Synthesis and Antitumor Characterization of Pyrazolic Analogues of the Marine Pyrroloquinoline Alkaloids: Wakayin and Tsitsikammamines

Laurent Legentil,[†] Laurent Benel,[‡] Viviane Bertrand,[‡] Brigitte Lesur,[‡] and Evelyne Delfourne*[,†]

Laboratoire SPCMIB, UMR-CNRS 5068, Université Paul Sabatier, 118 route de Narbonne, 31062, Toulouse Cedex 4, France, and Laboratoire Cephalon France, Centre de Recherches et de Développement, 19 avenue du Pr Cadiot, B.P. 22, 94701 Maisons-Alfort Cedex, France

Received December 15, 2005

A series of aza analogues of the marine alkaloids wakayin and tsitsikammamines A and B have been synthesized. The strategy used was based on [3 + 2] cycloaddition reactions involving 3-ethylamine-indole-4,7-dione and different diazo reagents. All the compounds were evaluated in vitro for antiproliferative activity against five distinct cancer cell lines and for their inhibitory effect on topoisomerase isoenzymes I and II. Some of the compounds inhibited the topoisomerase I and/or II catalyzed relaxation of supercoiled DNA at a concentration comparable to the drugs camptothecin and etoposide. Only a few of them exhibited cytotoxic activity with IC50 values in the micromolar range.

Introduction

Pyrrolo[4,3,2-*de*]quinolines, an important family of alkaloids isolated from marine source, recently received considerable attention due to their biological activities.[1] Wakayin **1**, isolated in 1991 from the ascidian *Clavelina* species, has been reported to have a diverse array of bioactivities including murine cell line cytotoxicity and topoisomerase I inhibition.[2] The closely structurally related tsitsikammamines A **2** and B **3**, isolated from a *Latrunculid* sponge, are cytotoxic but they inhibit neither topoisomerases I nor II[3] (Figure 1).

Synthesis of compounds structurally related to wakayin was reported by Zhang et al.,[4] Barret and Roue,[5] and most recently by Beneteau et al.[6] who also reported on their antiproliferative activity.

There are a wide range of topoisomerase I and II inhibitors reported in the literature, but very few possess dual inhibitory activities against I and II enzymes. Dual topoisomerase inhibitors should have a broader cell cycle activity and antitumor efficiency. They would prevent the frequently observed compensation process: alterations in expression of one isoform when the other is selectively inhibited; also they should display broader spectrum of activity with regards to tumor type.

As part of our research program on new potential anticancer drugs,[7] we focused our efforts on the preparation of analogues **a** and **b** of wakayin and tsitsikammamines in which one of the two pyrrole rings has been replaced by a pyrazole ring. We report herein the synthesis of these compounds and the results of their topoisomerases I and II inhibition along with their in vitro antitumor activity.

Chemistry

The retrosynthetic analysis to prepare the desired analogues was based on a [3 + 2] cycloaddition reaction between the fully protected dioxotryptamine derivative **4** and adequately substituted diazo compounds **6** to give rise to the corresponding tricyclic analogues **7**−**10**. Final cyclization will take place after cleavage of the Boc-protective group of the amine of the side chain (Scheme 1).

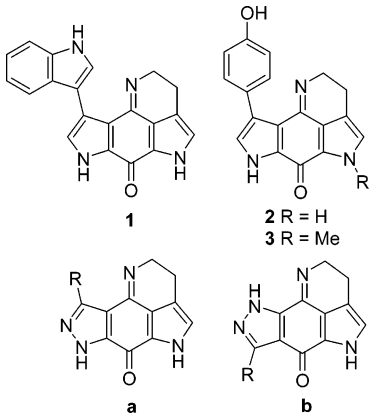


Figure 1. Natural compounds and analogues structures.

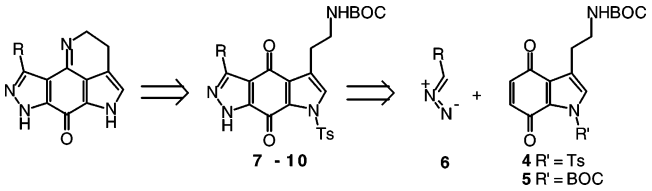
The synthesis of **4** (Scheme 2) was inspired by a recent work of Beneteau and Besson who described the preparation of a related compound **5** (Scheme 1).[8] The tosyl group was preferred to the Boc group as the protective group of the indolic nitrogen. Indeed these authors demonstrated that the final cyclization of similar N-protected indole only occurs if the N-indole is substituted by a strong electron-withdrawing group. The nitro derivative **14** precursor of compound **4** was prepared from 4,7-dimethoxyindole **12** prepared in turn from 2,5-dimethoxybenzaldehyde according to a five-step procedure previously published.[9] In our hands, the last step of this procedure, the Henry reaction on dimethoxyindole-3-carboxaldehyde **13**, gives rise to the dinitro derivative **15** as a side product. Compound **14** was obtained in 60% on a 1 g scale, but the yield drastically decreased upon scale-up. The production of **15** could be limited by using acetic acid as the solvent of the reaction. Unfortunately, in this case any yield improvement was observed due to the formation of several degradation products. The indolic nitrogen of compound **14** was further tosylated in 78% yield by treatment with potassium hydroxide and *p*-toluenesulfonyl chloride The resulting compound **16** was reduced into the corresponding amino derivative **17** by LAH in 95% yield.

The side chain amino group of **17** was then protected as Boc derivative **18** in 60% by treatment with sodium hydride and

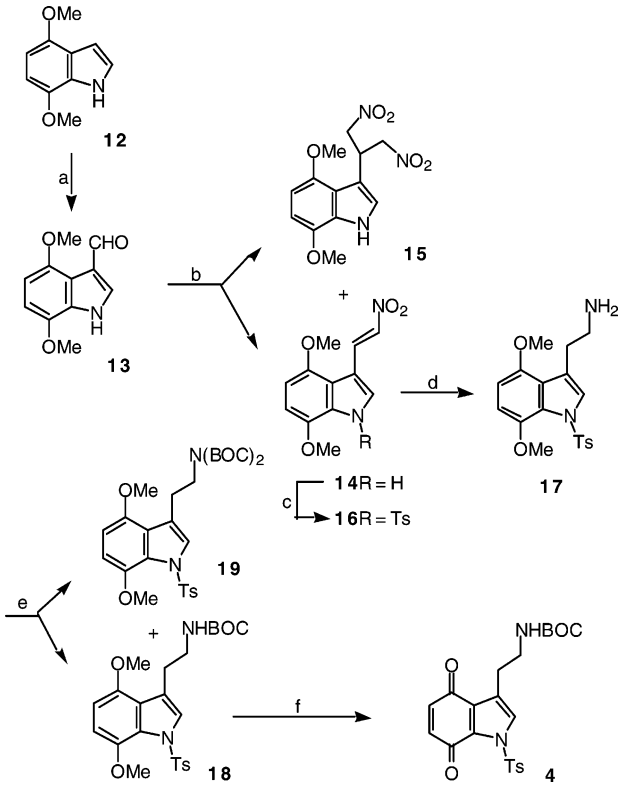
* Corresponding author. Phone: 33 561 55 62 93. Fax: 33 561 55 60 11. E-mail: delfourn@chimie.ups-tlse.fr.
[†] Université Paul Sabatier.
[‡] Centre de Recherches et de Développement.

10.1021/jm051247f CCC: $33.50
Published on Web 04/25/2006

Scheme 1. Retrosynthetic Scheme for the Preparation of Analogues



Scheme 2[a]



[a] (a) $POCl_3$/DMF, DMF, 0 °C, 1 h 30. (b) CH_3NO_2, NH_4OAc, AcOH, reflux, 3 h. (c) *p*-TsCl, KOH, THF, room temperature, 1 h. (d) LAH, CH_2Cl_2, Et_2O, 0 °C, 15 min. (e) Boc_2O, NaH, THF, room temperature, 1 h. (f) CAN, CH_3CN/H_2O, room temperature, 15 min.

