 (d, *J* = 8.0 Hz, 1H), 7.72–7.67 (m, 1H), 7.57–7.54 (d, *J* = 7.5 Hz, 1H), 7.38 (s, 2H), 7.22 (s, 3H), 7.01 (d, *J* = 7.0 Hz, 1H), 4.05 (s, 1H), 3.79 (s, 3H), 3.54 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.3, 159.3, 154.7, 150.5, 139.1, 138.6, 132.4, 128.7, 122.8, 122.6, 120.2, 115.6, 113.1, 110.7, 109.1, 58.1, 56.8, 37.0, 29.8; HRMS *m/z* (ESI) calcd for $C_{21}H_{16}BrN_3O_3$ ($M+Na^+$) 460.0273, found 460.0287.

2-Amino-4-(5-bromo-3-pyridinyl)-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinoline-3-carbonitrile (44)

85%; ^{1}H NMR (DMSO-d_6) δ 8.53 (d, *J* = 2.2 Hz, 1H), 8.50 (d, *J* = 1.6 Hz, 1H), 8.07 (d, *J* = 8.0 Hz, 1H), 7.84 (m, 1H), 7.74–7.69 (m, 1H), 7.56 (d, *J* = 7.6 Hz, 1H), 7.41–7.36 (m, 1H), 7.05 (s, 2H), 4.68 (s, 1H), 3.57 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 160.7, 159.8, 151.3, 149.2, 145.8, 142.4, 139.7, 132.4, 122.8, 115.5, 108.0, 58.2, 36.0, 29.8; HRMS *m/z* (ESI) calcd for $C_{19}H_{13}BrN_4O_2$ ($M+Na^+$) 431.0120, found 431.0139.

4-(2-Amino-3-cyano-6-methyl-5-oxo-5,6-dihydro-4*H*-pyrano[3,2-*c*]quinolin-4-yl)-2-bromo-6-methoxyphenyl acetate (45)

84%; ^{1}H NMR (DMSO-d_6) δ 8.04 (d, *J* = 8.0 Hz, 1H), 7.74–7.69 (m, 1H), 7.57 (d, *J* = 7.6 Hz, 1H), 7.42–7.37 (m, 1H), 7.25 (s, 2H), 7.03 (s, 1H), 7.00 (s, 1H), 4.61 (s, 1H), 3.76 (s, 3H), 3.57 (s, 3H), 2.29 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 168.3, 160.5,159.4, 153.2, 149.6, 142.3, 139.5, 132.8, 122.5, 122.0, 121.7, 120.4, 115.5, 108.1, 57.6, 36.8, 29.6, 20.4; HRMS *m/z* (ESI) calcd for $C_{23}H_{18}BrN_3O_5$ ($M+Na^+$) 518.0328, found 518.0334.

Cell Culture

Human T-cell leukemia cell line Jurkat (ATCC TIB-152, E6-1 clone) and human cervical cancer cell line HeLa (ATCC S3) were cultured in RPMI-1640 (Invitrogen) and supplemented with 10% FBS (Invitrogen), 100 mg/L penicillin G, 100 mg/L streptomycin, 1.0 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose (all from Sigma) at 37 °C in a humidified atmosphere with 10% CO_2. MCF-7 (human mammary carcinoma) were cultured in a mixture of DMEM and HAMF12 (50/50) (Invitrogen) supplemented with 250 IU/mL penicillin, 100 μg/mL streptomycin and 10% FBS.

Annexin V Apoptosis Assay

2×10^5 Jurkat cells/mL were plated in 24 well plates, treated with the required compounds and incubated for the necessary number of hours. The cells were centrifuged at 400 G for 1 min. The supernatant was discarded and the cells were resuspended in 100 μL per sample of Annexin-V-FITC/ propidium iodide solution in HHB (3 μL $CaCl_2$ (1.5 M) per mL HHB, 2 μL (10 mg/mL) propidium iodide (Sigma) per mL HHB and 20 μL Annexin-V-FITC (Southern Biotech, Birmingham, AL) per mL HHB). The samples in the labeling solution were transferred into Falcon tubes and incubated in a water bath at 37 °C for 20 min. The samples were then analyzed using a Becton Dickinson FACscan flow cytometer with CellQuest software. The results were tabulated as % of Annexin-V-FITC positive apoptotic cells.

