Synthesis and Biological Activity of (Hydroxymethyl)- and (Diethylaminomethyl)benzopsoralens

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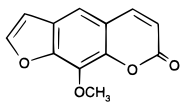
Some benzopsoralens, carrying a hydroxymethyl or a diethylaminomethyl group at the 3, 5, 8, and 11 positions, were prepared, and their biological activity was compared with that of 4-(hydroxymethyl)benzopsoralen (BP). 5-(Hydroxymethyl)benzopsoralen (**7b**), 11-(hydroxymethyl)benzopsoralen (**7c**), and 11-(diethylaminomethyl)benzopsoralen (**8c**) induced marked antiproliferative effects in mammalian cells by simple incubation in the dark; this activity appeared to be related to their ability to inhibit topoisomerase II. Benzopsoralens appeared to be more active, especially BP and **7c**, upon UVA activation. Compounds carrying a methyl group at the 4 position together with a hydroxymethyl or diethylaminomethyl at the 8 position (**7d** and **8d**, respectively) were also effective, although to a lower extent; instead, a substituent at the 3 position canceled all activity. Benzopsoralens did not induce interstrand cross-links in DNA in vitro, as seen in the induction of cytoplasmic ≪petite≫ mutations and double-strand breaks in yeast. This behavior is also compatible with their low mutagenic activity in *E. coli* WP2 and with the absence of any phototoxicity on the skin. For these features, benzopsoralens seem to be interesting potential drugs for PUVA photochemotherapy and photopheresis. The activity shown in the dark is not sufficient for their possible use as antitumor drugs, but it does offer a new model for the study of topoisomerase inhibitors.

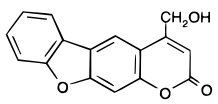
Introduction

Furocoumarins (psoralens) are well-known photosensitizing drugs which have been used in photomedicine for several years now, to cure a number of skin diseases such as psoriasis, mycosis fungoides, lupus erythematosus, etc.[1] This therapeutic treatment is generally called PUVA, from psoralen plus UVA (the wavelength range used, 320−400 nm); its therapeutic effectiveness, particularly to treat vitiligo, has been known for centuries.[2] In 1978, Edelson introduced extracorporeal photochemotherapy or photopheresis, in which lymphocytes are withdrawn, submitted to furocoumarin sensitization, and then returned to the patient.[3] This therapy was approved by the FDA for the treatment of T-cell lymphoma, but it is also successfully used for preventing rejection in organ transplants and to treat various autoimmune diseases.[4]

The compound mostly preferred for both PUVA and photopheresis is 8-methoxypsoralen (8-MOP), (Figure 1) but 5-methoxypsoralen (5-MOP) and 4,4′,8-trimethylpsoralen (TMP) are also used, particularly in some PUVA treatments.[5,6] All these compounds damage DNA, leading to monofunctional and bifunctional adducts (interstrand cross-links) with pyrimidine bases; the bifunctional damage is generally regarded as the main agent responsible for the side effects observed in PUVA therapy such as genotoxicity,[7,8] carcinogenicity,[9] and the formation of skin erythemas.[10] Recently, the formation of covalent adducts between DNA and proteins by



8-MOP



BP

Figure 1. Structures of 8-methoxypsoralen (8-MOP) and 4-(hydroxymethyl)benzopsoralen (BP).

8-MOP and by several other furocoumarins, a new aspect of their bifunctional behavior, has been reported.[11]

For these reasons, various monofunctional furocoumarins have been prepared and studied.[12,13] Among the various ways of obtaining monofunctional furocoumarins, we also studied the possibility of deleting one of the two photoreactive sites existing in the furocoumarin molecule (3,4 and 4′,5′ double bonds) by introducing a fourth nucleus into the furocoumarin skeleton at the furan ring. Following this research line, we prepared a series of compounds carrying a benzene or a cyclohexenyl ring fused at the furan side, thus obtaining benzopsoralens and tetrahydrobenzopsoralens.[14] Together with good photosensitizing activity, these derivatives also induce pronounced antiproliferative effects on mammalian cells in the dark,[15] i.e., without UVA activation; this property appears to be associated with an evident ability to inhibit topoisomerase II.[16] The presence of a hydroxymethyl group at the 4 position in the furocoumarin molecule is important for drug activity in both experimental conditions, upon UVA irradiation and by incubation in the dark.[15] Moreover, previous studies carried out on the influence of a methyl group inserted at various positions on the photosensitizing

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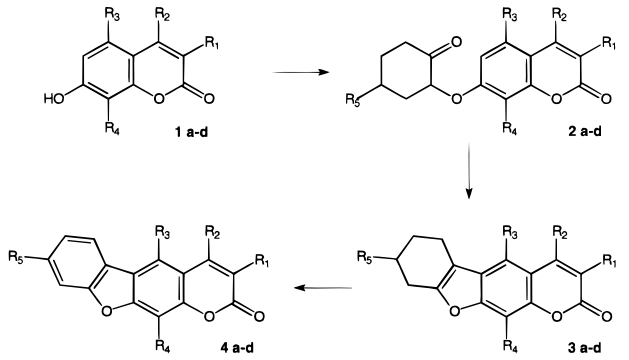
Scheme 1



	R_1	R_2	R_3	R_4	R_5
1-4 a	Me	-	-	-	-
1-4 b	-	-	Me	-	-
1-4 c	-	-	-	Me	-
1 d	-	Me	-	-	-
2-4 d	-	Me	-	-	Me

activity of both linear and angular furocoumarins[17,18] suggested that the introduction of the hydroxymethyl group variously in the benzopsoralen molecule could also modulate the activity and features of these drugs.

We therefore studied the main biological properties of a series of tetracyclic psoralen derivatives carrying a hydroxymethyl group at the 3, 5, 8, or 11 position. We also studied another series of benzopsoralens bearing a diethylaminomethyl substituent at the same positions; this group was introduced into the benzopsoralen molecule with the aim of improving its capacity to interact with DNA and, as a result, of increasing its activity in the dark.

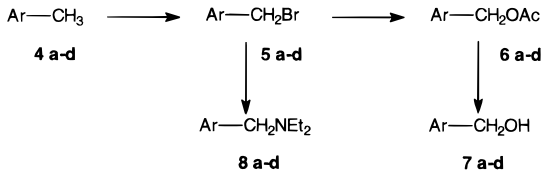
We tested all these compounds for their activity at the ground state, that is, by simple incubation in the dark, and upon UVA irradiation. As reference compounds we chose 8-MOP, a well-known photosensitizing furocoumarin widely used in photobiology and in photomedicine, and 4-(hydroxymethyl)benzopsoralen, as the most interesting tetracyclic psoralen derivative now known.[16]

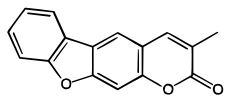
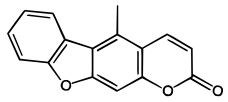
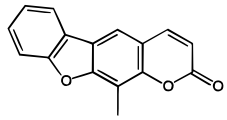
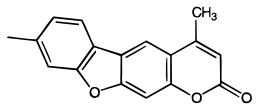
Chemistry

The synthetic route consists of building the tetracyclic nucleus already carrying the methyl group in the appropriate position, and then transforming the methyl function into a hydroxymethyl or diethylaminomethyl one. According to Scheme 1, starting materials were methyl-7-hydroxycoumarins **1a**−**d**, which were condensed with 2-chlorocyclohexanone to give the corresponding 7-*O*-(2′-oxocyclohexyloxy) ethers **2a**−**c** or with 2-bromo-4-methylcyclohexanone to give ether **2d**. Compounds **2a**−**d** were submitted to cyclization in alkaline medium,[19] yielding methyltetrahydrobenzopsoralens **3a**−**d**, which were aromatized in toluene solution by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) to the corresponding methylbenzopsoralens **4a**−**d**.

According to Scheme 2, compounds **4a**−**d** were reacted with *N*-bromosuccinimide, yielding bromination of the methyl substituents, giving bromomethyl groups.

Scheme 2



	Ar
4-8 a	
4-8 b	
4-8 c	
4-8 d	

Bromination of 4,8-dimethylbenzopsoralen (**4d**) occurs only in the 8 position, because the 4-methyl group is unaffected by substitution reactions.[20] Intermediates **5a**−**d** were submitted to acetylation, yielding the corresponding acetoxymethyl groups, which were hydrolyzed in alkaline conditions to the desired (hydroxymethyl)benzopsoralens **7a**−**d**. Direct alkaline hydrolysis of **5a**−**d** to **7a**−**d** was carried out, but the reaction time was longer and the yield lower than indirect hydrolysis through **6a**−**d**, owing to the formation of many degradation products. Alternatively, compounds **5a**−**d** were reacted with diethylamine to give the corresponding (diethylaminomethyl)benzopsoralens **8a**−**d**. Figure 1 shows the molecular structure of the two reference compounds, 8-methoxypsoralen (8-MOP) and 4-(hydroxymethyl)benzopsoralen (BP).

