New Furan Side Tetracyclic Allopsoralen Derivatives: Synthesis and Photobiological Evaluation

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Novel tetracyclic allopsoralen derivatives characterized by the condensation of a fourth cyclohexenylic (**5**−**7**) or benzenic (**8**−**10**) ring at the furan side and a methoxy (**5** and **8**), a hydroxy (**6** and **9**), or a dimethylaminopropoxy (**7** and **10**) side chain in the 10 position of the chromophore were prepared. Compounds **7** and **10** showed a strong photoantiproliferative activity, up to 3 orders of magnitude higher than that of the photochemotherapeutic drug 8-methoxypsoralen (8-MOP). The investigation into the mechanism of action demonstrated for **10** the capacity to intercalate between DNA base pairs in the ground state, to give rise to a covalent photoaddition upon UVA irradiation, and to inhibit polymerase chain reaction (PCR) in a sequence-specific manner. Conversely, compound **7** showed a limited capacity to form an intercalative complex and the lack of ability to photoadd to the macromolecule, thus revealing a novel and unusual behavior for an allopsoralen derivative.

Introduction

At present, PUVA therapy (psoralen plus UVA light) represents the branch of photochemotherapy devoted to the treatment of a variety of skin diseases (i.e., psoriasis, vitiligo, lichen planus) and of cutaneous T-cell lymphoma.[1−3] The psoralen tricyclic moiety constitutes the basic chromophore from which the drugs employed in this therapy were developed. In particular, the most widely used drug is 8-methoxypsoralen (8-MOP), even though 5-methoxypsoralen (5-MOP) and, to a lesser extent, 4,5′,8-trimethylpsoralen (TMP) are also successfully employed.[2,4] Nevertheless, the PUVA treatment presents some drawbacks. Indeed, both short-term (erythema, hyperpigmentation) and long-term (premalignant keratoses, skin cancers) side effects have been reported.[4,5] Nowadays, most of the research in this field is devoted to the development of new photochemotherapeutic compounds endowed with photoantiproliferative activity and with lower skin phototoxicity with respect to the above-mentioned drugs. In this regard, a number of chemical modifications on the linear psoralen moiety, such as the insertion of different groups or side chains in the various positions of the tricyclic nucleus, have made it possible to obtain a variety of new photoreactive compounds. Notable efforts were also addressed to the synthesis and study of the photobiological properties of the angular isomers of psoralen. In particular, the development of suitable synthetic methods allowed the preparation of angelicin (isopsoralen) and of many angelicin derivatives, the photobiological properties of which were well understood and extensively studied.[6,7] The chemical approaches allowed researchers also to obtain the allopsoralen moiety and in particular some allopsoralen derivatives characterized by having a number of methyl groups inserted in different positions of the tricyclic chromophore. Photobiological studies on allopsoralen analogues demonstrated their ability to interact with DNA by forming an intercalative complex in the ground state and by photoadding covalently to the macromolecule upon UVA irradiation, in accordance with the already well-established behavior of the linear isomer, psoralen.[8,9] Moreover, the study of the photobiological properties highlighted the lack of skin phototoxicity and a certain antiproliferative ability on various biological substrates.[10,11] Nevertheless, despite the interesting photobiological behavior shown by these allopsoralen derivatives, up to now only little attention has been devoted to this structural feature.

Within the attempt to obtain promising new photochemotherapeutic drugs for PUVA therapy, a noteworthy approach led to tetracyclic derivatives, that is, compounds characterized by the condensation of a fourth nucleus to the tricyclic psoralen chromophore.[12−15] In this connection, remarkable results were attained for both tetracyclic analogues characterized by the condensation of a benzenic or cyclohexenylic ring at the level of the 4′,5′ or 3,4 photoreactive double bond of the psoralen moiety and by the presence of a dimethylaminopropoxy side chain.[13,15] In particular, these new water-soluble structures appeared endowed with notable photoantiproliferative activity, significantly higher than that of 8-MOP, together with a diminished, in some cases absent, skin phototoxicity. In addition, their ability to interact with DNA by forming an intercalation complex in the ground state and by giving rise to a covalent photoadduct with thymine upon irradiation at 365 nm was demonstrated.[13,15]

On the basis of the above considerations, it appeared of interest to prepare and to study a chemical moiety characterized by the tetracyclic allopsoralen structure. In this paper, we report the synthesis and the photobiological properties of six new tetracyclic allopsoralen derivatives (**5**−**10**). The new chemical structures are characterized by the condensation of a fourth cyclohexenylic (**5**−**7**) or benzenic (**8**−**10**) ring at the level of the 4′,5′-double bond of the allopsoralen nucleus. Furthermore, in the 10 position of the polycyclic chromophore, a methoxy (**5** and **8**), a hydroxy (**6** and **9**), or a dimethylaminopropoxy (**7** and **10**) side chain is inserted. The photoantiproliferative activity on human tumor cell lines along with the appearance of skin

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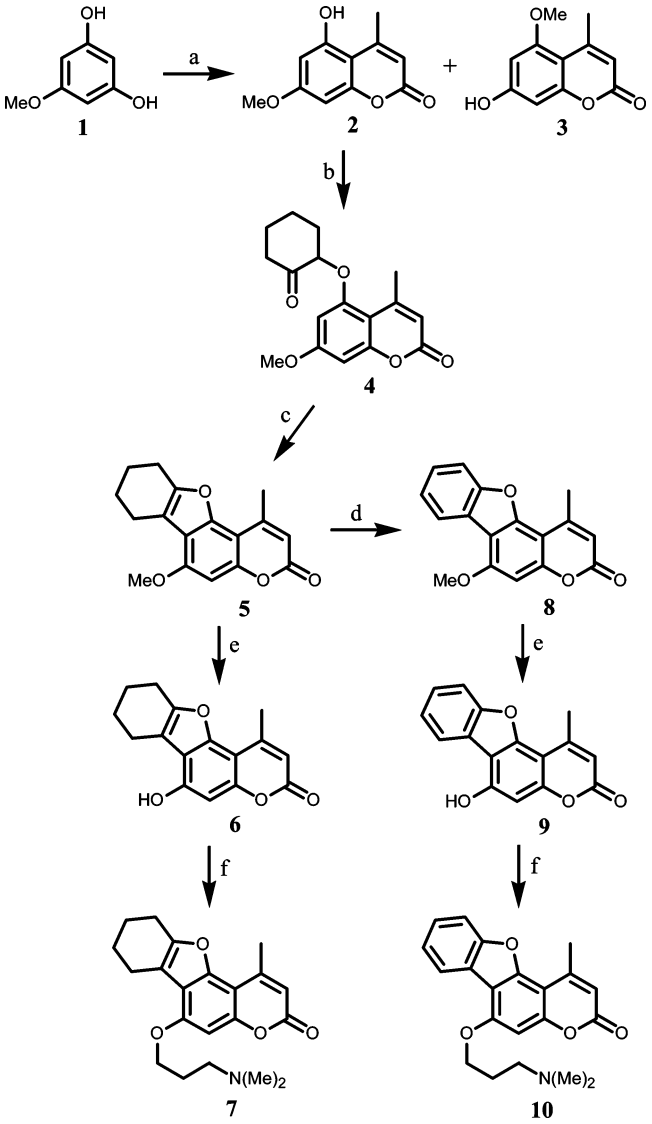
Scheme 1[a]



[a] Reagents: (a) ethyl acetoacetate, H_2SO_4; (b) 2-chlorocyclohexanone, K_2CO_3, acetone; (c) NaOH; (d) DDQ, toluene; (e) $AlCl_3$, CH_2Cl_2; (f) 3-chloro-*N*,*N*-dimethylpropylamine hydrochloride, NaH, NAI, DMF.

phototoxicity was determined. Moreover, the interaction with DNA was studied both in the dark and after UVA irradiation. In particular, for the most active compound **10**, the isolation and the in-depth characterization of the pyrone side photoadduct with the pyrimidine base thymine is reported. Finally, its ability to interact with the nuclear enzyme topoisomerase II in the dark was also investigated.

