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# DNA Binding Ellipticine Analogues: Synthesis, Biological Evaluation, and Structure–Activity Relationships

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*In connection with our interest in the synthesis and study of the biological properties of ellipticine analogues as anticancer agents, some 7H-pyrido[2,3-c]carbazoles (7H-PyC) and their corresponding tetrahydro derivatives (7H-THPyC) were synthesized. A common multistep pathway characterized by conventional reactions involving carbazole Fischer and Conrad–Limpach quinoline syntheses yielded the tetracyclic compounds. With the aim to improve the cytotoxic activity of the new 7H-PyC derivatives, we provided them with one or two diethylaminoethyl side chains. The new THPyCs, PyCs, and smaller pyrroloquinoline (PQ) derivatives were tested for their in vitro cytotoxic properties against several human tumor cell lines. Most of the compounds tested showed considerable cytotoxic activity, particularly compound* ***24****, which, with two basic alkylamino chains, has $IC_{50}$ values in the sub-micromolar range, about one order of magnitude lower than those of the reference compound, ellipticine. Chemosensitivity tests on cisplatin-, mitoxantrone-, and multidrug-resistant (MDR) phenotypes indicated that compound* ***24*** *shows no cross-resistance; this suggests that, besides not being a potential MDR substrate, it might act as a mixed inhibitor of topoisomerases I and II. Flow cytometric and caspase-3 activation analyses revealed that* ***24*** *induces a caspase-3-dependent apoptotic cell-death mechanism. Linear dichroism and unwinding experiments suggested that the most active compounds act as DNA intercalators. For compound* ***24****, an inhibitory concentration-dependent effect on topoisomerases II and I was demonstrated. Herein we discuss interesting structure–activity relationships with respect to molecular size and planarity, as well as the substitution and position of one side chain on the PyC nucleus, in comparison with corresponding smaller PQs.*

## Introduction

DNA topoisomerase II (topo II) inhibitors, used in human cancer chemotherapy,[1,2] constitute a group of structurally unrelated compounds that share a common property: the capacity to induce a significant increase in the number of covalent enzyme–DNA complexes (cleavable complexes) present on the cell chromatin at a given time, triggering a response that eventually leads to cell death by apoptosis.[3,4] However, probably as a consequence of their structural diversity, DNA topo II inhibitors have been shown to be active, at least in vitro, through different mechanisms.[5–8]

It is generally known that these compounds are involved in the formation of drug–enzyme–DNA ternary complexes. Some drugs enter this complex by interacting predominantly with the protein (etoposide)[9] or DNA (ellipticine),[10,11] but most DNA-intercalating drugs probably bind to both DNA, through intercalation of the chromophore at the enzyme–DNA interface, and the enzyme, through side chains, which are usually essential to their pharmacological activity.[12–17] Some drugs, such as etoposide and amsacrine (*N*-[4-(acridin-9-ylamino)-3-methoxyphenyl]methanesulfonamide {*m*-AMSA}), can inhibit the re-ligation of cleaved DNA, whereas others, such as ellipticine, genistein, and quinolones, are presumed to accelerate the forward rate of complex formation.[15]

In particular, ellipticine is a naturally occurring alkaloid of the 6*H*-pyridocarbazole (6*H*-PyC) family.[15] A number of successful ellipticine analogues have been designed and synthesized with improved cytotoxicity and anticancer activities. It has been shown that the presence of a dialkylaminoalkylamino side chain at position 1 of ellipticine significantly increases the antitumor properties of the DNA-base-intercalating heterocycle system (Figure 1).[18–21] A new series of 6*H*-pyrido[4,3-*b*]carbazole derivatives, characterized by a basic *N*-dialkylaminoalkylcarboxamido side chain grafted onto an olivacine moiety, has recently been characterized. Some of these compounds display remarkable activity against various experimental tumors. The most active of these derivatives, S16020-2 (NSC-659687) (Figure 1), has shown a broad range of antitumor activities against a panel of murine and human tumor xenografts.[22–24] It has also been suggested that DNA topo II is the intracellular target of S16020-2, and that the interaction of S16020-2 with this enzyme is strongly influenced by the *N*-[2(dimethylamino)ethyl]carbamoyl side chain.

In our ongoing search for new potential ellipticine-correlated anticancer agents,[25,26] we have developed a novel class of 7*H*-pyridocarbazole (7*H*-PyC) derivatives with a very interesting therapeutic profile which we describe herein. Several 7*H*-PyC

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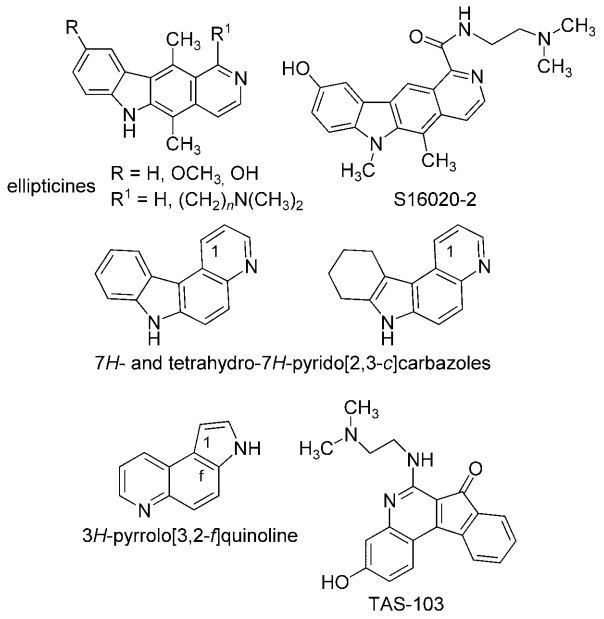



**Figure 1.** Structures of some key compounds mentioned in the text and general structures of synthesized PQ and PyC derivatives.

derivatives, both monomers and dimers, have been synthesized and studied for their DNA affinity, intercalating properties, and antitumor activity, and many of them exert high cytotoxicity.[27–32] It is suggested that the particular geometry and size of the 7*H*-PyC nucleus and the presence of some small substitutions (methyl or methoxy) at certain positions are the structural elements that determine the strong capacity to intercalate into the DNA double helix to a similar extent as ellipticine.[30] We examined 3-methyl-6-methoxy-7*H*-pyrido[2,3-*c*]carbazole as a basic nucleus and, with the aim of improving the cytotoxicity of the heterocycle system, we synthesized some new branched 7*H*-tetrahydro-PyCs (7*H*-THPyC) and 7*H*-PyCs (Figure 1) with one or two diethylaminoethyl side chains grafted onto PyC.

Herein we report the synthesis and characterization of the new THPyCs and PyCs and certain pyrroloquinoline (PQ) derivatives (Figure 1). We assessed the in vitro antitumor properties of these derivatives in a large panel of human cancer cell lines belonging to a variety of different tumor types, including cisplatin-, mitoxantrone-, and multidrug-resistant (MDR) phenotypes. DNA flow cytometry analysis and biochemical studies were performed to study the effects on cell cycle and the induction of apoptosis. Interaction with DNA was also studied by flow linear dichroism (LD), and the ability to inhibit topoisomerases I and II was evaluated.

## Results and Discussion

### Chemistry

The 6-methoxy-substituted 7*H*-PyC derivative was chosen as the new basic structure, because it had been designed as a precursor of the 6-branched compounds. Worth noting is the structural similarity between the 6-branched derivatives and TAS-103 (Figure 1), a known topo I and II dual inhibitor, a drug candidate.[33]

The presence of a methoxy group in the 6-position required total synthesis of the PyC ring. Among the various methods reported for synthesis of the 7*H*-PyC ring,[34–37] conventional Borsh–Fischer carbazole synthesis,[38] starting from the appropriate phenyl derivative, followed by the Conrad–Limpach[39] quinoline reaction, was chosen because all the differently branched PyCs could be obtained.

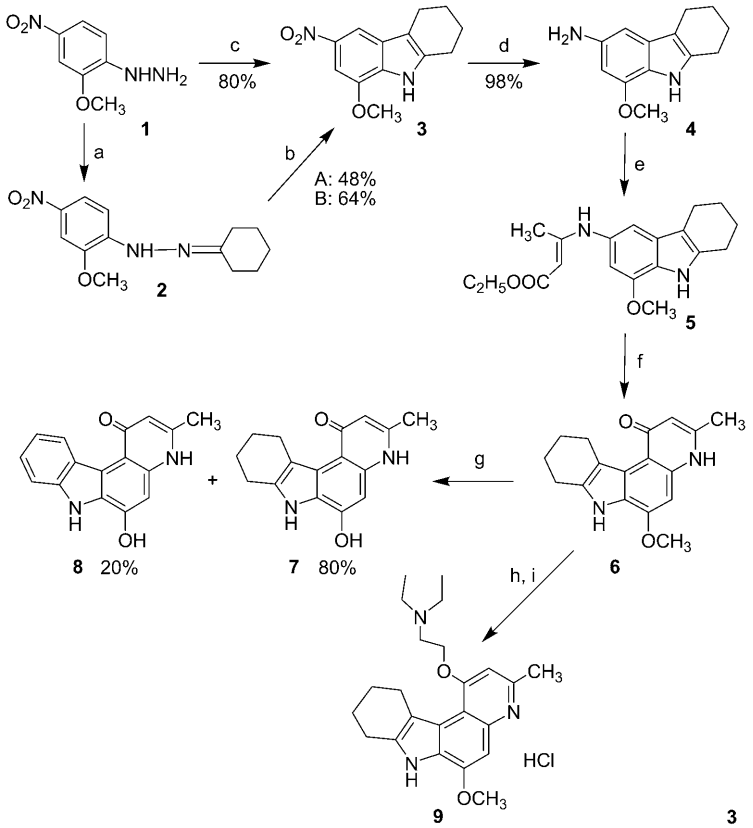
7*H*-pyrido[2,3-*c*]carbazoles branched at positions 1 and 6, or 1 and 7, and a PQ derivative branched at position 4 were obtained by the same alkylating method applied at the last step of the synthetic routes (Schemes 1–5). To obtain the best yields, alkylation reactions with chloroethyldiethylamine were performed each time by adopting different reagents and conditions (NaH/DMF, $K_2CO_3$/DMF, KOH/acetone).

Scheme 1 illustrates the synthesis of THPyC derivatives starting from 2-methoxy-4-nitrophenylhydrazine hydrochloride, prepared by following a previously described procedure,[40] and then treated with cyclohexanone to give *N*-cyclohexylidene-*N*′-(2-methoxy-4-nitrophenyl)hydrazine (**2**). Tetrahydrocarbazole **3** was obtained by acid cyclization following two previously reported methods: method A, consisting of cyclization by $H_2SO_4$ (4%), and method B, by ion-exchange resin, Amberlyst in aprotic media (toluene).[40] Both methods were quite advantageous (respective yields of 48 and 64%). The same compound **3** was also directly obtained from the starting hydrazine hydrochloride derivative and cyclohexanone in glacial acetic acid by a one-pot microwave-assisted reaction in high yield (80%), as previously reported.[41,42] The microwave procedure for tetrahydrocarbazole **3** was optimized for time, temperature, and pressure on a gram scale.[43] Then, 1-methoxy-3-nitro-6,7,8,9-tetrahydro-5*H*-carbazole was catalytically reduced to the amine derivative **4**, and this was condensed with ethyl acetoacetate to yield enamine compound **5**, which was cyclized at 250 °C to 1-oxo-3-methyl-6-methoxy-1,4,8,9,10,11-hexahydro-7*H*-pyrido[2,3-*c*]carbazole (**6**). To obtain 6-functionalized THPyC, compound **6** was treated with 48% HBr in acetic acid at reflux for variable periods; however, an irresolvable mixture of 6-hydroxy-THPyC **7** and its total aromatic analogue **8** always formed at a ratio of 80:20, as evaluated by $^1$H NMR integral data of the mixture. Because the $^1$H NMR spectrum of mixture was uncomplicated, we were able to attribute the signals to protons of the two compounds **7** and **8**.

Compound **6** was then alkylated with chloroethyldiethylamine chloride in *N*,*N*-dimethylformamide (DMF) and $K_2CO_3$, and only monofunctionalized derivative **9** was isolated from the reaction mixture by flash chromatography, confirmed by HRMS analysis. To elucidate its structure and the exact position of the side chain, 1D and 2D NMR experiments were performed (Supporting Information). Lastly, diethyl-[2-(6-methoxy-3-methyl-8,9,10,11-tetrahydro-7*H*-pyrido[2,3-*c*]carbazol-1-yloxy)-ethyl]amine (**9**) was transformed into its more soluble hydrochloride for biological assays.

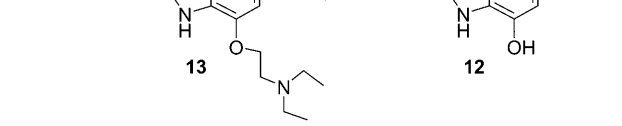
Scheme 2 shows how the 1-oxo group of **6** was eliminated, by first transforming 1-oxo compound **6** into 1-chloro deriva-



**Scheme 1.** Synthesis of 1-functionalized 8,9,10,11-7*H*-tetrahydropyrido[2,3-*c*]carbazole hydrochloride **9**. *Reagents and conditions*: a) cyclohexanone, EtOH, reflux; b) A. $H_2SO_4$ (4%), reflux; B. cyclohexanone, Amberlyst, toluene, reflux; c) cyclohexanone, $CH_3COOH$, 150 °C, 3 min; d) $H_2$, Pd/C 10%, room temperature; e) ethyl acetoacetate, EtOH, reflux; f) diphenyl ether, 250 °C; g) HBr (48%), $CH_3COOH$, reflux; h) chloroethyldiethylamine, $K_2CO_3$, DMF, i) HCl (37%).