Spectrophotometric Properties. Table 1 shows the spectrophotometric properties of the new benzopsoralens; because they are compounds designed for photochemotherapy and photopheresis, the most important wavelength range is 320−400 nm, and in particular 365 nm, the maximum of our UVA lamp emission. All benzopsoralens exhibit good absorption at this wavelength, with values similar to those of BP and higher than those of 8-MOP (the extinction coefficients of both compounds are reported in Table 1 for comparison). This result may be associated with higher photosensitizing activity.

Interaction with DNA in Vitro

Inhibition of Topoisomerase II. The ability of benzopsoralens to inhibit topoisomerase II was studied using the relaxation test of supercoiled DNA of PM2 phage, followed by electrophoretic separation on agarose gel. The data are reported in Figure 2. After incubation in the presence of topoisomerase II, the supercoiled form completely disappeared, but after incubation in the

Table 1. Spectrophotometric Properties of the New Benzopsoralen Derivatives

compd	wavelength (nm)	extinction coefficient ($M^{-1}\cdot cm^{-1}$)
7a	365	1570
	333	15040
	269	27690
	250	31230
7b	365	4200
	339	13050
	287	20000
	270	27350
	252	26850
7c	365	2970
	337	9990
	287	18010
	270	24160
	250	24210
7d	365	3400
	337	15940
	270	31030
	250	29760
BP	365	3050
8a	365	3080
	336	15620
	287	23550
	271	29080
	250	31010
8b	365	3890
	338	15080
	288	30470
	270	32140
	250	31050
8c	365	3040
	336	12010
	287	23210
	271	30880
	250	29570
8d	365	3580
	337	15000
	270	29660
	252	32070
8-MOP	365	990

presence of compounds **7b** and **7c**, the band corresponding to the supercoiled form of PM2 DNA was comparable to that of the control sample. The same migration pattern was obtained with BP (10 μM), used in the same experimental conditions as a reference. This derivative had already been assayed for the same activity, but by a slightly different assay.[16] All the benzopsoralen derivatives were assayed in biological studies, but sometimes, to simplify data display, only the most significant ones are reported.

Formation of Interstrand Cross-Links in DNA by UVA Irradiation. The induction of interstrand cross-links by photosensitization in PM2-linearized DNA in vitro was tested. The results obtained with compound **7c** and with BP and 8-MOP, used as references, are reported in Figure 3. The DNA treated with compound **7c** produced only the band for single-stranded DNA, identical to that of the reference nontreated DNA, thus suggesting its complete inability to induce interstrand cross-links. As expected, BP gave very similar results, whereas a significant amount of DNA irradiated in the presence of 8-MOP appeared to be cross-linked, and its amount increased with the UVA dose.

Experiments with Mammalian Cells

The antiproliferative activity of the new benzopsoralens on mammalian cells was assayed by two different

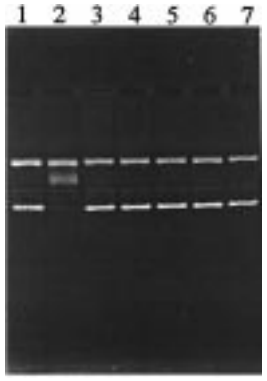


Figure 2. Inhibition of topoisomerase II activity studied by the DNA relaxation assay. Supercoiled PM2 DNA (0.125 μg) was incubated with topoisomerase II from *D. melanogaster* embryos in the presence of different concentrations of compound **7b** or **7c**. Agarose gel electrophoresis of the samples is shown. Lane 1: PM2 DNA alone, which migrates as two bands. Lane 2: PM2 DNA incubated in the presence of topoisomerase II, so showing only the relaxed form (the slower band). Lanes 3, 4: PM2 DNA incubated in the presence of topoisomerase II and compound **7b** (10 and 1 μM, respectively). Lanes 5, 6: PM2 DNA incubated in the presence of topoisomerase II and compound **7c** (10 and 1 μM, respectively). Lane 7: PM2 DNA incubated in the presence of topoisomerase II and BP, used as a reference (10 μM). The arrow indicates gel mobility.

Figure 3. Interstrand cross-links formed in vitro in PM2-linearized DNA by sensitization with benzopsoralen derivatives (2 drug molecules per base pair) detected by electrophoresis. 8-MOP and BP were also tested in the same experimental conditions. Lane 1: double-stranded DNA. Lane 2: denatured single-stranded DNA. Lanes 3, 4: DNA irradiated in the presence of 8-MOP and exposed to 3 and 5 $kJ\cdot m^{-2}$, respectively. Lanes 5, 6: DNA irradiated in the presence of compound **7c** and exposed to 3 and 5 $kJ\cdot m^{-2}$, respectively. Lanes 7, 8: DNA irradiated in the presence of BP and exposed to 3 and 5 $kJ\cdot m^{-2}$, respectively. The arrow indicates the direction of the electrophoretic migration.

experimental conditions: by incubation at the ground state keeping cells in the dark or by UVA irradiation. In both cases, inhibition of DNA synthesis and of clonal growth was evaluated.

At the Ground State. Table 2 summarizes the results obtained studying the antiproliferative activity of benzopsoralens in the dark. The most active compound appears to be BP, but compound **7c** also induced an evident antiproliferative effect; compounds **7b** and

Table 2. Antiproliferative Activity of the New Benzopsoralens in the Dark

	IC_{50}-dark ± SD[a]	
compd	DNA synthesis	clonogenic assay
8-MOP	>30	>30
BP	0.94 ± 0.23	3.8 ± 1.2
7a	>30	>30
7b	10.9 ± 0.31	25 ± 3.9
7c	3.0 ± 0.18	6.8 ± 1.9
7d	>30	>30
8a	>30	>30
8b	>30	>30
8c	10.7 ± 2	10.7 ± 2.1
8d	>30	>30

[a] The IC_{50}-dark is the drug concentration (μM) necessary to induce a 50% inhibition; the data were computed by probit analysis and are reported together with the standard deviation.

Table 3. Antiproliferative Activity of the New Benzopsoralens by UVA Irradiation

	IC_{50}-UVA ± SD[a]	
compd	DNA synthesis	clonogenic assay
8-MOP	3.59 ± 0.12	1.23 ± 0.21
BP	0.67 ± 0.27	0.11 ± 0.03
7a	16.8 ± 0.37	1.06 ± 0.18
7b	8.3 ± 0.23	0.23 ± 0.02
7c	0.97 ± 0.12	0.19 ± 0.02
7d	0.48 ± 0.13	0.16 ± 0.025
8a	>30	>30
8b	>30	>30
8c	9.3 ± 0.3	0.65 ± 0.19
8d	2.22 ± 0.17	1.83 ± 0.23

[a] The IC_{50}-UVA is the dose of UVA light ($kJ \cdot m^{-2}$) which in the presence of a fixed drug concentration (20 μM) induces a 50% inhibition; the data were computed by probit analysis and are reported together with the standard deviation.

8c showed moderate activity, while **7a**, **7d**, **8a**, **8b**, and **8d** were practically inactive.

By UVA Irradiation. Table 3 shows the data obtained by UVA irradiation; these experiments were carried out in the presence of a fixed compound concentration (20 μM) and increasing UVA doses. Therefore, in this case, an IC_{50} is the UVA dose which induces 50% inhibition when delivered in the presence of the compound studied. In addition, care was taken to avoid a dark effect, as described in the Experimental Section. Control experiments with UVA light alone were also carried out, without any detectable effects (data not shown). In these experimental conditions the new benzopsoralens showed pronounced antiproliferative activity; only two compounds appeared to be inactive, **8a** and **8b**. Again, BP appeared to be the most effective derivative, but compounds **7c** and **7d** showed comparable activity; even **7a**, **8c**, and **8d** induced evident antiproliferative effects.