Results and Discussion

Chemistry. The compounds studied were obtained as shown in Scheme 1. The preparation of the tetracyclic furocoumarin skeleton **5** was realized in three steps from 3-methoxyresorcinol (**1**) with 16% overall yield in accordance with a general method described elsewhere.[16] The series of tetrahydrobenzoallopsoralens was completed with the hydroxyderivative **6** and with compound **7**, which has a dimethylaminopropoxy side chain on the benzene ring. Compound **6** was prepared from the methoxy derivative **5** in almost quantitative yield by hydrolysis of the methoxy group to hydroxyl group with a refluxing mixture of aluminum trichloride and methylene chloride. This hydrolysis was also obtained with hydroiodic acid in a refluxing mixture of acetic acid and acetic anhydride[13] but, in this case, with only a 65% yield. Treatment of compound **6** with 3-chloro-*N*,*N*-dimethylpropylamine and NaH in the presence of NaI in refluxing dimethylformamide, so as to replace the hydroxyl group, gave compound **7** in 76.5% yield.

The first compound obtained in the benzoallopsoralen series was compound **8**. The unsubstituted terminal ring of **5** was aromatized by heating with DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) in refluxing toluene, which afforded benzofurocoumarin **8** in 90% yield. Compounds **9** and **10** were prepared from **8** in a similar way and with similar yields as compounds **6** and **7** from **5**.

Photobiological Activity. The ability of the new tetracyclic allopsoralen derivatives **5**−**10** to inhibit cell growth was evaluated on HeLa (human cervix adenocarcinoma) and HL-60 (human promyelocytic leukemia) cell lines. The results, expressed as IC_{50} values, that is, concentration (μM) of compound able to cause 50% of cell death with respect to the control culture, are shown in Table 1. The well-known drug 8-MOP was tested in the same experimental conditions and was used as reference compound.

The experiments performed in the presence of UVA light (365 nm, 0.789 J cm^{-2}) demonstrated the capacity for the compounds characterized by the insertion of a dimethylaminopropoxy side chain in the 10 position of the tetracyclic chromophore (**7** and **10**) to exert a strong antiproliferative activity on both cell lines. In particular, for the tetrahydrobenzoallopsoralen **7**, the IC_{50} values are 8 and 25 times lower in HL-60 and HeLa cells, respectively, than those evaluated for the reference drug. The most notable photoantiproliferative ability is shown by benzoallopsoralen **10**: for this new compound, IC_{50} values of 2 (HL-60) and 3 (HeLa) orders of magnitude lower than those found for 8-MOP, were obtained.

Conversely, the derivatives characterized by the presence of a methoxy or a hydroxy side chain, both tetrahydrobenzoallo- (**5** and **6**) and benzoallopsoralens (**8** and **9**), appeared unable to induce any cytotoxic effect at the tested concentrations.

The experiments performed in the dark, that is, in the absence of UVA light, confirmed the inactivity of derivatives **5**, **6**, **8**, and **9**, while for **7** and **10**, they revealed a certain cytotoxic effect. In detail, the tetrahydrobenzoallopsoralen **7** is inactive in HeLa cells, but it appeared able to induce cell death in the HL-60 cell line. Nevertheless, it has to be underlined that the IC_{50} value in this case is 1 order of magnitude higher than that attained after cell exposure to UVA irradiation. A similar behavior was observed also for **10**: indeed, the antiproliferative capacity shown in the dark appears to be significantly lower with respect to that exerted as a result of UVA irradiation. These results indicate that also for the tetrahydrobenzo- and benzoallopsoralen moiety, as already widely demonstrated for psoralen and a number of tetracyclic psoralen derivatives,[12−15,17] UVA light plays the main role in the occurrence of cellular antiproliferative events.

The occurrence of skin phototoxicity, the most commonly reported short-term adverse event in PUVA therapy, can be estimated as appearance of erythema on depilated guinea pig skin, according to the procedure described in the Experimental Section. The results reported in Table 1 indicate the absence of cutaneous photosensitization for the tetrahydrobenzoallopsoralens **5**−**7**, even if they are applied on skin at a concentration higher than that used for the reference drug. In particular, this result could be expected for the non-photoantiproliferative derivatives **5** and **6**, while it indicates a significant photobiological property for **7**, which, in contrast, is able to inhibit cell

Table 1. Cell Growth Inhibition and Skin Phototoxicity in Guinea Pigs in the Presence of Examined Compounds and 8-MOP as Reference Drug

	IC_{50} (μM)					
	HeLa		HL-60		formation of erythema	
compd	dark	UVA	dark	UVA	erythema intensity[a]	dose, μmol cm^{-2}
5	>20	>20	>20	>20	−	0.14
6	>20	>20	>20	>20	−	0.14
7	>20	0.40 ± 0.06	7.9 ± 0.6	0.61 ± 0.19	−	0.14
8	>20	>20	>20	>20	−	0.14
9	>20	>20	>20	>20	−	0.14
10	3.5 ± 0.6	0.0043 ± 0.0002	0.48 ± 0.01	0.0105 ± 0.0035	+ (with edema)	0.046
8-MOP[b]	>20	10 ± 3	>20	5.4 ± 0.7	+ (with edema)	0.046

[a] +, strong; − absent. [b] Taken from ref 15.

Table 2. Skin Phototoxicity in Guinea Pigs in the Presence of **10** and 8-MOP as Reference Drug

compd	dose, nmol cm^{-2}	erythema intensity[a]
8-MOP	46	+ + (with edema)
10	46	+ + (with edema)
	4.6	+ −
	0.46	− −

[a] + +, strong; + −, mild; − −, absent.

growth remarkably after UVA irradiation. With regard to the benzoallopsoralens **8**−**10**, the results shown in Table 1 indicate **8** and **9** as non-phototoxic. In contrast, compound **10** shows an effect similar to that of 8-MOP: indeed at the same concentration, both provoke marked skin phototoxicity. Nevertheless, it has to be taken into account that **10** is definitely more active in inducing the photoantiproliferative effect, showing an IC_{50} value more than 2 (HL-60) and 3 (HeLa) orders of magnitude lower than that of the reference drug. In this connection, to define more precisely the photobiological profile of **10**, it appeared of interest to test the skin phototoxic effect over a wider range of concentrations. The results obtained are reported in Table 2 and show that at a concentration 10 times lower than that of the reference drug, the cutaneous side effect induced by **10** is significantly dampened, while at a concentration 100 times lower it actually disappears.

Noncovalent Interaction with Salmon Testes DNA. It is well-known that the planar chromophore of psoralen allows the formation of a molecular complex with DNA following an intercalative mode of binding in the ground state. Furthermore, it has been established that the condensation of a fourth ring both at the level of the 4′,5′- and 3,4-double bond of the tricyclic nucleus does not compromise this ability: both tetrahydrobenzo- and benzopsoralen derivatives demonstrated the capacity to intercalate between base pairs.[12,13,15] In this connection, it appeared of interest to investigate also for the tetracyclic allopsoralen derivatives the ability to form a molecular complex with the macromolecule. For this purpose, flow linear dichroism (LD) analyses were performed and the spectra of DNA solutions in the presence of increasing amounts of **7** and **10** are reported in Figure 1, panels A and B, respectively.