DNA Laddering

Approximately 1×10^6 Jurkat cells were treated with the required compounds for 36 h. Cells were collected and centrifuged at 400 G for 1 min. The supernatant was discarded and the cells were gently resuspended in 20 μL of lysis buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, 0.5% SDS and 0.5 mg/mL proteinase K (Sigma)) on ice. The suspension of lysed

cells was first incubated at 55 °C for 60 min and then 5 μL of RNAse (Sigma) was added and incubated at 55 °C for another 60 min. The debris was collected by centrifugation and the supernatant collected. The lysate was heated to 70 °C and loading buffer (20 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA) and 10 μL FOG) was added. The samples were loaded on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide and run at 80V for approximately 1 h and visualized under UV light.

MTT Assay

HeLa and MCF-7 cells were transferred to microtiter plates in 100 μL of medium at a concentration of 2×10^4 cells/mL and then incubated for 24 h before treating with the required compounds to allow proper adhesion. Cells were further treated with the panel of test compounds and incubated for 48 h. 20 μL of MTT reagent (5 mg/mL) was added to each well and incubated for 2 h. The resulting formazan crystals were dissolved in 100 μL of DMSO and the OD was determined at a wavelength of 490 nm. The experiments were repeated at least three times for each compound per cell line.

Cell Cycle Analysis

Approximately 2×10^5 Jurkat cells/mL were treated with the respective compounds and incubated for 15 h. Cells were collected into microcentrifuge tubes and centrifuged at 400 G for 1 min. The supernantant was discarded and the cells were resuspended in 200 μL of HHB, Vybrant DyeCycle Orange stain (Invitrogen) solution (1 μL dye per 1 mL HHB). Samples were then transferred to Falcon tubes and incubated at 37 °C for 30 minutes while being protected from light. Samples were analyzed using a Beckon Dickinson FACscan flow cytometer with CellQuest software.

In Vitro Tubulin Polymerization Assay

The *in vitro* tubulin polymerization assay was conducted as described by the manufacturer (Cytoskeleton Inc.). In brief, paclitaxel, DMSO, **40**, **41** and podophyllotoxin were incubated with purified bovine tubulin and buffer containing 10% glycerol and 1 mM GTP at 37 °C each in a separate experiment. The effect of each agent on tubulin polymerization was monitored kinetically using a fluorescent plate reader.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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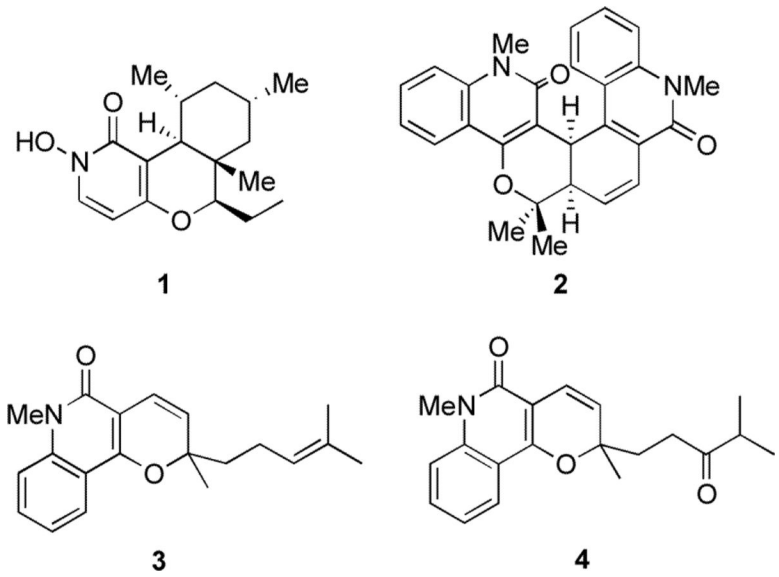


Figure 1.
Anticancer alkaloids based on pyrano[3,2-c]pyridone and pyrano[3,2-c]quinolone scaffolds.