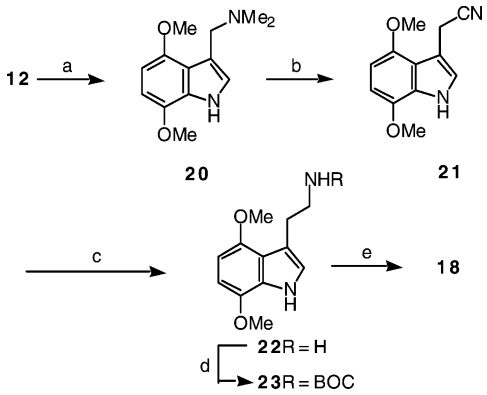
di-*tert*-butyl dicarbonate in THF along with the di-Boc compound **19** in 5% yield. The overall yield of the synthesis of **18** from 4,7-dimethoxyindole **12** was 20%. The oxidation of **18** by CAN gave rise to quinone **4** in 83% yield.

To improve the yield of the preparation of intermediate **18**, an alternative route derived from the method reported by Iwao et al. was examined (Scheme 3).[10]

It also started with compound **12**, which was further converted quantitatively into the gramine derivative **20** using a Mannich type reaction. This last compound was reacted with methyl iodide to give the quaternary salt, which was subsequently transformed into the cyano derivative **21** by action of cyanotrimethylsilane in the presence of tetrabutylammonium fluoride (90% yield). Despite what was reported by the authors, a silylated activating group on the nitrogen of the indole was not necessary to do this reaction.[11] It should be noted that compounds **20** and **21** have been previously described.[12] Compound **21** was then reduced by LAH in 86% yield to provide indole derivative **22**; the amino group of the side chain was further protected as a Boc derivative (compound **23**). The overall yield is 55% from **12** to **18** with no scale-up limitation.

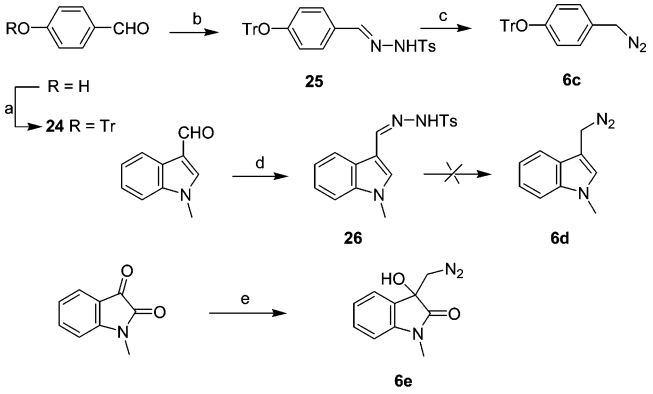
Aryldiazomethane derivatives **6b** (R = Ph) and **6c** were readily prepared by Banford−Stevens pyrolysis of the tosylhydrazones of corresponding aryl aldehydes in alcohol solvents. In this way, phenyldiazomethane **6b** was obtained as previously reported from the commercially available benzaldehyde tosylhydrazone,[13] whereas the diazoderivative **6c** (Scheme 4) was synthesized from 4-hydroxybenzaldehyde according to the reaction sequence described in Scheme 4.

Scheme 3[a]



[a] (a) Me_2NH, CH_2O, AcOH, room temperature, 3 h. (b) 1: MeI, CH_2Cl_2, toluene, room temperature, overnight. 2: TMSCN, 1 N TBAF in THF, THF, room temperature, 1 h. (c) LAH, CH_2Cl_2, Et_2O, room temperature, 1 h. (d) Boc_2O, CH_2Cl_2, room temperature, 1 h. (e) *p*-TsCl, NaH, room temperature, 30 min.

Scheme 4[a]



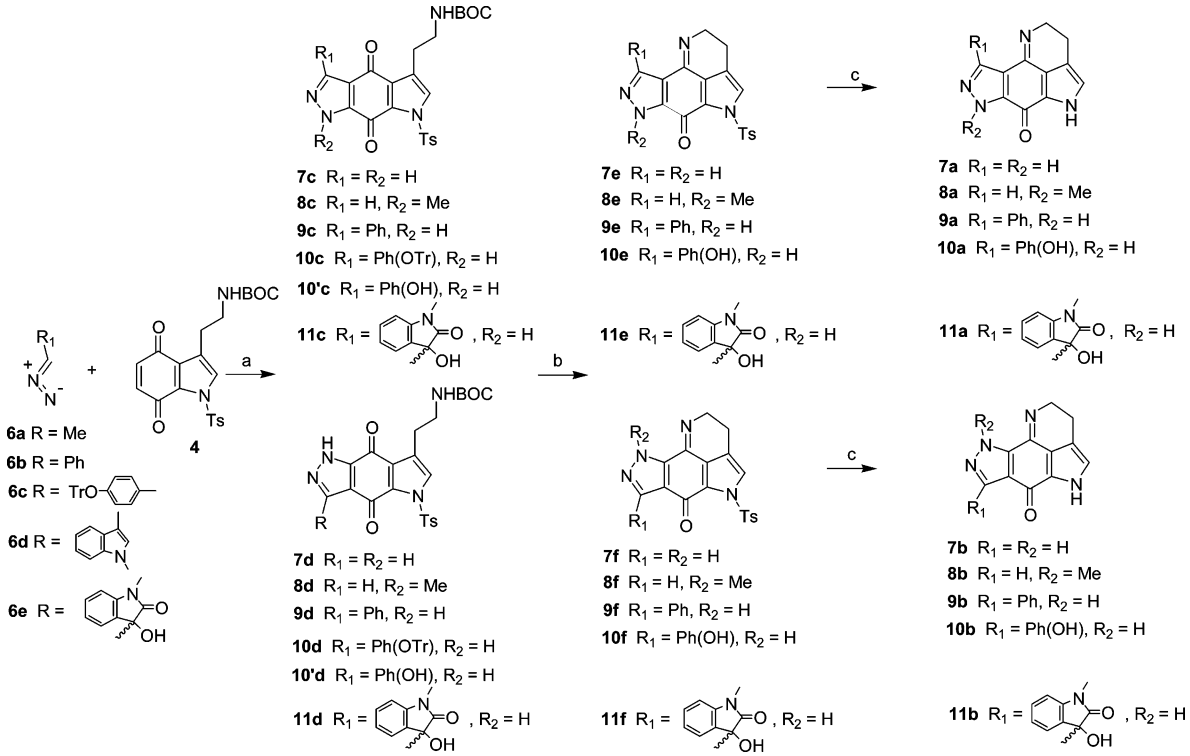
[a] (a) TrCl, Et_3N, DMAP, THF, reflux overnight. (b) $TsNHNH_2$, THF, room temperature, 1 h. (c) KOH/MeOH, THF, 90 °C, 1 h. (d) $TsNHNH_2$, EtOH, room temperature, 1 h. (e) CH_2N_2, THF, room temperature, 30 min.

The hydroxyl group was protected as a triphenylmethyl group (trityl or Tr) to give compound **24**, which was reacted with hydrazine to form the corresponding hydrazone **25**. In alkaline conditions, this last compound yielded the diazo derivative **6c**. Similarly, indole carboxaldehyde was easily transformed into the corresponding tosylhydrazone derivative **26**, but attempts to convert it into the diazo compound **6d** failed. An alternative way to introduce an indole moiety via a cycloaddition reaction was to generate the diazo species **6e** by action of diazomethane on isatine as previously reported by Eistert and Ganster.[14]

The [3 + 2] cycloaddition reactions of quinone **4** with the diazo derivatives diazomethane or aryldiazomethanes led as expected to a mixture of the two regioisomers (Scheme 5).