The DNA spectrum shown in Figure 1B (trace a) presents the typical negative dichroic signal at 260 nm, while in the presence of **10** (traces b−d) a second band, of increasing intensity as the concentration of the test compound increases, appears at higher wavelengths (310−400 nm). The presence of a LD signal in this latter spectral region, where only the added chromophore can absorb, is indicative of complexation with the macromolecule. Furthermore, the negative sign of this LD signal indicates an intercalative mode of binding, that is, with an orientation of the molecular plane of the chromophore preferentially parallel to the plane of the DNA bases.

In the case of the tetrahydrobenzoallopsoralen derivative **7** (Figure 1A), no significant spectral variation with respect to DNA can be detected at the lowest concentration tested in the 310−400 nm range (traces a and b). In this region, a detectable negative signal appears only at higher [drug]/[DNA] ratios (traces c and d).

In previous studies, a similar capacity to intercalate was demonstrated for the tetrahydrobenzopsoralen and the correspondent benzopsoralen moiety.[13,15] In contrast, **7** clearly

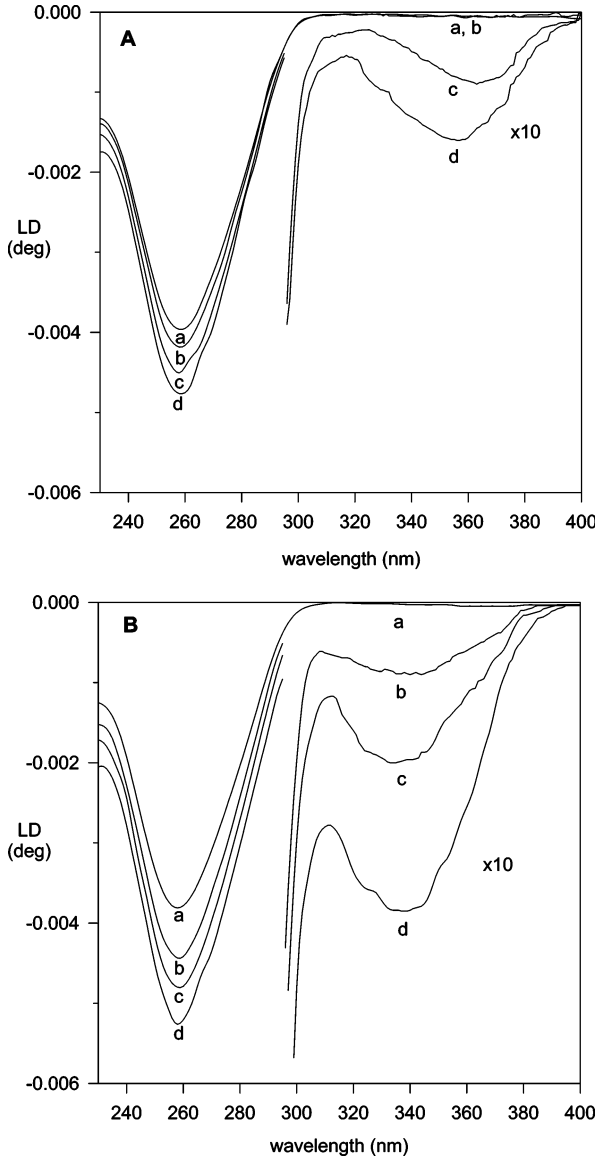


Figure 1. Linear flow dichroism spectra for compound **7** (A) and **10** (B) at different [drug]/[DNA] ratios: line a, 0; line b, 0.02; line c, 0.04; line d, 0.08. [DNA] = 1.6×10^{-3} M.

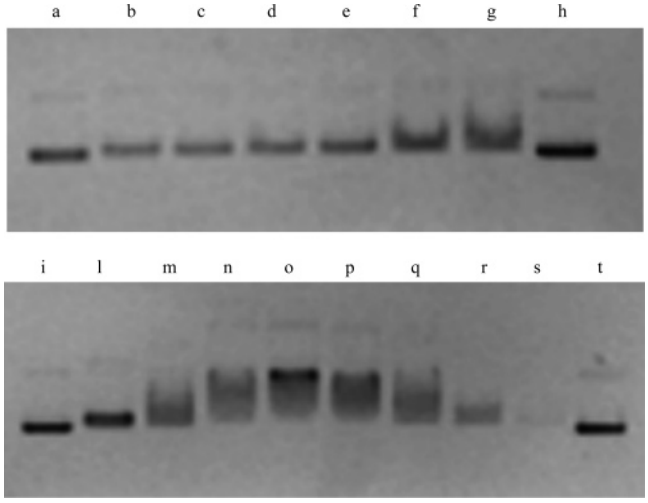


Figure 2. Effect of compound **7** and **10** on the supercoiling of pBR322 DNA: lanes a, h, i, and t, supercoiled pBR322 DNA; lanes b−g, compound **7** at 70, 80, 90, 100, 105, and 110 μM, respectively; lanes l−s, compound **10** at 1, 2, 5, 7, 10, 50, 70, and 100 μM, respectively.

shows greater difficulty to intercalate with respect to the congener **10**. The difference in behavior suggests a role for the two structural peculiarities of the tetrahydrobenzoallopsoralen moiety: the angular geometry and the condensation of a nonplanar fourth ring to the furocoumarin chromophore. In particular, both features can be considered essentially unfavorable for a proper insertion between parallel base pairs, and consequently, their coexistence in **7** could result in a molecular structure rather unsuitable for an intercalative binding process.

Noncovalent Interaction with Supercoiled pBR322 DNA. The difference in the ability of the two tetracyclic derivatives **7** and **10** to give rise to an intercalation complex with DNA base pairs was further confirmed by unwinding experiments performed with supercoiled plasmid pBR322 DNA. It is well-known that an increase in concentration of intercalating agents causes a slowing of the intrinsic electrophoretic mobility as a consequence of the unwinding process, which gives rise to the circular form of DNA. A further increase in drug concentration brings reversion to the original migration rate. The effect of increasing amounts of derivative **7** (lanes b−g) and **10** (lanes l−s) on supercoiled DNA (lanes a, h, i, and t) is shown in Figure 2. In the presence of **7**, a decrease in migration rate occurs at about 105 μM concentration (lane f). For compound **10**, on the other hand, the slowing of DNA migration begins at 2 μM (lane m) and reaches its maximum already at 7 μM (lane o); indeed, we obtained a complete titration curve for this tetracyclic derivative in the concentration range of 1−100 μM (lanes l−s). The significantly higher concentration at which **7** induces unwinding of supercoiled DNA proves its practically negligible capacity to form an intercalative complex with nucleic acid with respect to the aromatic analogue.

