Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 4591–4598

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Cytotoxic Activity of Some New Azapyranoxanthenone Aminoderivatives

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Received 17 June 2003; accepted 25 July 2003

Abstract—A series of novel azapyranoxanthenones, bearing structural similarity to the acridone alkaloid acronycine have been designed and synthesized. Their in vitro cytotoxicities against the murine L1210 leukemia and the human solid tumor HT-29 cell lines have been investigated. The new derivatives exhibited interesting cytotoxic activity and were more potent than the parent compound.

Introduction

Among the chemotherapeutic agents, DNA-interacting compounds constitute a significant class and many of them are in clinical use or in clinical trials for the treatment of cancer. Within this diverse group of compounds, molecules with quinone functionalities, such as the anthracycline antibiotics adriamycin and daunomycin and the anthracenedione mitoxantrone are representative examples of drugs that target DNA, interact with it by binding and intercalation and exert their critical cell-killing effects through many different mechanisms.[1] A great number of mitoxantrone related derivatives has been synthesized and studied, in attempts to reduce or remove undesirable side effects, mainly those associated with myelosuppression and cardiotoxicity.[2] These modified analogues are characterized by the presence of a planar or semi-planar lipophilic chromophore portion, substituted with one or two flexible basic side chains.[3] A common variation of known tricyclic antitumor agents has been the incorporation of a *N*-substituted pyrazolo ring fusion at the 1- and 9-positions and the resulting anthrapyrazoles are usually endowed with an increased spectrum of antitumor activity and reduced cardiotoxicity, compared with the corresponding quinone chemotypes.[4] Further improved pharmacological profile was achieved in some cases with different aza- and diaza-bioisosteres, derived through extensive structure–activity relationships studies, which clearly demonstrated that the cytotoxic effects are critically depended on the position of the nitrogen atom.[5]

We have already been involved in the synthesis and antiproliferative activity study of a number of cytotoxic pyranoxanthenones and pyranothioxanthenones,[6] which share close structural similarity with the acridone alkaloid acronycine (Fig. 1), a promising, broad-spectrum antitumor agent, which suffers from poor water solubility.[7]

The introduction of a flexible amino substituted side chain, or the incorporation of a fused amino substituted pyrazolo ring in the abovementioned molecules resulted in a noticeable increase of the cytotoxic activity against the murine leukemia L1210 and the human colon adenocarcinoma HT-29 cell lines.[8] As a continuation of these studies we decided to introduce a nitrogen atom into the xanthone chromophore, hence we present here the synthesis and in vitro cytotoxic activity evaluation of some new derivatives, in an effort to study the potential alteration of the biological activity, due to the replacement of the carbocyclic moiety by the isosteric pyridine heterocycle.

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doi:10.1016/S0968-0896(03)00503-0

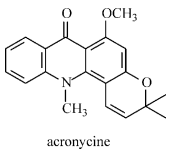
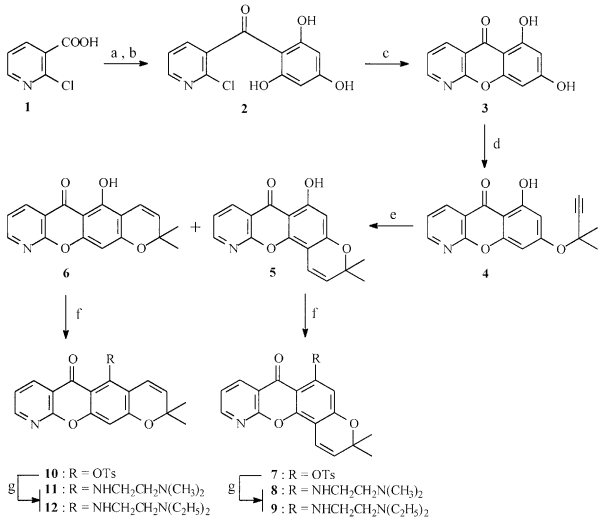


Figure 1.

Chemistry

For the preparation of the target derivatives we have used as starting material 2-chloronicotinic acid (**1**, Scheme 1), which was converted to the corresponding acylchloride after treatment with thionylchloride in refluxing toluene. Reaction of this chloride with 1,3,5-trihydroxybenzene in the presence of aluminum chloride in 1,2-dichloroethane provided the ketone **2** in good yield, which was then treated with aqueous sodium carbonate to result in the dihydroxy-derivative **3**. The preparation of this compound has been previously reported by a different procedure, through Ullmann condensation of 2-chloronicotinic acid with 3,5-dimethoxyphenol, followed by cyclization with PPA and *O*-demethylation.[9] However, the procedure reported herein is simpler and the product was obtained in a higher overall yield (51% vs 41%). The next step concerns the formation of the pyran ring. The most common method involves the etherification of the phenol hydroxyl, followed by thermal cyclisation of the resulting ether in boiling *N*,*N*-diethylaniline. In the case of compound **3** the deactivation of the 6-hydroxyl group, due to its chelation by the 5-carbonyl, made feasible the selective etherification of the 8-hydroxyl group with 3-chloro-3-methyl-1-butyne.

However, previous attempts to apply this condensation in the presence of anhydrous potassium carbonate and sodium iodide, in dry DMF at 70 °C, with various reaction periods, gave a single product, which proved to be the angular isomer **5**, in an optimized 20% yield.[9] No traces of the intermediate ether **4** or the linear isomer **6** were isolated. We have applied a slightly different procedure using CuI as catalyst for the etherification step[10] and we have obtained the ether **4** in a 76% isolated yield. This compound was then subjected to thermal cyclisation in boiling toluene to afford a 1:1 mixture of two four-ring isomers (**5** and **6**), which were separated by column chromatography and unambiguously identified on the basis of 1D and 2D-NMR spectral data.



Scheme 1. (a) $SOCl_2$, toluene dry, 90 °C, 1.5 h; (b) phloroglucinol, $AlCl_3$, 1,2-dichloroethane, 50 °C, 60 h; (c) 20% Na_2CO_3, 110 °C, 60 h; (d) 3-chloro-3-methyl-1-butyne, K_2CO_3, KI, CuI, DMF dry, 75 °C, 9 h; (e) toluene dry, reflux, 46 h; (f) TsCl, K_2CO_3, acetone dry, reflux, 3 h; (g) *N*,*N*-dimethylethylenediamine or *N*,*N*-diethylethylenediamine, DMSO dry, 100 °C, 1 h.