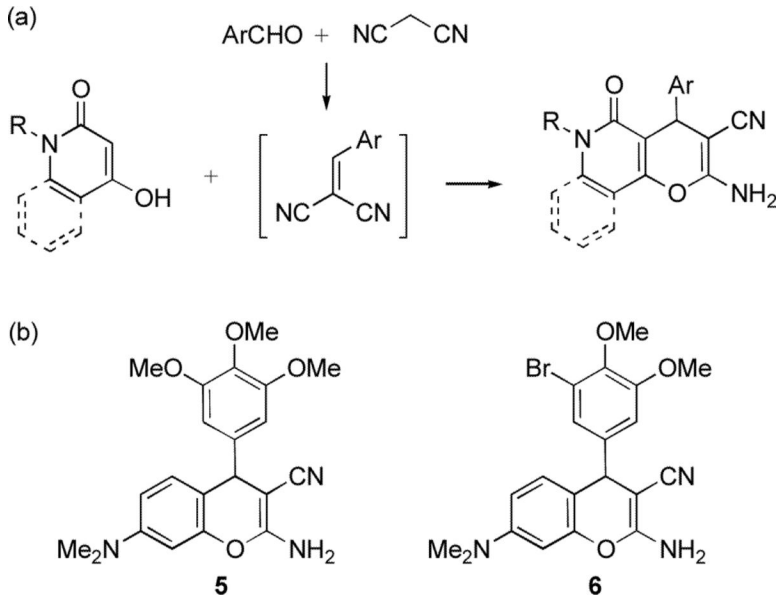


Figure 2.
(a) Proposed MCR for the preparation of pyrano[3,2-c]pyridone and pyrano[3,2-c]quinolone libraries. (b) Chromenes **5** and **6** investigated as anticancer agents.

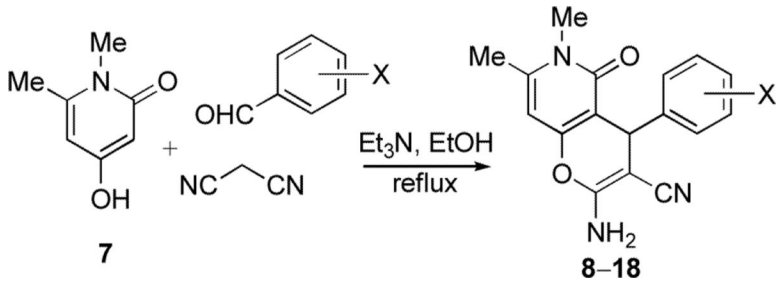


Figure 3.
Three-component synthesis of pyrano-[3,2-*c*]-pyridones.

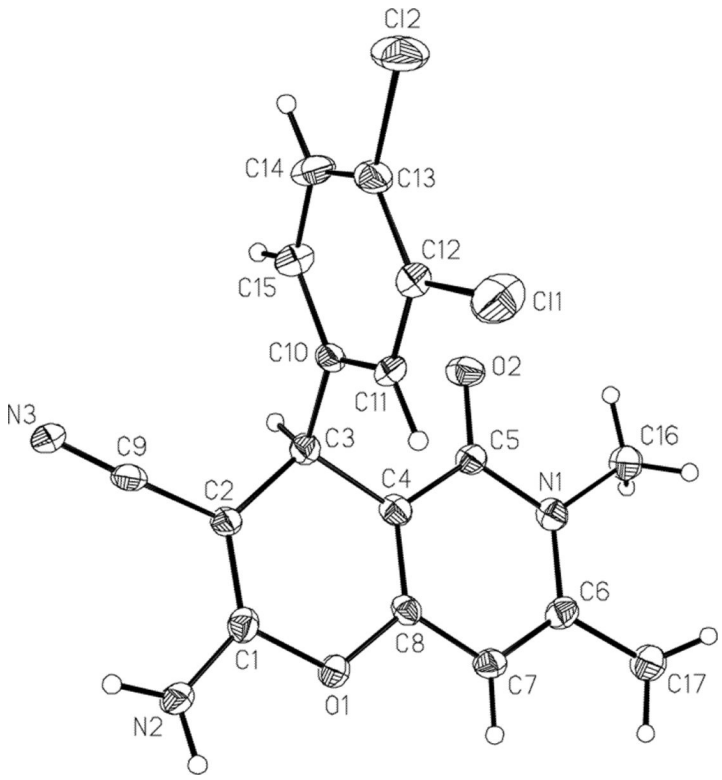


Figure 4.
X-ray structure of pyranopyridone **15** (50% probability thermal ellipsoids).

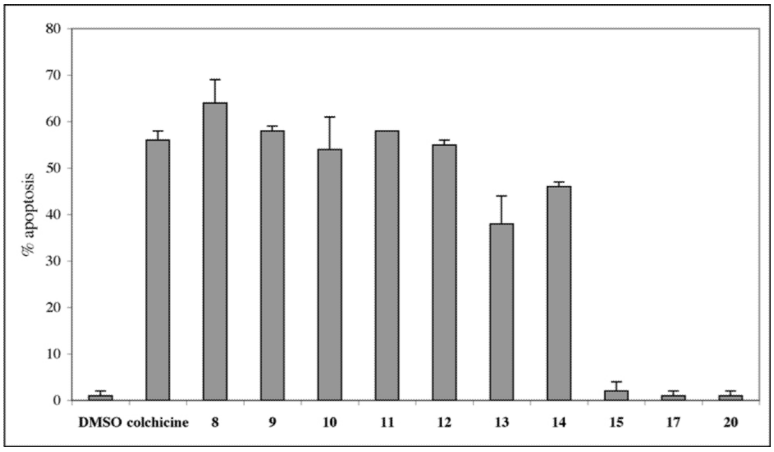


Figure 5.