Photoaddition to DNA. The formation of an intercalative complex constitutes the preliminary essential step for an effective covalent photoconjugation between the photoreactive 3,4- and 4′,5′-double bonds of the furocoumarin moiety and DNA bases. In this connection, the capacity of the tetracyclic benzoallopsoralen **10** to intercalate between base pairs prompted us to investigate the ability of the new derivative to photoreact with the macromolecule following UVA irradiation. The condensation of an aromatic ring to a photoreactive double bond in the tricyclic psoralen chromophore prejudices its ability to participate in a photocycloaddition.[12−15] Consequently, the photoaddition reaction between **10** and the macromolecule can give rise to a unique photoadduct, that is, that involving the 3,4-pyrone side double bond. It was previously demonstrated that the frequency of occurrence of this type of monoadduct is considerably lower with respect to that involving the 4′,5′-furan side,[18] and concerning benzopsoralen derivatives, up to now only a rough indication of the occurrence of the covalent photoaddition reaction at the level of 3,4-double bond has been reported.[12,13] In this connection, it appeared of particular interest to isolate and characterize in-depth the pyrone side photoadduct deriving from the photoaddition process between the benzoallopsoralen **10** and DNA. For this purpose, an aqueous solution of salmon testes DNA was irradiated at 365 nm for 120 min in the presence of **10**; the DNA was then precipitated and hydrolyzed, and the resultant photoproducts were separated, as reported in the Experimental Section. The major band isolated appeared devoid of fluorescence and the UV spectrum of the corresponding isolated product showed a remarkable decrease in the intensity of the absorption peak at 315 nm, characteristic of the parent compound (spectrum not shown). These experimental findings are both considered indicative of a saturation of the 3,4-double bond of the furocoumarin nucleus.[19] Moreover, the mass spectrometry analysis gives a peak showing a m/z = 478, which is consistent with a thymine−**10** photoadduct.

Structural characterization of the isolated adduct was obtained by NMR. The spectra contained several impurity peaks, but the identification of the signals of the adduct was easily achieved. The complete assignment of the proton and carbon resonances is given in Table 3.

In comparison with the ^{1}H spectrum of compound **10** in DMSO, we observed an upfield shift of proton 3-H (from 6.26 to 3.58 ppm) and of 4-CH_3 of compound **10** (from 2.70 to 1.87 ppm), confirming that the reaction occurred at the 3,4-double bond of the allopsoralen moiety. Correlations were found in the double-quantum filtered correlation (DQF−COSY) spectrum between 3-H and 6-H (T), confirming the site of the bond between the allopsoralen derivative and thymine. Homonuclear and heteronuclear correlations are consistent with the cycloaddition of thymine at the 3,4-double bond in the allopsoralen moiety giving a cyclobutane ring at the reaction site.

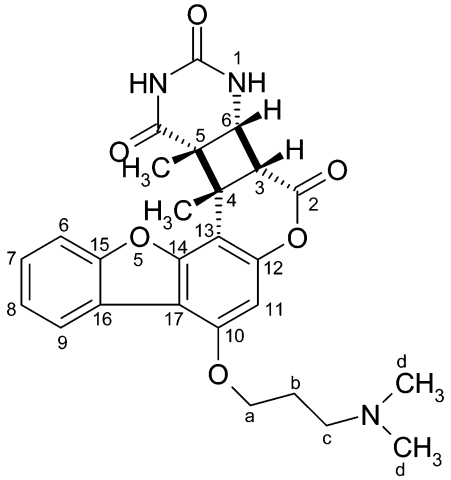
The photocycloaddition of the pyrimidine to the psoralen moiety can occur in either syn or anti configuration, and their relative position at the sides of the cyclobutane ring can be cis or trans.[18] The regiochemistry of this adduct was determined by the size of the coupling constants between 3-H and 6-H (T) protons and analysis of the correlations of all protons around the cyclobutane ring in the rotating-frame Overhauser enhancement (ROESY) spectrum.

The anti orientation can be excluded by the size of the coupling constant between 3-H and 6-H (T) (9.3 Hz). In such orientation, the protons would be positioned diagonally and give rise to a much smaller ^4J coupling. A 9.3 Hz coupling can only be ^3J, consistent only with a syn orientation.

Although in a cyclobutane ring $^3J_{cis}$ is generally larger than $^3J_{trans}$, the corresponding values overlap considerably ($^3J_{cis}$ = 4.6−11.4 Hz; $^3J_{trans}$ = 2.24−10.7 Hz).[20] The assignment of cis or trans stereochemistry cannot therefore be derived from the coupling constants.

Dipolar correlations, as found in the ROESY spectrum, between protons spatially close around the cyclobutyl system were a sensitive indicator of the stereochemistry in the substituted cyclobutane. Figure 3 shows the relevant region of the ROESY spectrum. The trans−syn configuration would produce a cross-peak between the 1-NH and 3-H: this peak

Table 3. ^{1}H and ^{13}C Assignment of the Thymine−Compound **10** Photoadduct in DMSO, Relative to the Solvent Peak (2.49 ppm)



position[a]	^{1}H (ppm)	^{13}C (ppm)
2		167.43
3	3.58 (d, $J = 9.3$ Hz)	46.17
4		40.36
4-CH_3	1.87 (s)	21.74
6	7.66 (d (overlapped))	111.34
7	7.46 (m)	125.96
8	7.39 (m)	123.50
9	8.03 (d)	121.99
11	6.66 (s)	95.28
13		102.67
14		149.97
15		155.32
17		109.85
1 (T)	8.09 (dd, $J = 4.9$ Hz, $J = 1.6$ Hz)	
3 (T)	9.78 (d, $J = 1.6$ Hz)	
4 (T)		172.3
5-CH_3 (T)	1.50 (s)	18.27
6 (T)	4.36 (dd, $J = 9.3$ Hz, $J = 4.9$ Hz)	51.25
a	4.28 (m)	65.78
b	2.29 (m)	23.61
c	3.28	
d	2.82 (s)	41.98

[a] T = thymine.

was not observed, although a cross-peak was found between more distant protons such as 4-CH_3 of compound **10** and 6-H (T).

From these observations, we concluded that the structure of the new photoadduct is cis−syn, which is also most consistent with the proposed model for psoralen−DNA interactions.[21]

Following the procedure reported in the Experimental Section, it was not possible to isolate any photoaddition product of derivative **7** with an UV and a mass spectrum consistent with those of a pyrone-side adduct. Furthermore, no fluorescent band was detected by TLC analysis of both organic and aqueous phases. This latter evidence seems to indicate the lack of occurrence of the furan-side monoadduct. As a whole, these data suggest the inability or a very limited ability of the tetrahydrobenzoallopsoralen derivative to give rise to a successful photoaddition reaction with the macromolecule. Because the intercalation constitutes an absolute requisite for the subsequent covalent C_4-cycloaddition, this behavior could be the consequence of the above-evidenced limited capacity of this new structural tetracyclic moiety to intercalate between base pairs.

Polymerase Stop Assay. The sites of DNA binding and covalent modification of derivative **10** were mapped using a polymerase chain reaction assay.[22−24] Comparison with compound **7** was performed to further confirm the differences between the mechanism of action of the two molecules. In a first set of experiments, inhibition of PCR was studied by preincubating target DNA sequence (a regulatory region of the F promoter of the human estrogen receptor gene containing putative binding sites of psoralen-related compounds) and, following UV irradiation, adding primers, buffers, and *Taq* DNA polymerase and performing 30 PCR cycles, as described in the Experimental Section. The results obtained are shown in Figure 4A and demonstrate that derivative **10** is at least 20-fold more active than derivative **7** in inhibiting PCR. Derivative **7** inhibits PCR only if added at very high concentration (5 mM). Furthermore, ^{32}P-labeled forward primer was used to identify the binding sites during the PCR assay. The results of the experiment are shown in Figure 4 (panels B and C). Addition of derivative **7** (1 and 5 mM) does not cause differences in premature termination sites with respect to control non-drug-treated DNA (compare lanes d and e with lane c in panel B of Figure 4). In contrast, several blocks were detected in PCR assays carried on using DNA pretreated with derivative **10** (used at 0.25 and 0.5 mM). The sequences of the blocks produced are shown in Figure 4B and boxed in Figure 4C. Interestingly, found binding sites contain AT-rich sequences, in agreement with the reported sequence selectivity of psoralen-related compounds.[24] In agreement with the finding reported in Table 1, inhibition of PCR by derivative **10** was found also in dark conditions, in the absence of UVA activation (data not shown).

