Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Medicinal Chemistry 41 (2006) 71–79
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://france.elsevier.com/direct/ejmech

Short communication

Design, synthesis and antiproliferative activity of some new azapyranoxanthenone aminoderivatives

George Kolokythas [a], Nicole Pouli [a], Panagiotis Marakos [a,*], Harris Pratsinis [b], Dimitris Kletsas [b]

[a] *Division of Pharmaceutical Chemistry, Department of Pharmacy, University of Athens, Panepistimiopolis-Zografou, Athens 15771, Greece*
[b] *Laboratory of Cell Proliferation and Ageing, Institute of Biology, NCSR "Demokritos", 15310 Athens, Greece*

Received 29 July 2005; received in revised form 10 October 2005; accepted 19 October 2005
Available online 21 November 2005

Abstract

Some novel aminosubstituted 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. Their eventual selective effect on a phase of the cell cycle was evaluated, using HT-29 cells. A number of the new derivatives exhibited interesting cytotoxic activity against the human solid tumor cell line.

Keywords: Azapyranoxanthenones; Cytotoxic activity; Cell-cycle effect

1. Introduction

Chemotherapeutic agents that target cellular DNA have been established as one of the most effective classes of drugs used in the clinic for the treatment of cancer. Representative examples include the anthracenedione derivatives doxorubicin [1] and mitoxantrone (Fig. 1) [2,3]. Several subsets of structurally related compounds have been also prepared and evaluated extensively for their cell-killing effects, which can be attributed to a number of different molecular mechanisms. Nevertheless, most of these molecules have been designed to exert cytotoxicity by interacting with DNA and subsequently causing extensive DNA damage, leading to induction of cell death. Within this chemically diverse group of compounds noticeable results have been obtained with DNA-intercalative agents, which are characterized by the presence of a polycyclic planar or semiplanar chromophore moiety [4].

In the field of mitoxantrone analogues, the anthacenedione nucleus has been modified to provide DNA binders with lowered cardiotoxicity, since quinone chemotypes induce cardiac damage through the generation of free radicals, when they undergo electrochemical reduction [5]. Within this context, a number of anthrapyrazoles [6,7], benzothiopyranoindazoles [8,9], and annelated acridine or acridone derivatives [10] have been successfully developed and some of them are currently under clinical evaluation. Structure–activity relationship studies of these series have shown a clear improvement of antiproliferative activity resulting from the introduction of one or two flexible aminoalkylamino substitutions on the chromophore nucleus. Further improved pharmacological profile was achieved in many cases with different aza- and diaza-bioistosteres, indicating that the cytotoxic properties of these derivatives are substantially affected by the location of the heteroatom [11].

As part of a program concerning the design and synthesis of cytotoxic pyranoxanthenones [12] and pyranothioxanthenones [13] bearing structural similarity with the acridone alkaloid acronycine (Fig. 1) [14], we have found that the introduction of a dialkylaminoethyllamino side chain substitution results in an improvement of the antiproliferative activity of these compounds [15]. The introduction of a methylene linker between the chromophore and the side chain is also favorable, yielding in interesting activity against the colorectal HT-29 cell line, where acronycine is completely inactive [16]. We were also interested in preparing some azapyranoxanthenone aminoderi-

* Correponding author.
E-mail address: marakos@pharm.uoa.gr (P. Marakos).

0223-5234/$ - see front matter © 2005 Elsevier SAS. All rights reserved.
doi:10.1016/j.ejmech.2005.10.011

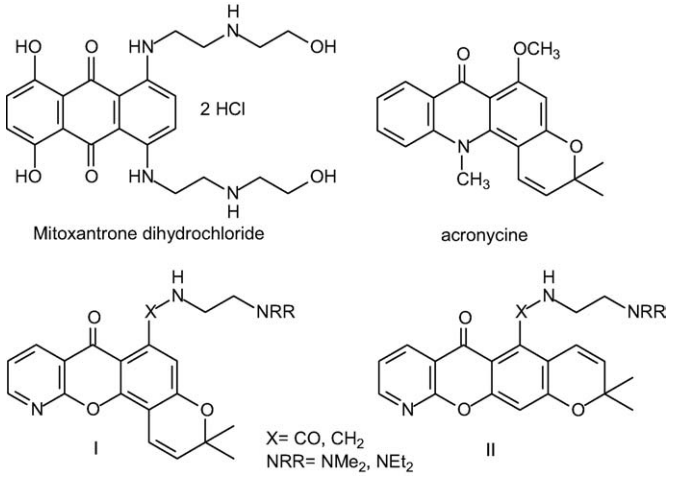

Fig. 1. Structures of mitoxantrone, acronycine and the synthesized compounds (**I**, **II**).

vatives, which demonstrated greater potency, in terms of IC_{50} values, against the HT-29 cell line, than against the leukemia L1210 cells [17]. Prompted by the above results, we decided to further investigate the azapyranoxanthenone chromophore, by side chain elongation (Fig. 1, structures I and II). By analogy to the previously reported compounds, we have introduced a methylene linker between the chromophore and the basic side chain substitution, which thus became more flexible. Alternatively, a more rigid amide link is introduced instead of the methylene, for comparative reasons concerning the structure–activity relationship studies.

2. Chemistry

For the preparation of the target derivatives we have used as starting material the isomeric chromenes **3** and **4** (Fig. 2). Thus, initial reaction of methyl 3,5-dihydroxybenzoate (**1**) with 3-chloro-3-methyl-1-butyne provided the corresponding ether **2**, which without further purification was ring-closed to result in the chromenes **3** and **4**, together with a small amount of compound **5** [16]. Compound **3** was then reacted with 2-chloronicotinic acid and the intermediate ether **6** was treated with phosphorous oxychloride to result in the carboxylate **7**. Unfortunately, the overall yield for the preparation of the ester **7** was too low (< 5%) and this compound could not serve as a useful intermediate for further group manipulation.

Consequently we have altered the synthetic procedure and used the carbinols **8** and **11** which were easily prepared upon reduction of the corresponding chromenes **3** and **4** (Fig. 3). Copper assisted condensation of the carbinols with 2-chloronicotinic acid followed by cyclodehydration in the presence of a mixture of trifluoroacetic acid and trifluoroacetic anhydride provided in one step the rather unstable azapyranoxanthenone trifluoroacetates **9** and **12**, respectively, which were subsequently hydrolyzed to the corresponding hydroxymethyl analogues **10** and **13**.

Jones oxidation of the carbinol **10** provided the carboxylic acid **14** in very good yield (Fig. 4), whereas our attempts to oxidize **10** by the use of PDC or PCC were not successful. The carboxylic acid **14** was then converted to the corresponding acylchloride **15**, which upon reaction with the suitable 2-dialkylaminoethylamine resulted in the target amides **16** and **17**. The carbinol **10** was subsequently converted to the chloride **18**, which was not isolated but used immediately for the preparation of the diamines **19** and **20** (Fig. 4). We have also attempted to prepare the abovementioned diamines through the mesylate or the trifluoroacetate of **10**, but without success, probably due to the instability of these intermediates.

Following analogous synthetic procedures we have also prepared the linear amides **23** and **24**, as well as the diamines **26** and **27** (Fig. 5).