Induction of apoptosis in Jurkat cells treated for 36 h with DMSO control, colchicine (5 μM) and selected pyridone library analogues (5 μM) in the flow cytometric annexin-V/propidium iodide assay. Error bars represent data from four replicates of a single experiment repeated twice with similar results.

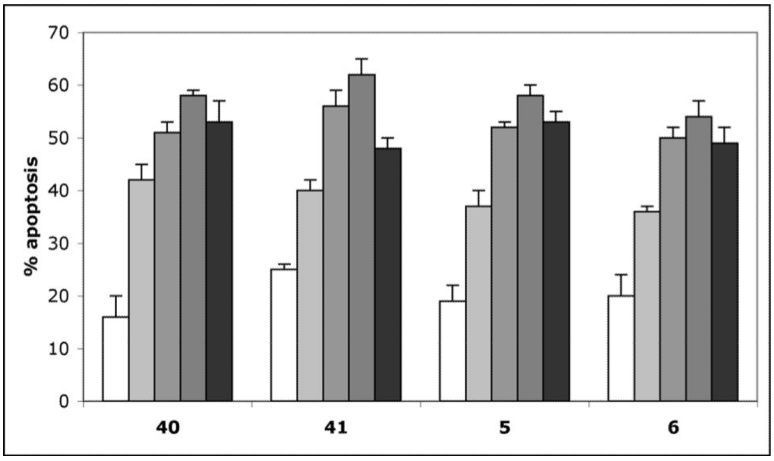


Figure 6.

Induction of apoptosis in Jurkat cells treated with **40**, **41**, **5** and **6** (all used at 1 μM) for 12 h (open columns), 24 h (light grey columns), 36 h (medium grey columns), 48 h (dark grey columns), 60 h (black columns), determined using the flow cytometric annexin-V/propidium iodide assay. Error bars represent data from four replicates of a single experiment repeated twice with similar results.

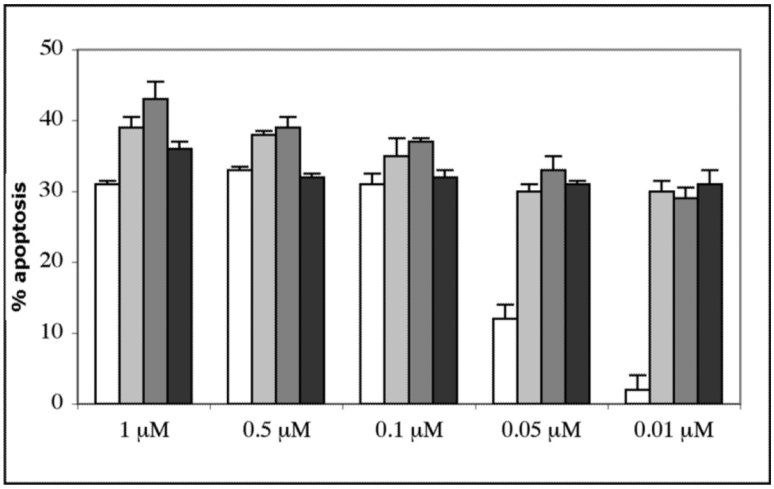


Figure 7.

Dose-dependence of apoptosis induction in Jurkat cells treated for 48 h with **40** (open columns), **41** (light grey columns), **5** (dark grey columns) and **6** (black columns), determined using the flow cytometric annexin-V/propidium iodide assay. Error bars represent data from four replicates of a single experiment repeated twice with similar results.