Cross-Linking. The ability to give rise to interstrand cross-links is a property of furocoumarins characterized by the concurrent availability of the two photoreactive double bonds and by a linear structure. Indeed, this capacity is compromised both in benzopsoralen derivatives and in angular furocoumarins. In the first case, both the steric effect and the electronic delocalization due to the condensation of an aromatic ring prevent the pertinent double bond from photoreacting. In the second case, as for angelicins[7] and allopsoralens,[10,11] once a monoadduct is formed, the angular structural geometry prevents the correct alignment of the remaining double bond with the base of the complementary strand.

Figure 5 shows the evaluation of the cross-linking ability, assayed by means of denaturation−renaturation experiments, for compounds **7** and **10** and 8-MOP, reported as reference drug. As expected, both the benzoallopsoralen derivative **10**, which possesses only one photoreactive double bond at the level of 3,4-pyrone side and the tetrahydroallopsoralen **7**, which is characterized by an angular geometry, demonstrated the inability to give rise to this DNA damage.

Inhibition of Topoisomerase II Activity. DNA topoisomerase II is a nuclear enzyme that catalyses the interconversion of different topological forms of DNA, an essential requirement for the faithful replication and segregation of chromosomes during the cell cycle. The crucial role played by this enzyme in cell division renders it a potential target for many antiproliferative agents. Indeed, a wide variety of antitumor drugs have been shown to influence the catalytic cycle of topoisomerase II. Specifically, intercalating agents, such as doxorubicin, mitoxantrone, and *m*-amsacrine (*m*-AMSA) exert their antitumor effect by interfering with topoisomerase II activity.

Because the newly synthesized benzoallopsoralen **10** is able to exert a certain antiproliferative activity on human tumor cell lines independent of UVA irradiation (see Table 1) and gives rise to an intercalative complex with DNA (Figures 1B and 2), it appeared of interest to investigate whether **10** interferes with DNA topoisomerase II activity.

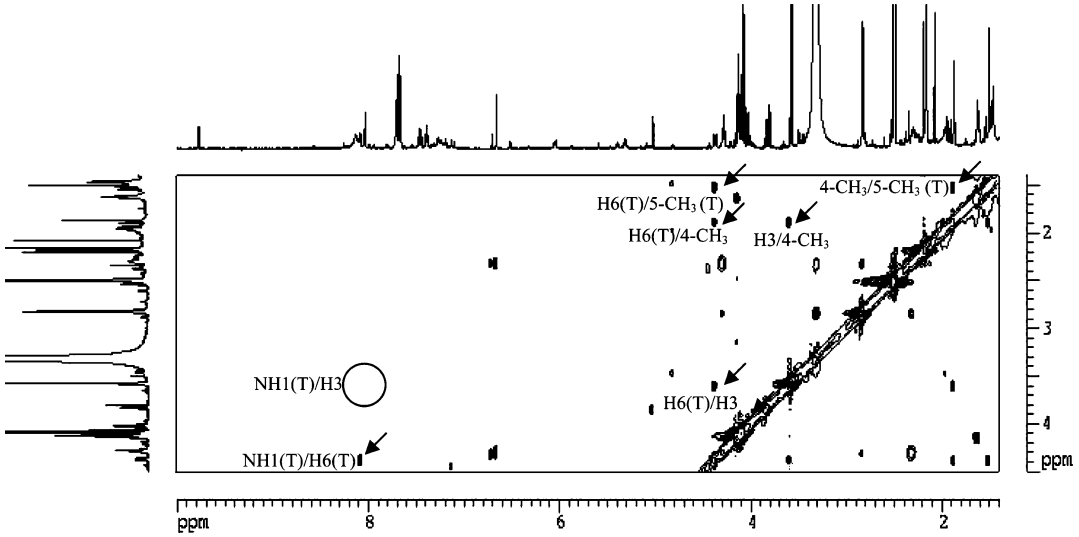


Figure 3. Region of the ROESY spectrum of the thymine−compound **10** photoadduct. The relevant peaks for the assignment of the stereochemistry are indicated with arrows or with a circle (missing peak).

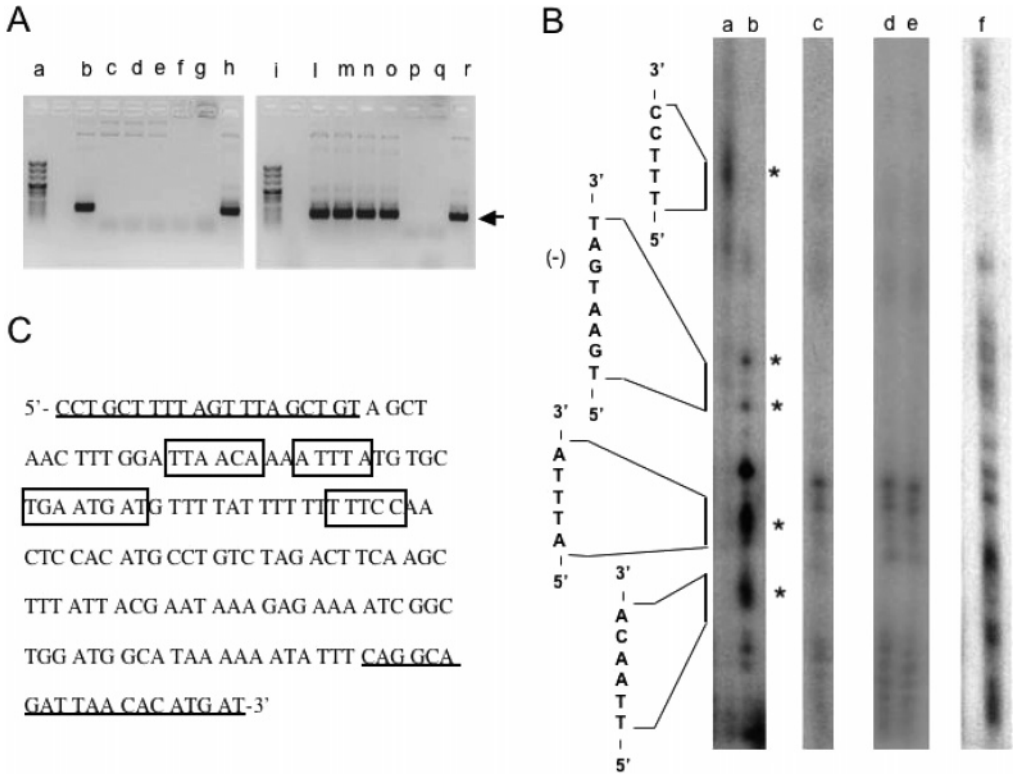


Figure 4. Effects of compound **7** and **10** on polymerase chain reaction: in panel A, DNA (100 ng/reaction) was reacted with 0.1 (b, l), 0.25 (c, m), 0.5 (d, n), 1 (e, o), 5 (f, p) and 10 (g, q) mM derivative **7** (l−q) and **10** (b−g), then samples were UV-irradiated, and PCR was performed. Control PCR reactions with unreacted DNA are in lanes h and r. Lanes a and i contain molecular weight marker (*Msp* I/*Dra* I digested pUC). PCR products (173 bp) are marked with arrows. Panels B and C illustrate detection of the PCR stop sites. DNA (100 ng/reaction) was reacted with 0.25 (a) and 0.5 (b) mM compound **10** or with 1 (d) and 5 (e) mM compound **7**. PCR reaction on control unreacted DNA is in lane c; Maxam−Gilbert G+A sequencing reaction is in lane f. Block sequences are shown in panel B and boxes in panel C, which reports the sequence of the 173 bp PCR product.