Mutagenic Activity

The genotoxic activity of benzopsoralens was detected in *E. coli* TM9, a strain very sensitive to the formation of C_4-cycloadducts of furocoumarins.[21] These experiments were also carried out at a fixed concentration of test compound (20 μM) and increasing the UVA dose. Figure 4 shows the results obtained with **7a**, **7b**, **7c**, BP, and 8-MOP, plotting the number of revertants per million survivors against the surviving fraction; thus, genotoxic activity is estimated at the same level of antiproliferative effect. Practically all the benzopsoralens (data relating to other derivatives are omitted for clarity) were capable of forming very similar levels of revertants. Instead, 8-MOP, tested in the same experimental conditions, appeared to be much more effective, forming a higher number of mutants: this result may be explained by recalling its ability to induce bifunctional adducts in DNA.[22]

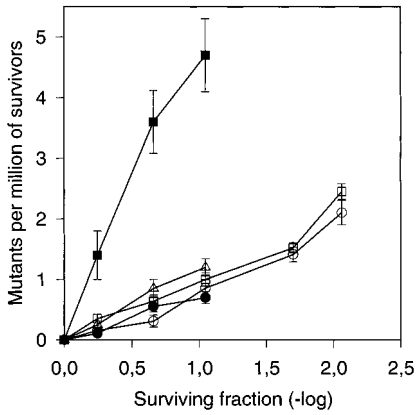


Figure 4. Mutagenicity in *E. coli* TM9 after sensitization with benzopsoralens. Bacteria were exposed to increasing UVA light doses in the presence of benzopsoralens (20 μM). The surviving fraction and the number of mutants/million survivors were determined. Results are reported plotting revertants against the surviving fraction obtained in the same treatment: Δ, **7a**; ○, **7b**; □, **7c**; ●, BP; ■, 8-MOP. The data related to the other compounds are not shown for clarity and because they are practically superimposable to that reported.

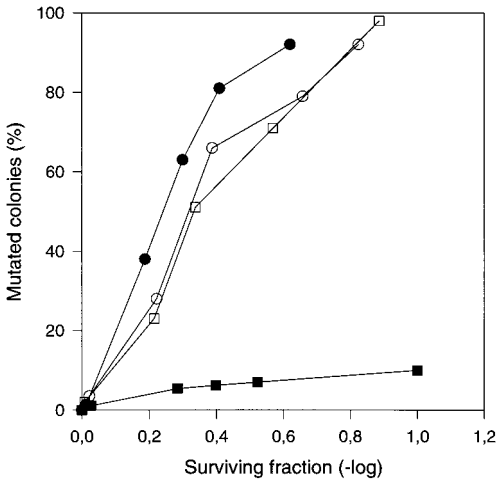


Figure 5. Formation of cytoplasmic ≪petite≫ mutants in *S. cerevisiae* D7 after sensitization with benzopsoralens. Yeast cells were irradiated with increasing UVA light doses in the presence of benzopsoralens (20 μM). The number of mutated colonies was plotted against the surviving fraction: ○, **7b**; □, **7c**; ●, BP; ■, 8-MOP.

Experiments with Yeast

Induction of Cytoplasmic ≪Petite≫ Mutations. Figure 5 shows the induction of cytoplasmic ≪petite≫ mutants in a diploid wild-type (D_7) strain of *Saccharomyces cerevisiae* as a function of survival after exposure to increasing doses of UVA light in the presence (20 μM) of compounds **7b**, and **7c**; 8-MOP and BP were used as references. Unlike 8-MOP, the mutagenic activity of benzopsoralens on mitochondrial DNA is very high; at

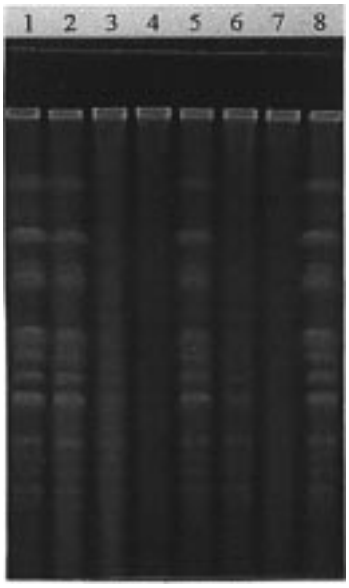


Figure 6. Induction of DNA double-strand breaks during posttreatment incubation of diploid yeast cells. Lane 1: untreated control. Lane 2: untreated control + 3 h of incubation. Lanes 3−5: cells treated with compound **7c** (1 μM) and UVA (3 kJ·m^{-2}) + no, + 15 min, and + 3 h of incubation, respectively. Lanes 6−8: cells treated with BP (1 μM) and UVA (3 kJ·m^{-2}) + no, + 15 min, and + 3 h of incubation, respectively.

50% of survival, BP, **7b**, **7c**, and 8-MOP produced respectively about 62%, 45%, 40%, and 5% of cytoplasmic ≪petite≫ mutations. These results fit the monofunctional activity of the new benzopsoralens. In fact, as is known, damage to mitochondrial DNA is not repaired; therefore, while interstrand cross-links forming in mitochondrial DNA are nearly always lethal, monoadducts can bypass and therefore induce mutants. Conversely, both lesions forming in chromosomal DNA are repaired, and thus the strong genotoxic activity of interstrand cross-links can be scored.[23]

Induction of DNA Double-Strand Breaks. Using pulsed-field gel electrophoresis (PFGE), the induction of double-strand breaks in DNA (DSB) in yeast strain D7 was determined. Figure 6 shows the data obtained by UVA irradiation in the presence of BP or **7c**. After treatment, but without any incubation after treatment (lanes 3, 6), the 12 bands corresponding to the 16 yeast chromosomes are clearly visible and their intensities are comparable with those obtained with untreated controls (lane 1). Conversely, when allowing for repair of potential lethal damage by holding treated cells in complete growth medium, a significant increase in DSB was observed. In fact, after 15 min of incubation postsensitization with compound **7c** or BP (lanes 4, 7), the first 7 chromosomal bands are scarcely visible; moreover, after 3 h of incubation, all bands totally disappeared (lanes 5, 8). These data suggest that neither compound **7c** nor BP directly induced DSB, which are formed by the enzymatic repair of the lesions induced in yeast DNA by the two benzopsoralen derivatives. This result is consistent with the data already obtained:[24] it is suggested that bifunctional damage (interstrand cross-links) induces much more DSB in DNA than the monofunctional one and that this fact is related to the different pathways of DNA repair of these two kinds of lesions. Even failing quantitative DSB determinations, it is clear that benzopsoralens are capable of inducing high DNA fragmentation. This observation, together with their monofunctional behavior, indicates that the number of monoadducts forming by benzopsoralens in DNA is very high.

Table 4. Phototoxicity on Guinea Pig Skin

compd	μM·cm^{-2}	UVA dose (kJ·m^{-2})	intensity of erythema[a]
BP	30	20	− − − −
7a	30	20	− − − −
7b	30	20	− − − −
7c	30	20	− − − −
7d	30	20	− − − −
8a	30	20	− − − −
8b	30	20	− − − −
8c	30	20	− − − −
8d	30	20	− − − −
8-MOP	2	5	− − − −
	4	5	+ − − −
	30	5	+ + + +

[a] Symbols: − − − −, no erythema; + − − −, barely visible erythema; + + − −, light erythema; + + + −, medium erythema; + + + +, strong erythema with edema.

Skin Phototoxicity

Skin phototoxicity was detected on albino guinea pig skin (Table 4); despite the severe experimental conditions used (compound concentration 30 μM·cm^{-2}; UVA light dose 20 kJ·m^{-2}) no erythemas were observed with any of the compounds tested, except 8-MOP, used as a positive reference. These results are consistent with the hypothesis of an association between the induction of bifunctional damage and the formation of skin erythemas.

Conclusions

The presence of a hydroxymethyl or diethylaminomethyl group at various positions in the benzopsoralen skeleton confers good antiproliferative activity on the molecule. However, the results are somewhat different according to the specific position involved and the experimental conditions considered, i.e., with or without UVA activation.

In fact, upon incubation in the dark, derivatives carrying the hydroxymethyl group at the 4, 5, or 11 position (BP, **7b**, **7c**) showed more pronounced antiproliferative activity, although to different extents; among the diethylaminomethyl series, only the 11-substituted derivative (**8c**) is effective. This activity appeared to be related to the capacity of such derivatives to inhibit topoisomerase II, as demonstrated in an in vitro assay.

In general, upon UVA irradiation, benzopsoralens induce higher antiproliferative effects than in the dark. The same derivatives regarding dark activity (BP, **7b**, **7c**, **8c**) appear to be good photosensitizing drugs; however, compounds **7d** and **8d** also showed marked activity. Among the 3-substituted benzopsoralens, only compound **7a** induced a significant antiproliferative effect, especially in the clonogenic assay.

Summarizing, in general we can say that, in both experimental conditions, in the dark and upon UVA irradiation, the hydroxymethyl group is a slightly better substituent than the diethylaminomethyl one. In the dark, the 4 position is certainly the best choice for the

hydroxymethyl-substituted compounds, followed by positions 11 and 5; in the same experimental conditions, only the 11-diethylaminomethyl derivative was active.