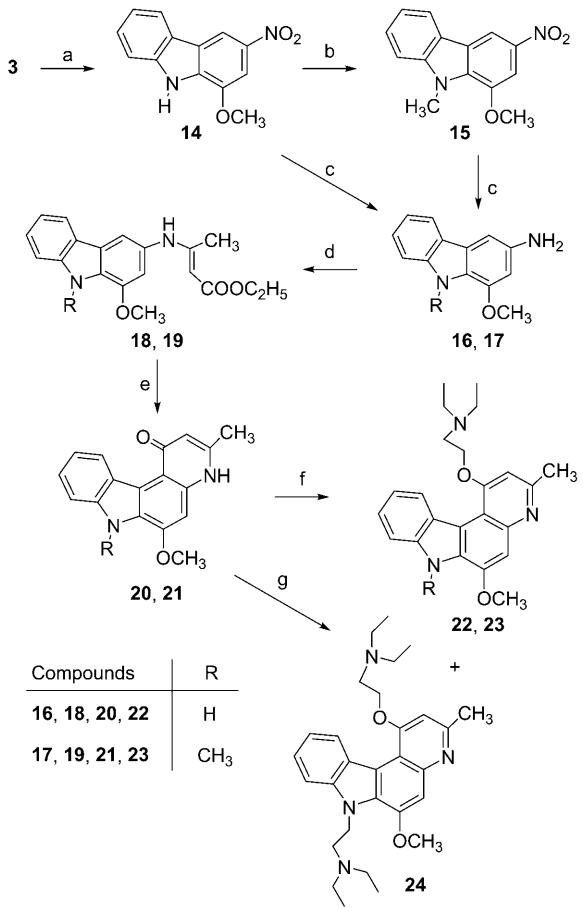
tive **10**, and then removing the chlorine atom by catalytic reduction to give 6-methoxy-3-methyl-8,9,10,11-tetrahydro-7*H*-pyrido[2,3-*c*]carbazole (**11**). The latter was demethoxylated with 48% HBr to 6-hydroxy derivative **12**. Treatment of **12** with chloroethyldiethylamine chloride yielded final compound **13**, the exact structure of which was again confirmed by 1D and 2D NMR experiments (Supporting Information). Lastly, diethyl-[2-(3-methyl-8,9,10,11-tetrahydro-7*H*-pyrido[2,3-*c*]carbazol-6-yloxy)-ethyl]amine hydrochloride **13** was transformed into its more soluble hydrochloride for biological assays.



**Scheme 2.** Synthesis of 6-functionalized 8,9,10,11-7*H*-tetrahydrorapyrido[2,3-*c*]carbazole hydrochloride **13**. *Reagents and conditions*: a) $POCl_3$, 110 °C; b) $H_2$, Pd/C 10%, room temperature; c) HBr (48%), $CH_3COOH$, reflux; d) chloroethyldiethylamine, $K_2CO_3$, DMF; e) HCl (37%).

The synthesis of some PyC derivatives is described in Scheme 3 in which compound **3** was oxidized to the total aromatic tetracycle **14** by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in benzene, and this was methylated at the *N*-pyrrole to give compound **15**. Both compounds **14** and **15** were subjected to the same series of transformations, catalytic reduction to **16** and **17**, condensation with ethyl acetoacetate to give **18** and **19**, and cyclization in diphenyl ether[39] to obtain the corresponding pyridinone derivatives **20** and **21**.

Both compounds **20** and **21** were alkylated with chloroethyldiethylenamine hydrochloride in DMF and $K_2CO_3$, and **22**, **23**, and **24** were isolated and pu-



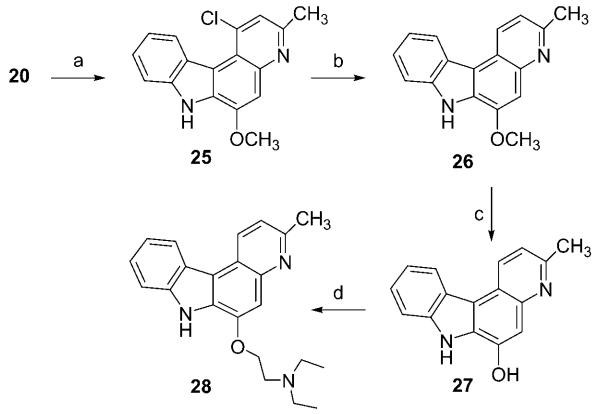
| Compounds | R |
|---|---|
| **16**, **18**, **20**, **22** | H |
| **17**, **19**, **21**, **23** | $CH_3$ |

**Scheme 3.** Synthesis of functionalized 7*H*-pyrido[2,3-*c*]carbazoles **22**–**24**. *Reagents and conditions*: a) DDQ, benzene, reflux; b) $CH_3I$, NaH, DMF; c) $H_2$, Pd/C 10%, room temperature; d) ethyl acetoacetate, EtOH, reflux; e) diphenyl ether, 250 °C; f) chloroethyldiethylamine, $K_2CO_3$, DMF; g) KOH, acetone, chloroethyldiethylamine, reflux.

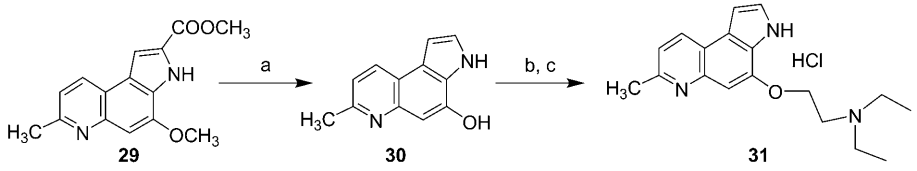
  

rified from the two reaction mixtures. Compound **24** was also obtained as the only product of the alkylation reaction with chloroethyldiethylenamine hydrochloride in acetone and KOH, in high yields (73%). The exact structure of **24**, which, on the basis of HRMS analysis, has two side chains, was confirmed by 1D and 2D NMR experiments (Supporting Information). The structures of **22** and **23**, with one side chain each (HRMS) at the 1-position, were determined by their own $^{1}H$ and $^{13}C$ NMR spectral data and by comparison with those of **24**.

As shown in Scheme 4, compound **20** was submitted to chlorination at C1 with phosphoryl chloride to give **25**, the hydrogenolysis of which yielded compound **26**. The methoxy group was deprotected with 48% HBr in acetic acid to furnish 6-hydroxy derivative **27**. The latter, upon treatment with chloroethyldiethylenamine hydrochloride in DMF and $K_2CO_3$, yielded **28**, in which the side chain is at the 6-position as shown by $^{1}H$ and $^{13}C$ NMR and IR data, and by comparison with data obtained for the corresponding tetrahydrogenated compound **13** (Scheme 2). Finally, in order to complete the series of PQ derivatives previously obtained,[40] we synthesized the pyrrolo[3,2-*f*]-quinoline analogues of **13** and **28** with one side chain at position 4, as shown in Scheme 5. Compound **29**, obtained by a previously reported procedure,[40] was demethoxylated with 48% HBr and simultaneously decarboxylated to the 4-hydroxy derivative **30**. The latter was treated with chloroethyldiethylamine chloride and then with 37% HCl to yield the final hydrochloride compound **31**.




**Scheme 4.** Synthesis of 6-functionalized 7*H*-pyrido[2,3-*c*]carbazole **28**. *Reagents and conditions*: a) $POCl_3$, 110 °C; b) $H_2$, Pd/C 10%, room temperature; c) HBr (48%), $CH_3COOH$, reflux; d) chloroethyldiethylamine, $K_2CO_3$, DMF.




**Scheme 5.** Synthesis of 3*H*-pyrrolo[3,2-*f*]quinoline derivative **31**. *Reagents and conditions*: a) HBr (48%), $CH_3COOH$, reflux; b) chloroethyldiethylamine, $K_2CO_3$, DMF; c) HCl (37%).

## Biology

### *Antiproliferative activity*

The newly synthesized compounds were evaluated for their cytotoxic activity toward a panel of human tumor cell lines containing examples of ovarian (2008), cervical (A431), lung (A549), colon (LoVo), and breast (MCF-7) cancers, as well as leukemia (HL60) and melanoma (A375). Cytotoxicity was evaluated by MTT tests after treatment for 72 h with increasing concentrations of the tested compounds. For comparison, the cytotoxicity of ellipticine was evaluated under the same experimental conditions. $IC_{50}$ values, calculated from dose–survival curves, are shown in Table 1. The non-branched PQ **32** proved to be ineffective in all tumor cell lines;[40] 4-branched **31** and 9-branched **33**[40] showed detectable cytotoxic activity, which was, however, markedly lower than that of the reference drug, with average $IC_{50}$ values across seven cell types exceeding 17 and 24 times those of ellipticine, respectively.

Among the PQ derivatives, **34**, which has two side chains at positions 3 and 9, showed the greatest in vitro antitumor efficacy, with a mean $IC_{50}$ value of 6.38 μM (0.91–9.54 μM), about three times higher than that of ellipticine.

All tetracyclic derivatives showed a marked inhibitory potency toward the various tumor cell types. Compounds **11**, **13**, **26**, and **28** showed in vitro antitumor activity very similar to and roughly 15-fold lower than that of ellipticine, the mean $IC_{50}$ (μM) values being 16.66 (11.54–24.50), 15.41 (9.64–20.54), 17.56 (12.01–27.19), 13.79 (10.16–18.47), and 1.77 (0.8–3.24) for **11**, **13**, **26**, **28**, and ellipticine, respectively.

Compounds **9**, **22**, and **23** showed quite similar patterns of response across the various cell lines. The cytotoxic activity of these three PyC derivatives appeared to be important but were still lower (about fourfold) than the reference drug, with mean $IC_{50}$ (μM) values of 8.51 (4.99–12.85), 7.07 (4.25–9.22), 8.91 (5.48–12.22) and 1.8 (0.8–3.24) for **9**, **22**, **23**, and ellipticine, respectively. Completely different remarks must be made about the growth inhibitory activity of PyC **24**, which carries two side chains and appears to be the most powerful derivative. Its in vitro antitumor activity was in the sub-micromolar range toward all tumor cell lines, exceeding that of ellipticine by factors ranging from about 2 to 9.

The very encouraging results obtained against the in-house panel of cell lines prompted us to test the cytotoxic activity of compound **24** on three additional human cell lines, characterized by acquired resistance to mitoxantrone (promyelocytic leukemia HL60/MX2 cells), cisplatin (ovarian adenocarcinoma C13* cells), and doxorubicin (colon adenocarcinoma LoVo/MDR cells), the latter as an example of a multidrug-resistance (MDR) phenotype.

Cytotoxicity was assessed by the MTT assay after exposure for 72 h. Cross-resistance profiles were evaluated by the resistance factor (RF), which is defined as the ratio between $IC_{50}$ values calculated for resistant cells and those arising from

**Table 1.** In vitro antitumor activity.

| Compd | $IC_{50}$ [μM][a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2008 | A431 | MCF-7 | HL60 | LoVo | A549 | A375 |
| **9** | 6.12 ± 1.64 | 12.85 ± 1.80 | 11.80 ± 2.63 | 9.73 ± 1.05 | 8.45 ± 1.89 | 5.64 ± 1.14 | 4.99 ± 0.34 |
| **11** | 18.19 ± 1.42 | 20.12 ± 1.90 | 11.54 ± 2.09 | 13.41 ± 1.85 | 24.50 ± 1.21 | 16.23 ± 1.84 | 12.66 ± 2.00 |
| **13** | 17.17 ± 2.00 | 20.54 ± 2.14 | 17.08 ± 1.05 | 9.64 ± 2.14 | 14.72 ± 1.20 | 11.83 ± 1.21 | 16.91 ± 2.03 |
| **22** | 5.54 ± 1.08 | 4.25 ± 3.10 | 7.15 ± 1.46 | 8.16 ± 2.93 | 8.72 ± 1.65 | 9.22 ± 1.01 | 6.51 ± 1.11 |
| **23** | 5.48 ± 2.38 | 11.57 ± 1.55 | 9.31 ± 0.47 | 7.86 ± 2.35 | 12.22 ± 1.19 | 8.83 ± 1.39 | 7.13 ± 0.26 |
| **24** | 0.19 ± 0.09 | 0.54 ± 0.08 | 0.18 ± 0.03 | 0.97 ± 0.08 | 0.32 ± 0.03 | 0.14 ± 0.01 | 0.10 ± 0.01 |
| **26** | 13.29 ± 2.05 | 14.17 ± 1.02 | 12.01 ± 1.09 | 14.60 ± 2.49 | 27.19 ± 1.22 | 17.54 ± 1.17 | 24.13 ± 2.41 |
| **28** | 10.16 ± 2.03 | 18.47 ± 3.52 | 14.56 ± 1.86 | 13.52 ± 2.62 | 11.37 ± 2.70 | 11.49 ± 1.22 | 17.02 ± 1.34 |
| **31** | 36.33 ± 2.37 | 22.54 ± 1.85 | 56.04 ± 2.31 | 43.91 ± 3.12 | 44.80 ± 1.56 | 49.53 ± 1.98 | 55.01 ± 2.14 |
| **32** | > 100 | > 100 | > 100 | > 100 | > 100 | > 100 | > 100 |
| **33** | 24.69 ± 2.20 | 19.65 ± 1.92 | 45.39 ± 3.26 | 19.82 ± 2.31 | 38.50 ± 2.21 | 29.30 ± 1.89 | 32.22 ± 1.34 |
| **34** | 7.68 ± 1.87 | 6.14 ± 1.78 | 8.56 ± 2.34 | 0.91 ± 0.09 | 5.65 ± 2.12 | 9.54 ± 1.98 | 6.22 ± 1.13 |
| ellipticine | 1.58 ± 0.68 | 3.24 ± 1.00 | 0.94 ± 0.14 | 2.03 ± 1.04 | 2.92 ± 1.28 | 1.00 ± 0.09 | 0.80 ± 0.12 |

[a] $IC_{50}$ values (± SD) were calculated by probit analysis ($P < 0.05$, $X_2$ test); cells ($3–8 \times 10^4$ mL$^{-1}$) were treated for 72 h with increasing concentrations of compounds tested. Cytotoxicity was assessed by MTT test.

chemosensitive cells (Table 2). Leukemic HL60/MX2 cells are described as displaying atypical multidrug resistance, the absence of P-glycoprotein (P-gp) associated with overexpression and altered topoisomerase II catalytic activity, and decreased levels of topoisomerase II α and β proteins.[44,45] Remarkably, on mitoxantrone-resistant cells, **24** showed activity levels similar to those exerted on the parental sensitive cell line, with an RF value > 50-fold lower than that of the reference drug, excluding the occurrence of cross-resistance phenomena.