Figure 6 shows the effect of the new derivative on topoisomerase II enzymatic activity, assayed by the ATP-dependent decatenation reaction of kinetoplast DNA. In particular, the formation of the decatenation products is still evident after incubation with 1 and 2 μM test compound (lanes c and d, respectively), while it clearly decreases in the presence of 4 and 8 μM concentrations (lanes e and f, respectively). The effect of the DNA topoisomerase II inhibitor *m*-AMSA, a well-known antiproliferative drug, is also reported at 4 and 8 μM concentrations (lanes g and h, respectively). The comparison between **10** and *m*-AMSA evidences a similar ability to inhibit the nuclear enzyme. These results suggest a likely involvement of the inhibition of topoisomerase II in the antiproliferative effect exerted by **10** in the dark, even though the contribution of other cellular events cannot be excluded.

Conclusions

The synthesis of tetracyclic allopsoralen derivatives was performed, and the biological activity was investigated. The tricyclic allopsoralen chromophore was widened by the con-

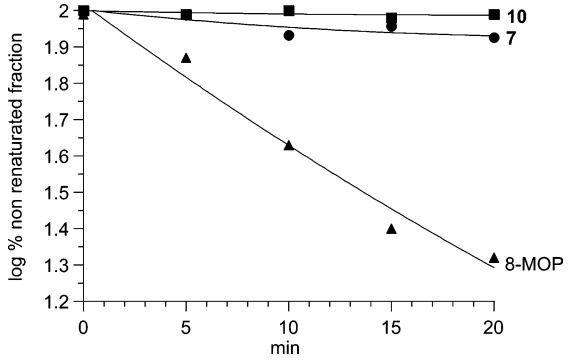


Figure 5. Cross-linking of compounds **7** and **10** and 8-MOP to double-stranded DNA from salmon testes as a function of irradiation time. [DNA]/[drug] = 75. [DNA] = 7.6×10^{-4} M.

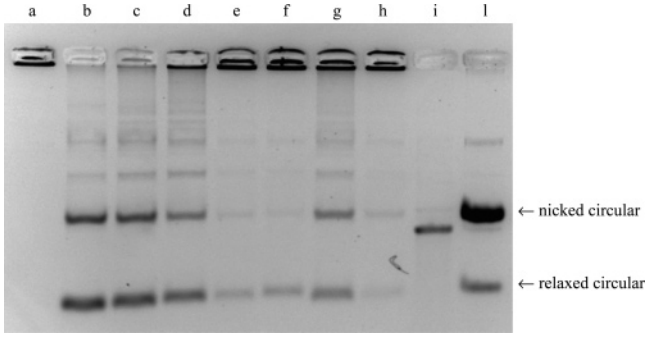


Figure 6. Topoisomerase II decatenation assay in the presence of compound **10**: lane a, KDNA control (no enzyme); lane b, KDNA and topoisomerase II; lanes c−f, same as lane b with 1, 2, 4 and 8 μM **10**, respectively; lanes g and h, same as lane b with 4 and 8 μM *m*-AMSA, respectively; lane i, linear KDNA standard; lane l, decatenated KDNA standard.

densation of a cyclohexane (compounds **5**−**7**) or a benzene (compounds **8**−**10**) moiety at the 4′,5′-double bond of the furan ring. Moreover, a methoxy (**5** and **8**), a hydroxy (**6** and **9**), or a dimethylaminopropoxy group (**7** and **10**) was inserted in position 10 of the chromophore.

The new derivatives characterized by the insertion of the dimethylaminopropoxy side chain, both tetrahydrobenzoallo- (**7**) and benzoallopsoralen (**10**), possess a noteworthy antiproliferative activity on human tumor cell lines after UVA irradiation (365 nm). In contrast, the compounds carrying the methoxy or the hydroxy group appear unable to exert any effect on cell growth.

The photoantiproliferative activity of compound **10** is from 2 to 3 orders of magnitude higher than that of the reference drug, 8-MOP. This remarkable effect, when compared with previous results on tetrahydrobenzo- and benzopsoralen derivatives,[13,15] allowed us to point out some structure−activity relationships. In detail, the widening of the tricyclic furocoumarin chromophore by the condensation of a fourth benzene ring at the 4′,5′-double bond, the insertion of a protonable dimethylaminopropoxy side chain, and finally the angular allopsoralen geometry appear as the most successful furocoumarin structural features in determining the photoantiproliferative effect. The evaluation of skin phototoxicity, which constitutes the most commonly reported adverse event in PUVA photochemotherapy, indicates that **10** induces a cutaneous photosensitization similar to that of 8-MOP when applied on skin at the same concentration. Nevertheless, on the basis of the notable photoantiproliferative activity, the phototoxic effect induced by **10** needed to be reconsidered. Indeed, when the concentration of **10** is decreased by 1 order of magnitude, the appearance of erythema diminishes significantly, and it disappears completely if a concentration 2 orders of magnitude lower is applied.

The mechanism of action of compound **10** is analogous to that of 8-MOP and of the tetracyclic psoralen derivatives developed up to now.[13−15] Compound **10** shows the ability to intercalate between DNA base pairs and, following UVA irradiation, to give rise to a C_4-cycloadduct with the DNA base thymine. This behavior was demonstrated by the isolation and the in-depth characterization of the pyrone-side cycloadduct between **10** and thymine and confirmed by the PCR stop assay shown in Figure 4.

Along with a remarkable photoantiproliferative activity, **10** is able to inhibit cell growth also independent of UVA light in both cell lines considered. This behavior prompted us to investigate the molecular mechanism accountable for this effect. Because **10** intercalates between DNA base pairs in the ground state, it appeared of interest to investigate a possible interference with the nuclear enzyme topoisomerase II. In fact, **10** is able to exert an inhibitory, concentration-dependent effect on the activity of the enzyme, assayed as decatenation ability of kinetoplast DNA. Although our results do not exclude additional effects of **10** on other molecular targets, the finding that this derivative inhibits topoisomerase II activity, similarly to *m*-AMSA, is the basis for explaining its antiproliferative activity.

The photobiological evaluation of tetrahydrobenzoallopsoralen **7** revealed some unexpected results. The estimation of the photoantiproliferative effect on cell lines indicated an ability to inhibit cell growth after UVA irradiation about 1 order of magnitude higher than that of 8-MOP. A certain activity in the dark was also observed on HL-60 cell line, but it is significantly less than that exerted upon UVA irradiation.

Experiments performed to establish the mechanism of action revealed a limited ability to intercalate inside the DNA double helix. Furthermore, the attempt to demonstrate the occurrence of photoaddition to DNA bases at the level of either the 4′,5′- or the 3,4-double bond of the allopsoralen chromophore, by isolating the furan side or the pyrone side photoadduct, was without result. Furthermore, no evident blocks in the PCR stop assay were noted for derivative **7** (Figure 4B, lanes d and e).