For biological evaluation purposes, the free base forms of the target diamines were converted into their water-soluble hydrochloride addition salts by treatment with hydrochloric acid in methanol. However, the target dialkylaminoethylamides (compounds **16**, **17**, **23** and **24**) were tested at the free base

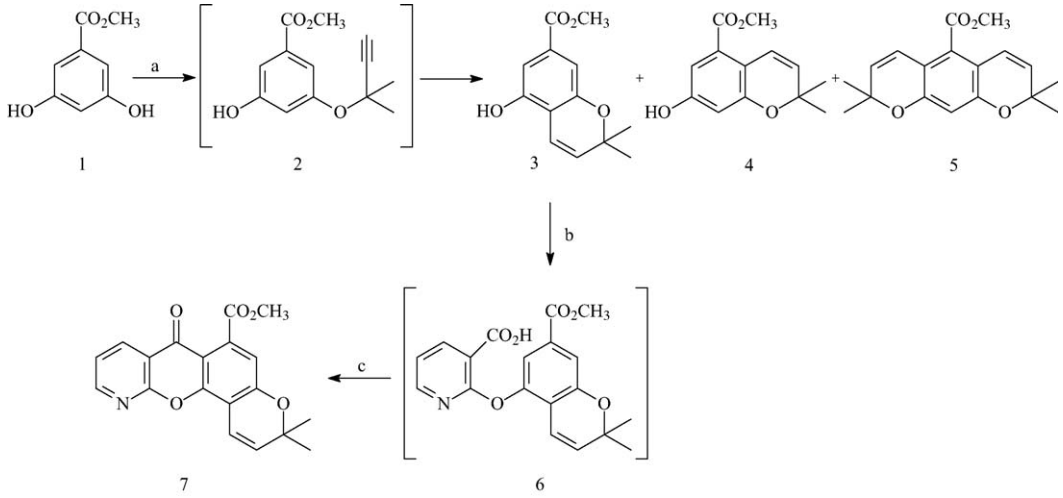

Fig. 2. a: 3-Chloro-3-methyl-1-butyne, K_2CO_3, KI, CuI, DMF dry, 75 °C, 24 h; b: 2-chloronicotinic acid, K_2CO_3, Cu, KI, DMF dry, reflux, 3 h; c: $POCl_3$, CCl_4 dry, reflux, 4 h.

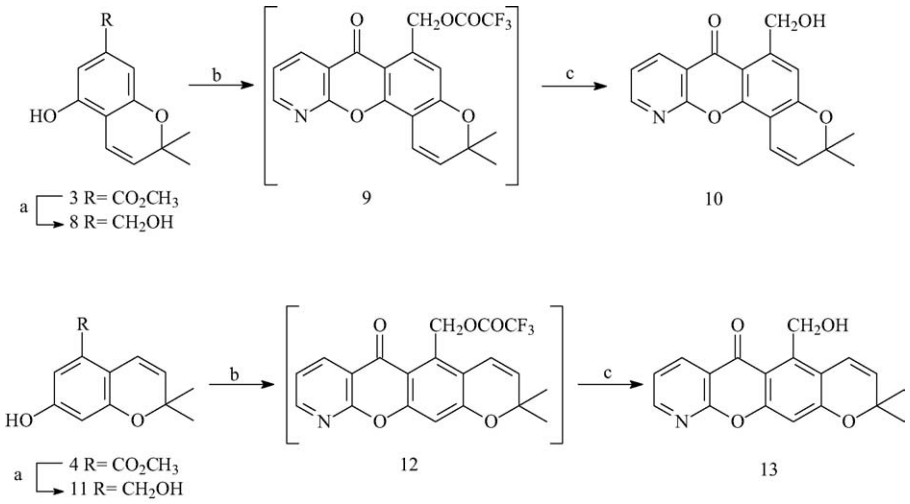

Fig. 3. a: $LiAlH_4$, THF dry, reflux, 3 h. b: 1) 2-chloronicotinic acid, K_2CO_3, Cu, KI, DMF dry, reflux, 90 min; 2) $(CF_3CO)_2O$, CF_3CO_2H, 50 °C, 6 h; c: EtOH, Na_2CO_3 20%, rt, 30 min.

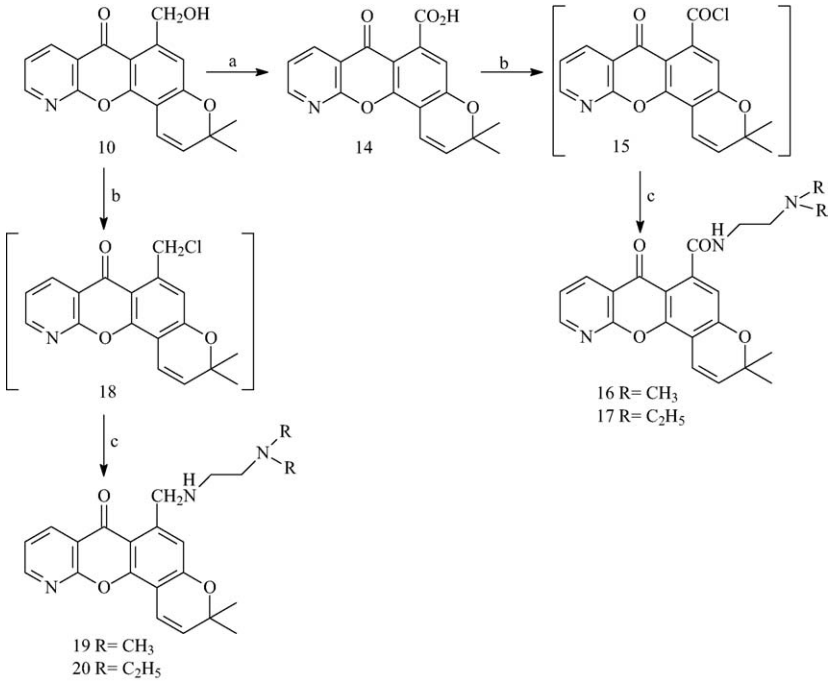

Fig. 4. a: Jones reagent, acetone, rt, 21 h; b: $SOCl_2$, toluene dry, 90 °C, 90 min; c: 2-dialkylaminoethylamine, toluene dry (for **16**, **17**) or THF dry (for **19**, **20**), 90 °C, 3 h.

form, since their hydrochloride, fumarate or malonate addition salts were highly hygroscopic.

3. Results and discussion

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

Among the new compounds the diamines **19**, **20**, **26** and **27** possess a certain degree of cytotoxicity against the L1210 cell line, although they were less active than the reference compound acronycine. On the contrary, the corresponding dialkylaminoethylamides **16**, **17**, **23** and **24** were inactive against the above mentioned cell line. All the compounds proved to be cytotoxic against the solid tumor HT-29 cell line, where acronycine is completely inactive. Notably, all compounds were more active against HT-29 cells, than against L1210, the diamines possessing the most interesting results, with IC_{50} values within 5.3–13.5 μM. The differences in activity between the linear and the corresponding angular diamines are not pronounced, with the exception of the diethylamino- substituted linear analogue **27**, which appear to be the most active compound in the series, possessing an IC_{50} of 5.3 μM.