In ovarian carcinoma C13* cells, cisplatin resistance has been correlated to decreased drug uptake, high cellular glutathione levels, and enhanced repair of DNA damage.[46] Tested on the 2008/C13* cell pair, the resistance factor of **24** was about 17-fold lower than that of cisplatin, clearly revealing no cross-resistance phenomena. Acquired MDR, whereby cells become refractory to multiple drugs, poses a most important challenge to the success of anticancer chemotherapy. The resistance of LoVo MDR cells to doxorubicin, a drug belonging to the MDR spectrum, has been associated with the overexpression of multi-specific drug transporters, such as the 170 kDa P-gp.[47] Cytotoxicity assays testing derivative **24** against this cell line pair showed a similar pattern of response across the parental and resistant sub-line (RF = 4.34 and 30.74 for **24** and doxorubicin, respectively) and allowed the calculation of an RF value about sevenfold lower than that obtained with doxorubicin, suggesting that this new PyC derivative is not a potential MDR substrate.

**Table 2.** Cytotoxicity and resistance factor data.

| Compd | $IC_{50}$ [μM][a] | $IC_{50}$ [μM][a] | RF |
|---|---|---|---|
| | Human promyelocytic leukemia cells | | |
| | HL60 | HL60 MX2 | |
| **24** | 0.97 ± 0.08 | 0.67 ± 0.09 | 0.69 |
| mitoxantrone | 0.33 ± 0.01 | 12.42 ± 0.27 | 37.63 |
| | Human ovarian adenocarcinoma cells | | |
| | 2008 | C13 | |
| **24** | 0.19 ± 0.09 | 0.11 ± 0.05 | 0.58 |
| cisplatin | 5.69 ± 1.3 | 59.18 ± 4.5 | 10.40 |
| | Human colon adenocarcinoma cells | | |
| | LoVo | LoVo MDR | |
| **24** | 0.32 ± 0.03 | 1.39 ± 0.53 | 4.34 |
| doxorubicin | 1.01 ± 0.11 | 31.17 ± 1.28 | 30.86 |

[a] $IC_{50}$ values (± SD) were calculated by probit analysis ($P < 0.05$, $X_2$ test); cells ($3–8 \times 10^4$ mL$^{-1}$) were treated for 72 h with increasing concentrations of tested compounds. Cytotoxicity was assessed by MTT test. [b] Resistance factor: ratio of $IC_{50}$ values calculated for resistant cells and those for chemosensitive cells.

### *Induction of apoptosis*

It has previously been reported that ellipticine induces apoptosis in cancer cells through the activation of caspases and induction of a cell block in the $G_2$M phase.[48] To characterize the cell-death pathway triggered by the most active derivative **24**, we examined its effects in terms of induction of apoptosis and cell cycle modifications.

As caspase-3 is a well-known executor enzyme in the apoptosis pathway, we investigated the ability of **24** to activate caspase-3 in 2008 human ovarian cancer cells. As shown in Figure 2, treatment with compound **24** at $IC_{50}$ concentrations for 24 h resulted in significant enhancement of enzyme activity, about 4.5- and 1.2-fold higher than those detected in untreated and ellipticine-treated cells, respectively.

### *Cell cycle analysis*

To examine the mechanism responsible for **24**-mediated cell growth inhibition, cell cycle distribution in 2008 cells was evaluated by flow cytofluorimetry analysis. Compared with control cells, treatment with an $IC_{50}$ concentration of **24** for 48 h caused a 1.6-fold decrease in the $G_1$ phase as well as a sensible enlargement (1.2-fold) of S, $G_2$, and M phases (Table 3). Treat-

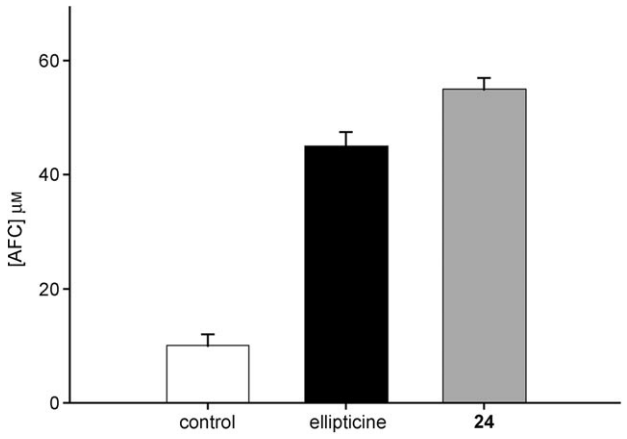



**Figure 2.** Induction of caspase-3: 2008 cells were incubated with compound **24** or ellipticine and then submitted to the test on caspase-3 induction as described in the Experimental Section. Data represent the mean of at least three independent experiments. Error bars indicate standard deviation; $^{*}P < 0.01$ relative to untreated cells.

**Table 3.** FACS analysis.[a]

| Phase | Control | **24** | Ellipticine |
|---|---|---|---|
| sub-$G_1$ | 2.9 ± 1.1 | 17.1 ± 2.2 | 15.3 ± 1.7 |
| $G_1$ | 59.6 ± 3.2 | 37.4 ± 1.9 | 34.6 ± 1.8 |
| S/$G_2$M | 36.1 ± 1.2 | 46.2 ± 2.0 | 49.8 ± 1.5 |

[a] Percentage of cells in different cell cycle phases after 48 h exposure to compound **24** or ellipticine (at $IC_{50}$ concentrations) versus untreated control cells.

ment also resulted in an appreciable increase in the number of 2008 cells with hypodiploid DNA (sub-$G_1$ phase). These results, indicating the classical signs of apoptosis, show that **24**-treated cells mainly died after induction of the apoptotic mechanism.

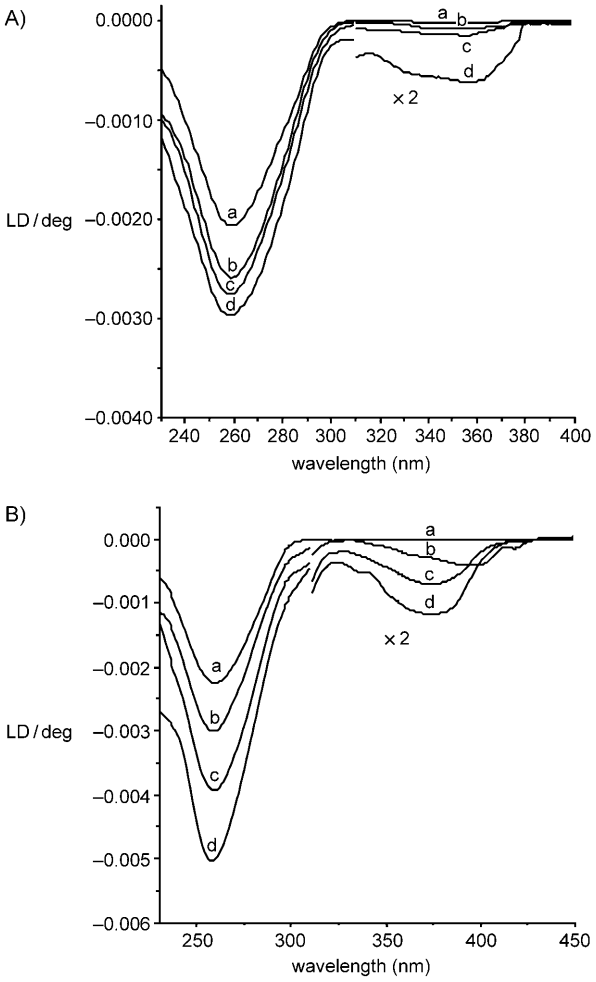



**Figure 3.** LD spectra: A) DNA alone (a), compounds **31** (b), **33** (c), and **34** (d); B) DNA alone (a), compounds **28** (b), **22** (c), and **24** (d). [DNA] = $1.47 \times 10^{-3}$ M, [drug]/[DNA] = 0.16.

### *Interaction with DNA*

To investigate the mechanism of action of the cytotoxic effect, the ability of the new compounds to interact with DNA was tested. Regarding the PQ derivatives, the ability of **34**, possessing two side chains, and **31** and **33**, with one side chain each, to form molecular complexes with DNA was assessed by flow linear dichroism (LD) experiments. Figure 3A shows the LD spectra of DNA solutions in the presence of the tested compounds at a [drug]/[DNA] ratio of 0.16. The DNA spectrum shows the typical negative signal at 260 nm (trace a) where, in the presence of the synthesized PQs, a further negative dichroic band appears at higher wavelengths (310–390 nm, traces b–d). Because small molecules cannot themselves become oriented in the flow field, the presence of a dichroic signal in this spectral region, where only the added chromophore can absorb, means that the PQ derivatives form complexes with DNA. The negative sign of this signal also matches the orientation of the molecular plane of the chromophore being preferentially parallel to the plane of the DNA bases, supporting an intercalative mode of binding.[49]

Interestingly, the intensity of the negative LD signals at 310–390 nm varies significantly between the compounds examined. In particular, for **34** (line d) a prominent negative band is observed, whereas weaker signals appear in the presence of **31** and **33** (traces b and c, respectively). These results for PQ compound **34**, characterized by two (2-diethylaminoethyl) side chains, suggest that it has a greater capacity to form an intercalative complex with DNA than do those compounds with only one such side chain, i.e., **31** and **33**.

Like the PQs, PyC derivative **24**, with two side chains, and **22** and **28**, with one side chain each, also form complexes with DNA, as established by LD experiments. The appearance of a negative dichroic signal in the 320–420 nm spectral range for compounds **24**, **22**, and **28** indicates their ability to form intercalative complexes with DNA (Figure 3B). The intensity of the signal increases in the order **24** > **22** > **28**, which suggests that compound **24** has a greater ability to intercalate between DNA base pairs.

Unwinding experiments with supercoiled plasmid DNA (pBR322) confirmed the behavior observed in the LD spectra. It is known that the intrinsic electrophoretic mobility of super-

coiled DNA decreases as a consequence of the unwinding process, which gives rise to the circular form, following an increase in concentration of an intercalating agent. A further increase in the concentration of intercalator present leads to an eventual return to the original migration rate. Figure 4 shows

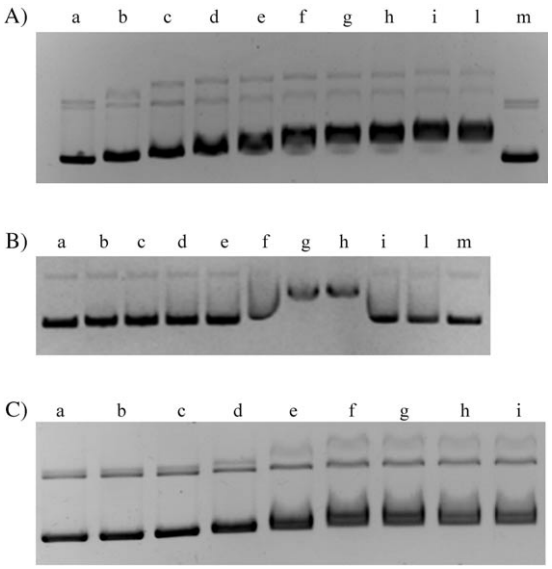



**Figure 4.** Effect of compounds **22**, **24**, and **34** on the supercoiling of pBR322 DNA. A) Lanes a and m: supercoiled pBR322 DNA; lanes b–l: compound **22** at 10, 50, 70, 80, 100, 150, 180, 210, and 250 μM, respectively. B) Lanes a and m: supercoiled pBR322 DNA; lanes b–l: compound **24** at 10, 30, 50, 60, 70, 80, 100, 120, and 150 μM, respectively. C) Lane a: supercoiled pBR322 DNA; lanes b–i: compound **34** at 1, 2.5, 5, 10, 50, 100, 200, and 400 μM, respectively.

the effect of increasing concentrations of derivatives **22**, **24**, and **34**. In the presence of **22**, the migration rate begins to decrease at about 70 μM, but a complete titration curve cannot be obtained until a concentration of 250 μM is reached (Figure 4A), and a similar trend was also observed for **28** (results not shown). For compound **24**, however, the DNA is already in the circular form at 80 μM (Figure 4B, lane g), and reversion to the original migration rate occurs at 120 μM. The inability of **22** to induce complete unwinding of supercoiled DNA at concentrations significantly higher than those used for the titration with **24** proves its capacity to intercalate between base pairs is lower than that of the analogue equipped with two side chains. Unwinding experiments also indicated that the intercalating capacity of the tricyclic PQ **34** is lower than that of the tetracyclic PyC **24**; indeed, no complete reversion of the DNA to its supercoiled form was observed up to concentrations as high as 400 μM (Figure 4C, lane i).

THPyC derivatives **9** and **13** show dichroic spectra practically overlapping that of DNA, thus indicating no detectable interaction with the macromolecule (spectra not shown). This behavior may be a consequence of two features that characterize the THPyC moiety: saturation of the fourth condensed ring, which renders it non-planar with the PyC tricyclic nucleus, and the angular geometry of the PyC chromophore. It is probable that the coexistence of both these unfavorable structural features prevents the chromophore from being inserted between base pairs.