Reaction of diazomethane **6a** with quinone **4** gave in 88% global yield the mixture of regioisomers **7c** and **7d** in a ratio of 60/40, the methylated analogues **8c**/**8d** being side products of the reaction. **8c**/**8d** were obtained in 97% yield when compounds **7c**/**7d** were reacted with excess diazomethane in methanol. The other diazo reagents including **6b**, **6c**, and **6e** led to the mixtures **9c**/**9d**, **10c**/**10d**, and **11c**/**11d**, respectively. It should be noted that the reaction leading to compounds **10c**/**10d** also gave a

Scheme 5[a]



[a] (a) THF, 30 min. (b) 1: TFA, CH_2Cl_2, room temperature, 30 min. 2: EtOH, 4A molecular sieve, reflux. (c) 1 N TBAF in THF, THF, reflux or 1 N NaOH, dioxane, room temperature, 24 h.

Table 1. Intramolecular Cyclization Reaction, Yields, and Ratios of Isomers (See Scheme 5)

compounds	R_1	R_2	% yield	Ratio of isomers c/d
7e/7f	H	H	40	90/10
8e/8f	H	Me	49	57/43
9e/9f	Ph	H	50	20/80[a]
10e/10f	Ph(OH)	H	55	76/34[a]
11e/11f		H	40	58/42[a]

[a] The ratio is indicated in the order of elution in HPLC.

small amount (10%) of the corresponding deprotected analogues **10′c**/**10′d**. Regioisomeric mixtures were not separated at this stage of our synthetic pathway.

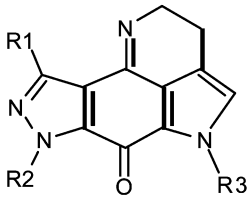
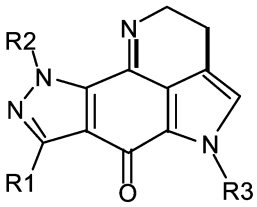
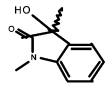
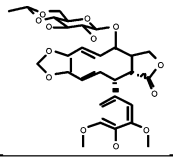
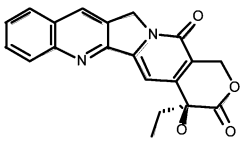
Cycloadducts were further converted to the final cyclized derivatives as previously described for products of the pyrroloquinoline family.[15] The cleavage of the Boc protection of the side chain amino group with trifluoroacetic acid in dicloromethane gave the corresponding trifluoroacetate in good yields (above 80%). For compounds **10c**/**10d**, the trityl-protective group was cleaved upon cleavage of the Boc group (**10e**/**10f**). The next cyclization step was achieved by reflux in ethanol in the presence of both 4 A molecular sieve and sodium bicarbonate. The yields of these reactions as well as the ratio of the different regioisomers which were separated are reported in Table 1. Finally, cleavage of the tosyl-protective group of compounds **7e**, **7f**, **8e**, **8f**, **9e**, and **9f** was performed with sodium hydroxide in dioxane to give **7a**, **7b**, **8a**, **8b**, **9a**, and **9b**, respectively. For compounds **10e** and **10f** this reaction was performed in the presence of tetrabutylammonium fluoride in THF in order to recover the phenolic form of the final product. In the case of compounds **11e** and **11f**, attempts to further convert the oxo-dihydroindolyl group into an indolic group[16] were unsuccessful. Further cleavage of the tosyl group under standard conditions as reported above give rise to corresponding **11a** and **11b** derivatives.

Structural Assignment. The structure assignment of each tetracyclic isomer **7e**/**7f** was first studied by 2D-NMR. Unfortunately, we were unable using HMBC experiments to show a J^3 long-distance coupling between the hydrogen on the pyrazole ring and C═N in one isomer or C═O in the other isomer. Finally, the structure of each isomer was assigned using comparison with related compounds. As we have previously reported in the case of quinolinic[17] or isoquinolinic[7c] derivatives, the proton close to the iminoquinone function (isomer **7e**) is deshielded compared to the same proton in the other isomer (**7f**). On that basis, the major isomer (which is also the more polar) in **7e**/**7f** mixture, with the proton of the pyrrazole being downfield shifted, would correspond to structure **7e**. The same analysis can be applied to **8e**/**8f**, compound **8f** being the major isomer of the two products. In the case of compounds **9e**/**9f**, **10e**/**10f**, and **11e**/**11f**, which do not have any proton on the pyrrazole, the structural assignment of each isomer has not been possible so far. Experiments are currently in progress to obtain a monocrystal of at least one component of each couple in order to perform X-ray analysis.

Pharmacology

Inhibition of Topoisomerases. Final cyclized derivatives **7a** and **7b**, **8a** and **8b**, **9a** and **9b**, and **10a** and **10b** and their tosylated precursors **7e** and **7f**, **8e** and **8f**, **9e** and **9f**, and **10e** and **10f**, respectively, and compound **11f** were evaluated in vitro for their ability to inhibit enzymatic cleavage of DNA by

Table 2. Effect on Topoisomerases I and II Enzymatic Activity[a]

STRUCTURE	cpd#	R1	R2	R3	Inhibition of DNA cleavage	
					hTopoI	hTopoII
	**7e**	H	H	Tosyl	~100	>100
	8e	H	Me	Tosyl	300	not tested
	9e	Phenyl	H	Tosyl	300	not tested
	10e	p-HOPhenyl	H	Tosyl	30	>100
	7a	H	H	H	>100	~100
	8a	H	Me	H	>100	>100
	9a	Phenyl	H	H	~100	>100
	10a	p-HOPhenyl	H	H	>100	~100
	**7f**	H	H	Tosyl	300	>300
	8f	H	Me	Tosyl	>100	>100
	9f	Phenyl	H	Tosyl	100	>100
	10f	p-HOPhenyl	H	Tosyl	10	100
	11f		H	Tosyl	>100	not tested
	7b	H	H	H	>100	~100
	8b	H	Me	H	>100	~100
	9b	Phenyl	H	H	>100	>100
	10b	p-HOPhenyl	H	H	~300	>300
	ETOPOSIDE				not tested	100
	CAMPTOTHECIN				100	not tested

[a] Values are expressed as the micromolar concentration of compound which induced complete, partial (~), or no observable (>) inhibition of DNA cleavage activity.

topoisomerase I and II isoenzymes. The results reported in Table 2 are the lowest concentration to achieve complete inhibition of topoisomerase I or II activity compared to those of etoposide and camptothecin (specific topoisomerase II and I inhibitor, respectively).

Activities for the natural compound wakayin reported in the literature are, respectively, 10 and 250 μM on topoisomerase I and II; unfortunately, this compound was not available for us to include in our test.

Compounds **10e** and **10f** are the most active compounds, demonstrating a 3−10-fold better activity, respectively, 30 and 10 μM on topoisomerase I compared to camptothecin and a topoisomerase II activity similar to the one of etoposide for **10f** (100 μM), while **10e** is less active (>100 μM).

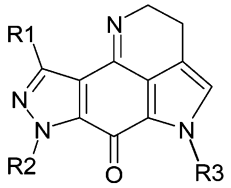
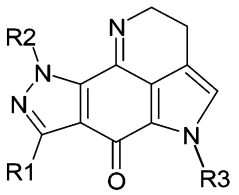
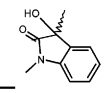
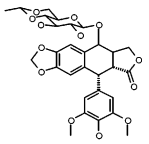
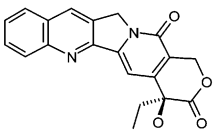
Compounds **7a**, **10a**, **7b**, and **8b** have topoisomerase II activity (around 100 μM) equivalent to the one observed with etoposide but are less active (>100 mM) than camptothecin on topoisomerase I activity.

Compounds **10f**, **10e**, and **9f** exhibited complete inhibition of DNA cleavage activity of human topoisomerase I at 10, 30, and 100 μM, respectively, being more or equally potent than the selective topoisomerase I inhibitor camptothecin. Compounds **9e**, **8e**, and **7f** have shown, but to a lesser extent, topoisomerase I inhibitory effect, with an active concentration of 300 μM. Among all the compounds cited above, **10f** is the sole one to also inhibit human topoisomerase II activity at a concentration comparable to that of the control drug etoposide (100 μM). For all the other tested compounds no relevant inhibition of topoisomerase I or II activity was observed at the maximum tested concentrations.