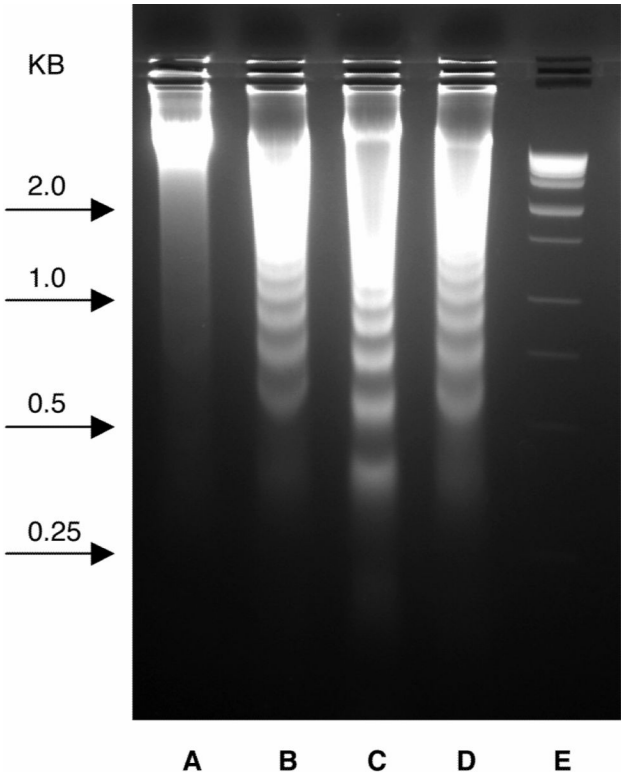


Figure 8.
DNA laddering in Jurkat cells after 36 h of treatment. (A) DMSO control, (B) **40** at 1.5 μM, (C) **41** at 1.5 μM, (D) paclitaxel at 0.4 μM, and (E) Molecular weight marker (KB).

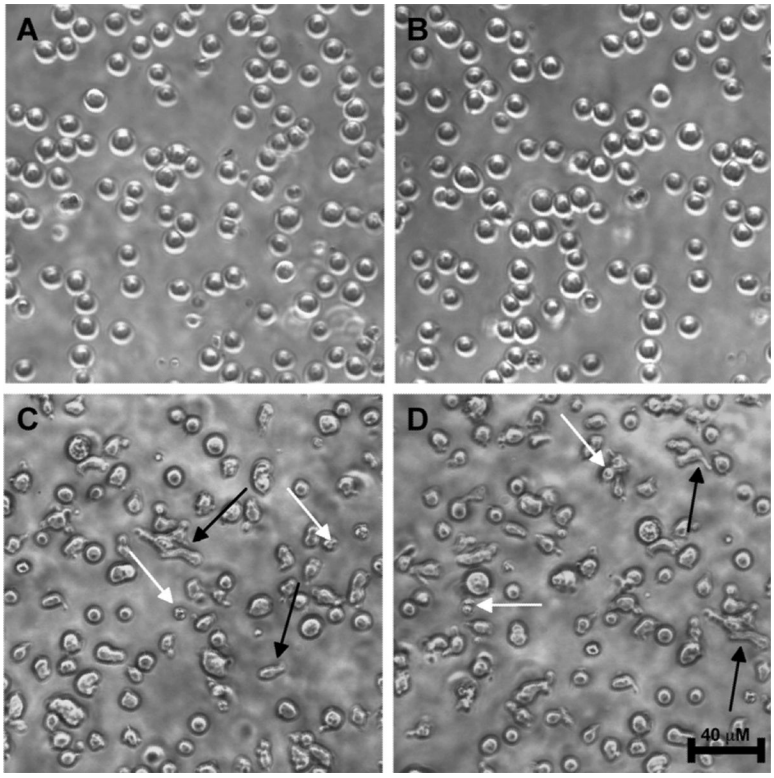


Figure 9.

Light microscopy pictures. Treatment of Jurkat cells with **40** (C) and **41** (D) for 2 hours induces formation of finger-like extensions (black arrows) and shriveling (white arrows). 0.1% DMSO (A) and **6** (B) were used as controls. All compounds are used at 1 μM. The scale bar indicates 40 μm.

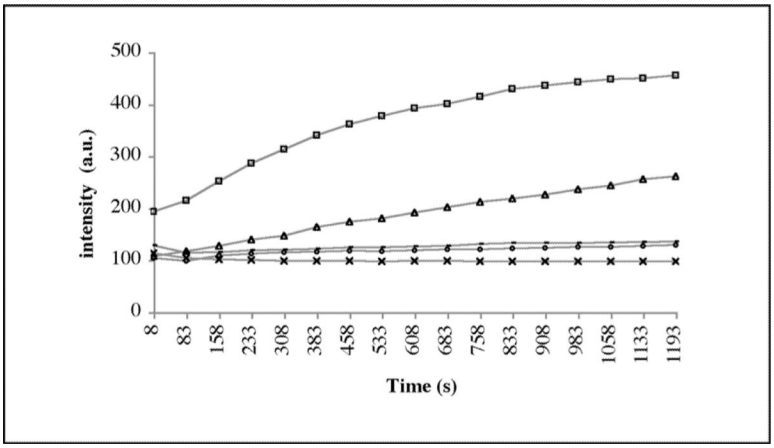
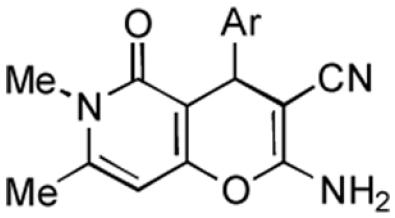