Each isomer was subsequently converted to the corresponding tosylate (**7** and **10**, respectively) by standard conditions. Treatment of the latter analogues with commercially available *N*,*N*-dialkylaminoethylenediamines in DMSO solution provided the target angular (**8**, **9**) and linear (**11**, **12**) diamines.

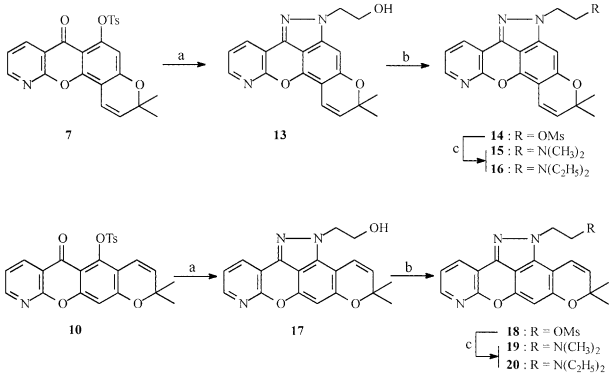
The synthesis of the pyrazolo-fuzed analogues is depicted in Scheme 2.

The angular tosylate **7** was treated with 2-hydroxyethylhydrazine and the resulted carbinol **13** was converted to the corresponding mesylate **14**. Nucleophilic substitution of the mesyl group with the appropriately substituted secondary amines gave the amines **15** and **16** in good yield. The linear derivatives **19** and **20**, were prepared by an analogous reaction sequence, starting from the tosylate **10**.

In order to examine the in vitro antineoplastic activity of the new compounds, the free base forms of the amines were converted into their water-soluble hydrochloride addition salts.

Results and Discussion

The cytotoxic activity of the new derivatives was evaluated in vitro in the established model of the murine L1210 leukemia cell line as well as against the human solid tumor HT-29 cell line, using acronycine and the drugs doxorubicin and mitoxantrone as reference compounds. The results are presented in Table 1.



Scheme 2. (a) 2-Hydroxyethylhydrazine, DMSO dry, 90 °C, 30 min; (b) MsCl, Et_3N, CH_2Cl_2 dry, rt, 2 h; (c) dimethylamine or diethylamine, EtOH, reflux, 20 h.

Table 1. Cytotoxic activity of the synthesized compounds

Compd	IC_{50} (μM)	
	L1210	HT-29
8	17.8 (±0.6)	11.8 (±5.2)
9	17.3 (±1.9)	6.15 (±2.85)
11	19.2 (±3.5)	15.3 (±2.5)
12	16.9 (±1.8)	6.95 (±2.45)
15	17.9 (±2.1)	15.9 (±1.1)
16	19.0 (±1.3)	5.15 (±0.15)
19	19.2 (±0.9)	8.85 (±3.25)
20	18.0 (±0.5)	5.25 (±2.65)
Acronycine	25.2 (±2.3)	>100
Mitoxantrone	nt	0.02 (±0.009)
Doxorubicin	0.08 (±0.01)	0.51 (±0.06)

Exponentially growing L1210 and HT-29 cells were incubated with serial dilutions of the indicated substances for 72 h. MTT-assay was performed as described in the Experimental. Values represent the mean (±standard deviation) of three independent experiments and were expressed as IC_{50}, the concentration that reduced by 50% the optical density of treated cells with respect to untreated controls. nt,

All the tested compounds proved to be more cytotoxic than acronycine against the L1210 cell line and at the same time they exhibit cytotoxicity against the HT-29 cell line, where the reference compound is inactive. This is consistent with our previous observations[8] and confirms the beneficial effects of the introduction of a dialkylaminoethylamino basic side chain for the cytotoxicity. In general, all the derivatives possess almost equal cytotoxicity against the L1210 cell line and the corresponding IC_{50} values were found to be in the range of 17–19 μM. On the contrary, concerning the HT-29 cell line, we observe that the *N*,*N*-diethylamino derivatives appear to be approximately twice more active than the corresponding *N*,*N*-dimethylamino analogues. Notably, in contrast to many chemotherapeutic agents, all compounds exhibit solid tumor selectivity, since they demonstrate greater potency, in terms of IC_{50} values, against the HT-29 cell line than against the leukemia L1210 cells.

From a comparison of the activity of the newly synthesized azapyranoxanthenones with the corresponding closely related analogues which lack the aza substitution,[8] we assume that the presence of the nitrogen atom results in a reduced cytotoxicity against the L1210 cell line, whereas the compounds remain equally active against the HT-29 cell line. It should be noted that the abovementioned comparison is not directly feasible for derivatives **19** and **20**, since this particular ring system has not been previously reported.

Table 2. Cell-cycle phase distribution (%)

	G0/G1	S	G2/M
Control	41.21	42.21	16.57
8	58.19	38.23	3.58
9	68.74	26.63	4.63
11	94.92	1.97	3.11
12	73.17	21.69	5.14
15	55.91	23.80	20.29
16	56.18	25.21	19.61
19	64.34	27.57	8.09
20	68.44	21.39	10.17
Doxorubicin	44.41	0.00	55.59
Mitoxantrone	2.55	1.70	95.76

Exponentially growing HT-29 cells received the indicated substances (20 μM, except doxorubicin 100 nM and mitoxantrone 80 nM). After incubation for 24 h, they were subjected to FACS analysis, as described in the Experimental. One representative determination out of three similar ones is demonstrated.

Cell-cycle perturbations induced by the new compounds were studied on the HT-29 cell line (Table 2).

As expected, both doxorubicin[11] and mitoxantrone[12] act as potent G2 blockers. On the other hand, all new derivatives seem to achieve their cytostatic effects by blocking the entry of the cells in the S-phase, the linear diamines **11** and **12** being the most effective. Furthermore, the angular pyrazoloderivatives **15** and **16** cause a slight increase of the G2 arrested cell-percentage, as well.

Experimental

All chemicals were purchased from Aldrich Chemical Co. Melting points were determined on a Büchi apparatus and are uncorrected. ^{1}H NMR spectra and 2D spectra were recorded on a Bruker Avanche 400 instrument, whereas ^{13}C NMR specra were recorded on a Bruker AC 200 spectrometer in deuterated solvents and were referenced to TMS (δ scale). The signals of ^{1}H and ^{13}C spectra were unambiguously assigned by using 2D NMR techniques: ^{1}H^{1}H COSY, NOESY HMQC and HMBC. Flash chromatography was performed on Merck silica gel 60 (0.040-0.063 mm). Analytical thin layer chromatography (TLC) was carried out on precoated (0.25 mm) Merck silica gel F-254 plates. Elemental analyses were performed at the Microanalytical Sections of the National Hellenic Research Foundation on a Perkin-Elmer PE 240C Elemental Analyzer (Norwalk, CT, USA) and were within ±0.4% of the theoretical values.