Upon UVA activation, again BP was the most effective derivative (even more than 8-MOP), but compound **7c** (having the hydroxymethyl group at the 11 position) showed similar activity, immediately followed by **7b**. Instead, compound **7a**, substituted at the 3 position, was poorly active. This last result is not surprising, considering the already known data on the photosensitizing activity of methylpsoralens[17] and methylangelicins.[18] The insertion of the substituent at the 3 position strongly reduces the sensitizing potency of the molecule, attributed to steric hindrance generated by the methyl group introduced near the photoreactive 3,4 double bond. It is probably the same for the hydroxymethyl group. Instead, the 5 and 11 positions in benzopsoralens correspond to the 5 and 8 positions in psoralens; the insertion of a methyl or hydroxymethyl group yields active photosensitizers.

With diethylaminomethyl-substituted derivatives, positive results were obtained only with compounds **8c** and **8d**. On the basis of these results, we can say that the kind of substituent and the position in which it is inserted are both very important for the best activity.

Because the formation of interstrand cross-links (ISC) in DNA is postulated to be mainly responsible for PUVA toxicity, we tested the ability of the new benzopsoralens to induce this lesion by an in vitro assay, using linearized DNA of PM2 plasmid and electrophoresis; all benzopsoralens appeared to be entirely incapable of inducing ISC. This result is consistent with the hypothesis of a complete block of the photoreactive site at the furan side by fusing the fourth benzene nucleus. To confirm the monofunctional behavior of benzopsoralens and to check their genotoxicity, additional experiments on bacteria were carried out. *E. coli* TM9 was chosen, a strain reverted by base substitution mutagens. Because it is defective in DNA repair (*uvrA*) and contains a plasmid (R46) coding error-prone DNA repair, it is very sensitive to the formation of furocoumarin C_4-cycloadducts. In such a system, benzopsoralens showed very similar, low mutagenic activity, while 8-methoxypsoralen, the drug currently used in human therapy (used here as a reference), appeared to be a very strong mutagen. This result may be explained by considering the ability of 8-methoxypsoralen to induce ISC, unlike benzopsoralens, which cannot form this lesion.

Benzopsoralens were also studied in yeast, scoring the cytoplasmic ≪petite≫ mutations, as consequences of damage to mitochondrial DNA. It is well-known that these mutations are better induced by monofunctional damage.[24] In fact, with bifunctional compounds (e.g., 8-methoxypsoralen), the main effects which are scored are the levels of chromosomal DNA.[25] Therefore, the high activity of benzopsoralens in inducing ≪petite≫ mutations consists of the induction of a monofunctional damage in DNA.

Using the technique of pulse field electrophoresis, the formation of double-strand breaks (DSB) was checked in yeast chromosomal DNA. DSBs are known to be severe lesions with very dangerous consequences, like the formation of chromosomal aberrations.[25] We ascertained that photochemical reactions caused by benzopsoralens upon UVA irradiation do not form DSBs; because DSBs can be formed by DNA repair,[26] their formation by incubating yeast cells after photosensitization with benzopsoralens was also studied. In these experimental conditions, the formation of DSBs, which are very probably formed by an enzymatic process of DNA repair, was observed. This result is consistent with the data already obtained by Averbeck et al.;[25] considering the reduced DSB frequency by monofunctional damage,[25] together with the high DNA fragmentation observed, we can conclude that benzopsoralens can induce a very high amount of monoadducts into DNA.

Last, we studied benzopsoralen phototoxicity in guinea pig skin, as the ability of bifunctional furocoumarins such as 8-methoxypsoralen to induce severe erythemas on the skin is a well-known and serious side effect of PUVA therapy.[11] Benzopsoralens appeared to be entirely unable to cause skin phototoxicity, even when tested in harsh experimental conditions.

Considering all these results, we can say that benzopsoralen derivatives are interesting and active potential drugs for PUVA therapy and photopheresis, showing good activity together with reduced side effects such as genotoxicity and skin phototoxicity in comparison with 8-methoxypsoralen, the drug presently used in this field. The activity shown by benzopsoralens in the dark is also interesting, although it is not sufficient to suggest their use as antitumor drugs. However, benzopsoralens can be good models for the study of new drugs having antitopoisomerase activity.

Experimental Section

Chemistry. Melting points were determined on a Gallenkamp MFB-595-010M melting point apparatus and are uncorrected. Analytical TLC was performed on precoated 60 F_{254} silica gel plates (0.25 mm; Merck) developing with an EtOAc/cyclohexane mixture (1:1) unless overwise indicated. Preparative column chromatography was performed using silica gel 60 (0.063−0.100 mm; Merck), eluting with $CHCl_3$. ^{1}H NMR spectra were recorded on a Varian Gemini-200 spectrometer with TMS as internal standard. UV spectra were taken in absolute ethanol using a Kontron UVIKON-930 UV−visible spectrophotometer. Elemental analyses were obtained on all intermediates and are within ±0.4% of theoretical values. Starting 7-hydroxycoumarins **1b**[27] and **1c**[28] were prepared according to literature methods.

3-Methyl-7-hydroxycoumarin (1a). A mixture of 2,4-dihydroxybenzaldehyde (25.0 g, 181.0 mmol), propionic anhydride (50.8 g, 50.0 mL, 389.9 mmol), sodium propionate (25.0 g), and piperidine (0.5 mL) was refluxed until aldehyde disappeared (1 h, TLC). H_2SO_4 (25 mL) was cautiously added to the reaction mixture, and the solution was kept at room temperature for 0.5 h. The mixture was poured into cold water (2 L), and the precipitate was collected, washed with water, and crystallized from MeOH to give **1a** (12.7 g, 40%): mp 232 °C; ^{1}H NMR (DMSO-d_6) δ 10.40 (br s, 1H, −OH), 7.77 (q, 1H, J = 1.2 Hz, 4-H), 7.75 (d, 1H, J = 8.4 Hz, 5-H), 6.78 (dd, 1H, J = 8.4, 2.3 Hz, 6-H), 6.71 (d, 1H, J = 2.3 Hz, 8-H), 2.05 (d, 3H, J = 1.2 Hz, 3-Me). Anal. ($C_{10}H_8O_3$) C,H.

Methyl-7-(2′-oxocyclohexyloxy)coumarins 2a−d. General Procedure. A mixture of **1** (70.0 mmol), 2-chlorocyclohexanone (105.0 mmol), and anhydrous K_2CO_3 (40.0 g) in acetone (800 mL) was refluxed until **1** disappeared (72 h, TLC). After cooling, the solid was filtered off and washed with fresh acetone. The solvent was evaporated from the combined filtrate and washings, and the residue was crystallized from MeOH to give **2**.

3-Methyl-7-(2′-oxocyclohexyloxy)coumarin (2a): yield 75%; mp 184 °C; ^{1}H NMR ($CDCl_3$) δ 7.43 (q, 1H, J = 1.3 Hz,

4-H), 7.29 (d, 1H, J = 8.5 Hz, 5-H), 6.80 (dd, 1H, J = 8.5, 2.4 Hz, 6-H), 6.69 (d, 1H, J = 2.4 Hz, 8-H), 4.72 (dd, 1H, J = 9.9, 5.5 Hz, 1′-H), 2.66−2.34 (m, 4H, 3′-H and 6′-H), 2.16 (d, 3H, J = 1.3 Hz, 3-Me), 2.13−1.74 (m, 4H, 4′-H and 5′-H). Anal. ($C_{16}H_{16}O_4$) C,H.

5-Methyl-7-(2′-oxocyclohexyloxy)coumarin (2b): yield 71%; mp 178 °C; ^{1}H NMR ($CDCl_3$) δ 7.80 (d, 1H, J = 9.9 Hz, 4-H), 6.68 (d, 1H, J = 2.2 Hz, 8-H), 6.51 (d, 1H, J = 2.2 Hz, 6-H), 6.24 (d, 1H, J = 9.9 Hz, 3-H), 4.73 (dd, 1H, J = 9.5, 5.5 Hz, 1′-H), 2.65−2.35 (m, 4H, 3′-H and 6′-H), 2.45 (s, 3H, 5-Me), 2.09−1.73 (m, 4H, 4′-H and 5′-H). Anal. ($C_{16}H_{16}O_4$) C,H.