### *Inhibition of DNA topoisomerase II*

The ability to interact efficiently with DNA suggests that the antiproliferative effect exerted by PyC derivative **24** comes from its ability to interfere with the enzymatic activity of topoisomerases, which regulate DNA topology and organization.[50] In particular, topo II catalyzes ATP-dependent relaxation of supercoiled DNA and decatenation of kinetoplast DNA.[51] For this purpose, the relaxation of supercoiled circular pBR322 plasmid DNA by topo II was studied with increasing concentrations of **24** (Figure 5A). The PyC derivative begins to inhibit relaxation

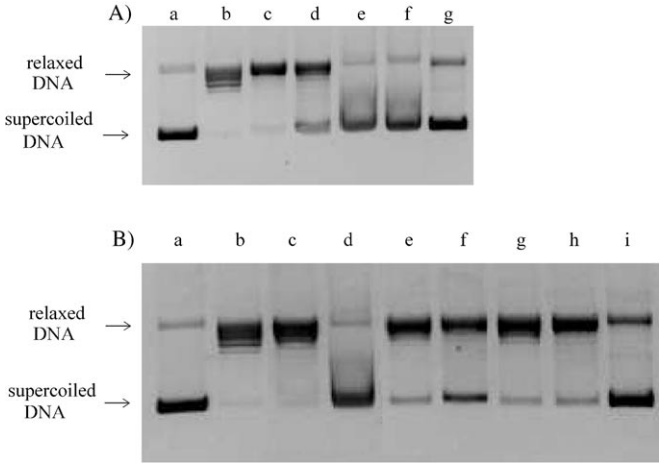



**Figure 5.** Inhibition of DNA topoisomerase II by PyC as measured by relaxation of supercoiled pBR322 DNA. A) Lane a: DNA control (no enzyme); lane b: DNA and topoisomerase II; lanes c–f: same as lane b with **24** at 0.5, 1, 5, or 10 μM; lane g: same as lane b with *m*-AMSA at 8 μM. B) Lane a: DNA control (no enzyme); lane b: DNA and topoisomerase II; lanes c and d: **24** at 1 and 5 μM; lanes e and f: **22** at 1 and 5 μM; lanes g and h: **28** at 1 and 5 μM; lane i: same as lane b with *m*-AMSA at 8 μM.

at a concentration of 1 μM (lane d), and the total absence of relaxed DNA was observed at 5 μM (lane e). Topo II inhibition by *m*-AMSA at 8 μM was reported as reference (lane g), and interestingly, the effect of **24** was similar to that of the drug. On the basis of these results, we were interested in comparing the effects of the closely related compounds **22** and **28**. Figure 5B shows the inhibition of topo II-mediated DNA relaxation induced by the three PyC derivatives assayed at the same concentrations. The results show that the degree of inhibition is much lower for **22** (lanes e and f) than for **24** (lanes c and d); **28** did not have any significant effect at the concentrations tested (lanes g and h). Thus, while derivative **24**, with two side chains, completely inhibits the formation of relaxed DNA, the congeners, with only one side chain each, show partial or no capacity to interfere with the relaxation activity of topo II. No significant inhibitory effects were observed with the PQ derivatives on topo II activity (data not shown). This supports their rather poor ability to form molecular complexes with DNA, as

demonstrated by LD and unwinding experiments (Figures 3 and 4).

Two classes of topoisomerase II-directed agents are known: poisons, which are able to stabilize covalent or cleavable DNA–topo II complexes, and inhibitors, which interfere with any of the other steps in the activity of the enzyme. A number of topo II poisons, including *m*-AMSA, are in clinical use as antitumor agents. To determine if **24** is able to induce the formation of a cleavable complex, the formation of linear from supercoiled DNA was investigated. Figure 6 shows a cleavable complex assay performed in the presence of **24** up to a concentration of 50 μM, and with 10 μM *m*-AMSA, taken as reference compound. The PyC derivative does not act as a poison, and indeed no appearance of linear DNA is observed. However, the presence of compound **24** elicits a certain effect on the mobility of DNA, due reasonably to its effective intercalative ability.

The ability of PyC derivative **24** to complex efficiently with DNA, together with its notable inhibition of topo II activity, prompted us to investigate its capacity to interfere with topo I as well. This enzyme is able to relax both positive and negative supercoiled DNA by an enzyme-mediated DNA single-strand break.[51] Figure 7 shows the relaxation of supercoiled pBR322 plasmid DNA catalyzed by topo I (lane b) and the effect of increasing amounts of **24** (lanes c–g). The figure clearly shows concentration-dependent inhibition of enzyme activity, although the degree of the effect on topo I is significantly lower than that observed for topo II. Indeed, complete inhibition of topo II occurs at 5 μM (Figure 5), whereas 50 μM is required to completely inhibit topo I (Figure 7).

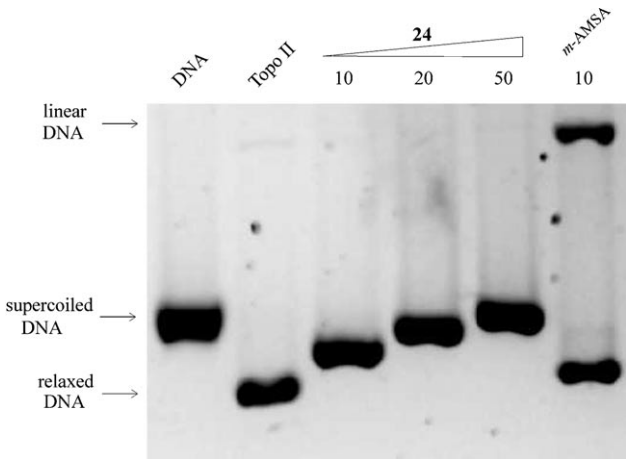



**Figure 6.** Effect of compound **24** on the activity of human recombinant topoisomerase II. Supercoiled pBR322 DNA was incubated without or with topo II in the absence or presence of test compound at the concentration indicated (μM) and 10 μM *m*-AMSA as reference. DNA cleavage products were separated by agarose gel electrophoresis in the presence of ethidium bromide.

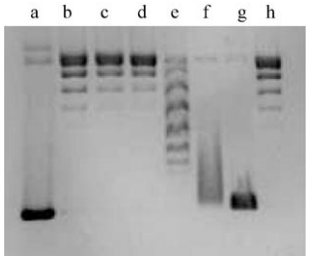



**Figure 7.** Inhibition of DNA topoisomerase I by **24** as measured by relaxation of supercoiled pBR322 DNA. Lane a: DNA control (no enzyme); lane b: DNA and topo I; lanes c–g: same as lane b with **24** at 0.5, 1, 10, 25, or 50 μM; lane h: same as lane b with solvent alone.

### *Structure–activity relationships*

All compounds investigated were subdivided into four groups (A–D, Figure 8) on the basis of structural similarity and increasing order of cytotoxic activity for SAR purposes in order to discuss the following three main structural elements:

1) Molecular size: tricycles versus tetracycles;
2) Aromaticity: tetrahydrogenated derivatives versus plain aromatic PyCs;
3) Substitutions: a) unsubstituted and substituted compounds, and b) one side chain and its substitution position versus two side chains.

#### *Unbranched compounds PQ 32, THPyC 11, and PyC 26*

Of these three unbranched compounds, only the fully aromatic **26** showed a certain weak cytotoxicity ($IC_{50}$: 12.01–27.19 μM), but neither intercalating ability nor inhibition of topo II or I were observed. We suggest that, in this group, molecular size and planarity size are not essential for significant cytotoxic activity.

#### *Compounds with one side chain at position 4 in PQ 31 and position 6 in PyCs 13 and 28*

Compounds **13** and **28** displayed similar weak cytotoxicity ($IC_{50}$: 9.64–20.54 and 10.16–18.47, respectively). Tricycle **31** did not have any significant activity, although $IC_{50}$ values were greatly decreased with respect to the unbranched **32**. This suggests that the introduction of an alkylamine side chain at those positions is a structural element essential for the in vitro cytotoxicity of both PQ and THPyC derivatives, but not for PyC **28**, as it is as active as **26**. The size of molecular planarity is not important for these tetracycles because the $IC_{50}$ values of **13** and **28** are practically the same. Notably, compound **28** showed weak but significant intercalative ability, without any topo I/II inhibition.

#### *Compounds with one alkylamine side chain at position 9 in PQ 33 and at position 1 in PyCs 9, 22, and 23*

All four structurally similar derivatives **33**, **9**, **22**, and **23** showed a significant increase in cytotoxic activity relative to the previous group. This is especially true for tetracycles, both THPyC or PyCs, the $IC_{50}$ values of which were halved. However, the $IC_{50}$ values shown by THPyC **9** and PyCs **22** and **23** were threefold smaller than those of the corresponding PQ **33**, rang-

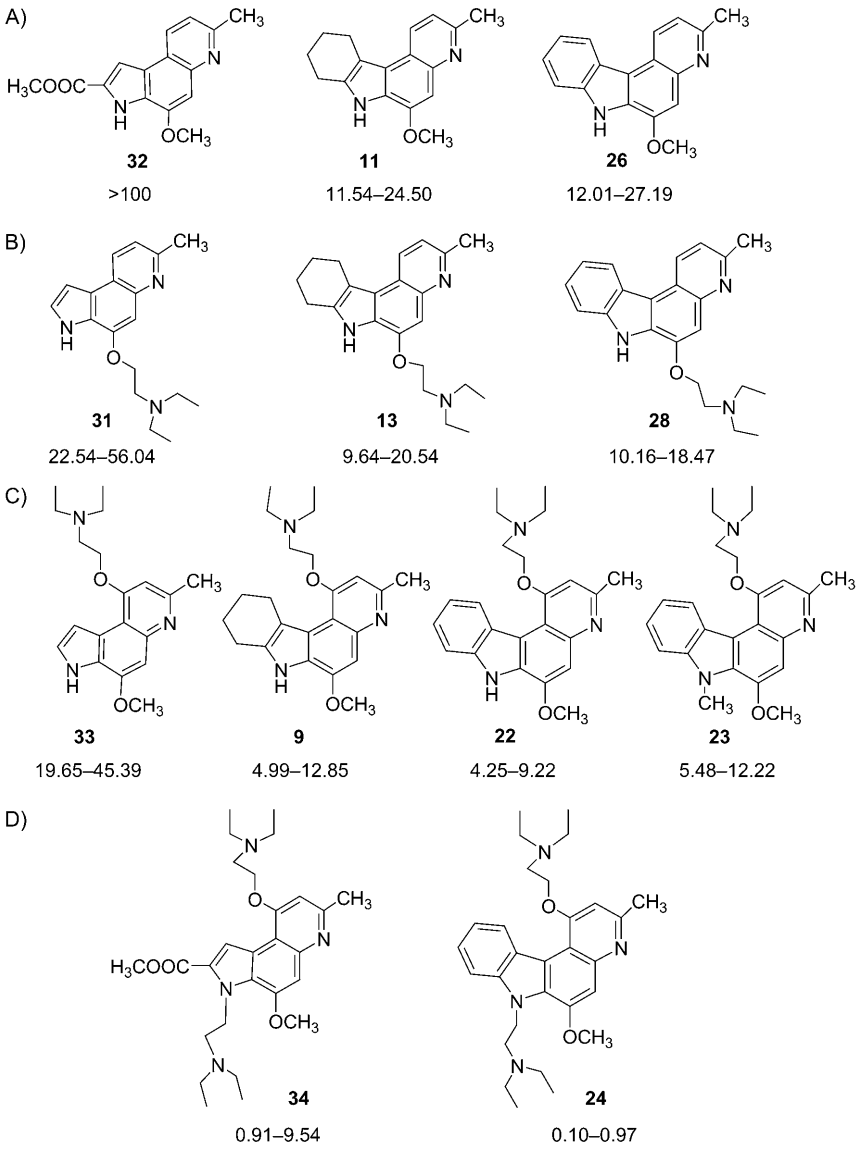



**Figure 8.** The tested PQ and PyC derivatives categorized on the basis of structural similarity and increasing order of cytotoxic activity; $IC_{50}$ value ranges (μM) are indicated.

ing from 4.99 to 12.85 μM, as compared with 19.65 to 45.39 μM, respectively. It is clear that the presence of one side chain at position 9 instead of 4 in PQ **33** and at position 1 in PyCs **22** and **23** favors cytotoxic activity more. Moreover, the similarity of $IC_{50}$ values for PyC derivatives **9**, **22**, and **23** again confirms that the planarity size is not determinant for the cytotoxicity of branched PyCs, whereas it is important for intercalation. Indeed, unlike **9**, **22** showed quite good intercalative activity. No significant inhibition of topo I or II was noted in this group.

### *Compounds bearing two side chains at positions 3 and 9 in PQ 34, and 1 and 7 in PyC 24*

Both **24** and **34**, each bearing two side chains, were the most cytotoxic compounds in the respective sets, even more active than the reference drug. Compound **34** displayed $IC_{50}$ values about half that of **33**, and PyC **24** was the most active compound, with $IC_{50}$ values in the nanomolar range against all human tumor cell lines. Intercalative activity was shown for both compounds, **24** being much more active than **34**, and compound **24** also inhibited both topo I and II.

## Conclusions

Some branched 3*H*-PQs and 7*H*-PyCs, and the corresponding THPyC derivatives, possessing one or two ethylaminoethyl side chains, were prepared and assayed for their antiproliferative activity, and some mechanistic aspects were investigated. Some structure–activity relationships, depending on molecular and planarity size and substitution with one or two diethylaminoethyl side chains were revealed. Taking into account all our results on the new branched compounds, it is clear that the presence of one basic side chain and its location on the PQ and PyC nuclei dramatically affect cytotoxicity and establish the ability to intercalate into the DNA double helix and to inhibit topo II activity. Moreover, the presence of two side chains in **24** and **34** markedly increases antiproliferative potency, thus demonstrating the effective participation of both charged side chain groups in the DNA binding process. Moreover, it is suggested that **24**-treated cells died mainly due to an apoptotic cell-death mechanism, and this agrees with the observed cell cycle block in sub-$G_1$ phase.

Some researchers have developed the paradigm of "interfacial inhibitor"[12–16] for inhibitory drugs that block the formation of macromolecular complexes by binding with high selectivity to binding sites involving two or more macromolecules. Starting from the exemplary case of camptothecin, an inhibitor of complex formation at the protein–DNA interface, they generalized the interfacial inhibitor concept to inhibitors of topo II and others.[16] In this context, we intend to continue our studies with branched 7*H*-PyCs, primarily with the more interesting compound **24**, to make more in-depth examinations of the mode of inhibiting enzymatic activities.

## Experimental Section

### *Chemistry*

Melting points were determined on a Gallenkamp MFB 595 010 M/B capillary melting point apparatus, and are not corrected. IR spectra were recorded on a PerkinElmer 1760 FTIR spectrometer, with samples in pressed KBr disks. UV/Vis spectra were recorded on a PerkinElmer Lambda UV/Vis spectrometer. $^1$H NMR spectra were recorded on a Bruker (300 MHz) spectrometer, using the indicated solvents; chemical shifts ($\delta$, ppm) are reported downfield from tetramethylsilane as internal reference. Coupling constants are given in Hz. For multiplets, the chemical shift quoted was measured from the approximate center. Integrals corresponded satisfactorily to those expected on the basis of compound structure. Elemental analyses were performed in the Microanalytical Laboratory, Department of Pharmaceutical Sciences, University of Padova, on a Perkin-Elmer Elemental Analyzer model 240B; results fell in the range $\pm$0.4% with respect to calculated values. Analytical data are presented in detail for each final compound. Mass spectra were obtained on a Mat 112 Varian Mat Bremen (70 eV) mass spectrometer and Applied Biosystems Mariner System 5220 LC–MS (nozzle potential 250.00 eV). Column flash chromatography was carried out on Merck silica gel (250–400 mesh, ASTM); reactions were monitored by analytical thin-layer chromatography (TLC) with Merck silica gel 60 $F_{254}$ glass plates. Microwave irradiation was performed in a Discover monomode reactor (IR detector for temperature from CEM corporation). Starting materials were purchased from Aldrich Chimica, Acros, and Riedel-de Haën, and solvents from Carlo Erba, Fluka, and Lab-scan. DMSO was made anhydrous by distillation under vacuum and was stored on molecular sieves.