(2-Chloropyridin-3-yl)-(2,4,6-trihydroxyphenyl)methanone (2). Thionyl chloride (5 mL) was added under argon to a suspension of 2-chloronicotinic acid (**1**, 3 g, 19.04 mmol) in dry toluene (5 mL) and the resulting solution was heated at 90 °C for 1.5 h. Excess thionyl chloride and the bulk of the solvent were evaporated under vacuum and then dry 1,2-dichloroethane (10 mL) was added to the white crystalline residue. This solution was added slowly under argon to a stirred mixture of phloroglucinol (2 g, 15.86 mmol) and aluminium trichloride (8.46 g, 63.48 mmol) in 1,2-dichloroethane (20 mL) at 0 °C. The mixture was stirred at rt for 10 min and then heated at 50 °C for 60 h. Upon cooling, a 9% HCl solution (40 mL) was added and the precipitate was filtrated, washed with water (2×40 mL) and dried over $CaCl_2$. The crude product was purified by column chromatography (silica gel, 18×2 cm) using ethyl acetate as the eluent to give **2** (2.20 g, 52%) as a pale yellow solid. Mp >250 °C, (EtOH). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 6.26 (d, J=2 Hz, 1H, H-3′), 6.48 (d, J=2 Hz, 1H, H-5′), 7.59 (m, 1H, H-5), 8.58 (m, 1H, H-6), 8.77 (m, 1H, H-4), 8.96 (s, 2H, D_2O exch., OH-4′,

OH-6′), 12.53 (s, 1H, D_2O exch., OH-2′). ^{13}C NMR (DMSO-d_6, 50 MHz) δ (ppm): 94.80 (C-5′), 98.58 (C-3′), 102.18 (C-1′), 115.19 (C-1), 121.70 (C-5), 136.14 (C-6), 154.45 (C-4), 157.02 (C-6′), 159.70 (C-2), 162.83 (C-2′), 166.43 (C-4′), 180.25 (–CO–). Anal. calcd for $C_{12}H_8ClNO_4$. Calcd (%): C: 54.25, H: 3.04, N: 5.27. Found (%): C: 54.12, H: 2.96, N: 5.21.

6,8-Dihydroxy-5*H*-[1]benzopyrano[2,3-*b*]pyridin-5-one (3). A suspension of **2** (4 g, 15.06 mmol) in 20% Na_2CO_3 solution (40 mL) was heated at 110 °C for 60 h. The mixture was then allowed to cool, neutralized with concentrated HCl and the precipitate was filtrated, washed with water (2×30 mL) and dried over $CaCl_2$. The crude product was purified by column chromatography (silica gel, 15×2 cm) using ethyl acetate as the eluent to give **3** (3.38 g, 98%) as a pale yellow solid. Mp >250 °C, (EtOH). ^1H NMR (DMSO-d_6, 400 MHz) δ (ppm): 6.25 (d, $J=2$ Hz, 1H, H-7), 6.48 (d, $J=2$ Hz, 1H, H-9), 7.59 (m, 1H, H-3), 8.57 (m, 1H, H-4), 8.77 (m, 1H, H-2), 11.28 (s, 1H, D_2O exch., OH-8), 12.53 (s, 1H, D_2O exch., OH-6). ^{13}C NMR (DMSO-d_6, 50 MHz) δ (ppm): 94.80 (C-9), 98.59 (C-7), 102.26 (C-5a), 115.20 (C-4a), 121.71 (C-3), 136.19 (C-4), 154.46 (C-2), 157.07 (C-9a), 159.72 (C-10a), 162.84 (C-6), 166.37 (C-8), 180.30 (C-5). Anal. calcd for $C_{12}H_7NO_4$. Calcd (%): C: 62.89, H: 3.08, N: 6.11. Found (%): C: 62.71, H: 3.03, N: 5.93.

6-Hydroxy-8-(1,1-dimethylpropyn-2-oxy)-5*H*-[1]benzopyrano[2,3-*b*]pyridin-5-one (4). 3-Chloro-3-methyl-1-butyne (3.17 mL, 30.98 mmol) was added under argon to a mixture of **3** (2.28 g, 9.95 mmol), anhydrous K_2CO_3 (1.65 g, 11.95 mmol), anhydrous KI (1.69 g, 10.18 mmol) and CuI (52 mg, 0.27 mmol) in dry DMF (20 mL) and the reaction was stirred at 75 °C for 9 h. The bulk of the solvent was then vacuum-evaporated, water was added to the residue and it was extracted with ethyl acetate. The organic phase was washed with water, a saturated NaCl solution, dried (Na_2SO_4) and evaporated to dryness. The residue was purified by column chromatography (silica gel, 15×1.5 cm) using a mixture of cyclohexane/ethyl acetate (9/1) as the eluent to give **4** (2.24 g, 76%) as a yellow solid. Mp 122–124 °C, (EtOAc–*n*-hexane). ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.74 (s, 6H, 2×gem CH_3), 2.70 (s, 1H, H-3′), 6.68 (d, $J=2$ Hz, 1H, H-9), 6.95 (d, $J=2$ Hz, 1H, H-7), 7.41 (m, 1H, H-3), 8.61 (m, 1H, H-4), 8.70 (m, 1H, H-2), 12.36 (s, 1H, D_2O exch., OH). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 29.51 (2×gem CH_3), 72.99 (C-1′), 75.60 (C-3′), 84.31 (C-2′), 97.88 (C-7), 101.88 (C-9), 104.05 (C-5a), 115.08 (C-4a), 121.03 (C-3), 136.51 (C-4), 154.19 (C-2), 156.54 (C-6), 160.41 (C-10a), 162.79 (C-9a), 163.89 (C-8), 181.02 (C-5). Anal. calcd for $C_{17}H_{13}NO_4$. Calcd (%): C: 69.15, H: 4.44, N: 4.74. Found (%): C: 69.41, H: 4.52, N: 4.68.