8-Methyl-7-(2′-oxocyclohexyloxy)coumarin (2c): yield 72%; mp 171 °C; ^{1}H NMR (DMSO-d_6) δ 7.97 (d, 1H, J = 9.4 Hz, 4-H), 7.47 (d, 1H, J = 8.8 Hz, 5-H), 6.89 (d, 1H, J = 8.8 Hz, 6-H), 6.29 (d, 1H, J = 9.4 Hz, 3-H), 5.23 (dd, 1H, J = 9.7, 5.7 Hz, 1′-H), 2.66−2.29 (m, 4H, 3′-H and 6′-H), 2.23 (s, 3H, 8-Me), 2.07−1.55 (m, 4H, 4′-H and 5′-H). Anal. ($C_{16}H_{16}O_4$) C,H.

4-Methyl-7-(2′-oxo-5′-methylcyclohexyloxy)coumarin (2d). This compound was prepared according to the general procedure, using 2-bromo-4-methylcyclohexanone. This reagent was obtained reacting pyrrolidone hydrotribromide (57.0 g, 115.0 mmol) with 4-methylcyclohexanone (12.9 g, 14.1 mL, 115.0 mmol) in THF (400 mL) at room temperature for 6 h, filtering off the solid and removing the solvent from the filtrate under reduced pressure: the residue so obtained was used without further purification. Compound **2d**: yield 87%; mp 207 °C; ^{1}H NMR ($CDCl_3$) δ 7.48 (d, 1H, J = 8.8 Hz, 5-H), 6.84 (dd, 1H, J = 8.8, 2.5 Hz, 6-H), 6.65 (d, 1H, J = 2.5 Hz, 8-H), 6.13 (q, 1H, J = 1.2 Hz, 3-H), 4.83 (dd, 1H, J = 12.2, 6.2 Hz, 1′-H), 2.52 (m, 2H, 3′-H), 2.43 (m, 1H, 6′-H), 2.38 (d, 3H, J = 1.2 Hz, 4-Me), 2.16−2.04 (m, 1H, 4′-H), 1.78−1.39 (m, 3H, 6′-H and 5′-H), 1.12 (d, 3H, J = 6.3 Hz, 5′-Me). Anal. ($C_{17}H_{18}O_4$) C,H.

Methyl-6,7,8,9-tetrahydro-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-ones 3a−d. General Procedure.[19] To an ethanolic solution (200 mL) of **2** (50.0 mmol) was added a 5% ethanolic potassium hydroxide solution (600 mL), and the mixture was refluxed in the dark for 1 h. The solution was cooled, diluted with water (1 L), and acidified with diluted HCl. The precipitate obtained was collected and crystallized from MeOH, to give **3**.

3-Methyl-6,7,8,9-tetrahydro-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (3a): yield 85%; mp 195 °C; ^{1}H NMR ($CDCl_3$) δ 7.58 (q, 1H, J = 1.2 Hz, 4-H), 7.36 (s, 1H, 5-H), 7.34 (s, 1H, 11-H), 2.77−2.70 (m, 2H, 9-H), 2.66−2.59 (m, 2H, 6-H), 2.21 (d, 3H, J = 1.2 Hz, 3-Me), 1.98−1.83 (m, 4H, 7-H and 8-H). Anal. ($C_{16}H_{14}O_3$) C,H.

5-Methyl-6,7,8,9-tetrahydro-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (3b): yield 57%; mp 201 °C; ^{1}H NMR ($CDCl_3$) δ 7.97 (d, 1H, J = 9.9 Hz, 4-H), 7.16 (s, 1H, 11-H), 6.30 (d, 1H, J = 9.9 Hz, 3-H), 2.89−2.83 (m, 2H, 9-H), 2.74−2.69 (m, 2H, 6-H), 2.67 (s, 3H, 5-Me), 1.95−1.83 (m, 4H, 7-H and 8-H). Anal. ($C_{16}H_{14}O_3$) C,H.

11-Methyl-6,7,8,9-tetrahydro-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (3c): yield 68%; mp 182 °C; ^{1}H NMR ($CDCl_3$) δ 7.77 (d, 1H, J = 9.5 Hz, 4-H), 7.28 (s, 1H, 5-H), 7.33 (d, 1H, J = 9.5 Hz, 3-H), 2.80−2.73 (m, 2H, 9-H), 2.66−2.59 (m, 2H, 6-H), 2.57 (s, 3H, 11-Me), 1.99−1.85 (m, 4H, 7-H and 8-H). Anal. ($C_{16}H_{14}O_3$) C,H.

4,8-Dimethyl-6,7,8,9-tetrahydro-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (3d): yield 82%; mp 192 °C; ^{1}H NMR ($CDCl_3$) δ 7.54 (s, 1H, 5-H), 7.36 (s, 1H, 11-H), 6.24 (q, 1H, J = 1.2 Hz, 3-H), 2.91−1.93 (m, 6H, 6-H, 7-H, 8-H and 9-H), 2.50 (d, 1H, J = 1.2 Hz, 4-Me), 1.58−1.46 (m, 1H, 8-H), 1.16 (d, 1H, J = 6.6 Hz, 8-Me). Anal. ($C_{17}H_{16}O_3$) C,H.

Methyl-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-ones 4a−d. General Procedure. A mixture of **3** (30 mmol) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (75 mmol) in anhydrous toluene (800 mL) was refluxed for 24 h. After cooling, the solid was filtered off and the solvent evaporated under reduced pressure. The residue was purified by column chromatography and crystallized from MeOH to give **4**.

3-Methyl-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (4a): yield 56%; mp 267 °C; ^{1}H NMR ($CDCl_3$) δ 7.96 (ddd, 1H, J = 7.5, 1.5, 0.7 Hz, 9-H), 7.95 (s, 1H, 5-H), 7.95 (qd, 1H, J = 1.4, 0.6 Hz, 4-H), 7.59 (ddd, 1H, J = 8.1, 1.4, 0.7 Hz, 6-H), 7.51 (d, 1H, J = 0.6 Hz, 11-H), 7.50 (ddd, 1H, J = 8.1, 7.1, 1.5 Hz, 7-H), 7.39 (ddd, 1H, J = 7.5, 7.1, 1.4 Hz, 8-H), 2.26 (d, 3H, J = 1.4 Hz, 3-Me). Anal. ($C_{16}H_{10}O_3$) C,H.

5-Methyl-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (4b): yield 87%; mp 241 °C; ^{1}H NMR ($CDCl_3$) δ 8.04 (d, 1H, J = 9.9 Hz, 4-H), 8.00 (ddd, 1H, J = 7.2, 1.4, 0.7 Hz, 9-H), 7.54 (ddd, 1H, J = 8.0, 1.5, 0.7 Hz, 6-H), 7.47 (ddd, 1H, J = 8.0, 6.9, 1.4 Hz, 7-H), 7.37 (ddd, 1H, J = 7.2, 6.9, 1.5 Hz, 8-H), 7.27 (s, 1H, 11-H), 6.36 (d, 1H, J = 9.9 Hz, 3-H), 2.87 (s, 3H, 5-Me). Anal. ($C_{16}H_{10}O_3$) C,H.

11-Methyl-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (4c): yield 76%; mp 224 °C; ^{1}H NMR ($CDCl_3$) δ 7.94 (ddd, 1H, J = 7.5, 1.5, 0.8 Hz, 9-H), 7.85 (s, 1H, 5-H), 7.84 (d, 1H, J = 9.5 Hz, 4-H), 7.61 (ddd, 1H, J = 8.1, 1.3, 0.8 Hz, 6-H), 7.50 (ddd, 1H, J = 8.1, 7.2, 1.4 Hz, 7-H), 7.38 (ddd, 1H, J = 7.5, 7.2, 1.3 Hz, 8-H), 6.40 (d, 1H, J = 9.5 Hz, 3-H), 2.67 (s, 3H, 11-Me). Anal. ($C_{16}H_{10}O_3$) C,H.

4,8-Dimethyl-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (4d): yield 88%; mp 237 °C; ^{1}H NMR ($CDCl_3$) δ 7.97 (s, 1H, 5-H), 7.79 (d, 1H, J = 7.9 Hz, 6-H), 7.40 (s, 1H, 11-H), 7.34 (dq, 1H, J = 0.7, 0.7 Hz, 9-H), 7.19 (dd, 1H, J = 7.9, 0.7 Hz, 7-H), 6.25 (q, 1H, J = 1.2 Hz, 3-H), 2.53 (d, 3H, J = 0.7 Hz, 8-Me), 2.52 (d, 3H, J = 1.2 Hz, 4-Me). Anal. ($C_{17}H_{12}O_3$) C,H.

(Bromomethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-ones 5a−d. General Procedure. A mixture of **4** (25 mmol) and *N*-bromosuccinimide (37.5 mmol) in anhydrous benzene (500 mL) was refluxed until starting product had disappeared (10−24 h, TLC: $CHCl_3$/MeOH, 95:5). After cooling, the solvent was evaporated under reduced pressure. The residue was purified by column chromatography and crystallized from EtOAc to give **5**.