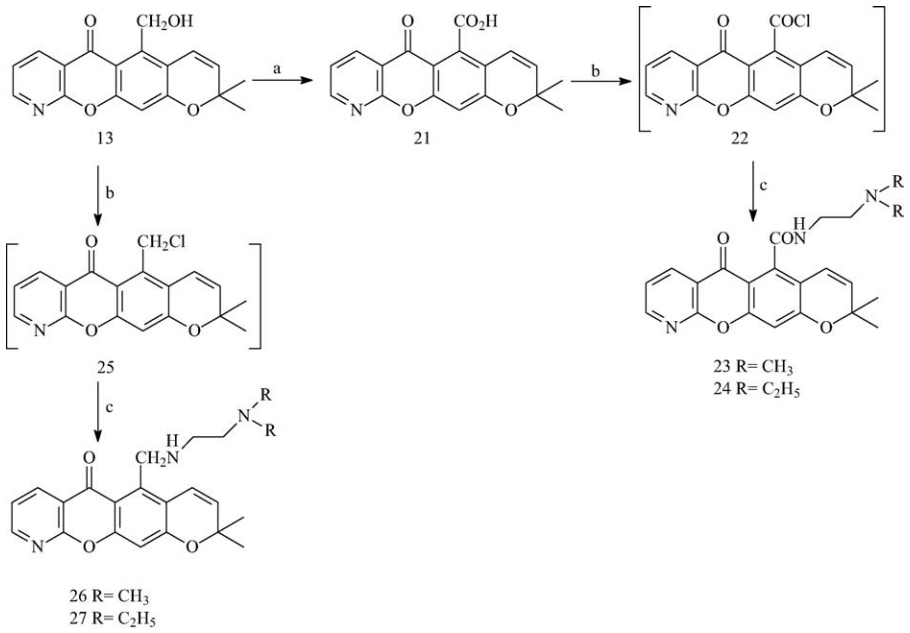

Fig. 5. a: Jones reagent, acetone, rt, 21 h; b: $SOCl_2$, toluene dry, 90 °C, 90 min; c: 2-dialkylaminoethylamine, toluene dry (for **23**, **24**) or THF dry (for **26**, **27**), 90 °C, 3 h.

Table 1
Inhibition of proliferation of the studied derivatives (IC_{50} values in μM) [a] and cell cycle phase distribution (%) [b]

Compound	IC_{50} (μM)		FACS analysis		
	L1210	HT-29	G_0/G_1	S	G_2/M
16 [d]	> 100	37.9 (4.32)	58.32 (2.45)	28.18 (2.91)	13.50 (3.17)
17 [d]	> 100	15.2 (2.33)	53.90 (2.37)	31.82 (2.87)	14.28 (1.45)
19 [c]	52.1 (3.82)	13.5 (1.10)	54.89 (2.28)	14.48 (1.11)	30.63 (1.31)
20 [c]	59.0 (4.14)	11.3 (1.69)	57.77 (1.43)	25.26 (2.27)	16.96 (1.12)
23 [d]	> 100	51.7 (4.76)	59.13 (1.15)	28.55 (1.88)	12.32 (1.29)
24 [d]	> 100	35.0 (6.5)	52.37 (1.60)	32.59 (2.18)	15.04 (2.00)
26 [c]	61.8 (4.68)	10.2 (1.41)	69.02 (1.55)	13.63 (2.40)	17.35 (1.16)
27 [c]	58.0 (2.65)	5.3 (1.13)	68.81 (2.39)	14.96 (3.43)	16.23 (1.05)
Acronycine	25.2 (2.3)	> 100			
Daunomycin HCl	0.601 (0.08)	0.028 (0.012)			
Control			57.73 (2.09)	30.55 (1.54)	11.72 (0.84)

[a] The results represent the mean (± standard deviation) of three independent experiments and are expressed as IC_{50}, the concentration that reduced by 50% the optical density of treated cells with respect to untreated controls.
[b] Mean (± standard deviation) of three independent experiments.
[c] Dihydrochloride.
[d] Free-base form.

We have previously reported [16] on the synthesis and cytotoxicity of the corresponding carbocyclic diamines, which showed very interesting results against the L1210 cell line, with IC_{50} values varying within 2–4 μM. The corresponding carbocyclic amides showed a trend towards decreasing cytotoxic potency, with greater IC_{50} values in the range of 9–16 μM, although they were still more active than the reference compound acronycine. An analogous observation was made concerning the cytotoxicity against the HT-29 cell line, where the carbocyclic diamines exhibited cytotoxicity in the range of 2–8 μM, while the corresponding amides demonstrated lowered potency, with IC_{50} values within 12–60 μM. On the other hand, the azaxanthenone aminoderivatives which lack the methylene linker displayed diminished cytotoxicity towards the L1210 cell line (IC_{50} values of 17–19 μM), but they retained considerable activity against the HT-29 cell line (IC_{50} values of 6–15 μM) [17]. Interestingly, both the linear and the angular diethylaminoethylamines proved to be the most cytotoxic derivatives within the above-mentioned series against the solid tumor cell line.

As a general remark, one could state that the insertion of nitrogen atom into this specific site of the xanthenone chromophore is not beneficial in terms of cytotoxicity. However, it is interesting to notice that the activity of the aza-substituted diamines against the solid tumor cell line is retained, without regard to the presence of the methylene linkage between the azaxanthenone chromophore and the basic side chain. On the basis of these data and taking under consideration that the position of the heteroatom affects the cytotoxicity of this class of compounds [11], it would be of interest to investigate in the future the potential activity of other structurally related azabioisosteric chemotypes.

Cell-cycle perturbations induced by the new compounds were studied on the HT-29 cell line. As can be seen in Table 1, and in agreement with their low cytotoxic activity, all dialkylaminoethylamides **16**, **17**, **23** and **24** were unable to alter the distribution of cell cycle phases. In contrast, the diamines provoked alterations in the cell cycle of HT-29 cells, with the exception of compound **20**, which did not affect cell cycle distribution. In particular, compounds **19**, **26** and **27** induced a decrease in the percentage of cells being in the S phase of the cell cycle, indicating a growth inhibitory effect. Among them, the angular diamine **19** induced a partial accumulation in the G_2/M phase. Interestingly, the linear diamines **26** and **27** were found to inhibit HT-29 cells by arresting them at the G_0/G_1 phase, as observed previously also in the case of the corresponding carbocyclic analogues [16], leading us to hypothesize two different inhibitory mechanisms for the angular and linear diamines.

4. Experimental protocols

4.1. Chemistry

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 spectra 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.

4.1.1. Methyl 2,2-dimethyl-5-hydroxy-2H-[1]benzopyrano-7-carboxylate (3) and methyl 2,2-dimethyl-7-hydroxy-2H-[1]benzopyrano-5-carboxylate (4)