The effort to understand this difference in behavior with respect to previously studied congeners[13−15] stimulated some considerations on the molecular structure of **7**. In particular, the tetrahydrobenzoallopsoralen moiety is characterized by two distinctive features: a fourth ring not planar with the allopsoralen tricyclic nucleus and an angular geometry. It is already well-established that neither feature alone compromises the ability of the chromophore to photoreact with DNA.[7−9] Nevertheless, the lower intrinsic binding constant of the tetrahydrobenzopsoralen moiety in comparison to the planar congener indicated a greater difficulty to intercalate between base pairs.[12] Moreover, it is known that the angular geometry entails a noncorrect alignment of the two reactive double bonds allowing only monoadduct formation.[7,10] The photobiological behavior of **7** suggests that the coexistence of these structural features could prevent the photoaddition reaction with the macromolecule. As a consequence, the notable photoantiproliferative activity exerted by **7** has to be attributed to cellular target(s) distinct from that of the tetrahydrobenzopsoralen derivatives studied so far.[13−15] This result, unusual for furocoumarin structures, encourages the development of more tetracyclic allopsoralen derivatives to identify the common structural features responsible for the lack of interaction with the macromolecule. The structure−activity relationships that will

derive from this comparison should constitute a useful tool for a rational prediction of the new cellular target.

In conclusion, the novelty of the photobiological behavior of **7**, along with the remarkably high photoantiproliferative activity of **10**, renders the new tetracyclic allopsoralen moiety a very interesting photobiological pattern deserving further development and investigation.

Experimental Section

Melting points are uncorrected and were determined with a Reichert Kofler thermopan or in capillary tubes in a Büchi 510 apparatus. IR spectra were recorded with a Perkin-Elmer 1640FT spectrometer (KBr disks, υ in cm^{-1}). ^1H NMR (300 MHz) and ^{13}C NMR (75.4 MHz) spectra of the synthetic compounds were recorded with a Bruker AMX spectrometer, using TMS as internal standard (chemical shifts in δ values, J in Hz). Mass spectrometry was carried out on a Kratos MS-50 or on a Varian AT-711 spectrometer. Elemental analyses were performed with a Perkin-Elmer 240B microanalyzer and were within ±0.4% of calculated values in all cases. Flash chromatography (FC) was performed on silica gel (Merck 60, 230−400 mesh); analytical TLC was performed on precoated silica gel plates (Merck 60 F254, 0.25 mm).

6,7,8,9-Tetrahydro-10-hydroxy-4-methylbenzofuro[2,3-*f*]coumarin (6). A mixture of $AlCl_3$ (514.0 mg, 3.861 mmol) and anhydrous CH_2Cl_2 (30 mL) was stirred for 2 h at room temperature. A solution of compound **5** (366.0 mg, 1.287 mmol) in anhydrous CH_2Cl_2 (20 mL) was added, and the mixture was stirred for another 3 h. The reaction mixture was then acidified with HCl and extracted with CH_2Cl_2 (4 × 35 mL), the extract was dried (Na_2SO_4), and the solvent was evaporated under reduced pressure, leaving a residue that upon purification by FC with 97:3 CH_2Cl_2/MeOH as eluent yielded pure **6** (341.0 mg, 98.5%): mp 308−310 °C (dec).

6,7,8,9-Tetrahydro-10-(3-dimethylaminopropoxy)-4-methylbenzofuro[2,3-*f*]coumarin (7). A mixture of hydroxytetrahydrobenzofurocoumarin **6** (120.0 mg, 0.443 mmol), 3-chloro-*N*,*N*-dimethylpropylamine hydrochloride (84.0 mg, 0.532 mmol), 60% NaH (32.0 mg, 1.331 mmol), NaI (79.0 mg, 0.532 mmol), and dimethylformamide (32 mL) was heated for 5 h at 100 °C and then concentrated under reduced pressure. The residue was purified by FC with 99:1 to 92:8 CH_2Cl_2/MeOH as eluent and then treated with ion-exchange resin Dowex SBR, giving pure **7** (120.0 mg, 76.5%): mp 118−119 °C.

10-Methoxy-4-methyl-benzofuro[2,3-*f*]coumarin (8). A solution of tetrahydrobenzoallopsoralen **5** (1.500 g, 5.275 mmol) and DDQ (2.395 g, 10.551 mmol) in toluene (125 mL) was refluxed for 5 h. The mixture was cooled, the precipitate was filtered, and the solvent was evaporated under reduced pressure. The resulting residue was purified by FC with 1:1 hexane/CH_2Cl_2 as eluent, giving pure **8** (1.452 g, 90%): mp 279 °C.

10-Hydroxy-4-methylbenzofuro[2,3-*f*]coumarin (9). This compound was prepared from **8** (430.0 mg, 1.534 mmol) in the same way as **6** from **5**. The crude product was purified by FC with 99:1 to 98:2 CH_2Cl_2/MeOH as eluent, yielding pure **9** (384.0 mg, 94%): mp 306 °C.

10-(3-Dimethylaminopropoxy)-4-methylbenzofuro[2,3-*f*]coumarin (10). This compound was prepared from **9** (120.0 mg, 0.450 mmol) in the same way as **7** from **6**. The crude product was purified by FC with 99:1 to 94:6 CH_2Cl_2/MeOH as eluent, giving pure **10** (101.0 mg, 64%): mp 242−243 °C.

Photobiological Methods. Cell Cultures. HL-60 and HeLa cells were grown in RPMI 1640 (Sigma Chemical Co.) supplemented with 15% heat-inactivated fetal calf serum (Seromed) and Nutrient Mixture F-12 [HAM] (Sigma Chemical Co.) supplemented with 10% heat-inactivated fetal calf serum (Seromed), respectively. Penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) (Sigma Chemical Co.) were added to both media. The cells were cultured at 37 °C in a moist atmosphere of 5% carbon dioxide in air.

Irradiation Procedure. Irradiations were performed by means of Philips HPW 125 lamps equipped with a Philips filter emitting over 90% at 365 nm. Irradiation intensity was checked on a UV-X radiometer (Ultraviolet Products Inc., Cambridge, U.K.) for each experimental procedure.

Inhibition Growth Assays. HeLa cells (10^5) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, the medium was replaced with an equal volume of Dulbecco's modified Eagle medium (DMEM, Sigma Chemical Co.) without phenol red, and various concentrations of the test agent were added. One hour later, the cells were irradiated with a UVA dose of 0.789 J cm^{-2}. After irradiation, the medium containing the compounds was removed, and the cells were incubated in complete F-12 medium for 24 h. In the case of the experiments carried out in the dark, the replacement of the incubation medium was omitted, and the cells were incubated in complete F-12 medium in the presence of the test compound for 24 h.

HL-60 cells (10^5) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, various concentrations of the test agents were added in complete medium. The cells were kept in the dark for 1 h, irradiated with a UVA dose of 0.789 J cm^{-2}, and then incubated for further 24 h.

A trypan blue assay was performed to determine cell viability. Cytotoxicity data were expressed as IC_{50} values, that is, the concentrations of the test agent inducing 50% reduction in cell numbers compared with control cultures.

Skin Phototoxicity. Skin phototoxicity was tested on depilated albino guinea pigs (outbred Dunkin−Hartley strain), as previously reported.[25] An ethanol solution of each new compound and 8-MOP was applied topically to the skin at the concentration indicated. The animals were then kept in the dark for 45 min, and the treated skin was irradiated with 20 kJ m^{-2} of UVA; erythema was scored after 48 h.

Nucleic Acids. Salmon testes DNA was purchased from Sigma Chemical Company. Its hypochromicity, determined according to Marmur and Doty,[26] was over 35%. The DNA concentration was determined using an extinction coefficient of 6600 M^{-1} cm^{-1} at 260 nm. pBR322 DNA was purchased from Fermentas Life Sciences. KDNA was supplied with the topoisomerase II assay kit (Topogen, Inc.).