3-(Bromomethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (5a): yield 81%; mp 262 °C; ^{1}H NMR ($CDCl_3$) δ 8.04 (s, 1H, 5-H), 8.02 (dt, 1H, J = 1.3, 0.6 Hz, 4-H), 7.97 (ddd, 1H, J = 7.6, 1.5, 0.7 Hz, 9-H), 7.61 (ddd, 1H, J = 8.2, 1.4, 0.7 Hz, 6-H), 7.54 (d, 1H, J = 0.6 Hz, 11-H), 7.53 (ddd, 1H, J = 8.2, 7.1, 1.5 Hz, 7-H), 7.41 (ddd, 1H, J = 7.6, 7.1, 1.4 Hz, 8-H), 4.49 (d, 2H, J = 1.3 Hz, 3-CH_2Br). Anal. ($C_{16}H_9BrO_3$) C,H,Br.

5-(Bromomethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (5b): yield 76%; mp 253 °C; ^{1}H NMR ($CDCl_3$) δ 8.16 (dd, 1H, J = 9.9, 0.7 Hz, 4-H), 8.12 (ddd, 1H, J = 7.5, 1.6, 0.8 Hz, 9-H), 7.65 (ddd, 1H, J = 8.0, 1.7, 0.8 Hz, 6-H), 7.58 (ddd, 1H, J = 8.0, 6.9, 1.6 Hz, 7-H), 7.52 (d, 1H, J = 0.7 Hz, 11-H), 7.48 (ddd, 1H, J = 7.5, 6.9, 1.7 Hz, 8-H), 6.56 (d, 1H, J = 9.9 Hz, 3-H), 5.15 (s, 2H, 5-CH_2Br). Anal. ($C_{16}H_9BrO_3$) C,H,Br.

11-(Bromomethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (5c): yield 72%; mp 267 °C; ^{1}H NMR ($CDCl_3$) δ 7.96 (s, 1H, 5-H), 7.94 (dd, 1H, J = 7.5, 1.4 Hz, 9-H), 7.83 (d, 1H, J = 9.6 Hz, 4-H), 7.66 (dd, 1H, J = 8.1, 1.2 Hz, 6-H), 7.53 (ddd, 1H, J = 8.1, 7.3, 1.4 Hz, 7-H), 7.41 (ddd, 1H, J = 7.5, 7.3, 1.2 Hz, 8-H), 6.43 (d, 1H, J = 9.6 Hz, 3-H), 5.04 (s, 2H, 11-CH_2-Br). Anal. ($C_{16}H_9BrO_3$) C,H,Br.

8-(Bromomethyl)-4-methyl-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (5d): yield 57%; mp 245 °C; ^{1}H NMR ($CDCl_3$) δ 8.11 (s, 1H, 5-H), 7.94 (dd, 1H, J = 8.0, 0.6 Hz, 6-H), 7.63 (dd, 1H, J = 1.5, 0.6 Hz, 9-H), 7.51 (s, 1H, 11-H), 7.43 (dd, 1H, J = 8.0, 1.5 Hz, 7-H), 6.31 (q, 1H, J = 1.3 Hz, 3-H), 4.67 (s, 2H, 8-CH_2Br), 2.58 (d, 3H, J = 1.3 Hz, 4-Me). Anal. ($C_{17}H_{11}$-BrO_3) C,H,Br.

(Acetoxymethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-ones 6a−d. General Procedure. A mixture of **5** (10 mmol) and anhydrous AcONa (1.0 g) in acetic anhydride (50 mL) was refluxed for 1 h. The mixture was cautiously diluted with water (50 mL) and poured into cold water (250 mL). The precipitate obtained was filtered, washed with water, and crystallized from MeOH to give **6**.

3-(Acetoxymethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (6a): yield 91%; mp 230 °C; ^{1}H NMR ($CDCl_3$) δ 8.05 (t, 1H, J = 1.0 Hz, 4-H), 7.96 (ddd, 1H, J = 7.7, 1.5, 0.7 Hz, 9-H), 7.93 (s, 1H, 5-H), 7.61 (ddd, 1H, J = 8.2, 1.4, 0.7 Hz, 6-H), 7.53 (s, 1H, 11-H), 7.52 (ddd, 1H, J = 8.2, 7.1, 1.5 Hz, 7-H),

7.41 (ddd, 1H, J = 7.7, 7.1, 1.4 Hz, 8-H), 5.11 (d, 2H, J = 1.0 Hz, 3-CH_2OAc), 2.18 (s, 3H, Ac). Anal. ($C_{18}H_{12}O_5$) C,H.

5-(Acetoxymethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (6b): yield 98%; mp 248 °C; ^{1}H NMR ($CDCl_3$) δ 8.31 (d, 1H, J = 9.9 Hz, 4-H), 8.13 (ddd, 1H, J = 7.5, 1.3, 0.8 Hz, 9-H), 7.62 (ddd, 1H, J = 8.1, 1.7, 0.8 Hz, 6-H), 7.55 (ddd, 1H, J = 8.1, 7.0, 1.3 Hz, 7-H), 7.53 (s, 1H, 11-H), 7.44 (ddd, 1H, J = 7.6, 7.0, 1.6 Hz, 8-H), 6.50 (d, 1H, J = 9.9 Hz, 3-H), 5.82 (s, 2H, 5-CH_2OAc), 2.10 (s, 3H, Ac). Anal. ($C_{18}H_{12}O_5$) C,H.

11-(Acetoxymethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (6c): yield 85%; mp 204 °C; ^{1}H NMR ($CDCl_3$) δ 8.04 (s, 1H, 5-H), 7.96 (dd, 1H, J = 7.5, 1.4 Hz, 9-H), 7.87 (d, 1H, J = 9.6 Hz, 4-H), 7.64 (dd, 1H, J = 8.1, 1.3 Hz, 6-H), 7.53 (ddd, 1H, J = 8.1, 7.2, 1.4 Hz, 7-H), 7.41 (ddd, 1H, J = 7.5, 7.2, 1.3 Hz, 8-H), 6.44 (d, 1H, J = 9.6 Hz, 3-H), 5.70 (s, 2H, 11-CH_2-OAc), 2.13 (s, 3H, Ac). Anal. ($C_{18}H_{12}O_5$) C,H.

8-(Acetoxymethyl)-4-methyl-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (6d): yield 95%; mp 220 °C; ^{1}H NMR ($CDCl_3$) δ 8.10 (s, 1H, 5-H), 7.94 (dd, 1H, J = 7.9, 0.6 Hz, 6-H), 7.59 (dd, 1H, J = 1.4, 0.6 Hz, 9-H), 7.48 (s, 1H, 11-H), 7.39 (dd, 1H, J = 7.9, 1.4 Hz, 7-H), 6.29 (q, 1H, J = 1.3 Hz, 3-H), 5.27 (s, 2H, 8-CH_2OAc), 2.56 (d, 3H, J = 1.3 Hz, 4-Me), 2.15 (s, 3H, Ac). Anal. ($C_{19}H_{14}O_5$) C,H.

(Hydroxymethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-ones 7a−d. General Procedure. To a methanolic solution (100 mL) of **6** (9.0 mmol) was added a 5% methanolic potassium hydroxide solution (100 mL), and the mixture was refluxed in the dark for 1 h. The solution was cooled, diluted with cold water (500 mL), and acidified with diluted HCl. The precipitate obtained was collected and crystallized from EtOAc to give **7**.

3-(Hydroxymethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (7a): yield 59%; mp 272 °C; ^{1}H NMR (DMSO-d_6) δ 8.55 (s, 1H, 5-H), 8.17 (ddd, 1H, J = 7.5, 1.6, 0.7 Hz, 9-H), 8.12 (t, 1H, J = 1.5 Hz, 4-H), 7.85 (s, 1H, 11-H), 7.76 (ddd, 1H, J = 8.1, 1.2, 0.7 Hz, 6-H), 7.59 (ddd, 1H, J = 8.1, 7.4, 1.6 Hz, 7-H), 7.48 (ddd, 1H, J = 7.5, 7.4, 1.2 Hz, 8-H), 5.51 (t, 1H, J = 5.5 Hz, −OH), 4.42 (dd, 2H, J = 5.5, 1.5 Hz, 3-CH_2OH). Anal. ($C_{16}H_{10}O_4$) C,H.