To a solution of **1** (5 g, 29.73 mmol) in dry DMF (20 ml) were added under argon 3-chloro-3-methyl-1-butyne (3.3 ml, 32.25 mmol), anhydrous K_2CO_3 (8.29 g, 60.04 mmol), anhydrous KI (8.47 g, 51.04 mmol) and CuI (166.7 mg, 0.87 mmol) and the mixture was heated at 75 °C for 24 h. The bulk of DMF was evaporated under reduced pressure and the residue was poured into ice-water and was extracted with ethyl acetate. The combined organic extracts were dried (Na_2SO_4), the solvent was vacuum-evaporated and the residue was purified by column chromatography (silica gel), using a mixture of cyclohexane/EtOAc (97:3 to 94:6) as the eluent. First was eluted compound **5** as a pale yellow oil (1.25 g, 14%). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.45 (s, 12H, 4 × CH_3), 3.96 (s, 3H, $COOCH_3$), 5.60 (d, J = 10 Hz, 2H, H-3, H-7), 6.40 (d, J = 10 Hz, 2H, H-4, H-6), 6.42 (s, 1H, H-10). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 27.67 (4 × CH_3), 51.93 ($COO\underline{C}H_3$), 75.93 (C-2, C-8), 106.74 (C-10), 112.38 (C-4a, C-5a), 119.12 (C-4, C-6), 126.25 (C-5), 129.12 (C-3, C-7), 153.86 (C-9a, C-10a), 167.79 ($\underline{C}OOCH_3$). By increasing the polarity, compound **4** was eluted as an oil (2.78 g, 40%). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.41 (s, 6H, 2 × gem CH_3), 3.85 (s, 3H, $COOCH_3$), 5.60 (d, J = 10 Hz, 1H, H-3), 6.50 (d, J = 2 Hz, 1H, H-8), 6.97 (d, J = 2 Hz, 1H, H-6), 7.09 (d, J = 10 Hz, 1H, H-4). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 27.74 (2 × gem CH_3), 52.15 ($COO\underline{C}H_3$), 76.04 (C-2), 114.27 (C-8), 116.15 (C-4), 119.64 (C-6), 119.97 (C-4a), 126.84 (C-5), 132.17 (C-3), 149.89 (C-7), 154.56 (C-8a), 166.58 ($\underline{C}OOCH_3$). The most polar compound was **3**, which was also isolated as an oil (1.81 g, 26%). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.40 (s, 6H, 2 × gem CH_3), 3.86 (s, 3H, $COOCH_3$), 5.67 (d, J = 10 Hz, 1H, H-3), 6.38 (d, J = 2 Hz, 1H, H-8), 6.65 (d, J = 10 Hz, 1H, H-4), 7.10 (d, J = 2 Hz, 1H, H-6). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 27.87 (2 × gem CH_3), 52.42 ($COO\underline{C}H_3$), 75.98 (C-2), 109.13 (C-8), 110.85 (C-4), 114.09 (C-4a), 116.29 (C-6), 130.05 (C-7), 131.62 (C-3), 153.75 (C-5), 155.04 (C-8a), 167.84 ($\underline{C}OOCH_3$).

4.1.2. Methyl 3,3-dimethyl-7-oxo-3H,7H-pyrano[2′,3′:7,8]benzopyrano[2,3-b]pyridine-6-carboxylate (7)

To a solution of 2-chloronicotinic acid (202 mg, 1.28 mmol) in dry DMF were added the chromene **3** (300 mg, 1.28 mmol), anhydrous K_2CO_3 (354 mg, 2.56 mmol), anhydrous KI (21 mg, 0.13 mmol) and Cu (8 mg, 0.13 mmol) and the mixture was heated at reflux for 3 h. The organic solvent was vacuum evaporated, EtOAc (50 ml) was added to the residue and it was extracted with a 15% aqueous Na_2CO_3 solution (2 × 50 ml). The combined aqueous phase was made strongly acidic (pH 2) by the addition of a 9% hydrochloric acid solution and was then extracted with CH_2Cl_2 (3 × 50 ml). The organic extracts were dried (Na_2SO_4) and the solvent was vacuum-evaporated to provide compound **6** as an oily residue. This residue was diluted in carbon tetrachloride (50 ml), phosphorus oxychloride (1.78 ml, 19.20 mmol) was added and the mixture was heated at reflux for 4 h. The solvent was vacuum-evaporated, water (50 ml) was added to the residue and it was extracted with CH_2Cl_2 (3 × 50 ml). The organic phase was dried (Na_2SO_4) and evaporated to dryness and the residue was purified by column chromatography (silica gel), using a mixture of cyclohexane/EtOAc (90:10) as the eluent to provide compound **7** (37 mg, 9%), as an oil. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.49 (s, 6H, 2 × gem CH_3), 3.95 (s, 3H, $COOCH_3$), 6.17 (d, J = 10 Hz, 1H, H-2), 6.73 (s, 1H, H-5), 6.89 (d, J = 10 Hz, 1H, H-1), 7.63 (m, 1H, H-9), 8.59 (m, 1H, H-8), 8.81 (m, 1H, H-10). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 27.96 (2 × gem CH_3), 52.98 ($COO\underline{C}H_3$), 77.13 (C-3), 109.45 (C-12b), 110.92 (C-6a), 111.76 (C-5), 114.12 (C-1), 116.02 (C-7a), 122.00 (C-9), 131.72 (C-2), 133.75 (C-6), 136.71 (C-8), 151.29 (C-12a), 154.30 (C-10), 157.69 (C-4a), 158.88 (C-11a), 167.49 ($\underline{C}OOCH_3$), 175.03 (C-7).

4.1.3. 2,2-Dimethyl-5-hydroxy-2H-[1]benzopyrano-7-methanol (8)

A solution of the chromene **3** (300 mg, 1.28 mmol) in dry THF (10 ml) was added drop-wise under argon, to a suspen-

sion of $LiAlH_4$ (58 mg, 1.53 mmol) in dry THF (5 ml). The mixture was heated at reflux for 3 h and was then hydrolyzed with water and a 15% NaOH solution under ice-cooling. The inorganic precipitate was filtered off, washed with THF and methanol and the filtrate was evaporated under vacuum. The residue was purified by column chromatography (silica gel) using EtOAc as the eluent to obtain compound **8** as an oil (224 mg, 85%). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.36 (s, 6H, 2 × gem CH_3), 4.42 (s, 2H, CH_2), 5.51 (d, J = 10 Hz, 1H, H-3), 6.18 (d, J = 2 Hz, 1H, H-8), 6.28 (d, J = 2 Hz, 1H, H-6), 6.57 (d, J = 10 Hz, 1H, H-4). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 27.62 (2 × gem CH_3), 64.91 (CH_2), 75.90 (C-2), 106.44 (C-8), 107.36 (C-6), 109.02 (C-4a), 116.51 (C-4), 128.90 (C-3), 141.69 (C-7), 151.95 (C-8a), 153.82 (C-5).

*4.1.4. 3,3-Dimethyl-7-oxo-3H,7H-pyrano[2′,3′:7,8] benzopyrano[2,3-b]pyridine-6-methanol (**10**)*

To a solution of the carbinol **8** (1.77 g, 8.58 mmol) and 2-chloronicotinic acid (1.36 g, 8.63 mmol) in dry DMF (15 ml), were added K_2CO_3 (2.50 g, 17.85 mmol), Cu (56 mg, 0.87 mmol) and KI (143 mg, 0.87 mmol) and the mixture was heated at reflux for 90 min. The solvent was vacuum evaporated, EtOAc (40 ml) was added to the residue and it was extracted with a 15% aqueous Na_2CO_3 solution (2 × 30 ml). The combined aqueous phase was made strongly acidic (pH 2) with the addition of a 9% HCl solution and was then extracted with CH_2Cl_2 (3 × 50 ml). The organic extracts were dried (Na_2SO_4) and the solvent was vacuum-evaporated to provide a dark oily residue which was dissolved into a mixture of trifluoroacetic acid (4 ml) and trifluoroacetic anhydride (2 ml). The resulting solution was heated at 50 °C for 6 h, the solvents were then vacuum-evaporated and to the residue were added ethanol (5 ml) and a 20% Na_2CO_3 solution (5 ml). The mixture was stirred at room temperature for 30 min, the organic solvent was evaporated and the aqueous phase was extracted with EtOAc (3 × 30 ml). The organic extracts were dried (Na_2SO_4) and evaporated to dryness and the residue was purified by column chromatography (silica gel), using a mixture of CH_2Cl_2/EtOAc (95:5) as the eluent to provide compound **10** (1.43 g, 54%). m. p. 212–214 °C (EtOAc-*n*-hexane). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.45 (s, 6H, 2 × gem CH_3), 4.67 (s, 1H, D_2O exchangeable, OH), 4.83 (s, 2H, C$\underline{H}_2$OH), 5.70 (d, J = 10 Hz, 1H, H-2), 6.79 (s, 1H, H-5), 6.97 (d, J = 10 Hz, 1H, H-1), 7.38 (m, 1H, H-9), 8.60 (m, 1H, H-8), 8.65 (m, 1H, H-10). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 28.30 (2 × gem CH_3), 65.09 (CH_2OH), 78.40 (C-3), 109.05 (C-12b), 113.46 (C-6a), 115.04 (C-1), 115.63 (C-5), 116.81 (C-7a), 121.18 (C-9), 130.15 (C-2), 137.36 (C-8), 144.38 (C-6), 153.68 (C-10), 154.09 (C-12a), 158.75 (C-4a), 159.45 (C-11a), 178.56 (C-7). Anal. for $C_{18}H_{15}NO_4$. Calc. (%): C, 69.89; H, 4.89; N, 4.53. Found (%): C, 69.71; H, 4.94; N, 4.42.