6-Hydroxy-3,3-dimethyl-3*H*,7*H*-pyrano[2′,3′:7,8]benzopyrano[2,3-*b*]pyridin-7-one (5) and 5-hydroxy-2,2-dimethyl-2*H*,6*H*-pyrano[3′,2′:6,7]benzopyrano[2,3-*b*]pyridin-6-one (6). A solution of **4** (2.24 g, 7.59 mmol) in dry toluene (50 mL) was heated under reflux for 46 h. The solvent was then evaporated to dryness and the residue was purified by column chromatography (silica gel, 25×1.5 cm) using a mixture of cyclohexane/ethyl acetate (85/15) as the eluent to give **5** (0.92 g, 41%) and 6 (1.07 g, 48%) as yellow solids. Data for **5**: Mp 208–210 °C, (EtOAc–*n*-hexane), ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.45 (s, 6H, 2×gem CH_3), 5.60 (d, $J=10$ Hz, 1H, H-2), 6.23 (s, 1H, H-5), 6.87 (d, $J=10$ Hz, 1H, H-1), 7.38 (m, 1H, H-9), 8.56 (m, 1H, H-8), 8.65 (m, 1H, H-10), 12.53 (s, 1H, D_2O exch., OH). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 28.45 (2×gem CH_3), 78.58 (C-3), 99.86 (C-5), 101.59 (C-12b), 103.39 (C-6a), 114.74 (C-1), 115.63 (C-7a), 121.07 (C-9), 127.61 (C-2), 136.39 (C-8), 151.02 (C-12a), 153.92 (C-10), 160.02 (C-11a), 161.61 (C-4a), 162.93 (C-6), 180.68 (C-7). Anal. calcd for $C_{17}H_{13}NO_4$. calcd (%): C: 69.15, H: 4.44, N: 4.74. Found (%): C: 68.87, H: 4.36, N: 4.70. Data for **6**: Mp 245–247 °C, (EtOAc–*n*-hexane). ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.42 (s, 6H, 2×gem CH_3), 5.59 (d, $J=10$ Hz, 1H, H-3), 6.43 (s, 1H, H-12), 6.67 (d, $J=10$ Hz, 1H, H-4), 7.38 (m, 1H, H-8), 8.57 (m, 1H, H-7), 8.66 (m, 1H, H-9), 12.73 (s, 1H, D_2O exch., OH). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 28.48 (2×gem CH_3), 78.69 (C-2), 96.00 (C-12), 103.46 (C-5a), 105.08 (C-4a), 115.04 (C-4), 115.71 (C-6a), 121.00 (C-8), 127.95 (C-3), 136.36 (C-7), 154.01 (C-9), 156.51 (C-11a), 157.57 (C-5), 160.18 (C-10a), 161.51 (C-12a), 180.80 (C-6). Anal. calcd for $C_{17}H_{13}NO_4$. calcd (%): C: 69.15, H: 4.44, N: 4.74. Found (%): C: 68.96, H: 4.29, N: 4.65.

6-[(4-Methylphenyl)sulfonyl]oxy-3,3-dimethyl-3*H*,7*H*-pyrano[2′,3′:7,8]benzopyrano[2,3-*b*]pyridin-7-one (7). Anhydrous potassium carbonate (1.28 g, 9.30 mmol) and p-toluenesulfonyl chloride (1.20g, 6.28 mmol) were added to a solution of **5** (550 mg, 1.86 mmol) in dry acetone (50 mL) and the mixture was heated under reflux for 3 h. Upon cooling the precipitate was filtered off, washed with acetone (40 mL), the filtrates were vacuum-evaporated and the residue was purified by column chromatography (silica gel, 10×1.5 cm) using a mixture of cyclohexane/ethyl acetate (75/25) as the eluent to give **7** (611 mg, 73%) as a yellow solid. Mp 170–172 °C, (EtOAc–*n*-pentane). ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.39 (s, 6H, 2×gem CH_3), 2.30 (s, 3H,-$SO_2C_6H_4C\underline{H}_3$), 5.66 (d, $J=10$ Hz, 1H, H-2), 6.49 (s, 1H, H-5), 6.86 (d, $J=10$ Hz, 1H, H-1), 7.24 (d, $J=8$ Hz, 2H, H-5′, H-3′), 7.31 (m, 1H, H-9), 7.84 (d, $J=8$ Hz, 2H, H-6′, H-2′), 8.49 (m, 1H, H-8), 8.53 (m, 1H, H-10). ^{13}C HMR ($CDCl_3$, 50 MHz) δ (ppm): 21.31 (–$SO_2C_6H_4\underline{C}H_3$), 27.96 (2×gem CH_3), 78.62 (C-3), 108.43 (C-12b), 108.91 (C-5), 109.46 (C-6a), 114.31 (C-1), 116.70 (C-7a), 121.07 (C-9), 128.42 (C-6′, C-2′), 129.31 (C-5′, C-3′), 130.11 (C-2), 132.43 (C-1′), 136.99 (C-8), 145.15 (C-4′), 147.46 (C-6), 151.84 (C-12a), 153.05 (C-10), 157.79 (C-4a), 158.82 (C-11a), 173.78 (C-7). Anal. calcd for $C_{24}H_{19}NO_6S$. calcd (%): C: 64.13, H: 4.26, N: 3.12. Found (%): C: 63.91, H: 4.17, N: 2.97.

***N*,*N*-Dimethyl-*N*′-[3,3-dimethyl-7-oxo-3*H*,7*H*-pyrano[2′,3′:7,8]benzopyrano[2,3-*b*]pyridin-6-yl]ethanediamine (8).** *N*,*N*-Dimethylethylenediamine (337 μL, 3.10 mmol) was added under argon to a solution of **7** (140 mg, 0.31 mmol) in dry DMSO (6 mL) and the mixture was heated at 100 °C for 1 h. The mixture was then poured

into ice-water and it was extracted with ethyl acetate. The organic phase was dried (Na_2SO_4) and concentrated to dryness and the residue was purified by column chromatography (silica gel, 20×1 cm) using a mixture of dichloromethane/methanol (97/3) as the eluent to give **8** (100 mg, 88%) as a pale yellow oil. Mp (hydrochloride) >250 °C, (EtOH). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.43 (s, 6H, 2×gem CH_3), 2.28 [s, 6H, $N(CH_3)_2$], 2.60 (t, J=7 Hz, 2H, $NHCH_2\underline{C}H_2NMe_2$), 3.25 (q, J=7 Hz, 5 Hz, 2H, $NHC\underline{H}_2CH_2NMe_2$), 5.49 (d, J=10 Hz, 1H, H-2), 5.87 (s, 1H, H-5), 6.88 (d, J=10 Hz, 1H, H-1) 7.28 (m, 1H, H-9), 8.52 (m, 2H, H-10, H-8), 9.61 (t, J=5 Hz, 1H, D_2O exch., NH). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 28.30 (2×gem CH_3), 40.77 ($NHC\underline{H}_2CH_2NMe_2$), 45.41 [$N(CH_3)_2$], 57.61 ($NHCH_2\underline{C}H_2NMe_2$), 77.86 (C-3), 92.01 (C-5), 97.86 (C-12b), 101.55 (C-6a), 115.58 (C-1), 116.99 (C-7a), 120.54 (C-9), 125.35 (C-2), 136.34 (C-8), 152.43 (C-10), 152.46 (C-6), 152.87 (C-12a), 159.52 (C-11a), 160.55 (C-4a), 177.90 (C-7). Anal. Calcd for $C_{21}H_{23}N_3O_3$·HCl. calcd (%): C: 62.76, H: 6.02, N: 10.46. Found (%): C: 62.42, H: 6.06, N: 10.21.