5-(Hydroxymethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (7b): yield 70%; mp 252 °C; ^{1}H NMR (DMSO-d_6) δ 8.55 (dd, 1H, J = 10.0, 0.7 Hz, 4-H), 8.32 (ddd, 1H, J = 7.7, 1.5, 0.7 Hz, 9-H), 7.70 (ddd, 1H, J = 8.1, 1.3, 0.7 Hz, 6-H), 7.59 (ddd, 1H, J = 8.1, 7.2, 1.5 Hz, 7-H), 7.57 (d, 1H, J = 0.7 Hz, 11-H), 7.47 (ddd, 1H, J = 7.7, 7.2, 1.3 Hz, 8-H), 6.44 (d, 1H, J = 10.0 Hz, 3-H), 5.42 (s, 2H, 5-CH_2OH). Anal. ($C_{16}H_{10}O_4$) C,H.

11-(Hydroxymethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (7c): yield 34%; mp 253 °C; ^{1}H NMR (DMSO-d_6) δ 8.51 (s, 1H, 5-H), 8.33 (br s, 1H, −OH), 8.25 (d, 1H, J = 9.6 Hz, 4-H), 8.20 (dd, 1H, J = 7.5, 1.5 Hz, 9-H), 7.82 (dd, 1H, J = 8.1, 1.2 Hz, 6-H), 7.60 (ddd, 1H, J = 8.1, 7.4, 1.5 Hz, 7-H), 7.49 (ddd, 1H, J = 7.5, 7.4, 1.2 Hz, 8-H), 6.54 (d, 1H, J = 9.6 Hz, 3-H), 4.92 (s, 2H, 11-CH_2OH). Anal. ($C_{16}H_{10}O_4$) C,H.

8-(Hydroxymethyl)-4-methyl-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (7d): yield 91%; mp 279 °C; ^{1}H NMR (DMSO-d_6) δ 8.56 (s, 1H, 5-H), 8.17 (d, 1H, J = 7.9 Hz, 6-H), 7.78 (s, 1H, 11-H), 7.66 (d, 1H, J = 1.0 Hz, 9-H), 7.42 (dd, 1H, J = 7.9, 1.0 Hz, 7-H), 6.42 (q, 1H, J = 1.2 Hz, 3-H), 5.44 (t, 1H, J = 5.8 Hz, −OH), 4.69 (d, 2H, J = 5.8 Hz, 8-CH_2OH), 2.58 (d, 3H, J = 1.2 Hz, 4-Me). Anal. ($C_{17}H_{12}O_4$) C,H.

(Diethylaminomethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-ones 8a−d. General Procedure. A mixture of **5** (10 mmol) and diethylamine (100 mmol) in anhydrous toluene (200 mL) was refluxed for 10 h (TLC: AcOEt/cyclohexane, 80:20). After cooling, the solvent was evaporated under reduced pressure. The residue was purified by column chromatography and crystallized from MeOH to give **8**.

3-(Diethylaminomethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (8a): yield 73%; mp 124 °C; ^{1}H NMR ($CDCl_3$) δ 8.09 (t, 1H, J = 1.4 Hz, 4-H), 8.05 (s, 1H, 5-H), 7.60 (ddd, 1H, J = 7.5, 1.5, 0.8 Hz, 9-H), 7.52 (ddd, 1H, J = 8.1, 1.4, 0.8 Hz, 6-H), 7.52 (s, 1H, 11-H), 7.50 (ddd, 1H, J = 8.1, 7.2, 1.5 Hz, 7-H), 7.39 (ddd, 1H, J = 7.5, 7.2, 1.4 Hz, 8-H), 3.58 (d, 2H, J = 1.4 Hz, 3-CH_2−N−CH_2−CH_3), 2.67 (q, 4H, J = 7.1 Hz, −N−CH_2−CH_3), 1.12 (t, 6H, J = 7.1 Hz, −N−CH_2−CH_3). Anal. ($C_{20}H_{19}NO_3$) C,H,N.

5-(Diethylaminomethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (8b): yield 54%; mp 162 °C; ^{1}H NMR ($CDCl_3$) δ 8.59 (d, 1H, J = 9.9 Hz, 4-H), 8.23 (dd, 1H, J = 7.6, 1.3 Hz, 9-H), 7.60 (dd, 1H, J = 8.1, 1.3 Hz, 6-H), 7.51 (ddd, 1H, J = 8.1, 7.2, 1.3 Hz, 7-H), 7.45 (s, 1H, 11-H), 7.39 (ddd, 1H, J = 7.6, 7.2, 1.3 Hz, 8-H), 6.40 (d, 1H, J = 9.9 Hz, 3-H), 4.30 (s, 2H, 5-CH_2−N−CH_2−CH_3), 2.64 (q, 4H, J = 7.1 Hz, −N−CH_2−CH_3), 1.06 (t, 6H, J = 7.1 Hz, −N−CH_2−CH_3). Anal. ($C_{20}H_{19}NO_3$) C,H,N.

11-(Diethylaminomethyl)-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (8c): yield 45%; mp 148 °C; ^{1}H NMR ($CDCl_3$) δ 7.98 (s, 1H, 5-H), 7.96 (dd, 1H, J = 7.5, 1.4 Hz, 9-H), 7.87 (d, 1H, J = 9.6 Hz, 4-H), 7.63 (dd, 1H, J = 8.1, 1.2 Hz, 6-H), 7.52 (ddd, 1H, J = 8.1, 7.3, 1.4 Hz, 7-H), 7.40 (ddd, 1H, J = 7.5, 7.3, 1.2 Hz, 8-H), 6.43 (d, 1H, J = 9.6 Hz, 3-H), 4.27 (s, 2H, 11-CH_2−N−CH_2−CH_3), 2.79 (q, 4H, J = 7.3 Hz, −N−CH_2−CH_3), 1.26 (t, 6H, J = 7.3 Hz, −N−CH_2−CH_3). Anal. ($C_{20}H_{19}NO_3$) C,H,N.

8-(Diethylaminomethyl)-4-methyl-2*H*-benzofuro[3,2-*g*]-1-benzopyran-2-one (8d): yield 82%; mp 164 °C; ^{1}H NMR ($CDCl_3$) δ 8.05 (s, 1H, 5-H), 7.86 (dd, 1H, J = 7.9, 0.6 Hz, 6-H), 7.58 (dd, 1H, J = 1.4, 0.6 Hz, 9-H), 7.44 (s, 1H, 11-H), 7.37 (dd, 1H, J = 7.9, 1.4 Hz, 7-H), 6.26 (q, 1H, J = 1.3 Hz, 3-H), 3.72 (s, 2H, 8-CH_2−N−CH_2−CH_3), 2.58 (q, 4H, J = 7.1 Hz, −N−CH_2−CH_3), 2.54 (d, 3H, J = 1.3 Hz, 4-Me), 1.08 (t, 6H, J = 7.1 Hz, −N−CH_2−CH_3). Anal. ($C_{21}H_{21}NO_3$) C,H,N.

Biological Section. Compounds **7a−d** and **8a−d** were dissolved in dimethyl sulfoxide (DMSO; 4.5 mM) and the solutions kept at −20 °C in the dark. Just before the experiment, a calculated amount of compound solution was added in the dark to PBS or to the growth medium containing cells, to a final DMSO concentration which never exceeded 0.5%.

UVA Irradiation. Cell suspensions containing the test compound were incubated at room temperature for 15 min in the dark, put into Petri dishes (5 cm in diameter, 3 mL), and exposed to UVA light. UVA exposure were performed with a Philips HPW 125 lamp, provided with a built-in Philips filter. Emission was in the 320−400 nm range, with a maximum, over 90% of the total, at 365 nm; irradiation intensity, determined by a radiometer (model 97503, Cole-Parmer Instrument Co., Niles, IL), was 0.9×10^{-6} W·m^{-2}.

Interaction with DNA in Vitro: Inhibition of Topoisomerase II. The inhibition of topoisomerase II activity was studied using a purified enzyme from *Drosophila melanogaster* embryos (USB, Amersham Italia S.r.l.). PM2 DNA (0.125 μg; Boehringer Mannheim GmbH, Germany) was incubated for 15 min at 30 °C in the presence of 2 units of topoisomerase II (1 unit is defined as the activity capable of relaxing 0.3 μg of supercoiled DNA) in the reaction buffer containing 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, 1 mM ATP. Aliquots of compound solution (2 μL) were added to reach the following final concentrations: 1, 10, 20, 40, 160 μM. A suitable amount of reaction buffer was then added to every sample to reach the final volume of 20 μL. The reaction was blocked by adding 7 mM EDTA (3 μL) containing 0.77% SDS. Bromophenol blue (0.2 μL) containing 15% glycerol was added to the samples, which were then analyzed by agarose gel (0.7%) containing TAE (40 mM Tris-sodium acetate, pH 8.2; 1 mM EDTA) at 50 V for 90 min. The gel was stained for 1 h in aqueous ethidium bromide (0.5 μg·mL^{-1}) and then photographed by a Polaroid camera placed over a UV TM36 transilluminator (UVP Inc., San Gabriel, CA).