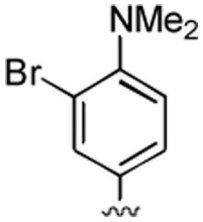
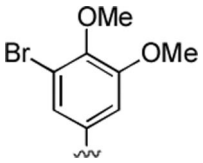
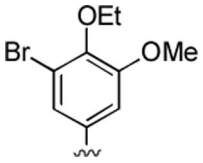
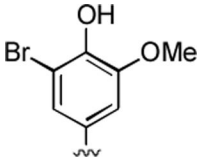
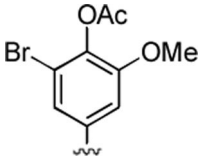
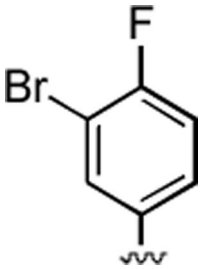
Figure 10.

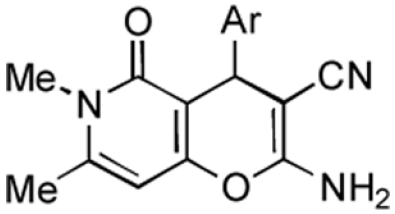
Effect of pyranoquinolones on *in vitro* tubulin polymerization. Paclitaxel (3 μM, square markers) promotes microtubule formation relative to 0.05% DMSO control (triangle markers). **40** (25 μM, dash markers), **41** (25 μM, circle markers) and podophyllotoxin (25 μM, cross markers) completely suppress tubulin polymerization. Each data point is a mean of two independent experiments producing similar results.

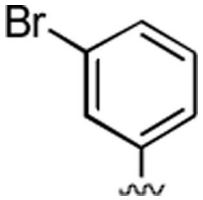
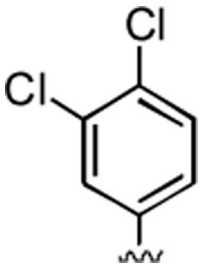
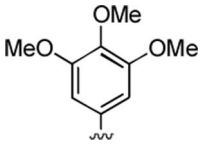
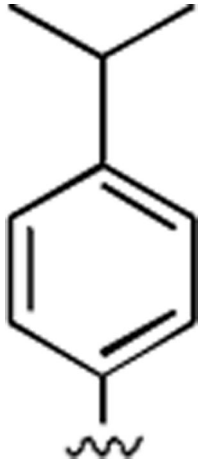
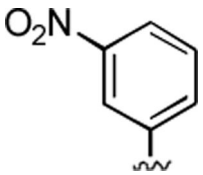
Table 1

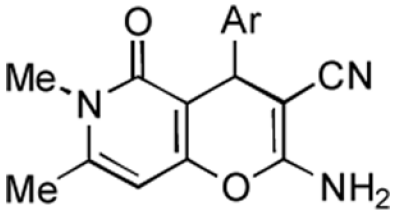
Synthetic yields and antiproliferative activity of pyrano-[3,2-*c*]-pyridones.

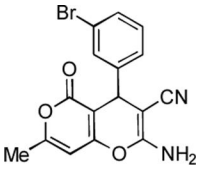
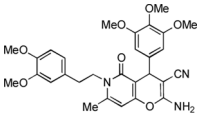


analogue	Ar	synthesis % yield	cell viability[a] GI_{50}, µM
8		83	0.33 ± 0.06
9		87	0.58 ± 0.14
10		88	1.1 ± 0.8
11		75	2.7 ± 1.1
12		83	3.5 ± 1.3
13		84	6.4 ± 1.1



analogue	Ar	synthesis: % yield	cell viability[a]: GI_{50}, µM
14		97	6.5 ± 1.3
15		98	18.3 ± 2.9
16		97	43.3 ± 5.1
17		81	> 100
18		97	35.0 ± 21.8