In Vitro Determination of the Drug-Induced Inhibition of Cancer Cell Line Growth. Compounds were further evaluated in vitro for their antiproliferative activity on three human cell lines (HCT15 and HT29, colon; PC3, prostate) and two murine cell lines (CT-26, colon; Renca, kidney). Camptothecin and etoposide selective topoisomerase I and II inhibitors, respectively, were included in the same experiment as comparators.

Compounds were tested for 72 h at nine concentrations ranging from 1 nM to 10 μM with a 0.1% DMSO final

Table 3. Characterization of the in Vitro Cytotoxic-Related Antitumor Effects

STRUCTURE	cpd#	R1	R2	R3	Inhibition of Cancer Cell proliferation or % inhibition@10μM IC50				
					HCT15	HT29	PC3	CT26	Renca
	**7e**	H	H	Tosyl	0%	0%	0%	0%	0%
	8e	H	Me	Tosyl	6.5μM	3.1μM	4.9μM	5.3μM	0.14μM
	9e	Phenyl	H	Tosyl	0%	0%	0%	0%	0%
	10e	p-HOPhenyl	H	Tosyl	0%	23%	0%	0%	0%
	7a	H	H	H	0%	0%	0%	0%	0%
	8a	H	Me	H	0%	4%	0%	7%	6%
	9a	Phenyl	H	H	0%	0%	0%	0%	0%
	10a	p-HOPhenyl	H	H	0%	0%	0%	0%	0%
	**7f**	H	H	Tosyl	0%	0%	0%	0%	0%
	8f	H	Me	Tosyl	5μM	1.9μM	5.8μM	4.9μM	4.6μM
	9f	Phenyl	H	Tosyl	0%	0%	0%	0%	0%
	10f	p-HOPhenyl	H	Tosyl	0%	41%	0%	0%	0%
	11f		H	Tosyl	1.6μM	2.9μM	5.2μM	2.9μM	1.9μM
	7b	H	H	H	25%	45%	0%	0%	0%
	8b	H	Me	H	0%	3.6μM	0%	7μM	6.3μM
	9b	Phenyl	H	H	2.7μM	47%	0%	0%	0%
	10b	p-HOPhenyl	H	H	0%	0%	0%	0%	0%
	ETOPOSIDE				4.5μM	6.6μM	7.8μM	0.2μM	2.9μM
	CAMPTOTHECIN				18nM	30nM	52nM	34nM	304nM

concentration in the growth medium. The results, expressed as IC50 (concentration reducing the cell proliferation rate by 50%), are reported in Table 3.

Cell growth inhibitory activities were reported in the literature for wakayin and tsitsikammamines in the HCT-116 cell line; IC50s of 1.53 μM for wakayin and of 1.4 and 2.38 μM for tsitsikammamines A and B were reported, respectively.

As presented in Table 3, compounds **8e**, **8f**, and **11f** inhibited the growth of the five cell lines with IC50 values in the micromolar range like what is observed for etoposide in the same conditions. Camptothecin inhibited the growth of these human and mouse tumoral cell lines at lower nanomolar range concentrations. Compound **8b** showed activity on three out the five tested cell lines. Quite unexpectedly, the most potent topoisomerase I inhibitors **10e** and **10f** are not inhibiting cell proliferation in any of the cell lines tested.

Discussion

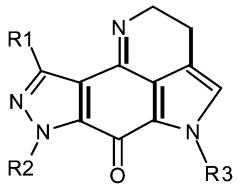
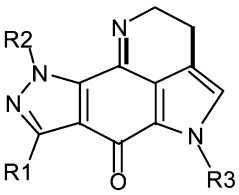
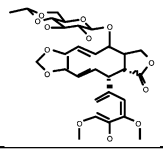
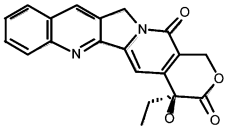
For the Topoisomerase Inhibition (Table 2). We have described a new series of aza analogues of the marine alkaloids wakayin and tsitsikammamines A and B and evaluated their inhibitory activities with regard to topoisomerases I and II. Compounds **10e** and **10f** structurally corresponding to tsitsikammamine A in which one pyrrole ring has been replaced by a pyrazole ring are the most active. This structural change seems to have increased the inhibitory effect on topoisomerase I, as the natural product tsitsikammamine is not an inhibitor of the enzyme. If compared to wakayin, the new analogues trend to show lower potency with regard to the topoisomerase inhibitory activities.

For the Cancer Cell Proliferation Inhibition (Table 3). On the basis of data from Table 3, camptothecin is 4- to 100-fold more efficient than etoposide in inhibiting, after a 72 h incubation period, the growth of the five tested tumoral cell lines. This may be due to a predominant topoisomerase I pattern of expression in those cell lines.

It has been reported that wakayin differs from camptothecin in that its cleavage complexes were much less stable than those of camptothecin and that wakayin poorly stabilizes cleavage complexes.[2] This could be a reason compounds such as **10e** and **10f** despite their interesting topoisomerases inhibitory activity profile are not inhibiting cell growth of the tested tumoral cell lines.

The noncorrelation of enzyme versus cell line inhibitory activities for **10e** and **10f** could also be due to a poor cell penetration of those two compounds. To investigate that hypothesis, in silico physicochemical properties calculations were performed for all tested compounds, etoposide and

Table 4. Calculated Values for log *P* and PSA

STRUCTURE	cpd#	R1	R2	R3	logP	PSA
	**7e**	H	H	Tosyl	2,11	165
	8e	H	Me	Tosyl	1,87	107
	9e	Phenyl	H	Tosyl	4,21	148
	10e	p-HOPhenyl	H	Tosyl	3,6	209
	7a	H	H	H	0,1	156
	8a	H	Me	H	-0,09	100
	9a	Phenyl	H	H	2,6	134
	10a	p-HOPhenyl	H	H	1,59	195
	**7f**	H	H	Tosyl	2,11	162
	8f	H	Me	Tosyl	1,87	112
	9f	Phenyl	H	Tosyl	4,21	145
	10f	p-HOPhenyl	H	Tosyl	3,6	206
	11f		H	Tosyl	1,78	135
	7b	H	H	H	0,1	156
	8b	H	Me	H	-0,09	104
	9b	Phenyl	H	H	2,19	140
	10b	p-HOPhenyl	H	H	1,6	190
	ETOPOSIDE				0,03	242
	CAMPTOTHECIN				0,9	133

camptothecin being included in the data set. Calculated values for log *P* and PSA (polar surface area) are presented in Table 4. PSA values for compounds **10e** and **10f** are above the acceptable threshold value of 160. Camptothecin (nanomolar inhibition activity on cell proliferation) is in the acceptable range for the PSA, while etoposide (micromolar inhibition activity on cell proliferation) also has a very high PSA value. Also, **10e** and **10f** with calculated log *P* values of 3.6 are more lipophilic which may correlate with a lower cell penetration than for camptothecin.

It is also interesting to mention that new compounds designed to target topoisomerase I were reported in the literature to be a substrate for P-glycoprotein (P-gp), while camptothecin and its neutral derivatives are not.[18] However, we have not tested our newly synthesized compounds with regard to P-gp.

Finally, compounds **8e**, **8f**, **11f** showed antiproliferative effect, blocking cell proliferation in the micromolar range, while no relevant inhibition of topoisomerase was detectable suggesting another mechanism for their cytotoxic activity against cancer cells. Further studies, based on the above findings, are in progress in our laboratory and will be reported in due course.

Experimental Section

A. Chemistry. A.1. Chemical Synthesis. ^{1}H and ^{13}C NMR spectra were performed on a JEOL 400 MHz spectrometer with the chemical shifts of the remaining protons of the deuterated solvents serving as internal standards. IR spectra were obtained on a Perkin-Elmer (1600 series FTIR) spectrometer. Mass spectra (MS) were recorded on an automass Unicam spectrometer. Reagents were purchased from commercial sources and used as received. Chromatography was performed on silica gel (15−40 μm) by means of the solvent systems indicated below.