Interaction with DNA in Vitro: Detection of Interstrand Cross-Links. Supercoiled circular DNA of PM2 bacteriophage (4 μg; Boehringer Mannheim GmbH, Germany) was linearized by incubating at 37 °C for 2 h with *Pst*I restriction enzyme (18 U·μL^{-1}; Amersham International Inc., U.K.) in 100 μL of aqueous solution containing 10 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 10 μg BSA. The solution was filtered through a Microcon 100 (Amicon, Beverly, MA), centrifuging at 500*g* for 5 min. The membrane was

washed by adding 100 μL of TE (40 mM Tris-HCl, pH 7.5; 1 mM EDTA), and the solution was stirred and centrifuged at 500*g* for a further 5 min. The solution over the membrane was then recovered by inverting the filter and centrifuging at 1000*g* for 3 min. The remaining solution was diluted by adding TE to obtain a DNA concentration of 80 ng·μL^{-1} and then stirred at 4 °C until use.

PM2-linearized DNA (2 μL for each sample) was added to a solution of the test compound (2 μL; 4× in DMSO) at the appropriate concentration, so that the final molar ratio drug/base pairs was 2; TE (4 μL) was then added to each sample (final volume 8 μL). Two samples were prepared for each compound: both were incubated at room temperature in the dark for 15 min and then exposed to UVA light. As a control, PM2-linearized DNA (2 μL) was added to DMSO (2 μL) and TE (4 μL) and incubated as described above, but not irradiated. Then 2-μL aliquots of a solution containing 1.5 M sodium acetate and 100 mM EDTA were added to the samples, which were then stirred and centrifuged at 10000*g* for 1 min. DNA was precipitated with three volumes (30 μL) of 95% ethanol, and the samples were kept at −80 °C for at least 1 h; DNA was recovered by centrifugation at 2000*g* for 20 min at 4 °C. Precipitated DNA was then suspended in TAE (7 μL) containing 0.04% bromophenol blue, 10% glycerol, and 30% DMSO, denatured by heating at 90 °C for 2 min, and quickly cooled in ice. Samples were analyzed by agarose gel (0.7%) electrophoresis in TAE containing ethidium bromide (0.5 μg·mL^{-1}) at 50 V for 3 h, to obtain good separation of denatured and nondenatured (cross-linked) DNA. The gel was photographed by a Polaroid camera, as previously described.

DNA Synthesis in Ehrlich Cells. DNA synthesis was assayed in Ehrlich cells (Lettrè strain) as already described.[15] Just after UVA irradiation (cell density: 2×10^7 cells·mL^{-1} in PBS), the samples (10^6 cells in 0.5 mL of PBS) were incubated for 30 min at 37 °C in the presence of 40 kBq·mL^{-1} [^{3}H]thymidine (4.77 TBq·mM^{-1}; Amersham International Inc., U.K.). The acid-insoluble fraction was precipitated by 5% ice-cold trichloroacetic acid and collected on Whatman GF/C filters (2.5 cm in diameter). After several washings with cold 1% trichloroacetic acid, the filters were dried and counted. In the experiments carried out in the dark, the irradiation step was omitted; the cells were treated with the compound at room temperature, incubated for 30 min at 37 °C in the dark in the presence of the radioactive precursor, and then processed as above. The results were calculated as the percentage of radioactivity incorporated into the DNA of untreated control cells (ca. 3−6 kBq). Filtrations were carried out using a Sample Manifold apparatus (Millipore Corp., Bedford, U.K.). Filters were counted by a toluene-based scintillation fluid (5 g PPO, 0.25 g dimethyl-POPOP, toluene up to 1 L of solution). All determinations were carried out on a Packard 4430 spectrometer. Every experiment was carried out at least three times.

Clonal Growth of HeLa Cells. HeLa cells were grown in nutrient mixture F-12 Ham medium (Sigma Chemical Co., St. Louis, MO) containing 5% fetal calf serum (increased to 10% in clonal growth) and supplemented with antibiotics. Cell growth was accomplished at 37 °C in a 5% carbon dioxide atmosphere.

HeLa cells ($1.5-2 \times 10^5$) were seeded in Petri dishes in growth medium (4 mL); after 24 h, the medium was replaced with a fresh one containing the test compound. For UVA irradiation, the cells were incubated in the dark for 15 min, to allow uptake of the compound. Samples were then exposed to UVA; in these experimental conditions, no significant absorption of UVA light by the medium was observed. For the experiments carried out in the dark, in which active cell metabolism is required, cells were incubated for 3 h at 37 °C in a 5% carbon dioxide atmosphere in growth medium supplemented with fetal calf serum and containing the test compound. In all cases, 0.5% DMSO was added to untreated controls. After treatment, aliquots of 200 cells were seeded in the same medium and incubated for 7 days, after which the colonies were stained and counted, colonies with less than 50 cells being discarded. The efficiency of clonal growth, that is, the ratio between the numbers of formed colonies and of cells seeded, was then calculated. Plating efficiency was about 80%. Every experiment was carried out at least three times.

Mutagenic Activity. The strain used was *E. coli* TM9 (*WP2, uvrA, R46*) carrying a nonsense mutation in the *trpE* gene, which is reverted by UV light and base pair substitution mutagens[21] and therefore by formation of C_4-cycloadducts of furocoumarins.[7] Bacteria were grown overnight in a minimal Davis-Mingioli salt glucose medium supplemented with tryptophan (20 mg·L^{-1}). *E. coli* cells were washed and then suspended in phosphate-buffered saline (pH 7.0) containing the test compound (2×10^{-5} M) at a density of 10^8 cells·mL^{-1}. Bacteria were irradiated with UVA, as described for DNA solutions. For the mutagenesis test, 0.1-mL aliquots of the irradiated suspensions were added to 2 mL of molten 0.6% top agar and poured onto plates containing 20 mL of SEM agar (MMA fortified with 0.1 mg·mL^{-1} Difco nutrient broth). To determine the surviving fraction, the irradiated cells (0.1 mL) were diluted with phosphate buffer, added to 2 mL of molten 0.6% agar, and plated on Davis-Mingioli minimal medium supplemented with tryptophan. Plates were incubated for 48 h at 37 °C in the dark, and the colonies were then counted. The mutation frequency was expressed as mutants per 10^6 survivors, computed by dividing the number of revertants observed per plate by the number of surviving bacteria at the same treatment and subtracting from the result the number of revertant colonies per million survivors observed in controls. In this test, all manipulations were done under red light.

Experiments with Yeast: Induction of Cytoplasmic ≪Petite≫ Mutations. Experiments with yeast were performed using diploid strain D7 (*S. cerevisiae*), a kind gift of Prof. F. K. Zimmermann (Technische Hochschule, Darmstadt, Germany). Yeast cells were grown in complete growth medium containing 1% yeast extract (Difco Laboratories, Detroit, MI), 2% bactopeptone (Difco), 2% glucose, and distilled water. Cells (10^7·mL^{-1}) were incubated in the dark for 15 min in the presence of 20 μM of the test compound and exposed to various doses of UVA light. Suitable dilutions of samples were plated on complete yeast-extract−peptone−glucose medium solidified by 2.6% bactoagar (Difco). Cytoplasmic ≪petite≫ mutants, i.e., respiratory-deficient mutants, were detected using the tetrazolium overlay technique.[23]

Experiments with Yeast: Detection of Double-Strand Breaks. Yeast chromosomes and the appearance of DNA DSBs were analyzed using a contour-clamped homogeneous-field electrophoresis apparatus (CHEF MAPPER, BIO-RAD, Richmond, VA). 1% agarose gels were made up from ultrapure DNA grade molecular biology certified agarose (BIO-RAD, Richmond, VA) and 0.5× TBE running buffer (5.4 g of Tris-Base, 2.75 g of boric acid, 2 mL of 0.5 EDTA, pH 8, 1 L of distilled water). Gels loaded with yeast chromosomal DNA were run for 22 h at 12 °C, using the CHEF apparatus at 160 mV, 170 mA, exponentially increasing pulse times. Gels were stained with ethidium bromide and photographed with a Polaroid camera, as previously described.

Skin Phototoxicity. Skin phototoxicity was tested on depilated albino guinea pigs (outbred Dunkin−Hartley strain), as described;[29] compounds were applied topically as 4.5 mM methanol solutions.

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