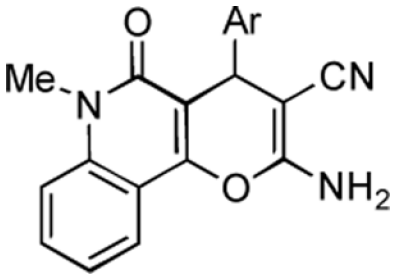


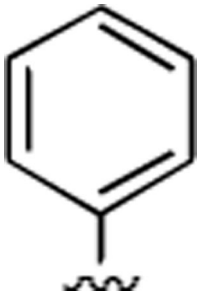
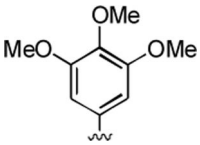
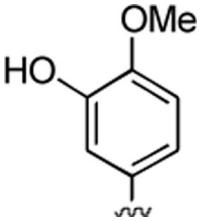
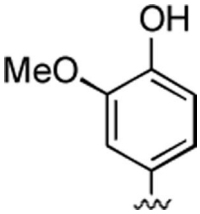
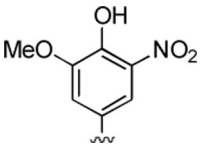
analogue	Ar	synthesis: % yield	cell viability[a]: GI_{50}, µM
19		87	> 100
20		80	22.7 ± 6.4

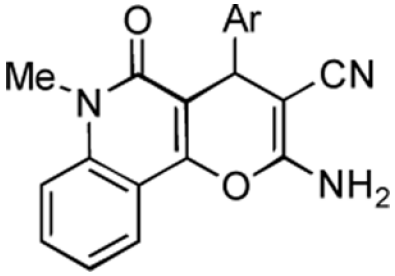
[a]Concentration required to reduce the viability of HeLa cells by 50% after 48 h of treatment with indicated compounds relative to DMSO control ± SD from three independent experiments, each performed in 4 replicates, determined by MTT assay.

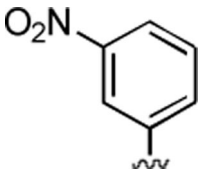
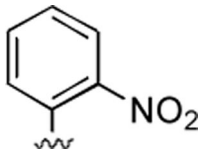
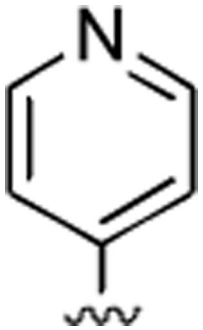
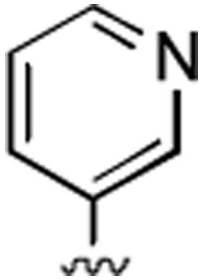
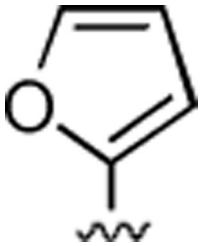
Table 2

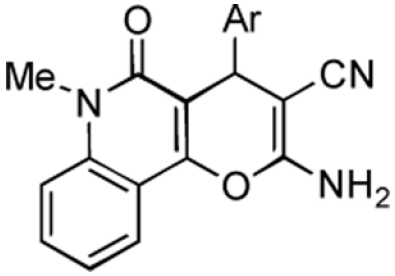
Synthetic yields and antiproliferative activity of pyrano-[3,2-*c*]-quinolones.

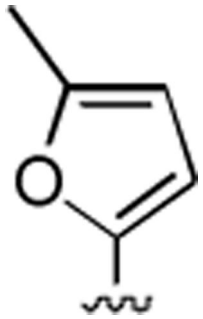
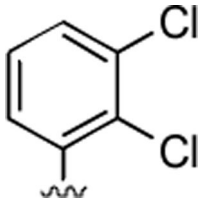
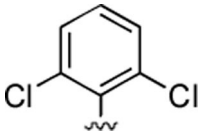
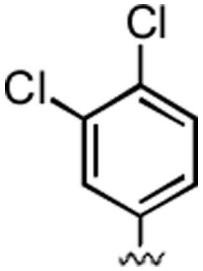
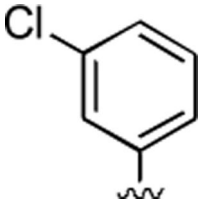


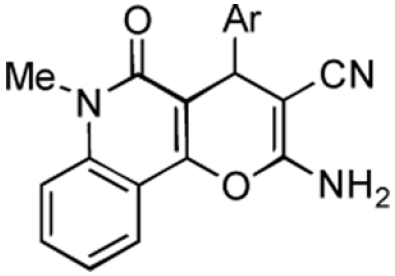
analogue	Ar	synthesis % yield	cell viability[a] GI_{50}, μM HeLa	MCF-7
21		78	0.39 ± 0.01	3.38 ± 0.53
22		86	0.24 ± 0.02	1.0 ± 0.3
23		82	> 50	> 50
24		84	0.63 ± 0.05	0.5 ± 0.14
25		89	0.63 ± 0.02	0.71 ± 0.12