***N*-Cyclohexylidene-*N*′-(2-methoxy-4-nitrophenyl)hydrazine (2)**.[44] A mixture of 2-methoxy-4-nitrophenylhydrazine chloride[39] (2.826 g 12.86 mmol) dissolved in 50 mL absolute EtOH, cyclohexanone (1.33 mL, $d$ = 0.947, 12.86 mmol), triethylamine (1.790 mL, $d$ = 0.726, 12.86 mmol), and Drierite (300 mg) was held at reflux for 3 h. After cooling, it was filtered and the filtrate evaporated to dryness to give an almost pure orange solid which was purified by recrystallization from MeOH. Yield: 75%; mp: 128–130 °C; $R_f$ = 0.70 (*n*-hexane/EtOAc 1:1); $^1$H NMR ([D$_6$]DMSO): $\delta$ = 1.6 (m, 6H, 3×$CH_2$), 2.32 (t, 2H, $J$ = 6.48 Hz, $CH_2$), 2.41 (t, 2H, $J$ = 6.48 Hz, $CH_2$), 3.94 (s, 3H, $OCH_3$), 7.28 (d, 1H, $J$ = 8.96 Hz, H-2), 7.66 (d, 1H, $J$ = 2.29 Hz, H-6), 7.86 (dd, 1H, $J$ = 8.96 and 2.29 Hz, H-5), 8.79 ppm (s, 1H, NH); HRMS: $m/z$ [$M$+H$^+$] calcd for $C_{14}H_{18}N_3O_3$ 264.135, found 264.283.

**6,7,8,9-Tetrahydro-1-methoxy-3-nitro-5*H*-carbazole (3)**. *Method A*: A suspension of hydrazone **2** (2.388 g, 9.07 mmol) in 250 mL $H_2SO_4$ (4%) was heated at reflux for ~1 h. At the end (TLC, *n*-hexane/EtOAc 7:3) the solution was made basic with NaOH (20%) and extracted with EtOAc. The organic layer was dried over $Na_2SO_4$ and evaporated to dryness to give a raw material which was purified by recrystallization from EtOH/$H_2O$ 6:4 to yield pure orange product. Yield: 48%. *Method B*: The cyclization reaction was performed by cation-exchange resin Amberlyst: a mixture of hydrazone **2** (3.615 g, 13.64 mmol) in dry toluene and Amberlyst (10-fold excess over hydrazone by weight) was heated at 115 °C for the time needed to complete the reaction (2–4 h) (TLC). At the end, the resin was filtered off and washed repeatedly with EtOAc. The organic layer was evaporated, and the residue, 8-methoxy-6-nitro-2,3,4,9-tetrahydro-1*H*-carbazole, was pure enough to proceed with synthesis. Yield: 65%; mp: 155–160 °C; $R_f$ = 0.64 (*n*-hexane/EtOAc 7:3); IR (KBr): $\tilde{\nu}$ = 3156 cm$^{-1}$; $^1$H NMR ([D$_6$]DMSO): $\delta$ = 1.82 (m, 4H, 2×$CH_2$), 2.67 (m, 4H, 2×$CH_2$), 3.98 (s, 3H, $OCH_3$), 7.43 (d, 1H, $J$ = 1.7 Hz, H-2), 8.03 (d, 1H, $J$ = 2.01 Hz, H-4), 11.58 ppm (br s, 1H, NH); $^{13}$C NMR ([D$_6$]DMSO): $\delta$ = 20.3 (C5), 22.5 (C8), 22.6 (C6), 22.8 (C7), 55.76 ($OCH_3$), 96.3 (C2), 108.5 (C4b), 111.7 (C4), 127.0 (C8b), 129.0 (C4a), 134,6 (C8a), 138.1 (C3), 144.9 ppm (C1); HRMS: $m/z$ [$M$+H]$^+$ calcd for $C_{13}H_{15}N_2O_3$ 247.108, found 247.089.

**6,7,8,9-Tetrahydro-1-methoxy-3-amino-5*H*-carbazole (4)**. A solution of nitrocarbazole **3** (1.07 g, 4.34 mmol) in 400 mL EtOH was added dropwise to a suspension of 10% Pd/C (125 mg) saturated with $H_2$ in EtOH (200 mL). The mixture was stirred at 50 °C and with $H_2$ at atmospheric pressure for 3 h. The catalyst was filtered off and the solution evaporated under reduced pressure to give the corresponding aminocarbazole **4** as a greenish solid. Yield: 98%; mp: 142–144 °C; $R_f$ = 0.23 (*n*-hexane/EtOAc 1:1); IR (KBr): $\tilde{\nu}$ = 3420 cm$^{-1}$; $^1$H NMR ([D$_6$]DMSO): $\delta$ = 1.74 (m, 4H, $(CH_2)_2$), 2.47 (m, 2H, $H_2$C-5), 2.58 (m, 2H, $H_2$C-8), 4.62 (br s, 2H, $NH_2$), 6.01 (d, 1H, $J$ = 1.52 Hz, H-2), 6.10 (d, 1H, $J$ = 1.52 Hz, H-4), 10.06 ppm (br s, 1H, NH); HRMS: $m/z$ [$M$+H]$^+$ calcd for $C_{13}H_{17}N_2O$ 217.134, found 216.165.

**3-(1-Methoxy-6,7,8,9-tetrahydro-5*H*-carbazol-3-ylamino)but-2-enoic acid ethyl ester (5)**. Aminocarbazole **4** (0.88 g, 4.07 mmol) was condensed with ethyl acetoacetate (0.78 mL, 6.1 mmol, $d$ = 1.021) in absolute EtOH (100 mL) in the presence of acetic acid as catalyst and Drierite (200 mg). The mixture was held at reflux for 5–6 h, checking the end of the reaction by TLC (*n*-hexane/EtOAc 1:1). The solid was filtered off and the solvent evaporated to dryness to yield a brown residue, which was crystallized from MeOH. Yield: 72%; mp: 130–138 °C; $R_f$ = 0.87 (*n*-hexane/EtOAc 1:1); $^1$H NMR ([D$_6$]DMSO): $\delta$ = 1.19 (t, 3H, $J$ = 6.87 Hz, $CH_3$), 1.78 (m, 4H, $(CH_2)_2$), 1.93 (s, 3H, $CH_3$), 2.56 (br t, 2H, $H_2$C-5), 2.65 (br t, 2H, $H_2$C-8), 3.88 (s, 3H, $OCH_3$), 4.05 (q, 2H, $J$ = 7.1 Hz, $CH_2$), 4.58 (s, 1H, CH), 6.41 (d, 1H, $J$ = 1.8 Hz, H-2), 6.74 (d, 1H, $J$ = 1.8 Hz, H-4), 10.23 (s, 1H, NH), 10.76 ppm (s, 1H, pyrrolic NH); HRMS: $m/z$ [$M$+H]$^+$ calcd for $C_{19}H_{25}N_2O_3$ 329.187, found 329.178.

**6-Methoxy-3-methyl-8,9,10,11-tetrahydro-4*H*,7*H*-pyrido[2,3-*c*]carbazol-1-one (6)**. In a two-necked round-bottom flask, diphenyl ether (70 mL) was heated at reflux, and then carbazole derivative **5** (2.16 g, 6.75 mmol) was added portion-wise and the mixture was maintained at reflux for 30 min. After cooling to 60 °C, the separated precipitate was collected by filtration and washed several times with $Et_2O$ (1.12 g). Yield: 58%; mp: > 300 °C; $R_f$ = 0.58 ($CHCl_3$/MeOH 8:2); IR (KBr): $\tilde{\nu}$ = 3417, 3160, 1630 cm$^{-1}$; $^1$H NMR ([D$_6$]DMSO): $\delta$ = 1.71 (m, 4H, $(CH_2)_2$), 2.25 (s, 3H, $CH_3$), 2.69 (m, 2H, $H_2$C-11), 3.26 (m, 2H, $H_2$C-8), 3.95 (s, 3H, $OCH_3$), 5.72 (s, 1H, H-2), 6.58 (s, 1H, H-5), 11.00 (br s, 1H, NH), 11.03 ppm (br s, 1H, carbazole NH); $^{13}$C NMR ([D$_6$]DMSO): $\delta$ = 19.8 ($CH_3$), 21.4 (C9), 22.8 (C10), 23.8 (C8), 26.5 (C11), 55.2 ($OCH_3$), 98.9 (C5), 107.5 (C2), 111.7 (C11c), 116.9 (C11a), 120.3 (C11b), 123.9 (C6a), 134.7 (C7a), 138.8 (C4a), 148.9 (C3), 153.9 (C6), 202.5 ppm (C1); HRMS: $m/z$ [$M$+H]$^+$ calcd for $C_{17}H_{19}N_2O_2$ 283.145, fond 283.143.

**6-Hydroxy-3-methyl-8,9,10,11-tetrahydro-4*H*,7*H*-pyrido[2,3-*c*]carbazol-1-one (7 and 8)**. A solution of **6** (0.300 g, 1.06 mmol) in HBr 48% (23 mL) and acetic acid (10 mL) was heated at 50 °C for 3 h, at 80 °C for 5 h, and at reflux for 12 h. At the end (TLC), the acids were evaporated off, and the residue was taken up with $H_2O$ (30 mL) and neutralized with $NH_4OH$ (28%). A precipitate formed by cooling at 0 °C, which was collected by filtration and subjected to silica gel flash chromatography ($CHCl_3$/MeOH 8:2). An inseparable mixture of hydroxy derivative **7** and its corresponding aromatic compound **8** was obtained. Yield: 79%; $R_f$ = 0.41 for **7** and 0.35 for **8** ($CHCl_3$/MeOH 8:2); **7**: $^1$H NMR ([D$_6$]DMSO): $\delta$ = 6.78 (s, 1H, H-5), 10.20 (s, 1H, OH), 11.00 (br s, 1H, NH), 11.03 ppm (br s, 1H, carbazole NH); **8**: $^1$H NMR ([D$_6$]DMSO): $\delta$ = 7.07 (t, 1H, $J$ = 8.02 Hz, H-10),

7.32 (t, 1H, $J=7.3$ Hz, H-9), 7.49 (d, 1H, $J=8.07$ Hz, H-8), 9.78 ppm (d, 1H, $J=8.3$ Hz, H-11); HRMS: (**7**): $m/z$ $[M+H]^+$ calcd for $C_{16}H_{17}N_2O_2$ 269.129, found 269.118; (**8**): $m/z$ $[M+H]^+$ calcd for $C_{16}H_{13}N_2O_2$ 265.097, found 265.150.

**1-(2-Diethylaminoethoxy)-6-methoxy-3-methyl-8,9,10,11-tetrahydro-7*H*-pyrido[2,3-*c*]carbazole hydrochloride (9)**. A mixture of pyridinone tetracycle **6** (0.110 g, 0.39 mmol) and $K_2CO_3$ (0.231 g, 1.75 mmol) in DMF (15 mL) was stirred at room temperature for 30 min under $N_2$ atmosphere, then diethylchloroethylamino chloride (0.072 g, 0.42 mmol) was added and both were held at reflux for 12 h (TLC, $CHCl_3$/MeOH 8:2). After cooling, $H_2O$ was added, the mixture was extracted with $CHCl_3$, and the combined organic extracts were dried with $Na_2SO_4$ and evaporated to dryness. The residue was purified by silica gel flash chromatography ($CHCl_3$/MeOH 8:2) to give a yellow solid. Yield: 14%; mp: $>300\,^\circ$C; $R_f=0.12$ ($CHCl_3$/MeOH 8:2); IR (KBr): $\tilde{\nu}=3162$ cm$^{-1}$; $^1$H NMR ($CDCl_3$): $\delta=1.08$ (t, 6H, $J=7.49$ Hz, 2×$CH_3$), 1.85 (m, 2H, $H_2$C-10), 1.87 (m, 2H, $H_2$C-9), 2.65 (q, 4H, $J=7.23$ Hz, 2×$CH_2$), 2.67 (s, 3H, $CH_3$), 2.85 (t, 2H, $J=5.7$ Hz, $H_2$C-8), 3.04 (t, 2H, $J=7.43$ Hz, N-$CH_2$), 3.14 (t, 2H, $J=5.7$ Hz, $H_2$C-11), 4.04 (s, 3H, $OCH_3$), 4.37 (t, 2H, $J=7.4$ Hz, O-$CH_2$), 6.68 (s, 1H, H-2), 7.36 (s, 1H, H-5), 8.63 ppm (br s, 1H, NH); $^{13}$C NMR ($CDCl_3$): $\delta=11.74$ (2×$CH_3$), 22.4 ($CH_3$), 22.8 (C11), 23.8 (C9), 23.9 (C10), 26.8 (C8), 47.7 (2×$CH_2$), 51.2 (N-$CH_2$), 55.6 ($OCH_3$), 67.3 ($OCH_2$), 99.1 (C5), 100.6 (C2), 110.7 (C11c), 114.2 (C11a), 120.03 (C11b), 125.0 (C6a), 134.7 (C7a), 144.3 (C4a), 149.9 (C6), 153.1 (C3), 164.3 ppm (C1); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{23}H_{32}N_3O_2$ 382.249, found 382.247; anal.: C, H, N (Supporting Information). The free base was transformed into the hydrochloride by dissolving it in $CHCl_3$ and then adding HCl (37%). The precipitate was collected and dried to give the monochloride salt. Yield: 86%; anal.: C, H, N, Cl.