Linear Flow Dichroism. Linear dichroism (LD) measurements were performed on a Jasco J500A circular dichroism spectropolarimeter converted for LD and equipped with an IBM PC and a Jasco J interface.

Linear dichroism is defined as

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

where $A_\parallel$ and $A_\perp$ correspond to the absorbances of the sample when polarized light is oriented parallel or perpendicular to the flow direction, respectively. The orientation is produced by a device designed by Wada and Kozawa[27] at a shear gradient of 500−700 rpm, and each spectrum was accumulated four times.

A solution of salmon testes DNA (1.6×10^{-3} M) in ETN buffer (containing 10 mM TRIS, 10 mM NaCl, and 1 mM EDTA, pH = 7) was used. Spectra were recorded at 25 °C at different [drug]/[DNA] ratios.

Preparation of the Adducts. Volumes of concentrated solutions of the examined compounds were added to salmon testes DNA in ETN solution (1.5×10^{-3} M) to achieve a DNA/compound ratio of about 40. The mixture was irradiated in a glass dish with four Philips HPW 125 lamps, arranged two above and two below the dish, at a distance of 7 cm for 120 min at room temperature. After irradiation, the DNA was precipitated with NaCl (up to 1 M concentration) and cold ethanol (2 volumes); the precipitated DNA was collected, washed with 80% ethanol, dried, and then dissolved in a measured volume of buffer. The final solution was made 0.5 N with HCl, heated at 100 °C for 90 min, neutralized, and extracted exhaustively with $CHCl_3$. After this procedure, the organic layers were collected and dried under high vacuum, the residue was dissolved in ethanol, and the adduct was separated on TLC plates and eluted with $CHCl_3$/ethanol/ammonium hydroxide 9:0.8:0.2.

For compound **7**, the aqueous phase obtained after extraction with $CHCl_3$ was lyophilized and resuspended in ethanol. and then the photoproducts were separated on TLC plates by eluting with $CHCl_3$/ethanol 8:2.

UV spectra were recorded on a Perkin-Elmer model Lambda 5 spectrophotometer.

Mass spectrometry measurements were performed on an electrospray ionization (ESI) time-of-flight (ToF) instrument (mod. Mariner, Perseptive-Biosystem) by dissolving the samples in water/acetonitrile/formic acid (50:49:1) solution.

NMR Characterization of the Adduct. The NMR spectra were acquired in DMSO-d_6, at 298 K, on a Bruker Avance DMX 600 spectrometer (600.09 MHz proton frequency) equipped with a 5 mm TXI probe. Data processing was performed on an Indy workstation using XWIN NMR software. ^{1}H and ^{13}C resonance assignment was obtained using 2D homonuclear correlation spectroscopy (DQF−COSY[28] and ROESY[29] experiments) and 2D heteronuclear correlation spectroscopy [heteronuclear multiple quantum correlation (HMQC)[30] and heteronuclear multiple bond correlation (HMBC)[31]]. The proton and carbon chemical shifts were referenced to the residual solvent signal at 2.49 ppm (^{1}H) and at 39.5 ppm (^{13}C).

The ^{1}H 1D spectrum was acquired with 32 transients, a spectral width of 6613 Hz, and 16 384 data points. DQF−COSY and ROESY spectra were recorded using a total of 512 and 400 experiments, respectively, of 32 scans each; 2048 data points were recorded in the acquisition dimension (F2), and the spectral width was 14 ppm. The data were zero-filled in both dimensions to final sizes of 2048 × 1024 points. Multiplication with a Gaussian function in F2 and a sin function (DQF−COSY) or cos^2 function (ROESY) in F1 were performed prior to 2D Fourier transformation. In the ROESY experiment, a spin lock sequence of small flip angle pulses was applied for a mixing period of 300 ms.[32]

The ^{1}H−^{13}C HMQC spectrum was acquired using a total of 200 experiments of 200 scans each. The time proportional phase incrementation (TPPI) method was used,[33] and decoupling of ^{13}C during acquisition was achieved with a Globally optimized Alternating phase Rectangular Pulse (GARP) sequence. The spectral width was 11 ppm in F2 and 200 ppm in F1. The data were zero-filled in both dimensions to final sizes of 2048 × 512 points. Multiplication with a Gaussian function (F2) and a cos^2 function (F1) was performed prior to 2D Fourier transformation.

The ^{1}H−^{13}C HMBC was acquired using a total of 256 experiments of 300 scans each and processed in the magnitude mode. The evolution delay for ^{1}H−^{13}C long-range coupling was 67 ms; the spectral width was 11 ppm in F2 and 320 ppm in F1. The data were zero-filled in both dimensions to final sizes of 2048 × 1024 points. Multiplication with a Gaussian function (F2) and a cos^2 function (F1) was performed prior to 2D Fourier transformation.

Polymerase Stop Assay. The target DNA sequence (a regulatory region of the promoter F of the human estrogen receptor gene containing putative binding sites of psoralen-related compounds) was the plasmid PGL-3 862;[34] DNA (100 ng/reaction) was incubated with compounds **7** and **10** for 10 min in the dark, and then UV exposure was performed for 10 min at 365 nm. The primers were the forward 5′-CCT GCT TTT AGT TTA GCT GT-3′ and the reverse 5′-ATC ATG TGT TAA TCT GCC TG-3′ synthetic oligonucleotides. DNA amplification was carried out in PCR buffer with 0.25 ng of primers, 250 μM of each dNTP and 1 U of *Taq* DNA polymerase with 1 μg of drug-treated DNA. PCR assay was carried out for 30 cycles, each consisting of 1 min denaturation at 95 °C, 1 min annealing at 60 °C, and 1 min elongation at 72 °C. In the experiments performed to determine the PCR blocks, a ^{32}P-labeled forward primer was employed. After the last cycle, samples were chilled on ice, phenol-extracted, precipitated with ethanol, and analyzed by polyacrylamide gel electrophoresis as previously reported.[24] After electrophoresis, gels were transferred to filter papers and dried, and autoradiography was performed.

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

Evaluation of Interstrand Cross-Links in Vitro. Evaluation of cross-links was carried out by measuring the renaturation capacity of cross-linked DNA after thermal denaturation. Aliquots of aqueous solution of salmon testes DNA and examined compound, [DNA]/[drug] = 75, were introduced into calibrated glass tubes, immersed in a thermostatically controlled bath, and then irradiated for various periods of time. After irradiation, the samples were thermally denatured (95 °C for 15 min) and quickly cooled in ice. The renaturation capacity of DNA, due to cross-link formation, was investigated by recording the absorbance at 260 nm. Data were expressed in terms of log % of nonrenaturated fraction of irradiated compound−DNA complex relative to irradiated DNA, as suggested by Blais et al.[35]

Decatenation Assay. Topoisomerase II activity was measured by the ATP-dependent decatenation of kinetoplast DNA (KDNA). The decatenation assay was performed using the topoisomerase II assay kit (TopoGEN, Inc.) and human topoisomerase II (TopoGEN, Inc.). Briefly, 20 μL reaction volumes containing 0.15 μg of KDNA, test compound as indicated, and 1 U of topoisomerase II were incubated for 1 h at 37 °C. After this time, 4 μL of stop buffer and 50 μg/mL of proteinase K (Sigma Chemical Company) were added, and the mixture was incubated for further 30 min at 37 °C. The samples were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide, 0.5 μg/mL in TAE buffer (40 mM Tris, 30 mM glacial acetic acid, 1 mM EDTA, pH = 8). After electrophoresis, gels were destained in distilled water for 30 min at room temperature.

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Supporting Information Available: Elemental analysis, NMR, IR, and mass spectrometry data of all the new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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