A.2. 4,7-Dimethoxy-3-(2-nitro-1-nitromethyl-ethyl)-1H-indole (15). To a suspension of compound **13**[8] (4 g, 19.5 mmol) in nitromethane (100 mL) was added ammonium acetate (900 mg, 11.6 mmol). The mixture was refluxed for 1 h. After concentration on vacuum, CH_2Cl_2 (50 mL) was added to the residue. The mixture was separated, washed with H_2O, and dried over $MgSO_4$. After concentration the crude product was purified by flash chromatography (CH_2Cl_2) to give the expected product as an orange solid (3.62 g, 60%), mp 145 °C.

A.3. 4,7-Dimethoxy-3-(2-nitro-vinyl)-1-(toluene-4-sulfonyl)-1H-indole (16). To a solution of compound **14** (1.12 g, 20 mmol) in anhydrous THF (40 mL) was added at 0 °C, under nitrogen atmosphere, powder KOH (967 mg, 24.2 mmol). After 5 min of stirring, *p*-toluenesulfonyl chloride (850 mg, 4.45 mmol) was added, and the mixture was stirred for additional 1 h. AcOEt (80 mL) was added, and the organic layer was washed with H_2O (3 × 50 mL). After drying over $MgSO_4$ and concentration, the crude product was purified by flash chromatography (CH_2Cl_2) to give the product as an orange solid (880 mg, 54%), mp 195 °C.

A.4. 2-[4,7-Dimethoxy-1-(toluene-4-sulfonyl)-1H-indol-3-yl]-ethylamine (17). To a solution of $LiAlH_4$ (283 mg, 7.46 mmol) in diethyl ether (10 mL) was added dropwise, at 0 °C, a solution of

compound **16** (500 mg, 1.24 mmol) in CH_2Cl_2 (30 mL). The mixture was stirred for 15 min, and H_2O (0.5 mL) was added. After filtration over Celite, the filtrate was dried over $MgSO_4$ and concentrated to give the product as an unstable brown solid (402 mg, 86%).

A.5. *tert*-Butyl(2-{4,7-dimethoxy-1-[(4-methylphenyl)sulfonyl]-1*H*-indol-3-yl}ethyl)carbamate (18) and Di-*tert*-butyl(2-{4,7-dimethoxy-1-[(4-methylphenyl)sulfonyl]-1*H*-indol-3-yl}ethyl)-imidodicarbonate (19). To a solution of compound **17** (260 mg, 0.69 mmol) in anhydrous THF (10 mL) was added sodium hydride (60% dispersion in oil, 55 mg, 1.39 mmol) under nitrogen atmosphere. After 5 min of stirring, di-*tert*-butyl-dicarbonate (227 mg, 1.04 mmol) was added, and the stirring was maintained for 1 h. Water (1 mL) and CH_2Cl_2 (10 mL) were added, and the organic layer was separated. It was then washed with water (3 × 10 mL), dried over $MgSO_4$, and concentrated on vacuum. The crude product was purified by flash chromatography (CH_2Cl_2/MeOH 99:1) to give **18** as an oil (200 mg, 60%) and **19** as an oil (20 mg, 5%).

A.5.1. Compound 18 Was Also Obtained from Compound 23. To a solution of compound **21** (1.15 g, 3.59 mmol) in anhydrous THF (30 mL) was added under nitrogen atmosphere NaH (60% dispersion in oil, 720 mg, 17.97 mmol). The mixture was stirred for 1 h. *p*-TsCl (753 mg, 3.95 mmol) was added, and stirring was maintained for 30 min. H_2O (2 mL) and CH_2Cl_2 (50 mL) were added. After drying over MgSO4 and concentrating, the crude product was purified by flash chromatography (CH_2Cl_2/MeOH 99:1) to give the product (1.53 g, 90%).

A.6. 1-(4,7-Dimethoxy-1*H*-indol-3-yl)-*N*,*N*-dimethylmethanamine (20). A 50% aqueous solution of dimethylamine (3.81 mL, 29.94 mmol), acetic acid (4 mL), and formaldehyde (37% in H_2O, 2.19 mL, 28.81 mmol) was added to compound **12** (5 g, 28.25 mmol). The reaction media was stirred at room temperature for 3 h. The solution was poured into a mixture of 10% NaOH (50 mL)/ice (50 g), and it was extracted with CH_2Cl_2 (3 × 50 mL). The organic layers were dried over $MgSO_4$ and concentrated to give the expected product as an oil (6.61 g, 100%).

A.7. 4,7-Dimethoxy-1*H*-indol-3-yl)acetonitrile (21). To a suspension of compound **20** (6.61 g, 28.25 mmol) in a mixture CH_2Cl_2/toluene (80/150 mL) was added methyl iodide (7.02 mL, 56.49 mmol). The mixture was stirred at room temperature overnight. The solution was then concentrated, and THF (130 mL) was added to the residue. TMSCN (4.91 mL, 42.37 mmol) was added to the resulted suspension, and TBAF (1 N in THF, 85 mL, 85 mmol) was also added dropwise. After 1 h of additional stirring, the solution was concentrated in vacuum. CH_2Cl_2 (200 mL) was added to the residue. The mixture was washed with H_2O (3 × 100 mL), dried over $MgSO_4$, and evaporated. The residue was purified by flash chromatography (CH_2Cl_2) to give the expected product as a white solid (5.67 g, 94%), mp 140 °C.

A.8. 2-(4,7-Dimethoxy-1*H*-indol-3-yl)ethanamine (22). To a suspension of $LiAlH_4$ (2.25 g, 59.2 mmol) in Et_2O (90 mL) was added dropwise at 0 °C a solution of compound **21** (1.83 g, 8.47 mmol) in CH_2Cl_2 (30 mL). The mixture was stirred at room temperature for 30 min. After this time, H_2O (2 mL), 10% NaOH (2 mL), and again H_2O (5 mL) were added, and stirring was maintained for an additional 10 min. The mixture was filtered over Celite, and the filtrate was concentrated on vacuum to give the expected product as an oil (1.6 g, 86%). This product, which is unstable, was engaged in the next step without further purification.

A.9. *tert*-Butyl(2-{4,7-dimethoxy-1*H*-indol-3-yl}ethyl)carbamate (23). To a solution of compound **22** (1.76 g, 8.02 mmol) in CH_2Cl_2 (45 mL) was added di-*tert*-butyl dicarbonate (1.75 g, 8.02 mmol). The mixture was stirred at room temperature for 1 h, and it was left in the refrigerator overnight. After filtration, the expected product was obtained as a white solid (2.30 g, 90%), mp 180 °C.

A.10. *tert*-Butyl(2-{1-[(4-methylphenyl)sulfonyl]-4,7-dioxo-4,7-dihydro-1H-indol-3-yl}ethyl)carbamate (4). A mixture of compound **18** (4 g, 8.4 mmol) and CAN (13.88 g, 25.3 mmol) in CH_3CN/H_2O (280 mL/140 mL) was stirred at room temperature for 15 min. The solution was then extracted with CH_2Cl_2 (3 × 200 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuum to give the expected compound as an orange oil (3.60 g, 95%).

A.11. 4-Trityloxy-benzaldehyde (24). A solution of hydroxybenzaldehyde (500 mg, 4.09 mmol), trytil chloride (1.37 g, 4.91 mmol), triethylamine (1.71 mL, 12.28 mmol), and DMAP (50 mg, 0.41 mmol) in THF (30 mL) was refluxed overnight. CH_2Cl_2 (50 mL) was added, and this organic layer was washed with water (3 × 50 mL). The organic layer was dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography (pentane/AcOEt 80:20) to give the expected product as a white solid (1.57 mg, 92%), mp 119 °C.

A.12. 4-Trityloxy-benzaldehyde-(4-toluenesulfonyl)-hydrazone (25). To a solution of compound **24** (13.7 g, 41.4 mmol) in THF (500 mL) was added tosylhydrazine (7.7 g, 41.4 mmol). The mixture was stirred at room temperature for 30 min. After concentration, the crude product was purified by flash chromatography (petroleum ether/AcOEt 70:30) to give quantitatively the expected product as a white solid, mp 76 °C.