**1-Chloro-6-methoxy-3-methyl-8,9,10,11-tetrahydro-7*H*-pyrido-[2,3-*c*]carbazole (10)**. A mixture of **6** (0.299 g, 1.06 mmol) and $POCl_3$ (1.5 g) was held at reflux at 130 °C for 1 h (TLC) and, after cooling, was poured into an ice/$H_2O$ bath under stirring. The solution was made alkaline with $NH_4OH$ (28%) and a yellow precipitate formed, which was collected by filtration, washed with $H_2O$, and dried. Yield: 90%; mp: $>300\,^\circ$C; $R_f=0.91$ ($CHCl_3$/MeOH 8:2); IR (KBr): $\tilde{\nu}=3158$ cm$^{-1}$; $^1$H NMR ([D$_6$]DMSO): $\delta=1.72$ (m, 2H, $CH_2$), 1.82 (m, 2H, $CH_2$), 2.56 (s, 3H, $CH_3$), 2.76 (t, 2H, $J=5.53$ Hz, $H_2$C-8), 3.21 (t, 2H, $J=5.53$ Hz, $H_2$C-11), 4.05 (s, 3H, $OCH_3$), 7.06 (s, 1H, H-2), 7.35 (s, 1H, H-5), 11.65 ppm (br s, 1H, NH); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{17}H_{18}ClN_2O$ 301.112, found 301.120.

**6-Methoxy-3-methyl-8,9,10,11-tetrahydro-7*H*-pyrido[2,3-*c*]carbazole (11)**. Chloro derivative **10** (0.290 g, 0.96 mmol) in 100 mL EtOH was added dropwise into a suspension of 10% Pd/C (100 mg) saturated with $H_2$ in EtOH (200 mL). The mixture was stirred at room temperature and under $H_2$ at atmospheric pressure for 10 h. The catalyst was filtered off, and the solution was evaporated at reduced pressure and room temperature, to give a raw brown material, which was crystallized from $CHCl_3$/MeOH 7:3. Yield: 85%; mp: 225–228 °C; $R_f=0.57$ ($CHCl_3$/MeOH 8:2); IR (KBr): $\tilde{\nu}=3156$ cm$^{-1}$; $^1$H NMR ([D$_6$]DMSO): $\delta=1.89$ (m, 4H, -$CH_2$-$CH_2$-), 2.70 (m, 2H, $H_2$C-8), 2.86 (s, 3H, $CH_3$), 3.03 (m, 2H, $H_2$C-11), 4.14 (s, 3H, $OCH_3$), 7.31 (s, 1H, H-5), 7.65 (d, 1H, $J=8.3$ Hz, H-2), 8.97 (d, 1H, $J=8.3$ Hz, H-1), 12.11 ppm (br s, 1H, NH); $^{13}$C NMR ([D$_6$]DMSO): $\delta=17.8$ ($CH_3$), 22.3 (C11), 24.9 (C9), 25.6 (C10), 25.7 (C8), 59.1 ($OCH_3$), 93.0 (c-5), 115.2 (C11a), 121.4 (C2), 123.7 (C11c), 125.9 (C11b), 132.9 (C4a), 139.9 (C7a), 140.4 (C1), 141.8 (C3), 152.6 ppm (C6 and C6a); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{17}H_{19}N_2O$ 267.149, found 267.152; anal.: C, H, N (Supporting Information).

**6-Hydroxy-3-methyl-8,9,10,11-tetrahydro-7*H*-pyrido[2,3-*c*]carbazole (12)**. The dealkylation of methoxy derivative **12** was carried out with HBr (48%), as for **6**. Yield: 67%; mp: 251–253 °C; $R_f=0.32$ ($CHCl_3$/MeOH 8:2); $^1$H NMR ([D$_6$]DMSO): $\delta=10.20$ ppm (br s, 1H, OH); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{16}H_{17}N_2O$ 253.134, found 253.135.

**6-(2-Diethylaminoethoxy)-3-methyl-8,9,10,11-tetrahydro-7*H*-pyrido[2,3-*c*]carbazole hydrochloride (13)**. NaH (0.020 g, 0.83 mmol), NaI (0.49 g, 0.33 mmol), and diethylchloroethylamino chloride (0.057 g, 0.33 mmol) were added to a solution of the 6-hydroxy tetracyclic derivative **13** (0.070 g, 0.277 mmol) in 10 mL DMF. The mixture was heated at 100 °C for 12 h in $N_2$ atmosphere, and the reaction was monitored by TLC ($CHCl_3$/MeOH 8:2). After cooling, $H_2O$ was added, and the resulting mixture was extracted with EtOAc, the combined extracts washed with $H_2O$, dried with $Na_2SO_4$ and evaporated to dryness. The residue was purified by silica gel flash chromatography ($CHCl_3$/MeOH 8:2). Yield: 18%; mp: $>300\,^\circ$C; $R_f=0.3$ ($CHCl_3$/MeOH 8:2); $^1$H NMR ($CD_3OD$): $\delta=1.39$ (t, 6H, $J=7.3$ Hz, 2×$CH_3$), 1.84 (m, 4H, $H_2$C-10, $H_2$C-8), 2.88 (m, 2H, $H_2$C-11), 3.08 (m, 2H, $H_2$C-8), 3.79 (t, 2H, $J=6.3$ Hz, N-$CH_2$), 4.75 (t, 2H, $J=6.3$ Hz, O-$CH_2$), 7.15 (s, 1H, H-5), 7.65 (d, 1H, $J=8.25$ Hz, H-2), 8.97 ppm (d, 1H, $J=8.25$ Hz, H-1); $^{13}$C NMR ($CD_3OD$): $\delta=13.90$ (2×$CH_3$), 21.6 (C11), 22.6 (C9 and C10), 22.8 ($CH_3$), 23.5 (C8), 46.7 (2×$CH_2$), 48.8 (N-$CH_2$), 62.8 ($OCH_2$), 89.7 (C5), 112.8 (C11a), 118.6 (C11c), 118.1 (C2), 120.1 (C11b), 136.5 (C4a), 137.4 (C6a), 139.2 (C1), 148.8 (C3), 149.0 (C7a), 151.1 ppm (C6); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{22}H_{30}N_3O$ 352.239, found 352.230; anal.: C, H, N (Supporting Information). The free base was transformed into the hydrochloride by dissolving it in $CHCl_3$ and then adding HCl (37%). The precipitate was collected and dried to give the monochloride salt. Yield: 75%.

**1-Methoxy-3-nitro-9*H*-carbazole (14)**. DDQ (2.578 g, 11.36 mmol) was added to a solution of the tetrahydrocarbazole derivative **3** (1.398 g, 5.68 mmol) in 20 mL benzene, and the mixture was held at reflux for 3 h in $N_2$ atmosphere (TLC, *n*-hexane/EtOAc 1:1). After filtering, the filtrate was evaporated under vacuum, and the residue was purified by flash chromatography (*n*-hexane/EtOAc 1:1). Yield: 36%; mp: 208–210 °C (EtOH); $R_f=0.84$ (*n*-hexane/EtOAc 1:1); IR (KBr): $\tilde{\nu}=3157$ cm$^{-1}$; $^1$H NMR ([D$_6$]DMSO): $\delta=4.11$ (s, 3H, $OCH_3$), 7.28 (t, 1H, $J=7.2$ Hz, H-6), 7.49 (t, 1H, $J=7.06$ Hz, H-7), 7.58 (d, 1H, $J=7.44$ Hz, H-5), 7.81 (d, 1H, $J_m=1.95$ Hz, H-2), 8.33 (d, 1H, $J=8.41$ Hz, H-8), 8.85 (d, 1H, $J_m=1.95$ Hz, H-4), 12.18 ppm (br s, 1H, NH); $^{13}$C NMR ([D$_6$]DMSO): $\delta=55.8$ ($OCH_3$), 100.7 (C2), 110.6 (C4b), 111.5 (C4), 111.9 (C8), 120.2 (C7), 121.7 (C5), 121.9 (C6), 127.9 (C4a), 133.4 (C8a), 140.2 (C3), 144.6 ppm (C1); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{13}H_{11}N_2O_3$ 243.077, found 243.072.

**1-Methoxy-9-methyl-3-nitro-9*H*-carbazole (15)**. NaH (0.39 g, 2.75 mmol) and $CH_3I$ (0.546 g, 3.85 mmol) were added to a solution of methoxynitrocarbazole **14** (0.300 g, 1.24 mmol) in 10 mL DMF, and the mixture was held at reflux for 7 h. At the end (TLC), the mixture was poured into ice/$H_2O$ and extracted with $CHCl_3$, and the organic phase was dried on $Na_2SO_4$ and evaporated to yield the *N*-methyl derivative. Yield: 75%; mp: 185–187 °C; $R_f=0.77$ (*n*-hexane/EtOAc 1:1); $^1$H NMR ([D$_6$]DMSO): $\delta=4.09$ (s, 3H, $OCH_3$), 4.20 (s, 3H, N-$CH_3$), 7.34 (td, 3H, $J_o=8.01$ Hz, $J_m=0.95$ Hz, H-7), 7.59 (td, 1H, $J_o=8.01$ Hz, $J_m=0.95$ Hz, H-6), 7.70 (d, 1H, $J=8.39$ Hz, H-8), 7.81 (d, 1H, $J=2.29$ Hz, H-2), 8.38 (d, 1H, $J=8.2$ Hz, H-5), 8.86 ppm (d, 1H, $J=2.1$ Hz, H-4); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{14}H_{13}N_2O_3$ 257.093, found 257.082.

**1-Methoxy-3-amino-9*H*-carbazole (16)**. The reduction of nitrocarbazole **14** was carried out as for **3**, but at 50 °C for 4 h. Yield: 91%; mp: 189–3 °C; $R_f=0.21$ (*n*-hexane/EtOAc 1:1);$^1$H NMR ([D$_6$]DMSO):

$\delta = 3.89$ (s, 3H, $OCH_3$), 5.20 (br s, 2H, $NH_2$), 6.43 (d, 1H, $J = 1.7$ Hz, H-2), 6.83 (d, 1H, $J = 1.5$ Hz, H-4), 7.01 (td, $J = 6.84$ and 1.14 Hz, H-6), 7.25 (td, 1H, $J = 6.80$ and 1.14 Hz, H-7), 7.35 (d, 1H, $J = 7.44$ Hz, H-5), 7.83 (d, 1H, $J = 7.44$ Hz, H-8) 10.75 ppm (br s, 1H, NH); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{13}H_{13}N_2O$ 213.103, found 213.091.

**1-Methoxy-9-methyl-3-amino-9*H*-carbazole (17)**. The reduction of nitrocarbazole derivative **15** was carried out as for **3**, for 4 h. Yield: 94%; mp: 174–6 °C; $R_f = 0.22$ (*n*-hexane/EtOAc 1:1); IR (KBr): $\tilde{\nu} = 3279$ cm$^{-1}$; $^1$H NMR ([D$_6$]DMSO): $\delta = 3.88$ (s, 3H, $OCH_3$), 3.98 (s, 3H, N-$CH_3$), 5.01 (br s, 2H, $NH_2$), 6.45 (d, 1H, $J = 2.1$ Hz, H-2), 6.68 (d, 1H, $J = 2.1$ Hz, H-4), 7.04 (td, 1H, $J_o = 8.01$ Hz, $J_m = 0.95$ Hz, H-6), 7.34 (td, 1H, $J_o = 8.01$ Hz, $J_m = 0.95$ Hz, H-7), 7.41 (d, 1H, $J = 8.02$ Hz, H-8), 7.87 ppm (d, 1H, $J = 8.01$ Hz, H-5); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{14}H_{15}N_2O$ 227.118, found 227.114.

**3-(1-Methoxy-9*H*-carbazol-3-ylamino)but-2-enoic acid ethyl ester (18)**. The condensation reaction of **16** with ethyl acetoacetate was carried out as for carbazole derivative **4**. Yield: 70%; mp: 245–248 °C; $R_f = 0.78$ (*n*-hexane/EtOAc 1:1); $^1$H NMR ([D$_6$]DMSO): $\delta = 1.21$ (t, 3H, $J = 7.06$ Hz, $CH_3$), 1.99 (s, 3H, $CH_3$), 3.99 (s, 3H, $OCH_3$), 4.06 (q, 2H, $J = 7.06$ Hz, $CH_2$), 4.66 (s, 1H, CH), 6.84 (d, 1H, $J = 1.7$ Hz, H-2), 7.13 (td, 1H, $J_m = 1.1$ Hz, $J_o = 8.21$ Hz, H-6), 7.37 (td, 1H, $J_m = 1.1$ Hz, $J_o = 8.21$ Hz, H-7), 7.47 (d, 1H, $J = 8.1$ Hz, H-8), 7.57 (d, 1H, $J = 1.62$ Hz, H-4), 8.08 (d, 1H, $J = 8.1$ Hz, H-5), 10.36 ppm (s, 1H, NH), 11.35 (s, 1H, pyrrolic NH); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{19}H_{21}N_2O_3$ 325.155, found 325.154.

**3-(1-Methoxy-9-methyl-9*H*-carbazol-3-ylamino)but-2-enoic acid ethyl ester (19)**. The condensation reaction of aminocarbazole derivative **17** and ethyl acetoacetate was performed as for **4**. Yield: 72%; mp: 195–197 °C; $R_f = 0.90$ (*n*-hexane/EtOAc 1:1); $^1$H NMR ([D$_6$]DMSO): $\delta = 1.21$ (t, 3H, $J = 7.06$ Hz, $CH_3$), 2.00 and 2.08 (2 s, 3H, $CH_3$), 3.94 and 3.97 (2 s, 3H, $OCH_3$), 4.06–4.11 (m, 5H, $CH_2$ and N-$CH_3$), 4.66 and 4.74 (2 s, 1H, CH), 6.79 and 6.80 (2d, 1H, $J = 2.1$ Hz, H-2), 7.18 (td, 1H, $J_m = 1.1$ Hz, $J_o = 8.04$ Hz, H-6), 7.46 (td, 1H, $J_m = 1.2$ Hz, $J_o = 8.04$ Hz, H-7), 7.56 (m, 1H, H-8), 7.62 (d, 1H, $J = 1.52$ Hz, H-4), 8.11 (m, 1H, H-5), 8.50 and 10.34 ppm (2 s, 1H, NH); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{20}H_{23}N_2O_3$ 339.171, found 339.160.