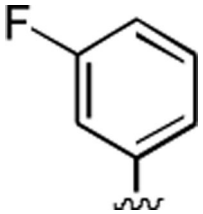
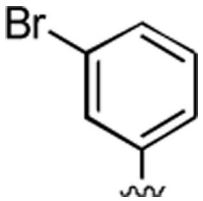
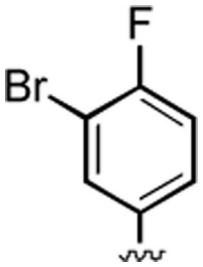
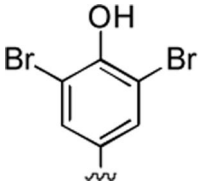
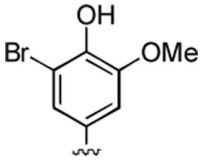
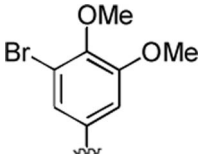


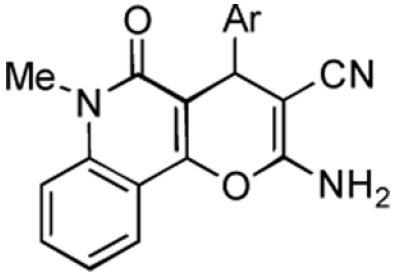
analogue	Ar	synthesis % yield	cell viability[a] GI_{50}, μM HeLa	MCF-7
26		77	0.32 ± 0.02	1.1 ± 0.1
27		73	5.0 ± 1.4	3.5 ± 0.3
28		79	> 50	32 ± 2
29		81	2.0 ± 0.1	> 50
30		75	22 ± 1	35 ± 2

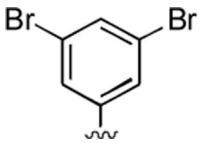
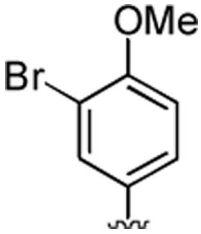
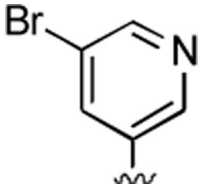
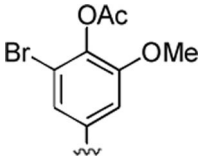


analogue	Ar	synthesis % yield	cell viability[a] GI_{50}, μM HeLa	MCF-7
31		78	3.9 ± 0.2	5.1 ± 0.1
32		95	1.8 ± 0.2	> 50
33		88	> 50	36 ± 2
34		97	0.3 ± 0.03	2.5 ± 0.3
35		91	0.15 ± 0.04	2.2 ± 0.2



analogue	Ar	synthesis % yield	cell viability[a] GI_{50}, μM HeLa	MCF-7
36		90	0.30 ± 0.01	1.0 ± 0.3
37		93	0.74 ± 0.03	0.003 ± 0.001
38		95	0.27 ± 0.03	0.81 ± 0.08
39		92	0.27 ± 0.02	0.43 ± 0.01
40		94	0.047 ± 0.010	0.39 ± 0.16
41		95	0.014 ± 0.003	0.38 ± 0.03



analogue	Ar	synthesis % yield	cell viability[a] GI_{50}, μM HeLa	MCF-7
42		82	0.077 ± 0.006	0.075 ± 0.007
43		64	0.41 ± 0.04	0.5 ± 0.1
44		85	0.013 ± 0.003	0.015 ± 0.008
45		84	0.18 ± 0.02	0.025 ± 0.06
5	NA	NA	0.017 ± 0.004	0.50 ± 0.05
6	NA	NA	0.007 ± 0.002	0.50 ± 0.14

[a]Concentration required to reduce the viability of cells by 50% after 48 h of treatment with indicated compounds relative to DMSO control ± SD from two independent experiments, each performed in 8 replicates, determined by MTT assay.

Table 3

Flow cytometric cell cycle analysis of Jurkat cells.

compound	% relative DNA content[a]		
	G0/G1	S	G2/M
DMSO	56 ± 2	21 ± 3	20 ± 2
40	27 ± 3	22 ± 2	47 ± 3
41	20 ± 2	28 ± 2	49 ± 2
5	19 ± 1	37 ± 3	42 ± 1
6	19 ± 2	36 ± 2	41 ± 3

[a]% Relative DNA content ± SD after 24 h treatment of Jurkat cells with indicated compounds from two independent experiments each performed in triplicate. Compounds **40**, **41**, **5** and **6** are used at 1 μM, obtained using the flow cytometric Vybrant Orange staining assay. The remaining % DNA content is found in sub G0/G1 region.