***N*,*N*-Diethyl-*N*′-[3,3-dimethyl-7-oxo-3*H*,7*H*-pyrano-[2′,3′:7,8]benzopyrano[2,3-*b*]pyridin-6-yl]ethanediamine (9).** This compound was prepared by a procedure analogous to that of **8**. Yield: 90%. Mp (hydrochloride) 233–235 °C, (EtOH). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.04 [t, J=7 Hz, 6H, $N(CH_2C\underline{H}_3)_2$], 1.42 (s, 6H, 2×gem CH_3), 2.58 [q, J=7 Hz, 4H, $N(C\underline{H}_2CH_3)_2$], 2.74 (t, J=7 Hz, 2H, $NHCH_2C\underline{H}_2NEt_2$), 3.24 (q, J=7 Hz, 5 Hz, 2H, $NHC\underline{H}_2CH_2NEt_2$), 5.49 (d, J=10 Hz, 1H, H-2), 5.88 (s, 1H, H-5), 6.88 (d, J=10 Hz, 1H, H-1), 7.28 (m, 1H, H-9), 8.52 (m, 2H, H-10, H-8), 9.58 (t, J=5 Hz, 1H, D_2O exch., NH). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 11.72 [$N(CH_2\underline{C}H_3)_2$], 28.30 (2×gem CH_3), 41.09 ($NH\underline{C}H_2CH_2NEt_2$), 46.97 [$N(\underline{C}H_2CH_3)_2$], 51.05 ($NHCH_2\underline{C}H_2NEt_2$), 77.81 (C-3), 92.11 (C-5), 97.80 (C-12b), 101.59 (C-6a), 115.59 (C-1), 117.03 (C-7a), 120.48 (C-9), 125.30 (C-2), 136.36 (C-8), 152.39 (C-10), 152.50 (C-6), 152.87 (C-12a), 159.52 (C-11a), 160.51 (C-4a), 177.79 (C-7). Anal. calcd for $C_{23}H_{27}N_3O_3$·HCl. Calcd (%): C: 64.25, H: 6.56, N: 9.77. Found (%): C: 64.09, H: 6.51, N: 9.94.

5-[(4-Methylphenyl)sulfonyl]oxy-2,2-dimethyl-2*H*,6*H*-pyrano[3′,2′:6,7]benzopyrano[2,3-*b*]pyridin-6-one (10). This compound was prepared by a procedure analogous to that of **7**. Yield: 75%. Mp 203–205 °C, (EtOAc–*n*-pentane). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.32 (s, 6H, 2×gem CH_3), 2.31 (s, 3H, $-SO_2C_6H_4C\underline{H}_3$), 5.41 (d, J=10 Hz, 1H, H-3), 6.09 (d, J=10 Hz, 1H, H-4), 6.78 (s, 1H, H-12), 7.22 (d, J=8 Hz, 2H, H-5′, H-3′), 7.30 (m, 1H, H-8), 7.77 (d, J=8 Hz, 2H, H-6′, H-2′), 8.49 (m, 1H, H-7), 8.54 (m, 1H, H-9). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 21.36 ($-SO_2C_6H_4\underline{C}H_3$), 27.90 (2×gem CH_3), 78.03 (C-2), 103.68 (C-12), 110.44 (C-5a), 113.97 (C-4a), 115.15 (C-4), 116.84 (C-6a), 120.85 (C-8), 128.49 (C-6′, C-2′), 129.45 (C-5′, C-3′), 131.03 (C-3), 132.53 (C-1′), 136.98 (C-7), 142.42 (C-5), 145.18 (C-4′), 153.00 (C-9), 156.79 (C-11a), 158.77 (C-12a, C-10a), 173.84 (C-6). Anal. Calcd for $C_{24}H_{19}NO_6S$. calcd (%): C: 64.13, H: 4.26, N: 3.12. Found (%): C: 63.97, H: 4.31, N: 3.05.

***N*,*N*-Dimethyl-*N*′-[2,2-dimethyl-6-oxo-2*H*,6*H*-pyrano-[3′,2′:6,7]benzopyrano[2,3-*b*]pyridin-5-yl]ethanediamine (11).** This compound was prepared by a procedure analogous to that of **8**. Yield: 92%. Mp (hydrochloride) >250 °C, (EtOH). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.40 (s, 6H, 2×gem CH_3), 2.24 [s, 6H, $N(CH_3)_2$], 2.48 (t, J=7 Hz, 2H, $NHCH_2C\underline{H}_2NMe_2$), 3.48 (q, J=7 Hz, 5 Hz, 2H, $NHC\underline{H}_2CH_2NMe_2$), 5.42 (d, J=10 Hz, 1H, H-3), 6.23 (s, 1H, H-12), 6.53 (d, J=10 Hz, 1H, H-4) 7.26 (m, 1H, H-8), 8.50 (m, 2H, H-7, H-9), 9.57 (t, J=5 Hz, 1H, D_2O exch., NH). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 27.08 (2×gem CH_3), 45.53 [$N(CH_3)_2$], 47.23 ($NHC\underline{H}_2CH_2NMe_2$), 59.87 ($NHCH_2\underline{C}H_2NMe_2$), 76.34 (C-2), 94.02 (C-12), 103.80 (C-5a), 103.87 (C-4a), 116.81 (C-6a), 120.30 (C-4), 120.37 (C-8), 124.16 (C-3), 136.47 (C-7), 151.65 (C-5), 152.65 (C-9), 158.34 (C-11a), 159.34 (C-10a), 160.92 (C-12a), 178.12 (C-6). Anal. calcd for $C_{21}H_{23}O_3N_3$·HCl·H_2O. Calcd (%): C: 60.07, H: 6.24, N: 10.01. Found (%): C: 59.72, H: 6.21, N: 9.88.