**6-Methoxy-3-methyl-4*H*,7*H*-pyrido[2,3-*c*]carbazol-1-one (20)**. The cyclization reaction of **18** was carried out in diphenyl ether as for **6**. Yield: 86%; mp: 292–295 °C; $R_f = 0.56$ ($CHCl_3$/MeOH 8:2); IR (KBr): $\tilde{\nu} = 3402$, 3220, 1622 cm$^{-1}$; $^1$H NMR ([D$_6$]DMSO): $\delta = 2.34$ (s, 3H, $CH_3$), 4.09 (s, 3H, $OCH_3$), 5.95 (s, 1H, H-2), 7.04 (s, 1H, H-5), 7.07 (t, 1H, $J = 8.02$ Hz, H-10), 7.32 (t, 1H, $J = 7.3$ Hz, H-9), 7.49 (d, 1H, $J = 8.07$ Hz, H-8), 9.78 (d, 1H, $J = 8.3$ Hz, H-11), 11.43 (br s, 1H, pyrrole NH), 11.66 ppm (br s, 1H, pyridinone NH); $^{13}$C NMR ([D$_6$]DMSO): $\delta = 19.2$ ($CH_3$), 56.0 $OCH_3$), 95.7 (C5), 110.14 (C2), 11.3 (C8), 116.14 (C11c), 118.8 (C9), 123.9 (C11), 125.1 (C10), 129.0 (C6a), 130.3 (C11a), 138.2 (C7a), 139.9 (C4a), 146.0 (C3), 149.2 (C6), 177.77 ppm (C1); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{17}H_{15}N_2O_2$ 279.113, found 279.104.

**6-Methoxy-3,7-dimethyl-4*H*-pyrido[2,3-*c*]carbazol-1(7*H*)-one (21)**. The cyclization reaction of **19** was carried out in diphenyl ether as for **6**. Yield: 77%; mp: >300 °C; $R_f = 0.67$ ($CHCl_3$/MeOH 8:2); IR (KBr): $\tilde{\nu} = 3408$, 1614 cm$^{-1}$; $^1$H NMR ([D$_6$]DMSO): $\delta = 2.38$ (s, 3H, $CH_3$), 4.08 (s, 3H, $OCH_3$), 4.21 (s, 3H, N-$CH_3$), 6.07 (s, 1H, H-2), 7.10 (s, 1H, H-5), 7.15 (t, 1H, $J = 7.62$ Hz, H-9), 7.45 (t, 1H, $J = 7.82$ Hz, H-10), 7.59 (d, 1H, $J = 8.39$ Hz, H-8), 9.84 (d, 1H, $J = 8.39$ Hz, H-11), 11.72 ppm (br s, 1H, NH); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{18}H_{17}N_2O_2$ 293.129, found 293.121.

**General procedure for the synthesis of mono- and bifunctionalized 7*H*-pyrido[2,3-*c*]carbazole derivatives 22–24**. *Method A*: After stirring for 30 min, $K_2CO_3$ (3.5 mmol) and chloroethyldiethylamine hydrochloride (1.06 mmol) were added to a solution of 7*H*-pyrido-[2,3-*c*]carbazol-1-one derivatives **20** or **21** (0.71 mmol) in 15 mL DMF. The temperature was then slowly raised to reflux. The ongoing reaction was monitored by TLC. After ~8 h, the cooled mixture was treated with ice/$H_2O$ (30 g) and extracted with $CHCl_3$ (3× 50 mL). The combined extracts were washed with $H_2O$, dried with $Na_2SO_4$, and evaporated to dryness. The residue was purified by silica gel flash chromatography ($CHCl_3$/MeOH/$NH_3$ 9:1:0.01). Yields: 33% of **22** as the only product separating from reaction with **20**; 22% of **23** and 8% of **24** as products separated from **21**.

**1-(2-Diethylaminoethoxy)-6-methoxy-3-methyl-7*H*-pyrido[2,3-*c*]-carbazole (22)**. Yield: 33%; $R_f = 0.37$; mp: >300 °C; $^1$H NMR ($CDCl_3$): $\delta = 1.02$ (t, 6H, $J = 7.15$ Hz, 2×$CH_3$), 2.72 (s, 3H, $CH_3$), 2.73 (q, 4H, $J = 7.20$ Hz, 2×$CH_2$) 3.20 (t, 2H, $J = 6.86$ Hz, $NCH_2$), 4.10 (s, 3H, $OCH_3$), 4.48 (t, 2H, $J = 6.86$ Hz, $OCH_2$), 6.80 (s, 3H, H-2), 7.26 (td, 1H, $J = 8.20$ and 2.03 Hz, H-10), 7.41 (s, 1H, H-5), 7.41 (dd, 1H, $J = 8.01$ and 2.04 Hz, H-9), 7.53 (d, 1H, $J = 8.01$ Hz, H-8), 8.95 (br s, 1H, NH), 9.02 ppm (d, 1H, $J = 8.20$ Hz, H-11); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{23}H_{24}N_3O_2$ 374.187, found 374.208; anal.: C, H, N (Supporting Information).

**1-(2-Diethylaminoethoxy)-6-methoxy-3,7-dimethyl-7*H*-pyrido-[2,3-*c*]carbazole (23)**. Yield: 22%; mp: >300 °C; $R_f = 0.43$ ($CHCl_3$/MeOH 8:2); IR (KBr): $\tilde{\nu} = 1618$ cm$^{-1}$; $^1$H NMR ([D$_6$]DMSO): $\delta = 0.93$ (t, $J = 7.02$ Hz, 6H, 2×$CH_3$), 2.00 (s, 3H, $CH_3$), 2.54 (m, 4H, 2×$CH_2$), 3.02 (t, 2H, $J = 6.92$ Hz, N-$CH_2$), 3.94 (s, 3H, $OCH_3$), 4.13 (s, 3H, N-$CH_3$), 4.31 (t, 2H, $J = 6.92$ Hz, $OCH_2$), 6.62 (s, 1H, H-2), 7.09 (td, 1H, $J_m = 2.3$ and $J_o = 8.2$ Hz, H-10), 7.32 (m, 2H, H-8 and H-9), 7.10 (s, 1H, H-5), 8.89 ppm (d, 1H, $J = 8.2$ Hz, H-11); $^{13}$C NMR ($CDCl_3$): $\delta = 13.9$ (2×$CH_3$), 23.9 ($CH_3$), 46.6 (2×$CH_2$), 51.52 (N-$CH_2$ and N-$CH_3$), 54.8 ($OCH_3$), 67.8 ($OCH_2$), 100.2 (C5), 107.1 (C11b), 110.2 (C2), 114.5 (C11c), 117.7 (C8), 122.5 (C11), 123.5 (C9), 125.6 (C10), 128.9 (C11a and C6a), 140.0 (C7a and C4a), 145.7 (C3), 149.3 (C6), 178.2 ppm (C1); HRMS: $m/z$ $[M+H^+]^+$ calcd for $C_{24}H_{30}N_3O_2$ 392.234, found 392.292; anal.: C, H, N (Supporting Information).

**1-(2-Diethylaminoethoxy)-7-(2-diethylaminoethyl)-6-methoxy-3-methyl-7*H*-pyrido[2,3-*c*]carbazole (24)**. Yield: 8%; mp: >300 °C; $R_f = 0.48$ ($CHCl_3$/MeOH 7:3); $^1$H NMR ($CDCl_3$): $\delta = 1.08$ (t, 6H, $J = 7.15$ Hz, 2×$CH_3$), 1.10 (t, 6H, $J = 7.15$ Hz, 2×$CH_3$), 2.17 (s, 3H, $CH_3$), 2.72 (m, 8H, 4×$CH_2$), 2.92 (t, 2H, $J = 8.11$ Hz, $NCH_2$), 3.21 (t, 2H, $J = 6.96$ Hz, $NCH_2$), 4.17 (s, 3H, $OCH_3$), 4.51 (t, 2H, $J = 6.96$ Hz, $OCH_2$), 4.92 (t, 2H, $J = 8.01$ Hz, $NCH_2$), 6.81 (s, 1H, H-2), 7.29 (m, 1H, $J = 8.79$, 7.80 and 1.03 Hz, H-10), 7.65 (s, 1H, H-5), 7.50 (ddd, 1H, $J = 8.20$, 7.80 and 0.78 Hz, H-9), 7.61 (dd, 1H, $J = 8.20$ and 1.03 Hz, H-8), 9.02 ppm (dd, 1H, $J = 8.79$ and 0.78 Hz, H-11); $^{13}$C NMR ($CDCl_3$): $\delta = 13.7$ (4×$CH_3$), 23.4 ($CH_3$), 48.8 (4×$CH_2$-N), 51.8 ($CH_2$-N), 52.7 ($CH_2$-N), 56.3 ($CH_2$-N, $CH_3$-O), 67.8 ($CH_2$-O), 101.6 (C2), 104.4 (C5), 109.1 (C8), 112.9 (C11c), 116.3 (Cb), 119.3 (C10), 122.1 (C11), 123.4 (Ca), 125.2 (C9), 126.3 (C6a), 142.3 (C7a), 148.2 (C4a), 154.1 (C6), 163.8 (C1), 168.5 ppm (C3); HRMS: $m/z$ $[M+H]^+$ calcd for $C_{29}H_{41}N_4O_2$ 477.323, found 477.343; anal.: C, H, N (Supporting Information).

**1-(2-Diethylaminoethoxy)-7-(2-diethylaminoethyl)-6-methoxy-3-methyl-7*H*-pyrido[2,3-*c*]carbazole (24)**. *Method B*: Powdered KOH (200 mg) was added to a cooled solution of pyridocarbazole derivative **20** (100 mg, 0.22 mmol) in 15 mL acetone. The mixture was maintained under stirring at 0 °C until the color changed from brown to green (15–20 min). Chlorodiethylethylamine hydrochloride (200 mg, 1.16 mmol) was then added, and after 30 min, the temperature was increased to reflux under an inert ($N_2$) atmosphere. At the end of the reaction (TLC, $CHCl_3$/MeOH/$NH_3$ 90:1:0.2) toluene was added, and the formed precipitate was filtered off.

The organic solution was dried with anhydrous $Na_2SO_4$ and evaporated to dryness. The solid residue was purified by flash chromatography ($CHCl_3$/MeOH/$NH_3$ 90:1:0.1). Yield: 73%.

**1-Chloro-6-methoxy-3-methyl-7*H*-pyrido[2,3-*c*]carbazole (25)**. The halogenation of **17** was carried out with $POCl_3$ at 110 °C for 7 h and worked up as for **7** and **8**. Yield: 90%; mp: >300 °C; $R_f$ = 0.85 ($CHCl_3$/MeOH 8:2); IR (KBr): $\tilde{\nu}$ = 3205 $cm^{-1}$; $^1H$ NMR ([$D_6$]DMSO): $\delta$ = 2.64 (s, 3H, $CH_3$), 4.17 (s, 3H, $OCH_3$), 7.23 (td, 1H, $J_m$ = 1.4 Hz, $J_o$ = 8.4 Hz, H-10), 7.41 (td, 1H, $J_m$ = 1.4 Hz, $J_o$ = 8.4 Hz, H-9), 7.44 (s, 1H, H-5), 7.55 (s, 1H, H-2), 7.6 (d, 1H, $J$ = 8.4 Hz, H-8), 8.86 (d, 1H, $J$ = 8.4 Hz, H-11), 12.29 (br s, 1H, NH); HRMS: *m/z* $[M+H]^+$ calcd for $C_{17}H_{14}ClN_2O$ 297.079, found 297.069.

**6-Methoxy-3-methyl-7*H*-pyrido[2,3-*c*]carbazole (26)**. The removal of the Cl group of **18** was carried out as for **11**. Yield: 87%; mp: 285–288 °C; $R_f$ = 0.77 ($CHCl_3$/MeOH 8:2); IR (KBr): $\tilde{\nu}$ = 3179 $cm^{-1}$; $^1H$ NMR ([$D_6$]DMSO): $\delta$ = 2.93 (s, 3H, $CH_3$), 4.22 (s, 3H, $OCH_3$), 7.38 (t d, 1H, $J_m$ = 1.1 Hz, $J_o$ = 8.21 Hz, H-10), 7.54 (td, 1H, $J_m$ = 1.1 Hz, $J_o$ = 8.2 Hz, H-9), 7.73 (d, 1H, $J$ = 8.39 Hz, H-11), 7.77 (s, 1H, H-5), 7.80 (d, 1H, $J$ = 8.58 Hz, H-2), 8.62 (d, 1H, $J$ = 8.2 Hz, H-8), 9.55 (d, 1H, $J$ = 8.58 Hz, H-1), 12.31 ppm (s, 1H, NH); $^{13}C$ NMR ([$D_6$]DMSO): $\delta$ = 23.4 ($CH_3$), 57.0 ($OCH_3$), 113.2 (C5), 114.7 (C11a), 119.6 (C2), 119.7 (C9), 121.0 (C11), 121.1 (C2), 121.9 (C10), 122.3 (C11c), 126.3 (C7a), 129.7 (C11b), 137.3 (C1), 138.9 (C7a), 139.5 (C4a), 151.1 (C3), 152.9 ppm (C6); HRMS: *m/z* $[M+H]^+$ calcd for $C_{17}H_{15}N_2O$ 263.118, found 263.114; anal.: C, H, N (Supporting Information).

**6-Hydroxy-3-methyl-7*H*-pyrido[2,3-*c*]carbazole (27)**. The dealkylation of **26** was carried out under the same conditions as those for **12**. Yield: 48%; mp: >300 °C; $R_f$ = 0.2 ($CHCl_3$/MeOH 8:2); $^1H$ NMR ([$D_6$]DMSO): $\delta$ = 2.66 (s, 3H, $CH_3$), 7.26 (s, 1H, H-5), 7.29 (td, 1H, $J_m$ = 1.1 Hz, $J_o$ = 8.21 Hz, H-10), 7.38 (td, 1H, $J_m$ = 1.1 Hz, $J_o$ = 8.2 Hz, H-9), 7.44 (d, 1H, $J$ = 8.2 Hz, H-11), 7.65 (d, 1H, $J$ = 8.58 Hz, H-2), 8.53 (d, 1H, $J$ = 8.39 Hz, H-8), 9.55 (d, 1H, $J$ = 8.58 Hz, H-1), 12.18 ppm (s, 1H, NH). HRMS: *m/z* $[M+H]^+$ calcd for $C_{16}H_{13}N_2O$ 249.103, found 249.098.