A.13. 4-Methyl-*N*′-[(1*E*)-(1-methyl-1*H*-indol-3-yl)methylene]-benzenesulfonohydrazide (26). A solution of 1-methylindole-3-carboxaldehyde (500 mg, 3.24 mmol) and tosylhydrazine (585 mg, 3.24 mmol) in EtOH (50 mL) was stirred at room temperature for 30 min. Filtration of the mixture gave the expected product as a pale-yellow solid (850 mg, 83%), mp 143 °C.

A.14. *tert*-Butyl(2-{7-[(4-methylphenyl)sulfonyl]-4,8-dioxo-1,4,7,8-tetrahydro-pyrrolo[3,2-*f*]indazol-5-yl}ethyl)carbamate (7c) and *tert*-Butyl(2-{5-[(4-methylphenyl)sulfonyl]-4,8-dioxo-1,4,5,8-tetrahydro-pyrrolo[2,3-*f*]indazol-7-yl}ethyl)carbamate (7d). To a solution of compound **4** (500 mg, 1.13 mmol) in THF (50 mL) was added, at 0 °C in the dark, the solution of diazomethane (1 equiv), and the mixture was stirred for 30 min. The solvent was evaporated over vacuum, and the residue was purified by flash chromatography (CH_2Cl_2/MeOH 98:2) to give the mixture of the two isomers as an orange solid (480 mg, 88%): isomer 1, 60%; isomer 2, 40%.

A.15. *tert*-Butyl(2-{1-methyl-7-[(4-methylphenyl)sulfonyl]-4,8-dioxo-1,4,7,8-tetrahydro-pyrrolo[3,2-f]indazol-5-yl}ethyl)-carbamate (8c) and *tert*-Butyl(2-{1-methyl-5-[(4-methylphenyl)-sulfonyl]-4,8-dioxo-1,4,5,8-tetrahydro-pyrrolo[2,3-f]indazol-7-yl}ethyl)carbamate (8d). To a solution of compounds **7c**/**7d** (1 g, 2.07 mmol) in MeOH (20 mL) was added diazomethane (2 equiv). The mixture was stirred for 2 h and then was concentrated on vacuum to give the expected products as a yellow solid (998 mg, 97%): isomer 1, 60%; isomer 2, 40%.

A.16. *tert*-Butyl(2-{7-[(4-methylphenyl)sulfonyl]-4,8-dioxo-3-phenyl-1,4,7,8-tetrahydro-pyrrolo[3,2-f]indazol-5-yl}ethyl)-carbamate (9c) and *tert*-Butyl(2-{1-methyl-5-[(4-methylphenyl)-sulfonyl]-4,8-dioxo-3-phenyl-1,4,5,8-tetrahydro-pyrrolo[2,3-f]indazol-7-yl}ethyl)carbamate (9d). To a solution of benzaldehyde tosylhydrazone (670 mg, 2.43 mmol) in THF (40 mL) was added a solution of KOH (340 mg, 6.08 mmol) in MeOH (10 mL). The mixture was warmed at 90 °C for 1 h. Et_2O (60 mL) was added, and the solution was washed with brine (3 × 50 mL). This phenyldiazomethane **6b** solution was added to a solution of quinone **4** (900 mg, 203 mmol) in THF (100 mL). After 30 min of stirring, the solution was concentrated in vacuum. The crude product was purified by flash chromatography (CH_2Cl_2/MeOH 99:1) to give the two expected isomers as a yellow solid (555 mg, 49%): isomer 1, 90%; isomer 2, 10%.

A.17. *tert*-Butyl(2-{7-[(4-methylphenyl)sulfonyl]-4,8-dioxo-3-(4-hydroxyphenyl)-1,4,7,8-tetrahydro-pyrrolo[3,2-f]indazol-5-yl}ethyl)carbamate (10c), *tert*-Butyl(2-{1-methyl-5-[(4-methylphenyl)sulfonyl]-4,8-dioxo-3-(4-hydroxyphenyl)-1,4,5,8-tetrahydro-pyrrolo[2,3-f]indazol-7-yl}ethyl)carbamate (10d), tert-Butyl(2-{7-[(4-methylphenyl)sulfonyl]-4,8-dioxo-3-(4-trityloxyphenyl)-1,4,7,8-tetrahydro-pyrrolo[3,2-f]indazol-5-yl}ethyl)carbamate (10′c), and *tert*-Butyl(2-{1-methyl-5-[(4-methylphenyl)sulfonyl]-4,8-dioxo-3-(4-trityloxyphenyl)-1,4,5,8-tetrahydro-pyrrolo[2,3-f]indazol-7-yl}ethyl)carbamate (10′d). To a solution of compound **25** (1.80 g, 3.38 mmol) in THF (60 mL) was added a solution of KOH (380 mg, 6.78 mmol) in MeOH (40

mL). The mixture was warmed at 90 °C for 1 h. Et_2O (100 mL) was added, and the solution was washed with brine (3 × 100 mL). This solution of trityloxyphenyldiazomethane **6c** was added to a solution of compound **4** (1 g, 2.25 mmol) in THF (100 mL). After 30 min of stirring, the solution was concentrated on vacuum. The crude product was purified by flash chromatography (petroleum ether/AcOEt 70:30 and 50:50) to give **10c** and **10d** as an orange solid (920 mg, 50%).

Isomer 1 (60%), Isomer 2 (40%). And **(10′c and 10′d)**: separated Isomer 1 (30 mg, 0.6%), mp 187 °C. Isomer 2: (20 mg, 0.4%), mp 198 °C.

A.18. *tert*-Butyl(2-{3-(3-hydroxy-1-methyl-2-oxo-2,3-dihydro-1H-indol-3-yl)-7-[(4-methylphenyl)sulfonyl]-4,8-dioxo-1,4,7,8-tetrahydro-pyrrolo[3,2-f]indazol-5-yl}ethyl)carbamate (11c) and *tert*-Butyl(2-{3-(3-hydroxy-1-methyl-2-oxo-2,3-dihydro-1H-indol-3-yl)-5-[(4-methylphenyl)sulfonyl]-4,8-dioxo-3-(4-hydroxyphenyl)-1,4,5,8-tetrahydropyrrolo[2,3-f]indazol-7-yl}ethyl)carbamate (11d). To a solution of *N*-methylisatine (599 mg, 3.72 mmol) in THF (30 mL) was added, at 0 °C in the dark, diazomethane (1.5 equiv) in Et_2O (60 mL). The mixture was stirred for 1 h. After concentration up to 1/4, the solution was added to a solution of quinone **4** (1.5 g, 3.38 mmol) in THF (30 mL). The mixture was stirred for an additional 1 h. After concentration in vacuum, the crude product was purified by flash chromatography (CH_2Cl_2/MeOH 98:2) to give a mixture of the first isomer with an impurity as an orange solid (730 mg) comprising 70% of isomer 1 (23% yield) and isomer 2 as an orange solid (360 mg, 17%), mp 185 °C.

A.19. 5-[(4-Methylphenyl)sulfonyl]-2,3,5,7-tetrahydro-6H-pyrazolo[3,4-h]pyrrolo[4,3,2-de]quinolin-6-one (7e) and 5-[(4-Methylphenyl)sulfonyl]-2,3,5,9-tetrahydro-6H-pyrazolo[4,3-h]-pyrrolo[4,3,2-de]quinolin-6-one (7f). To a solution of compounds **7c**/**7d** (700 mg, 1.40 mmol) in CH_2Cl_2 (30 mL) was added dropwise TFA (3 mL). After 30 min of stirring the mixture was concentrated over vacuum to yield the corresponding trifluoroacetate salt. A suspension of this compound and $NaHCO_3$ (300 mg) and 4 A molecular sieve (600 mg) in EtOH (50 mL) was refluxed in the dark for 3 h. The molecular sieve was filtered off, and the filtrate was concentrated over vacuum. The crude product was purified by flash chromatography (CH_2Cl_2/MeOH 97:3) to give the two separated isomers: **7f** (80 mg, 12%), mp 264 °C, Anal. ($C_{18}H_{14}N_4O_3S$) C, H, N; **7e**, white solid (180 mg, 28%), mp 248 °C, Anal. ($C_{18}H_{14}N_4O_3S$) C, H, N.