***N*,*N*-Diethyl-*N*′-[2,2-dimethyl-6-oxo-2*H*,6*H*-pyrano-[3′,2′:6,7]benzopyrano[2,3-*b*]pyridin-5-yl]ethanediamine (12).** This compound was prepared by a procedure analogous to that of **8**. Yield: 90%. Mp (hydrochloride) >250 °C, (EtOH). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.96 [t, J=7 Hz, 6H, $N(CH_2C\underline{H}_3)_2$], 1.40 (s, 6H, 2×gem CH_3), 2.51 [q, J=7 Hz, 4H, $N(C\underline{H}_2CH_3)_2$], 2.60 (t, J=7 Hz, 2H, $NHCH_2C\underline{H}_2NEt_2$), 3.46 (q, J=7 Hz, 5 Hz, 2H, $NHC\underline{H}_2CH_2NEt_2$), 5.42 (d, J=10 Hz, 1H, H-3), 6.22 (s, 1H, H-12), 6.53 (d, J=10 Hz, 1H, H-4), 7.25 (m, 1H, H-8), 8.49 (m, 2H, H-7, H-9), 9.51 (t, J=5 Hz, 1H, D_2O exch., NH). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 11.50 [$N(CH_2\underline{C}H_3)_2$], 27.01 (2×gem CH_3), 47.04 [$N(\underline{C}H_2CH_3)_2$], 47.52 ($NH\underline{C}H_2CH_2NEt_2$), 53.44 ($NH\underline{C}H_2CH_2NEt_2$), 76.37 (C-2), 93.94 (C-12), 103.76 (C-5a), 103.83 (C-4a), 116.77 (C-6a), 120.30 (C-4), 120.34 (C-8), 124.05 (C-3), 136.36 (C-7), 151.65 (C-5), 152.54 (C-9), 158.23 (C-11a), 159.30 (C-10a), 160.81 (C-12a), 177.94 (C-6). Anal. calcd for $C_{23}H_{27}O_3N_3$,·HCl·3/2H_2O. calcd (%): C: 60.59, H: 6.63, N: 9.22. Found (%): C: 60.80, H: 6.65, N: 9.13.

2-[6,9-Dihydro-9,9-dimethylpyrano[3,2-*f*]pyrano-[2′,3′:2,3]pyridine[4,3,2-*c*,*d*]indazol-6-yl]-1-ethanol (13). 2-Hydroxyethylhydrazine (515 μL, 7,6 mmol) was added under argon to a solution of **7** (340 mg, 0.76 mmol) in dry DMSO (5 mL) and the mixture was heated at 90 °C for 30 min. The reaction mixture was then poured into ice-water (20 mL) and the precipitate was filtered, washed with water (20 mL) and dried over $CaCl_2$, to give after recrystalyzation pure **13** (240 mg) (95%) as a pale yellow solid. Mp 188–190 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 1.41 (s, 6H, 2×gem CH_3), 3.79 (q, J=5.5 Hz, 4 Hz, 2H, $CH_2\underline{C}H_2OH$), 4.28 (t, J=5.5 Hz, 2H, $C\underline{H}_2CH_2OH$), 4.87 (t, J=4 Hz, 1H, D_2O exch., OH), 5.82 (d, J=10 Hz, 1H, H-10), 6.53 (s, 1H, H-7), 6.65 (d, J=10 Hz, 1H, H-11), 7.34 (m, 1H, H-3), 8.20 (m, 1H, H-4), 8.28 (m, 1H, H-2). ^{13}C NMR (DMSO-d_6, 50 MHz) δ (ppm): 27.82 (2×gem CH_3), 51.60 ($\underline{C}H_2CH_2OH$), 61.75 ($CH_2\underline{C}H_2OH$), 77.05 (C-9), 89.68 (C-7), 101.22 (C-11a), 111.51 (C-11c), 114.57 (C-4a), 115.30 (C-11), 120.89 (C-3), 128.72 (C-10), 132.06

(C-4), 137.87 (C-4b), 140.11 (C-6a), 143.46 (C-11b), 147.72 (C-2), 156.95 (C-7a), 160.18 (C-12a). Anal. Calcd for $C_{19}H_{17}N_3O_3$. calcd (%): C: 68.05, H: 5.11, N: 12.53. Found (%): C: 67.88, H: 5.17, N: 12.39.

2-[6,9-Dihydro-9,9-dimethylpyrano[3,2-*f*]pyrano[2′,3′:2,3]pyridine[4,3,2-*c*,*d*]indazol-6-yl]-1-ethanol methanesulfonate (14). Triethylamine (232 μL, 1.67 mmol) and methanesulfonyl chloride (128 μL, 1.64 mmol) were added to a solution of **13** (210 mg, 0.63 mmol) in dry CH_2Cl_2 (20 mL) at 0 °C and the mixture was stirred for 10 min at 0 °C and for an additional 1 h at rt. Water (20 mL) was then added to the mixture and it was extracted with dichloromethane. The organic phase was dried (Na_2SO_4) and concentrated to dryness and the residue was purified by column chromatography (silica gel, 12×1 cm) using a mixture of cyclohexane/ethyl acetate (1/9) as the eluent to give **14** (190 mg, 73%) as a yellow solid. Mp 152–154 °C, (AcOEt/*n*-hexane). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.40 (s, 6H, 2×gem CH_3), 2.78 (s, 3H, OSO_2CH_3), 4.46 (t, J=5 Hz, 2H, $CH_2CH_2OSO_2CH_3$), 4.61 (t, J=5 Hz, 2H, CH_2CH_2O-SO_2CH_3), 5.64 (d, J=10 Hz, 1H, H-10), 6.31 (s, 1H, H-7), 6.73 (d, J=10 Hz, 1H, H-11), 7.15 (m, 1H, H-3), 8.09 (m, 1H, H-4), 8.23 (m, 1H, H-2). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 27.71 (2×gem CH_3), 37.37 (OSO_2CH_3), 48.37 ($CH_2CH_2OSO_2CH_3$), 67.33 ($CH_2CH_2OSO_2CH_3$), 77.08 (C-9), 89.79 (C-7), 101.41 (C-11a), 111.66 (C-11c), 114.46 (C-4a), 115.12 (C-11), 120.92 (C-3), 128.86 (C-10), 132.10 (C-4), 138.53 (C-4b), 140.37 (C-6a), 143.38 (C-11b), 148.02 (C-2), 157.17 (C-7a), 160.26 (C-12a). Anal. calcd for $C_{20}H_{19}N_3O_5S$. Calcd (%): C: 58.10, H: 4.63, N: 10.16. Found (%): C: 57.94, H: 4.57, N: 10.02.