*4.1.5. 2,2-Dimethyl-7-hydroxy-2H-[1]benzopyrano-5-methanol (**11**)*

This compound was prepared by a procedure analogous to that of **8**, starting from the chromene **4**. Yield 87% (oil). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.35 (s, 6H, 2 × gem CH_3), 4.55 (s, 2H, CH_2), 5.40 (d, J = 10 Hz, 1H, H-3), 6.22 (d, J = 2 Hz, 1H, H-8), 6.31 (d, J = 2 Hz, 1H, H-6), 6.41 (d, J = 10 Hz, 1H, H-4). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 27.64 (2 × gem CH_3), 62.15 (CH_2), 75.93 (C-2), 103.58 (C-8), 107.77 (C-6), 112.29 (C-4a), 118.17 (C-4), 128.39 (C-3), 136.88 (C-5), 154.45 (C-8a), 156.43 (C-7).

*4.1.6. 2,2-Dimethyl-6-oxo-2H,6H-pyrano[3′,2′:6,7] benzopyrano[2,3-b]pyridine-5-methanol (**13**)*

This compound was prepared by a procedure analogous to that of **10**, starting from **11**. Yield 87%, m.p. 168–170 °C (EtOAc-*n*-hexane). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.41 (s, 6H, 2 × gem CH_3), 4.68 (s, 1H, D_2O exchangeable, OH), 4.89 (s, 2H, CH_2OH), 5.78 (d, J = 10 Hz, 1H, H-3), 6.75 (d, J = 10 Hz, 1H, H-4), 6.82 (s, 1H, H-12), 7.33 (m, 1H, H-8), 8.54 (m, 1H, H-7), 8.60 (m, 1H, H-9). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 28.11 (2 × gem CH_3), 57.66 (CH_2OH), 77.40 (C-2), 104.71 (C-12), 114.12 (C-5a), 116.84 (C-6a), 117.58 (C-4), 118.13 (C-4a), 120.92 (C-8), 132.43 (C-3), 137.17 (C-7), 138.27 (C-5), 153.57 (C-9), 158.38 (C-10a), 159.19 (C-11a), 159.96 (C-12a), 178.52 (C-6). Anal. for $C_{18}H_{15}NO_4$. Calc. (%): C, 69.89; H, 4.89; N, 4.53. Found (%): C, 69.98; H, 4.83; N, 4.37.

*4.1.7. 3,3-Dimethyl-7-oxo-3H,7H-pyrano[2′,3′:7,8] benzopyrano[2,3-b]pyridine-6-carboxylic acid (**14**)*

Freshly prepared Jones reagent (16 ml, 2 mmol/ml) was added drop-wise to a stirred solution of the carbinol **10** (400 mg, 1.29 mmol) in acetone (20 ml) at 0 °C and the resulting mixture was stirred at room temperature for 21 h. The excess reagent was destroyed by drop-wise addition of isopropanol (10 ml) under cooling, stirring was continued at room temperature for 10 min and the inorganic layer was then filtered off and washed with acetone. The filtrate was vacuum-evaporated and the residue was dissolved in CH_2Cl_2 (50 ml) and extracted with a 20% Na_2CO_3 solution (3 × 50 ml). The aqueous phase was made strongly acidic (pH 2) by the addition of a 9% HCl solution and was then extracted with CH_2Cl_2 (3 × 50 ml). The organic extracts were dried (Na_2SO_4) and the solvent was vacuum-evaporated to provide pure the carboxylic acid **14** (310 mg, 74%), m.p. 212–214 °C (EtOH). ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 1.49 (s, 6H, 2 × gem CH_3), 6.03 (d, J = 10 Hz, 1H, H-2), 6.85 (s, 1H, H-5), 6.93 (d, J = 10 Hz, 1H, H-1), 7.60 (m, 1H, H-9), 8.56 (m, 1H, H-8), 8.78 (m, 1H, H-10). ^{13}C-NMR (DMSO-d_6, 50 MHz): δ 27.83 (2 × gem CH_3), 78.70 (C-3), 109.50 (C-12b), 111.37 (C-6a), 112.25 (C-5), 114.05 (C-1), 116.11 (C-7a), 122.03 (C-9), 131.95 (C-2), 136.03 (C-6), 136.84 (C-8), 151.17 (C-12a), 154.15 (C-10), 157.75 (C-4a), 159.41 (C-11a), 169.11 (COOH), 174.84 (C-7). Anal. for $C_{18}H_{13}NO_5$. Calc. (%): C, 66.87; H, 4.05; N, 4.33. Found (%): C, 66.71; H, 3.96; N, 4.12.

*4.1.8. 3,3-Dimethyl-N-[2-(dimethylamino)ethyl]-7-oxo-3H,7H-pyrano[2′,3′:7,8]benzopyrano[2,3-b]pyridine-6-carboxamide (**16**)*

Thionyl chloride (2 ml) was added under argon to a suspension of the carboxylic acid **14** (100 mg, 0.31 mmol) in dry

toluene (3 ml) and the mixture was heated 90 °C for 90 min. The solvent and the excess of thionyl chloride were vacuum-evaporated to provide the chloride **15**, as a light yellow oil, which without purification was dissolved in dry toluene (10 ml) and the resulting solution was treated with 2-(dimethylamino)ethylamine (507 μl, 4.65 mmol) and heated at 90 °C for 3 h. The solvent was then vacuum-evaporated and the residue was dissolved in EtOAc (50 ml), washed with a 15% Na_2CO_3 solution (2 × 50 ml), dried (Na_2SO_4) and evaporated to dryness. The residue was purified by column chromatography (silica gel), using a mixture of CH_2Cl_2/MeOH (95:5) as the eluent to provide compound **16** (90 mg, 74%), as an oil. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.43 (s, 6H, 2 × gem CH_3), 2.21 [s, 6H, $N(CH_3)_2$], 2.56 [t, J = 7 Hz, 2H, $NHCH_2C\underline{H}_2NMe_2$), 3.56 (q, J = 5 Hz, 7 Hz, 2H, $NHC\underline{H}_2CH_2NMe_2$), 5.71 (d, J = 10 Hz, 1H, H-2), 6.54 (t, J = 5 Hz, 1H, D_2O exch., NH), 6.71 (s, 1H, H-5), 6.95 (d, J = 10 Hz, 1H, H-1), 7.33 (m, 1H, H-9), 8.53 (m, 1H, H-8), 8.59 (m, 1H, H-10). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 28.19 (2 × gem CH_3), 37.23 ($NH\underline{C}H_2CH_2NMe_2$), 44.95 [$N(CH_3)_2$], 57.37 ($NHCH_2\underline{C}H_2NMe_2$), 78.40 (C-3), 110.01 (C-12b), 111.92 (C-6a), 113.94 (C-5), 114.79 (C-1), 116.62 (C-7a), 121.11 (C-9), 130.70 (C-2), 137.21 (C-8), 138.27 (C-6), 151.73 (C-12a), 153.31 (C-10), 158.20 (C-4a), 159.56 (C-11a), 169.00 (CONH), 175.25 (C-7). Anal. for $C_{22}H_{23}N_3O_4$. Calc. (%): C, 67.16; H, 5.89; N, 10.68. Found (%): C, 66.93; H, 5.81; N, 10.82.