**6-(2-Diethylaminoethoxy)-3-methyl-7*H*-pyrido[2,3-*c*]carbazole hydrochloride (28)**. The alkylation reaction of **20** was carried out as for **13**. Yield: 32%; mp: >300 °C; $R_f$ = 0.15 ($CHCl_3$/MeOH 7:3); $^1H$ NMR ([$D_6$]DMSO): $\delta$ = 1.21 (t, 6H, $J$ = 7.06 Hz, 2×$CH_3$), 2.77 (s, 3H, $CH_3$), 2.87 (q, 4H, $J$ = 7.06 Hz, 2×$CH_2$), 3.16 (t, 2H, $J$ = 5.60 Hz, $NCH_2$), 4.43 (t, 2H, $J$ = 5.60 Hz, $OCH_2$), 7.36 (m, H-9 and H-8), 7.40 (s, 1H, H-5), 7.46 (td, 1H, $J$ = 7.75 and 1.14 Hz, H-10), 7.66, d, 1H, $J$ = 8.01 Hz, H-2), 8.42 (d, 1H, $J$ = 7.82 Hz, H-11), 8.84 (d, 1H, $J$ = 8.01 Hz, H-1), 10.37 ppm (br s, 1H, NH); $^{13}C$ NMR ([$D_6$]DMSO): $\delta$ = 13.9 (2× $CH_3$), 23.7 ($CH_3$), 46.7 (2×$CH_2$), 48.8 ($CH_2$-N), 62.7 ($OCH_2$), 113.2 (C5), 114.7 (C11a), 119.6 (C2), 119.7 (C9), 121.0 (C11), 121.1 (C2), 121.9 (C10), 122.3 (C11c), 126.3 (C7a), 129.7 (C11b), 137.3 (C1), 138.9 (C7a), 139.5 (C4a), 151.1 (C3), 152.9 ppm (C6); HRMS: *m/z* $[M+H]^+$ calcd for $C_{22}H_{26}N_3O$ 348.208, found 348.194; anal.: C, H, N (Supporting Information). The free base was transformed into its hydrochloride by dissolving it in $CHCl_3$ and then adding HCl (37%). The precipitate was collected and dried to give the monohydrochloride salt. Yield: 89%.

**Synthesis of 4-hydroxy-7-methyl-3*H*-pyrrolo[3,2-*f*]quinoline 30**. A solution of **29**[40] (0.300 g, 1.11 mmol) in HBr (48%, 30 mL) and acetic acid (15 mL) was heated at 50 °C for 5 h, at 80 °C for 5 h, and at reflux for 12 h. At the end (TLC) the volatiles were evaporated off, and the residue was taken up with $H_2O$ (30 mL) and neutralized with $NH_4OH$ (28%). The mixture was then extracted with $CHCl_3$ (3× 50 mL) and the extract was washed with $H_2O$ and evaporated to dryness. The residue was subjected to silica gel flash chromatography ($CHCl_3$/MeOH 8:2). Yield: 30%; mp: 250–251 °C; $R_f$ = 0.40 ($CHCl_3$/MeOH 8:2); $^1H$ NMR ([$D_6$]DMSO): $\delta$ = 2.66 (s, 3H, $CH_3$), 6.97 (dd, 1H, $J$ = 2.86 and 2.29 Hz, H-1), 7.26 (s, 1H, H-5), 7.32 (dd, 1H, $J$ = 2.86 and 3.2 Hz, H-2), 7.65 (d, 1H, $J$ = 8.58 Hz, H-8), 9.55 (d, 1H, $J$ = 8.58 Hz, H-9), 11.25 ppm (s, 1H, NH); HRMS: *m/z* $[M+H]^+$ calcd for $C_{12}H_{11}N_2O$ 199.087, found 199.105.

**Synthesis of 4-(2-diethylaminoethoxy)-7-methyl-3*H*-pyrrolo[3,2-*f*]-quinoline (31)**. A solution of hydroxy derivative **30** (0.060 g, 0.30 mmol) in 10 mL DMF and $K_2CO_3$ (0.063 g, 0.45 mmol) was stirred at room temperature for 30 min, and chloroethyldiethylamino chloride (0.062 g, 0.36 mmol) was then added. The mixture was slowly heated at 70 °C and maintained at that temperature under $N_2$ atmosphere until the starting material had disappeared as monitored by TLC ($CHCl_3$/MeOH 8:2). Yield: 34%; mp: 230–33 °C; $R_f$ = 4.6 ($CHCl_3$/MeOH 8:2); $^1H$ NMR ($CDCl_3$): $\delta$ = 1.14 (t, 6H, $J$ = 7.25 Hz, 2×$CH_3$), 2.72 (s, 3H, $CH_3$), 2.78 (q, 4H, $J$ = 7.12 Hz, 2×$CH_2$), 3.06 (t, 2H, $J$ = 5.63 Hz, $CH_2$), 4.36 (t, 2H, $J$ = 5.64 Hz, $CH_2$), 6.97 (dd, 1H, $J$ = 2.86 and 2.29 Hz, H-1), 7.13 (s, 1H, H-5), 7.21 (d, 1H, $J$ = 8.20 Hz, H-8), 7.29 (dd, 1H, $J$ = 2.86 and 3.02 Hz, H-2), 8.32 (d, 1H, $J$ = 8.20 Hz, H-9), 10.12 ppm (br s, 1H, NH), HRMS: *m/z* $[M+H]^+$ calcd for $C_{18}H_{24}N_3O$ 298.102, found 298.218; anal.: C, H, N (Supporting Information).

## *Biology*

### *Experiments with human tumor cell lines*

THPyC, PyC, and PQ derivatives were dissolved in DMSO just before the experiments, and a calculated amount of drug solution was added to the growth medium containing cells to a final solvent concentration of 0.5%; this had no discernible effect on cell viability. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), ellipticine, *m*-AMSA, mitoxantrone, and doxorubicin were obtained from Sigma Chemical Co., St. Louis, MO (USA).

**Cell cultures**. Human lung (A549) and breast (MCF-7) carcinoma cell lines, together with melanoma (A375), promyelocytic leukemia (HL60), and the corresponding mitoxantrone-resistant variant HL60/MX2 cell lines, were obtained from ATCC, Rockville, MD (USA). A431 human cervix carcinoma cells were kindly provided by Prof. Zunino, Division of Experimental Oncology B, Istituto Nazionale dei Tumori, Milan (Italy). The human ovarian cancer cell lines 2008 and its cisplatin-resistant variant C13* were kindly provided by Prof. G. Marverti, Department of Biomedical Science, University of Modena (Italy). The LoVo human colon carcinoma cell line and its derivative multidrug-resistant sub-line (LoVo MDR) were kindly provided by Prof. Franca Majone, Department of Biology, University of Padova (Italy). Cell lines were maintained in the logarithmic phase at 37 °C under a 5% $CO_2$ atmosphere with the following culture media: 10% fetal calf serum (Biochrom-Seromed GmbH & Co., Berlin, Germany), antibiotics (50 U $mL^{-1}$ penicillin and 50 μg $mL^{-1}$ streptomycin), and 2 mM L-glutamine: 1) RPMI-1640 medium (Euroclone, Celbio, Milan, Italy) with 25 mM HEPES buffer for HL60, MCF-7, 2008, C13*, and A431 cells; 2) Ham's F-12 (Sigma Chemical Co.) for LoVo and LoVo MDR cells (LoVo MDR culture medium also contained 0.1 μg $mL^{-1}$ doxorubicin); 3) DMEM (Euroclone) for A549 and A375 cells.

**Cytotoxicity assays**. The growth inhibitory effect toward tumor cell lines was evaluated by the tetrazolium salt reduction (MTT) assay.[52] Briefly, 3–8×$10^3$ cells per well, depending on the growth characteristics of the cell line, were seeded in 96-well microplates in growth medium (100 μL) and then incubated at 37 °C in a 5% $CO_2$ atmosphere. After 24 h, the medium was removed and re-

placed with fresh medium containing the compound to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After 72 h, each well was treated with 10 μL of a 5 mg mL$^{-1}$ MTT saline solution, and after 5 h of incubation, 100 μL of a sodium dodecylsulfate (SDS) solution in 0.01 M HCl was added. After incubation overnight, the inhibition of cell growth induced by the tested derivatives was detected by measuring the absorbance of each well at 570 nm with a Bio-Rad 680 microplate reader. The mean absorbance for each drug dose was expressed as a percentage of the untreated control well absorbance and plotted as a function of drug concentration. $IC_{50}$ values represent the drug concentrations that decreased the mean absorbance at 570 nm to 50% of those in the untreated control wells.

**Flow cytometric analysis**. Drug-induced cell cycle effects and DNA fragmentation were analyzed by flow cytometry after DNA staining with propidium iodide (PI) according to Nicoletti et al.[53] Briefly, 2008 cells ($5\times10^5$ cells) were exposed for 1–48 h to tested compound concentrations corresponding to $IC_{50}$ values. PI solution (1 mL) containing 50 μg mL$^{-1}$ PI, 0.1% *w/v* Triton X-100 and 0.01% *w/v* sodium citrate, was added to the cells and then incubated for 25 min at 4 °C in the dark. Induced cell death was determined as a percentage of hypodiploid nuclei counted out of the total cell population, measured on a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) on a 550–600 nm filter. Analysis was performed by Cell Quest software (Becton-Dickinson).

**Caspase-3 activity**. Caspase-3 activity was detected with the Apo-Alert Caspase-3 Fluorescent Assay Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's recommended procedures. Following 12 or 24 h incubation with tested compounds (at concentrations corresponding to $IC_{50}$ values) 2008 cells (~$10^6$ cells) were collected and lysed on ice in 50 μL lysis buffer for 10 min and then treated with 50 μL reaction buffer containing dithiothreitol (DTT) and 5 μL caspase-3 substrate solution (Asp-Glu-Val-Asp-(7-amino-4-trifluoromethylcoumarin) [DEVD-AFC], Clontech). Fluorescence was determined on a PerkinElmer 550 spectrofluorimeter ($\lambda_{ex}=440$ nm, $\lambda_{em}=505$ nm). Caspase-3 activity was expressed as the increase in AFC-emitted fluorescence. Student's *t* test was used for data analysis.

### *Interactions with DNA*

**Nucleic acids**. Salmon testes DNA was purchased from Sigma. Its hypochromicity, determined according to Marmur and Doty,[54] was >35%. DNA concentration was determined with $\varepsilon=6600\ M^{-1}\,cm^{-1}$ at 260 nm. pBR322 plasmid DNA was purchased from Fermentas Life Sciences, Burlington, ON, Canada).

**Flow linear dichroism**. LD measurements were performed on a Jasco J500A circular dichroism spectropolarimeter, converted for LD and equipped with an IBM PC and a Jasco J interface. Linear dichroism is defined in Equation (1) as:

$$LD_{(\lambda)} = A_{\parallel(\lambda)} - A_{\perp(\lambda)} \quad (1)$$

in which $A_{\parallel}$ and $A_{\perp}$ correspond to the absorbance of the sample with polarized light oriented parallel or perpendicular to the flow direction, respectively. The orientation is produced by a device designed by Wada and Kozawa[55] at a shear gradient of 500–700 rpm, and each spectrum was accumulated four times.

A solution of salmon testes DNA ($1.47\times10^{-3}$ M) in ETN buffer (containing 10 mM TRIS, 10 mM NaCl, and 1 mM EDTA, pH 7) was used. Spectra were recorded at 25 °C at various [drug]/[DNA] ratios.

**Unwinding assay**. Solutions of tested drugs were prepared at the required concentrations; 0.5 μL solution was mixed with 150 ng supercoiled pBR322 DNA in TAE buffer (40 mM TRIS, 20 mM glacial acetic acid, 1 mM EDTA, pH 8) to reach a final reaction volume of 10 μL. Complexes were incubated for 30 min at 37 °C. Following incubation, 3 μL loading buffer (50% glycerol, 10% bromophenol blue) were added, and the samples were loaded onto a 0.8% agarose gel. Electrophoresis continued for 90 min at 74 V at room temperature. After electrophoresis, the gel was stained for 30 min in a TAE bath containing ethidium bromide (1 μg mL$^{-1}$), and then destained in a TAE bath for an additional 20 min. The gel was trans-illuminated by UV light, and fluorescence emission was visualized by a CCD camera coupled to a Bio-Rad Gel Doc 1000 apparatus.

**DNA topoisomerase relaxation assay**. DNA topoisomerase II relaxation activity was assayed by the relaxation of a supercoiled DNA substrate. In detail, 0.25 μg pBR322 plasmid DNA (Fermentas Life Sciences) was incubated with 1 U topoisomerase II (USB) and the test compound as indicated, for 60 min at 37 °C in 20 μL reaction buffer consisting of 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg mL$^{-1}$ BSA, and 1 mM ATP.

The reaction was stopped by adding 4 μL stop buffer (5% SDS, 0.125% bromophenol blue, and 25% glycerol), 50 μg mL$^{-1}$ proteinase K (Sigma) and incubating for a further 30 min at 37 °C. The samples were separated by electrophoresis on a 1% agarose gel. The gel was stained with ethidium bromide (1 μg mL$^{-1}$) in TAE buffer and trans-illuminated by UV light; fluorescence emission was visualized by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

The relaxation activity of 2 U topoisomerase I (Amersham Biosciences, Piscataway, NJ, USA) was performed under the same experimental conditions, replacing the reaction buffer with the following: 35 mM Tris-HCl (pH 8), 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine, 0.01% BSA.

**DNA topoisomerase II cleavage reaction**. The reaction conditions were the same as for the relaxation assay except that 10 U DNA topoisomerase II were used. The DNA cleavage products were separated by electrophoresis on a 1% agarose gel containing 0.5 μg mL$^{-1}$ ethidium bromide in TBE buffer. The gel was trans-illuminated by UV light, and fluorescence emission was visualized by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

Structure assignments for compounds **9**, **13**, and **24** by 1D and 2D NMR experiments and a table of elemental analysis data for the tested compounds are given in the Supporting Information

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