A.20. 7-Methyl-5-[(4-methylphenyl)sulfonyl]-2,3,5,7-tetrahydro-6H-pyrazolo[3,4-h]pyrrolo[4,3,2-de]quinolin-6-one (8e) and 9-Methyl-5-[(4-methylphenyl)sulfonyl]-2,3,5,9-tetrahydro-6H-pyrazolo[4,3-h]pyrrolo[4,3,2-de]quinolin-6-one (8f). The same procedure was used as for compounds **7e**/**7f** involving compounds **8c**/**8d** (800 mg, 1.61 mmol), CH_2Cl_2 (50 mL), TFA (5 mL), 30 min of stirring; $NaHCO_3$ (300 mg), 4 A molecular sieve (600 mg), and EtOH (100 mL), 3 h of reflux. Purification by flash chromatography (CH_2Cl_2/MeOH 99:1) gave the two separated isomers: **8f**, yellow solid (130 mg, 21%), mp 117 °C, Anal. ($C_{19}H_{16}N_4O_3S$) C, H, N; **8e**, yellow solid (170 mg, 28%), mp 120 °C, Anal. ($C_{19}H_{16}N_4O_3S$) C, H, N.

A.21. 5-[(4-Methylphenyl)sulfonyl]-9-phenyl-2,3,5,7-tetrahydro-6H-pyrazolo[3,4-h]pyrrolo[4,3,2-de]quinolin-6-one (9e) and 5-[(4-Methylphenyl)sulfonyl]-7-phenyl-2,3,5,9-tetrahydro-6H-pyrazolo[4,3-h]pyrrolo[4,3,2-de]quinolin-6-one (9f). The same procedure was used as for compounds **7e**/**7f** involving compounds **9c**/**9d** (630 mg, 1.13 mmol), CH_2Cl_2 (40 mL), TFA (4 mL), 1 h of stirring; $NaHCO_3$ (200 mg), 4 A molecular sieve (400 mg), and EtOH (50 mL), 4 h of reflux. Purification by flash chromatography (CH_2Cl_2/MeOH 98:2) gave the two separated isomers: isomer 1, pale-yellow solid (50 mg, 10%), mp 236 °C, Anal. ($C_{24}H_{18}N_4O_3S$) C, H, N; isomer 2, pale-yellow solid (200 mg, 40%), mp 254 °C, Anal. ($C_{24}H_{18}N_4O_3S$) C, H, N.

A.22. 5-[(4-Methylphenyl)sulfonyl]-9-(4-hydroxyphenyl)-2,3,5,7-tetrahydro-6H-pyrazolo[3,4-h]pyrrolo[4,3,2-de]quinolin-6-one (10e) and 5-[(4-Methylphenyl)sulfonyl]-7-(4-hydroxyphenyl)-2,3,5,9-tetrahydro-6H-pyrazolo[4,3-h]pyrrolo[4,3,2-de]quinolin-6-one (10f). The same procedure was used as for compounds **7e**/**7f** involving compounds **10c**/**10d** (580 mg, 0.71 mmol), CH_2Cl_2 (30 mL), TFA (3 mL), 1 h of stirring; 4 A molecular sieve (600 mg), $NaHCO_3$ (100 mg), and EtOH (50 mL), 12 h of reflux. Purification by flash chromatography (CH_2Cl_2/MeOH 98:2) gave the two separated isomers: isomer 1, pale-yellow solid (130 mg, 40%), mp 186 °C, Anal. ($C_{24}H_{18}N_4O_4S$) C, H, N; isomer 2, pale-yellow solid (40 mg, 12%), mp 192 °C, Anal. ($C_{24}H_{18}N_4O_4S$) C, H, N.

A.23. 9-(3-Hydroxy-1-methyl-2-oxo-2,3-dihydro-1H-indol-3-yl)-5-[(4-methylphenyl)sulfonyl]2,3,5,7-tetrahydro-6H-pyrazolo-[3,4-h]pyrrolo[4,3,2-de]quinolin-6-one (11e) and 9-(3-Hydroxy-1-methyl-2-oxo-2,3-dihydro-1H-indol-3-yl)-5-[(4-methylphenyl)-sulfonyl]2,3,5,9-tetrahydro-6H-pyrazolo[4,3-h]pyrrolo[4,3,2-de]-quinolin-6-one (11f). From isomer 1: the same procedure was used as for compounds **7e**/**7f** involving the mixture comprising isomer 1 of compound **11** (700 mg which corresponded to 1.08 mmol of isomer 1), CH_2Cl_2 (20 mL), TFA (2 mL), 30 min of stirring; 4 A molecular sieve (100 mg), $NaHCO_3$ (100 mg), and EtOH (25 mL), 1 h 30 min of reflux. Purification by flash chromatography (CH_2Cl_2/MeOH 97:3) gave isomer 1 of compound **11** as a yellow solid (400 mg, 70%), mp 172 °C, Anal. ($C_{27}H_{21}N_5O_5S$) C, H, N. From isomer 2: the same procedure was used as for compounds **7e**/**7f** involving isomer 2 of compound **11** (250 mg, 0.39 mmol), CH_2Cl_2 (10 mL), TFA (1 mL), 30 min of stirring; 4 A molecular sieve (100 mg), $NaHCO_3$ (50 mg), and EtOH (10 mL), 1 h 30 min of reflux. Purification by flash chromatography (CH_2Cl_2/MeOH 97:3) gave isomer 2 of compound **11** as a yellow solid (100 mg, 49%), mp 182 °C, Anal. ($C_{27}H_{21}N_5O_5S$) C, H, N.

A.24. 2,3,5,7-Tetrahydro-6*H*-pyrazolo[3,4-*h*]pyrrolo[4,3,2-*de*]-quinolin-6-one (7a). A solution of compound **7e** (80 mg, 0.22 mmol) and tetrabutylammonium fluoride (200 μL of a 1 N solution in THF) in THF (10 mL) was refluxed for 2 h. After concentration to dryness, H_2O (20 mL) was added to the residue. The precipitate was filtered and washed with H_2O and finally with Et_2O to give the expected compound as a dark yellow solid (25 mg, 55%), mp 182 °C, Anal. ($C_{11}H_9N_4O$) C, H, N.

A.25. 2,3,5,9-Tetrahydro-6*H*-pyrazolo[4,3-*h*]pyrrolo[4,3,2-*de*]-quinolin-6-one (7b). The same procedure was used as for compound **7a** involving compound **7f** (60 mg, 0.16 mmol) and tetrabutylammonium fluoride (150 μL of a solution 1 N in THF) in THF (10 mL), reflux time of 2 h. After concentration to dryness, H_2O (20 mL) was added to the residue. After workup, the expected product was obtained (20 mg, 60%), mp 198 °C, Anal. ($C_{11}H_9N_4O$) C, H, N.

A.26. 7-Methyl-2,3,5,7-tetrahydro-6*H*-pyrazolo[3,4-*h*]pyrrolo-[4,3,2-*de*]quinolin-6-one (8a). A solution of compound **8e** (500 mg, 1.31 mmol) and 1 N NaOH (10 mL) in dioxane (10 mL) was stirred at room temperature for 24 h. After concentration to dryness, the residue was purified by flash chromatography on C8 silica gel (H_2O/MeOH 80:20, 0.1% TFA) to give the expected product as a red solid (220 mg, 74%), mp 186 °C, Anal. ($C_{12}H_{11}N_4O$) C, H, N.

A.27. 9-Methyl-2,3,5,9-tetrahydro-6*H*-pyrazolo[4,3-*h*]pyrrolo-[4,3,2-*de*]quinolin-6-one (8b). The same procedure was used as for compound **8a** involving compound **8f** (100 mg, 0.26 mmol), 1 N NaOH (5 mL), and dioxane (5 mL). Flash chromatography on C8 silica gel (H_2O/MeOH 80:20, 0.1% TFA) gave the expected compound as a red solid (30 mg, 50%), mp 196 °C, Anal. ($C_{12}H_{11}N_4O$) C, H, N.