*4.1.9. 3,3-Dimethyl-N-[2-(diethylamino)ethyl]-7-oxo-3H,7H-pyrano[2′,3′:7,8]benzopyrano[2,3-b]pyridine-6-carboxamide (**17**)*

This compound was prepared by a procedure analogous to that of **16**. Yield 77% (oil). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.91 [t, J = 7 Hz, 6H, $N(CH_2C\underline{H}_3)_2$], 1.42 (s, 6H, 2 × gem CH_3), 2.49 [q, J = 7 Hz, 4H, $N(C\underline{H}_2CH_3)_2$], 2.65 [t, J = 7 Hz, 2H, $NHCH_2C\underline{H}_2NEt_2$), 3.53 (q, J = 5 Hz, 7 Hz, 2H, $NHC\underline{H}_2CH_2NEt_2$), 5.70 (d, J = 10 Hz, 1H, H-2), 6.51 (t, J = 5 Hz, 1H, D_2O exchangeable, NH), 6.69 (s, 1H, H-5), 6.94 (d, J = 10 Hz, 1H, H-1), 7.32 (m, 1H, H-9), 8.52 (m, 1H, H-8), 8.59 (m, 1H, H-10). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 11.31 [$N(CH_2\underline{C}H_3)_2$], 28.15 (2 × gem CH_3), 37.15 ($NH\underline{C}H_2CH_2NEt_2$), 46.31 [$N(\underline{C}H_2CH_3)_2$], 51.01 ($NHCH_2\underline{C}H_2NEt_2$), 78.40 (C-3), 110.01 (C-12b), 111.88 (C-6a), 113.83 (C-5), 114.79 (C-1), 116.62 (C-7a), 121.11 (C-9), 130.67 (C-2), 137.13 (C-8), 138.42 (C-6), 151.73 (C-12a), 153.31 (C-10), 158.20 (C-4a), 159.56 (C-11a), 168.86 (CONH), 175.18 (C-7). Anal. for $C_{24}H_{27}N_3O_4$. Calc. (%): C, 68.39; H, 6.46; N, 9.97. Found (%): C, 68.54; H, 6.38; N, 10.12.

*4.1.10. N,N-dimethyl-N′-[[3,3-dimethyl-7-oxo-3H,7H-pyrano[2′,3′:7,8]benzopyrano[2,3-b]pyridin-6-yl]methyl]ethane-1,2-diamine (**19**)*

Thionyl chloride (1 ml) was added under argon to a solution of the carbinol **10** (90 mg, 0.29 mmol) in dry toluene (5 ml) and the mixture was heated at 90 °C for 90 min. The solvent and the excess of thionyl chloride were vacuum-evaporated to provide the chloride **18**, as an oil, which without purification was dissolved in dry THF (10 ml) and the resulting solution was treated with 2-(dimethylamino)ethylamine (315 μl, 2.90 mmol) and heated at reflux for 4 h. The solvent was then vacuum-evaporated, water was added to the residue and it was extracted with EtOAc (3 × 50 ml). The organic phase was dried (Na_2SO_4) and evaporated to dryness and the residue was purified by column chromatography (silica gel), using a mixture of CH_2Cl_2/EtOAc (96:4) as the eluent to provide the diamine **19** (89 mg, 81%) as an oil, m.p. (dihydrochloride) > 250 °C (EtOH). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.43 (s, 6H, 2 × gem CH_3), 2.15 [s, 6H, $N(CH_3)_2$], 2.41 [t, J = 7 Hz, 2H, $NHCH_2C\underline{H}_2NMe_2$), 2.72 (t, J = 7 Hz, 2H, $NHC\underline{H}_2CH_2NMe_2$), 4.24 (s, 2H, $ArC\underline{H}_2NH$), 5.67 (d, J = 10 Hz, 1H, H-2), 6.86 (s, 1H, H-5), 6.98 (d, J = 10 Hz, 1H, H-1), 7.33 (m, 1H, H-9), 8.56 (m, 1H, H-8), 8.59 (m, 1H, H-10). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 28.26 (2 × gem CH_3), 45.42 [$N(CH_3)_2$], 46.60 ($NH\underline{C}H_2CH_2NMe_2$), 53.18 ($Ar\underline{C}H_2NH$), 59.06 ($NHCH_2\underline{C}H_2NMe_2$), 78.03 (C-3), 108.43 (C-12b), 113.21 (C-6a), 115.23 (C-1), 115.71 (C-5), 117.10 (C-7a), 120.89 (C-9), 129.71 (C-2), 137.21 (C-8), 144.27 (C-6), 152.94 (C-12a), 153.16 (C-10), 158.12 (C-4a), 159.37 (C-11a), 177.68 (C-7). Anal. for $C_{22}H_{27}Cl_2N_3O_3$. Calc. (%): C, 58.41; H, 6.02; N, 9.29. Found (%): C, 58.18; H, 5.87; N, 8.98.

*4.1.11. N,N-diethyl-N′-[[3,3-dimethyl-7-oxo-3H,7H-pyrano[2′,3′:7,8]benzopyrano[2,3-b]pyridine-6-yl]methyl]ethane-1,2-diamine (**20**)*

This compound was prepared by a procedure analogous to that of **19**. Yield 83 %, m.p. (dihydrochloride) 244–246 °C (EtOH). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.92 [t, J = 7 Hz, 6H, $N(CH_2C\underline{H}_3)_2$], 1.43 (s, 6H, 2 × gem CH_3), 2.44 [q, J = 7 Hz, 4H, $N(C\underline{H}_2CH_3)_2$], 2.54 [t, J = 7 Hz, 2H, $NHCH_2C\underline{H}_2NEt_2$), 2.68 (t, J = 7 Hz, 2H, $NHC\underline{H}_2CH_2NEt_2$), 4.24 (s, 2H, $ArC\underline{H}_2NH$), 5.67 (d, J = 10 Hz, 1H, H-2), 6.85 (s, 1H, H-5), 6.98 (d, J = 10 Hz, 1H, H-1), 7.34 (m, 1H, H-9), 8.56 (m, 1H, H-8), 8.60 (m, 1H, H-10). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 11.46 [$N(CH_2\underline{C}H_3)_2$], 28.30 (2 × gem CH_3), 46.49 ($NH\underline{C}H_2CH_2NEt_2$), 46.78 [$N(\underline{C}H_2CH_3)_2$], 52.59 ($NHCH_2\underline{C}H_2NEt_2$), 53.07 ($Ar\underline{C}H_2NH$), 78.10 (C-3), 108.50 (C-12b), 113.21 (C-6a), 115.19 (C-1), 115.85 (C-5), 117.10 (C-7a), 120.92 (C-9), 129.78 (C-2), 137.21 (C-8), 143.97 (C-6), 153.05 (C-12a), 153.23 (C-10), 158.16 (C-4a), 159.35 (C-11a), 177.70 (C-7). Anal. for $C_{24}H_{31}Cl_2N_3O_3$. Calc. (%): C, 60.00; H, 6.50; N, 8.75. Found (%): C, 59.81; H, 6.44; N, 8.93.