Dimethyl-[2-[6,9-dihydro-9,9-dimethylpyrano[3,2-*f*]pyrano[2′,3′:2,3]pyridine[4,3,2-*c*,*d*]indazol-6-yl]ethyl]amine (15). A 33% solution of dimethylamine in dry ethanol (327 μL, 2.4 mmol) was added to a solution of **14** (100 mg, 0.24 mmol) in dry ethanol (20 mL) and the mixture was stirred under reflux for 20 h. The solvent was then vacuum-evaporated, water (20 mL) was added to the residue and it was extracted with dichloromethane. The organic phase was dried (Na_2SO_4) and concentrated to dryness and the residue was purified by column chromatography (silica gel, 18×1 cm) using a mixture of ethyl acetate/methanol (95/5) as the eluent to give **15** (70 mg, 79%) as a pale yellow solid. Mp (hydrochloride) >250 °C, (EtOH). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.40 (s, 6H, 2×gem CH_3), 2.26 [s, 6H, $N(CH_3)_2$], 2.77 (t, J=7 Hz, 2H, $CH_2CH_2NMe_2$), 4.25 (t, J=7 Hz, 2H, $CH_2CH_2NMe_2$), 5.61 (d, J=10 Hz, 1H, H-10), 6.27 (s, 1H, H-7), 6.74 (d, J=10 Hz, 1H, H-11), 7.11 (m, 1H, H-3), 8.10 (m, 1H, H-4), 8.19 (m, 1H, H-2). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 27.71 (2×gem CH_3), 45.46 [$N(CH_3)_2$], 47.67 ($CH_2CH_2NMe_2$), 58.14 ($CH_2CH_2NMe_2$), 76.85 (C-9), 89.57 (C-7), 100.97 (C-11a), 111.85 (C-11c), 114.97 (C-4a), 115.34 (C-11), 120.78 (C-3), 128.42 (C-10), 131.92 (C-4), 137.54 (C-4b), 139.63 (C-6a), 143.57 (C-11b), 147.54 (C-2), 156.65 (C-7a), 160.26 (C-12a). Anal. calcd for $C_{21}H_{22}N_4O_2$.HCl·3/2H_2O. Calcd (%): C: 59.22, H: 6.15, N: 13.16. Found (%): C: 59.08, H: 6.23, N: 13.17.

Diethyl-[2-[6,9-dihydro-9,9-dimethylpyrano[3,2-*f*]pyrano[2′,3′:2,3]pyridine[4,3,2-*c*,*d*]indazol-6-yl]ethyl]amine (16). This compound was prepared by a procedure analogous to that of **15**. Yield: 82%. Mp (hydrochloride) >250 °C, (EtOH). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.98 [t, J=7 Hz, 6H, $N(CH_2CH_3)_2$], 1.42 (s, 6H, 2×gem CH_3), 2.56 [q, J=7 Hz, 4H, $N(CH_2CH_3)_2$], 2.90 (t, J=7 Hz, 2H, $CH_2CH_2NEt_2$), 4.24 (t, J=7 Hz, 2H, $CH_2CH_2NEt_2$), 5.63 (d, J=10 Hz, 1H, H-10), 6.29 (s, 1H, H-7), 6.76 (d, J=10 Hz, 1H, H-11), 7.13 (m, 1H, H-3), 8.13 (m, 1H, H-4), 8.21 (m, 1H, H-2). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 11.83 [$N(CH_2CH_3)_2$], 27.71 (2×gem CH_3), 47.41 [$N(CH_2CH_3)_2$], 48.14 ($CH_2CH_2NEt_2$), 52.11 ($CH_2CH_2NEt_2$), 76.89 (C-9), 89.83 (C-7), 101.00 (C-11a), 111.85 (C-11c), 115.00 (C-4a), 115.41 (C-11), 120.85 (C-3), 128.53 (C-10), 131.92 (C-4), 137.50 (C-4b), 139.78 (C-6a), 143.57 (C-11b), 147.55 (C-2), 156.62 (C-7a), 160.33 (C-12a). Anal. calcd for $C_{23}H_{26}N_4O_2$·HCl. Calcd (%): C: 64.70, H: 6.37, N: 13.12. Found (%): C: 64.61, H: 6.35, N: 13.07.

2-[1,10-Dihydro-10,10-dimethylpyrano[2,3-*g*]pyrano[2′,3′:2,3]pyridine[4,3,2-*c*,*d*]indazol-1-yl]-1-ethanol (17). This compound was prepared by a procedure analogous to that of **13**. Yield: 40%. Mp 183–185 °C (dec), (CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.41 (s, 6H, 2×gem CH_3), 4.12 (t, J=5 Hz, 2H, CH_2CH_2OH), 4.44 (t, J=5 Hz, 2H, CH_2CH_2OH), 5.47 (d, J=10 Hz, 1H, H-11), 6.12 (s, 1H, H-8), 6.67 (d, J=10 Hz, 1H, H-12), 7.05 (m, 1H, H-4), 7.98 (m, 1H, H-3), 8.14 (m, 1H, H-5). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 27.12 (2×gem CH_3), 53.29 (CH_2CH_2OH), 61.63 (CH_2CH_2OH), 76.34 (C-10), 95.23 (C-8), 99.35 (C-12a), 111.74 (C-12c), 113.87 (C-2b), 116.73 (C-12), 120.52 (C-4), 126.33 (C-11), 131.88 (C-3), 136.66 (C-12b), 136.95 (C-2a), 147.50 (C-5), 148.09 (C-7a), 156.73 (C-8a), 159.56 (C-6a). Anal. calcd for $C_{19}H_{17}N_3O_3$. Calcd (%): C: 68.05, H: 5.11, N: 12.53. Found (%): C: 68.14, H: 5.08, N: 12.41.