A.28. 9-Phenyl-2,3,5,7-tetrahydro-6H-pyrazolo[3,4-h]pyrrolo-[4,3,2-de]quinolin-6-one (9a) and 7-Phenyl-2,3,5,9-tetrahydro-6H-pyrazolo[4,3-h]pyrrolo[4,3,2-de]quinolin-6-one (9b). From isomer 1: the same procedure was used as for compound **8a** involving compound **9e** (80 mg, 0.18 mmol), 1 N NaOH (5 mL), and dioxane (5 mL). Flash chromatography on C8 silica gel (H_2O/MeOH 80:20, 0.1% TFA) gave the expected compound as yellow solid (39 mg, 75%), mp 171 °C, Anal. ($C_{17}H_{13}N_4O$) C, H, N. From isomer 2: the same procedure was used as for compound **8a** involving compound **9f** (50 mg, 0.11 mmol), 1 N NaOH (5 mL), and dioxane (5 mL). Flash chromatography on C8 silica gel (H_2O/MeOH 80:20, 0.1% TFA) gave the expected compound as a yellow solid (27 mg, 83%), mp 165 °C, Anal. ($C_{17}H_{13}N_4O$) C, H, N.

A.29. 9-(4-Hydroxyphenyl)-2,3,5,7-tetrahydro-6H-pyrazolo-[3,4-h]pyrrolo[4,3,2-de]quinolin-6-one (10a) and 7-(4-Hydroxyphenyl)-2,3,5,9-tetrahydro-6H-pyrazolo[4,3-h]pyrrolo[4,3,2-de]-quinolin-6-one (10b). From isomer 1: the same procedure was used as for compound **7a** involving isomer 1 of compound **10e**/**10f** (90 mg, 0.2 mmol) and tetrabutylammonium fluoride (420 μL of a solution 1 N in THF) in THF (5 mL), reflux time of 4 h. Purification by flash chromatography on C8 silica gel (H_2O/MeOH 80:20, 0.1% TFA) gave the product as a brown solid (30 mg, 50%), mp > 260 °C, Anal. ($C_{17}H_{13}N_4O_2$) C, H, N. From isomer 2: the same procedure was used as for compound **7a** involving isomer 2 of compound **10e**/**10f** (50 mg, 0.2 mmol) and tetrabutylammonium fluoride (5 mL of a solution 1 N in THF) in THF (5 mL), reflux time of 4 h. Purification by flash chromatography on C8 silica gel (H_2O/MeOH 80:20, 0.1% TFA) gave the product as a yellow solid (15 mg, 45%), mp > 260 °C, Anal. ($C_{17}H_{13}N_4O_2$) C, H, N.

A.30. 9-(3-Hydroxy-1-methyl-2-oxo-2,3-dihydro-1H-indol-3-yl)-2,3,5,7-tetrahydro-6H-pyrazolo[3,4-h]pyrrolo[4,3,2-de]quinolin-6-one (11a) and 9-(3-Hydroxy-1-methyl-2-oxo-2,3-dihydro-1H-indol-3-yl)-2,3,5,9-tetrahydro-6H-pyrazolo[4,3-h]pyrrolo[4,3,2-de]quinolin-6-one (11b). From isomer 1: the same procedure was used as for compound **8a** involving compound **11e** (100 mg, 0.19 mmol), 1 N NaOH (5 mL), and dioxane (5 mL). Flash chromatography on C_8 silica gel (H_2O/MeOH 70:30, 0.1% TFA) gave the expected compound as a red solid (51 mg, 72%), mp 193 °C, Anal. ($C_{20}H_{16}N_5O_3$) C, H, N. From isomer 2: the same procedure was used as for compound **8a** involving compound **11f** (90 mg, 0.17 mmol), 1 N NaOH (5 mL), and dioxane (5 mL). Flash chromatography on C_8 silica gel (H_2O/MeOH 70:30, 0.1% TFA) gave the expected compound as a red solid (51 mg, 72%), mp 199 °C, Anal. ($C_{20}H_{16}N_5O_3$) C, H, N.

B. Biological Studies. All compounds were weighed and prepared as a 10 mM stock solution in pure, analytical grade, DMSO. Small aliquots were stored at −80 °C and used on demand.

B.1. Evaluation of the Effects on Cell Proliferation. The cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA); HCT15 and HT29 colon carcinoma, PC3 prostate cancer, or mouse CT26 colon carcinoma or Renca renal cancer. All cell lines were checked for the absence of mycoplasma by an immunological method at the time of the experiments (mycoplasma detection kit, Boehringer Roche, Germany).

The cells were grown in RPMI (Invitrogen) medium supplemented with 10% heat-inactivated fetal calf serum (FCS), in absence of antibiotics to avoid any interference on growth, at 37 °C in a 95% air/5% CO_2 humidified atmosphere.

Exponentially growing cells (seeding density = 2000 cells/well for HCT15, PC3, CT26, RENCA; 1000 cells/well for HT29) were incubated, 24 h after seeding, for 72 h in the presence of the growth medium without phenol red containing 0.1% DMSO (control condition) or with nine concentrations of test compounds ranging from 1 nM to 10 μM with a fixed 0.1% final DMSO concentration. Cell proliferation was then assayed by the XTT reduction assay. Briefly, the cells were stained for 4 h with an XTT/PMS solution (XTT Sigma ×4626; PMS Sigma P9625). The absorbance was read using a microtiter plate reader Labsystem Multiskan (Ascent version 2.4.2) at 492 nm for four wells per concentration. The cell growth was expressed as percent (%) of the control (0.1% DMSO in the growth medium) and plotted as a function of compound concentration. Whenever possible, IC50 values were determined using a logistic four-parameter regression model (Sigmaplot, Jandel).

B.2. Inhibition of Human Topoisomerase Isoenzymes. This assay (adapted from refs 19 and 20) determines the ability of the test compounds to interfere with the cleavage catalyzed on specific sequences by the human topoisomerase isoenzymes I or II. The experiments were performed using the topoisomerase I kit (Topogen, 1018-1) + the human topoisomerase I enzyme (Topogen, 20005H-1) and the topoisomerase II kit (Topogen, 1009-1) + the human topoisomerase II enzyme (Topogen, 2000H-1), obtained from Topogen U.S.A., through Biovalley in France.

After preliminary experiments, procedures recommended by the manufacturer were modified to be as follows:

B.2.1. Topoisomerase II. An amount of 4 units of human topoisomerase II was incubated with 0.25 μg of pRYG DNA supplemented with ATP (0.5 mM final), at 37 °C, for 30 min in the absence or presence of the tested compound. The reactions were stopped by addition of SDS (1% final). After digestion with proteinase K (50 μg/mL final) at 37 °C for 15 min and addition of loading buffer, samples were extracted twice with CIA and loaded onto a 1% agarose gel. The gel was run for 3.5 h at 75 V; gels were run in the absence of ethidium bromide but stained for 45 min in a solution of ethidium bromide (0.5 μg/mL) and, finally, photographed and digitalized in a jpg image format.

B.2.2. Topoisomerase I. An amount of 2 units of topoisomerase I was incubated with 0.25 μg of DNA I at 37 °C for 30 min in the absence or presence of the compound. The reactions were stopped with SDS (1% final). After digestion with proteinase K (50 μg/mL final) at 37 °C for 15 min and addition of loading buffer, samples were extracted twice with CIA and loaded onto a 1% agarose gel. The gel was run for 3.5 h at 75 V like for topoisomerase II, in the absence of ethidium bromide, and stained, photographed, and digitalized in a jpg image format.

Addition of DMSO from 0.1% to 3% had no incidence on the activities of both enzymes (data not shown).

B.3. Active concentration was considered as the lowest concentration of test or reference compound for which complete inhibition of the enzymatic activity was observed, i.e., the absence of relaxed or linear DNA form on the gel.

Supporting Information Available: MS, NMR, IR, and elemental analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM051247F