*4.1.12. 2,2-Dimethyl-6-oxo-2H,6H-pyrano[3′,2′:6,7]benzopyrano[2,3-b]pyridine-5-carboxylic acid (**21**)*

This compound was prepared by a procedure analogous to that of **14** starting from the carbinol **13**. Yield 76%, m.p. 232–234 °C (EtOH). ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 1.46 (s, 6H, 2 × gem CH_3), 6.06 (d, J = 10 Hz, 1H, H-3), 6.36 (d, J = 10 Hz, 1H, H-4), 7.09 (s, 1H, H-12), 7.58 (m, 1H, H-8), 8.54 (m, 1H, H-7), 8.75 (m, 1H, H-9). ^{13}C-NMR (DMSO-d_6, 50 MHz): δ 28.09 (2 × gem CH_3), 78.55 (C-2), 104.50 (C-12), 111.33 (C-5a), 115.05 (C-4a), 116.08 (C-6a), 117.03 (C-4), 121.88 (C-8), 131.84 (C-5), 133.68 (C-3), 136.77 (C-7), 154.12 (C-9), 156.47 (C-11a), 158.93 (C-12a), 159.52 (C-

10a), 168.19 (COOH), 174.73 (C-6). Anal. for $C_{18}H_{13}NO_5$. Calc. (%): C, 66.87; H, 4.05; N, 4.33. Found (%): C, 67.14; H, 3.96; N, 4.08.

4.1.13. 2,2-Dimethyl-N-[2-(dimethylamino)ethyl]-6-oxo-2H,6H-pyrano[3′,2′:6,7]benzopyrano[2,3-b]pyridine-5-carboxamide (**23**)

This compound was prepared by a procedure analogous to that of **16**, starting from the carboxylic acid **21** Yield 82% (oil). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.42 (s, 6H, 2 × gem CH_3), 2.19 [s, 6H, $N(CH_3)_2$], 2.57 [t, $J = 7$ Hz, 2H, $NHCH_2CH_2NMe_2$), 3.59 (q, $J = 5$ Hz, 7 Hz, 2H, $NHCH_2CH_2NMe_2$), 5.72 (d, $J = 10$ Hz, 1H, H-3), 6.43 (d, $J = 10$ Hz, 1H, H-4), 6.52 (t, $J = 5$ Hz, 1H, D_2O exchangeable, NH), 6.82 (s, 1H, H-12), 7.30 (m, 1H, H-8), 8.50 (m, 1H, H-7), 8.58 (m, 1H, H-9). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 28.49 (2 × gem CH_3), 37.23 ($NHCH_2CH_2NMe_2$), 44.91 [$N(CH_3)_2$], 57.30 ($NHCH_2CH_2NMe_2$), 77.25 (C-2), 105.01 (C-12), 112.06 (C-5a), 116.62 (C-4a, C-6a), 117.80 (C-4), 120.96 (C-8), 132.39 (C-3), 133.86 (C-5), 137.09 (C-7), 153.30 (C-9), 156.94 (C-11a), 159.36 (C-12a), 159.62 (C-10a), 167.78 (CONH), 175.17 (C-6). Anal. for $C_{22}H_{23}N_3O_4$. Calc. (%): C, 67.16; H, 5.89; N, 10.68. Found (%): C, 67.02; H, 5.94; N, 10.47.

4.1.14. 2,2-Dimethyl-N-[2-(diethylamino)ethyl]-6-oxo-2H,6H-pyrano[3′,2′:6,7]benzopyrano[2,3-b]pyridine-5-carboxamide (**24**)

This compound was prepared by a procedure analogous to that of **16**, starting from the carboxylic acid **21**. Yield 85% (oil). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.88 [t, $J = 7$ Hz, 6H, $N(CH_2CH_3)_2$], 1.42 (s, 6H, 2 × gem CH_3), 2.47 [q, $J = 7$ Hz, 4H, $N(CH_2CH_3)_2$], 2.65 [t, $J = 7$ Hz, 2H, $NHCH_2CH_2NEt_2$), 3.58 (q, $J = 5$ Hz, 7 Hz, 2H, $NHCH_2CH_2NEt_2$), 5.71 (d, $J = 10$ Hz, 1H, H-3), 6.45 [m, 2H, H-4, NH (D_2O exchangeable)], 6.83 (s, 1H, H-12), 7.31 (m, 1H, H-8), 8.51 (m, 1H, H-7), 8.59 (m, 1H, H-9). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 11.17 [$N(CH_2CH_3)_2$], 28.44 (2 × gem CH_3), 36.93 ($NHCH_2CH_2NEt_2$), 46.23 [$N(CH_2CH_3)_2$], 51.09 ($NHCH_2CH_2NEt_2$), 78.21 (C-2), 105.01 (C-12), 111.99 (C-5a), 116.62 (C-4a, C-6a), 117.84 (C-4), 121.00 (C-8), 132.36 (C-3), 133.90 (C-5), 137.02 (C-7), 153.31 (C-9), 156.95 (C-11a), 159.41 (C-12a), 159.63 (C-10a), 167.61 (CONH), 175.18 (C-6). Anal. for $C_{24}H_{27}N_3O_4$. Calc. (%): C, 68.39; H, 6.46; N, 9.97. Found (%): C, 68.44; H, 6.33; N, 10.23.

4.1.15. N,N-dimethyl-N′-[[2,2-dimethyl-6-oxo-2H,6H-pyrano[3′,2′:6,7]benzopyrano[2,3-b]pyridin-5-yl]methyl]ethane-1,2-diamine (**26**)

This compound was prepared by a procedure analogous to that of **19**. Yield 82%, m.p. (dihydrochloride) 236–238 °C (dec) (EtOH). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.41 (s, 6H, 2 × gem CH_3), 2.13 [s, 6H, $N(CH_3)_2$], 2.39 [t, $J = 7$ Hz, 2H, $NHCH_2CH_2NMe_2$), 2.75 (t, $J = 7$ Hz, 2H, $NHCH_2CH_2NMe_2$), 4.27 (s, 2H, $ArCH_2NH$) 5.76 (d, $J = 10$ Hz, 1H, H-3), 6.76 (d, $J = 10$ Hz, 1H, H-4), 6.82 (s, 1H, H-12), 7.31 (m, 1H, H-8), 8.57 (m, 2H, H-9, H-7). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 28.52 (2 × gem CH_3), 45.39 [$N(CH_3)_2$], 46.05 ($ArCH_2NH$), 46.75 ($NHCH_2CH_2NMe_2$), 59.03 ($NHCH_2CH_2NMe_2$), 77.26 (C-2), 104.57 (C-12), 113.94 (C-5a), 117.25 (C-6a), 118.02 (C-4), 119.09 (C-4a), 120.74 (C-8), 132.06 (C-3), 137.25 (C-7), 137.98 (C-5), 153.16 (C-9), 158.34 (C-11a), 159.30 (C-10a, C-12a), 177.64 (C-6). Anal. for $C_{22}H_{27}Cl_2N_3O_3$. Calc. (%): C, 58.41; H, 6.02; N, 9.29. Found (%): C, 58.29; H, 6.07; N, 9.11.