2-[1,10-Dihydro-10,10-dimethylpyrano[2,3-*g*]pyrano[2′,3′:2,3]pyridine[4,3,2-*c*,*d*]indazol-1-yl]-1-ethanol methanesulfonate (18). This compound was prepared by a procedure analogous to that of **14**. Yield: 79%. Mp 158–160 °C, (AcOEt/*n*-hexane). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.40 (s, 6H, 2×gem CH_3), 2.79 (s, 3H, OSO_2CH_3), 4.62 (m, 4H, $CH_2CH_2OSO_2CH_3$), 5.50 (d, J=10 Hz, 1H, H-11), 6.20 (s, 1H, H-8), 6.59 (d, J=10 Hz, 1H, H-12), 7.12 (m, 1H, H-4), 8.04 (m, 1H, H-3), 8.21 (m, 1H, H-5). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 27.01 (2×gem CH_3), 37.23 (OSO_2CH_3), 49.76 ($CH_2CH_2OSO_2CH_3$), 67.37 ($CH_2CH_2OSO_2CH_3$), 76.37 (C-10), 95.60 (C-8), 99.27 (C-12a), 112.07 (C-12c), 113.87 (C-2b), 116.22 (C-12), 120.74 (C-4), 126.95 (C-11), 131.99 (C-3), 136.84 (C-12b), 138.05 (C-2a), 148.16 (C-5), 148.31 (C-7a), 156.91 (C-8a), 159.92 (C-6a). Anal. calcd for $C_{20}H_{19}N_3O_5S$. calcd (%): C: 58.10, H: 4.63, N: 10.16. Found (%): C: 57.94, H: 4.59, N: 10.09.

Dimethyl-[2-[1,10-dihydro-10,10-dimethylpyrano[2,3-*g*]-pyrano[2′,3′:2,3]pyridine[4,3,2-*c*,*d*]indazol-1-yl]ethyl]-amine (19). This compound was prepared by a procedure analogous to that of **15**. Yield: 89%. Mp

(hydrochloride) >250 °C, (EtOH). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.42 (s, 6H, 2×gem CH_3), 2.29 [s, 6H, $N(CH_3)_2$], 2.77 (t, J=7 Hz, 2H, $CH_2CH_2NMe_2$), 4.46 (t, J=7 Hz, 2H, $CH_2CH_2NMe_2$), 5.50 (d, J=10 Hz, 1H, H-11), 6.21 (s, 1H, H-8), 6.61 (d, J=10 Hz, 1H, H-12), 7.11 (m, 1H, H-4), 8.11 (m, 1H, H-3), 8.21 (m, 1H, H-5). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 27.13 (2×gem CH_3), 45.69 [$N(CH_3)_2$], 49.58 ($CH_2CH_2NMe_2$), 58.84 ($CH_2CH_2NMe_2$), 76.37 (C-10), 95.30 (C-8), 99.35 (C-12a), 112.47 (C-12c), 114.45 (C-2b), 116.55 (C-12), 120.70 (C-4), 126.80 (C-11), 131.98 (C-3), 136.25 (C-12b), 137.05 (C-2a), 147.79 (C-5), 148.59 (C-7a), 156.68 (C-8a), 160.06 (C-6a). Anal. calcd for $C_{21}H_{22}N_4O_2 \cdot HCl \cdot H_2O$. calcd (%): C: 60.50, H: 6.04, N: 13.44. Found (%): C: 60.22, H: 5.87, N: 13.30.

Diethyl-[2-[1,10-dihydro-10,10-dimethylpyrano[2,3-*g*]pyrano[2′,3′:2,3]pyridine[4,3,2 - *c*,*d*]indazol - 1 - yl]ethyl]amine (20). This compound was prepared by a procedure analogous to that of **15**. Yield: 92%. Mp (hydrochloride) >250 °C, (EtOH). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.98 [t, J=7 Hz, 6H, $N(CH_2CH_3)_2$], 1.43 (s, 6H, 2×gem CH_3), 2.56 [q, J=7 Hz, 4H, $N(CH_2CH_3)_2$], 2.87 (t, J=7 Hz, 2H, $CH_2CH_2NEt_2$), 4.43 (t, J=7 Hz, 2H, $CH_2CH_2NEt_2$), 5.50 (d, J=10 Hz, 1H, H-11), 6.22 (s, 1H, H-8), 6.67 (d, J=10 Hz, 1H, H-12), 7.13 (m, 1H, H-4), 8.13 (m, 1H, H-3), 8.22 (m, 1H, H-5). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 11.80 [$N(CH_2CH_3)_2$], 27.09 (2×gem CH_3), 47.49 [$N(CH_2CH_3)_2$], 50.28 ($CH_2CH_2NEt_2$), 52.78 ($CH_2CH_2NEt_2$), 76.34 (C-10), 95.19 (C-8), 99.46 (C-12a), 112.47 (C-12c), 114.52 (C-2b), 116.80 (C-12), 120.66 (C-4), 126.54 (C-11), 131.91 (C-3), 136.39 (C-12b), 136.98 (C-2a), 147.75 (C-5), 148.59 (C-7a), 156.57 (C-8a), 160.10 (C-6a). Anal. calcd for $C_{23}H_{26}N_4O_2 \cdot HCl$. calcd (%): C: 64.70, H: 6.37, N: 13.12. Found (%): C: 64.91, H: 6.33, N: 13.07.

Biological activity

The L1210 murine lymphoblastic leukemia cell line (strain: DBA subline 212) was obtained from ATCC (American Type Culture Collection, Rockville, MD, USA) and routinely cultured in RPMI 1640 supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% Fetal Bovine Serum (media and antibiotics from Biochrom KG, Berlin, Germany). Human colorectal adenocarcinoma HT-29 cells obtained from ECACC (European Collection of Cell Cultures, Salisbury, UK) were routinely cultured in DMEM supplemented with antibiotics and serum, as above, and subcultured using a trypsin 0.25%–EDTA 0.02% solution.

The cytotoxicity assay was performed by a modification of the MTT-method.[8b] Briefly, cells were plated at a density of approximately 5000 cells/well in 96-well microplates, and after 24 h the test compounds were added, appropriately diluted with DMSO. After 72 h incubation, the medium was replaced with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO, USA) dissolved at a final concentration of 1 mg/mL in serum-free, phenol-red-free RPMI 1640, for a further 4 h incubation. Then, the MTT-formazan was solubilized in isopropanol and the optical density was measured with a microplate reader at a wavelength of 550 nm (reference wavelength: 690 nm). Results were expressed as IC_{50}, that is the concentration that reduced by 50% the optical density of treated cells with respect to untreated controls.

Cell-cycle analysis was performed after incubation of exponentially growing HT-29 cells with the test compounds (20 μM) for 24 h. Treated cultures were then trypsinized, washed in PBS, fixed in 50% ethanol, and stained with an RNAse-containing propidium iodide solution (50 μg/mL). DNA content was analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer using the ModFit software.

This study was supported by a Research Grant provided by the Greek Ministry of Education (Business Program of Education and Initial Professional Preparing-EPEAEK).

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