4.1.16. N,N-diethyl-N′-[[2,2-dimethyl-6-oxo-2H,6H-pyrano[3′,2′:6,7]benzopyrano[2,3-b]pyridin-5-yl]methyl]ethane-1,2-diamine (**27**)

This compound was prepared by a procedure analogous to that of **19**. Yield 80 %, m.p. (dihydrochloride) 229–231 °C (EtOH). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.88 [t, $J = 7$ Hz, 6H, $N(CH_2CH_3)_2$], 1.40 (s, 6H, 2 × gem CH_3), 2.41 [q, $J = 7$ Hz, 4H, $N(CH_2CH_3)_2$], 2.52 [t, $J = 7$ Hz, 2H, $NHCH_2CH_2NEt_2$), 2.72 (t, $J = 7$ Hz, 2H, $NHCH_2CH_2NEt_2$), 4.29 (s, 2H, $ArCH_2NH$), 5.75 (d, $J = 10$ Hz, 1H, H-3), 6.76 (d, $J = 10$ Hz, 1H, H-4), 6.81 (s, 1H, H-12), 7.30 (m, 1H, H-8), 8.54 (m, 1H, H-7), 8.57 (m, 1H, H-9). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 11.28 [$N(CH_2CH_3)_2$], 28.08 (2 × gem CH_3), 45.68 ($ArCH_2NH$), 46.31 ($NHCH_2CH_2NEt_2$), 46.53 [$N(CH_2CH_3)_2$], 52.41 ($NHCH_2CH_2NEt_2$), 77.22 (C-2), 104.60 (C-12), 113.79 (C-5a), 117.14 (C-6a), 117.87 (C-4), 119.16 (C-4a), 120.74 (C-8), 132.17 (C-3), 137.17 (C-7), 137.28 (C-5), 153.16 (C-9), 158.31 (C-11a), 159.11 (C-10a), 159.23 (C-12a), 177.57 (C-6). Anal. for $C_{24}H_{31}Cl_2N_3O_3$. Calc. (%): C, 60.00; H, 6.50; N, 8.75. Found (%): C, 59.77; H, 6.39; N, 8.68.

4.2. Biological assays

4.2.1. Cell culture and assessment of cytotoxicity

The new compounds were tested for their cytotoxic activity on the murine leukemia cell line L1210 (American Type Culture Collection, Rockville, MD), as well as on the human colorectal adenocarcinoma HT-29 cell line (European Collection of Cell Cultures, Salisbury, UK). L1210 cells were cultured in RPMI 1640 medium (Gibco BRL, Paisley, U.K.) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (media and antibiotics from Biochrom KG, Berlin, Germany) in an environment of 5% CO_2, 85% humidity, and 37 °C. HT-29 cells were cultured in Dulbecco's minimal essential medium supplemented with antibiotics and serum (as above), and routinely subcultured using a 0.25% trypsin–0.02% EDTA solution. The cytotoxicity assay was performed by a modification of the MTT method [18]. Briefly, the cells were plated at a density of approximately 5000 cells per well in 96-well flat-bottomed microplates, and after 24 h the test compounds were added, appropriately diluted with DMSO. After a 72-h incubation, the medium was replaced with MTT (Sigma) dissolved at a final concentration of 1 mg/ml in serum-free, phenol-red-free RPMI (Biochrom KG) for a further 4 h incubation. Then, the MTT formazan was solubilized in 2-propanol, and the optical density was measured with a microplate analyzer at a wavelength of 550 nm (reference wavelength 690 nm). Daunomycin hydrochloride and acronycine were included in

the experiments as positive controls. The results represent the mean of three independent experiments and are expressed as IC_{50}, the concentration that reduced by 50% the optical density of treated cells with respect to untreated controls.

4.2.2. Cell cycle analysis

The effect of the studied compounds on cell cycle distribution was assayed in exponentially growing HT-29 cells. Eighteen hours after plating the test compounds were added at a concentration of 40 μM. After an incubation period of 30 h treated cells were washed in PBS, fixed in 50% ethanol, and stained with an RNAse-containing propidium iodide solution. DNA content was analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer using the ModFit software (Verity Software House, Topsham, ME, USA).

Acknowledgements

This study is a part of the Ph.D. thesis of G.K. and was supported by a Research Grant provided by the Greek Ministry of Education (Business Program of Education and Initial Professional Preparing, EPEAEK-IRAKLEITOS).

References

[1] G. Minotti, P. Menna, E. Salvatorelli, G. Cairo, L. Gianni, Pharmacol. Rev. 56 (2004) 185–229.
[2] T.D. Shenkenberg, D.D. Von Hoff, Ann. Intern. Med. 105 (1986) 67–81.
[3] D. Faulds, J.A. Balfour, P. Chrisp, H.D. Langtry, Drugs 41 (1991) 400–449.
[4] M.F. Brana, M. Cacho, A. Gradillas, B. De Pascual-Teresa, A. Ramos, Curr. Pharm. Des. 7 (2001) 1745–1780.
[5] A. Liwo, D. Jeziorek, T. Ossowski, D. Dyl, A. Tempczyk, J. Tarasiuk, M. Nowacka, E. Borowski, W. Woznicki, Acta Biochim. Pol. 42 (1995) 445–456.
[6] H.D. Showalter, J.L. Johnson, J.M. Hoftiezer, W.R. Turner, L.M. Werbel, W.R. Leopold, J.L. Shillis, R.C. Jackson, E.F. Elslager, J. Med. Chem. 30 (1987) 121–131.
[7] I.R. Judson, Anticancer Drugs 2 (1991) 223–231.
[8] H.D. Showalter, M.M. Angelo, E.M. Berman, G.D. Kanter, D.F. Ortwine, S.G. Ross-Kesten, A.D. Sercel, W.R. Turner, L.M. Werbel, D.F. Worth, E.F. Elslager, W.R. Leopold, J.L. Shillis, J. Med. Chem. 31 (1988) 1527–1539.
[9] C.A. Arndt, M.D. Krailo, W. Liu-Mares, P.M. Anderson, G.H. Reaman, Cancer 91 (2001) 1166–1169.
[10] I. Antonini, Curr. Med. Chem. 9 (2002) 1701–1716.
[11] C. Sissi, E. Leo, S. Moro, G. Capranico, A. Mancia, E. Menta, A.P. Krapcho, M. Palumbo, Biochem. Pharmacol. 67 (2004) 631–642.
[12] K. Ghirtis, N. Pouli, P. Marakos, A.L. Skaltsounis, S. Leonce, G. Atassi, D.H. Gaignard, J. Heterocycl. Chem. 38 (2001) 147–152.
[13] I.K. Kostakis, N. Pouli, P. Marakos, E. Mikros, A.L. Skaltsounis, S. Leonce, G. Atassi, P. Renard, Bioorg. Med. Chem. 9 (2001) 2793–2802.
[14] R.T. Dorr, J.D. Liddil, D.D. Von Hoff, M. Soble, C.K. Osborne, Cancer Res. 49 (1989) 340–344.
[15] I.K. Kostakis, K. Ghirtis, N. Pouli, P. Marakos, A.L. Skaltsounis, S. Leonce, D.H. Caignard, G. Atassi, Farmaco 55 (2000) 455–460.
[16] G. Kolokythas, I.K. Kostakis, N. Pouli, P. Marakos, A.L. Skaltsounis, H. Pratsinis, Bioorg. Med. Chem. Lett. 12 (2002) 1443–1446.
[17] G. Kolokythas, I.K. Kostakis, N. Pouli, P. Marakos, D. Kletsas, H. Pratsinis, Bioorg. Med. Chem. 11 (2003) 4591–4598.
[18] F. Denizot, R. Lang, J. Immunol. Meth. 89 (1986) 271–277.