4′-Methyl Derivatives of 5-MOP and 5-MOA: Synthesis, Photoreactivity, and Photobiological Activity

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The synthesis and photobiological activity of four new 4′-methyl derivatives of 5-MOP (5-methoxypsoralen) and 5-MOA (5-methoxyangelicin), i.e., 4,4′-dimethyl-5-methoxypsoralen, 3,4′-dimethyl-5-methoxypsoralen, 4,4′-dimethyl-5-methoxyangelicin, and 3,4′-dimethyl-5-methoxyangelicin, are described. All these compounds photobind efficiently to DNA. The DNA-photobinding process was investigated using various nucleic acid structures such as double-helix DNA, bacterial DNA, and synthetic polydeoxyribonucleotides. Photoreaction experiments showed that, unlike 8-MOP (8-methoxypsoralen) and 5-MOP, both angular derivatives bind thymine and cytosine with the same efficiency. The principal nucleoside−psoralen monoadducts were isolated and characterized after enzymatic digestion or acid hydrolysis. Biological activity studies revealed a good correlation with the extent of covalent photoaddition. Moreover, the two angular derivatives and the 4,4′-dimethyl-5-methoxypsoralen were unable to induce skin erythema, in striking contrast with the reference drugs, 8-MOP and 5-MOP; only the 3,4′-dimethyl-5-methoxypsoralen caused erythema, although to a substantially lower extent than that induced by the two parent compounds.

Introduction

The photosensitizing furocoumarins 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), and 4,5′,8-trimethylpsoralen (TMP) are widely used in PUVA therapy (psoralen plus ultraviolet-A radiation) as effective treatment for various skin diseases and, more recently, for cutaneous T-cell lymphoma by means of photopheresis, an extracorporeal form of photochemotherapy.[1−3] The biological activity of these derivatives has been correlated to their ability to photoreact with DNA; photoaddition involves the 5,6 double bond of the pyrimidine bases of the macromolecule and the 3,4 and/or 4′,5′ psoralen double bonds, leading to the formation of mono- and diadducts in DNA.[4,5] This damage to the macromolecule is also responsible for some of their therapeutic effects. Both mono- and diadducts are effective in inhibiting DNA synthesis, although decreased photoinduced mutagenicity has been found in the case of monofunctional as opposed to bifunctional psoralens.[6] Thus, a general attempt has been made to find new compounds which only give rise to monofunctional photobinding with DNA, with the aim of reducing undesired side effects such as skin phototoxicity and genotoxicity. We have studied a number of derivatives of 8-MOP bearing one or two methyl groups at positions 3 or 4 and 4′ of the tricyclic structure. In particular, one of these compounds, 3,4′-dimethyl-8-MOP, showed intense antiproliferative activity without mutagenicity and skin toxicity. This compound is essentially monofunctional, and its unusual behavior is due to its ability to form the furan side adduct having particular characteristics.[7]

More recently, especially in Europe, 5-MOP has efficiently been used in the oral therapy of psoriasis, being less toxic and better tolerated by patients than 8-MOP.[5,8,9] In light of this new evidence, it appeared interesting to examine new derivatives of 5-MOP. Moreover, remarkable attention has been directed to angelicins, which are monofunctional agents:[10] a number of methylangelicins have been synthesized and investigated. They show significant antiproliferative activity, lower genotoxicity than psoralens, and, in general, lack of skin phototoxicity.[11−14] Nevertheless, no investigation has ever been made on the presence of a methoxy group in the 5 position with methylangelicins.

In this paper we describe the preparation of four 4′-methyl derivatives of 5-MOP (compounds **1** and **3**) and 5-MOA (compounds **2** and **4**), all carrying a methyl group at position 4′ and a further methyl at position 3 or 4 (see Schemes 1 and 2); their photoreactivity with nucleic acids and their photobiological activity were studied.

We also report an in-depth investigation of photoaddition toward bacterial nucleic acids and synthetic polydeoxyribonucleotides, highlighting the fact that, unlike 8-MOP and 5-MOP, 4,4′-dimethyl-5-methoxyangelicin binds both thymine, the DNA base usually preferred by furocoumarins, and cytosine with the same efficiency. We therefore isolated and characterized the principal nucleoside−psoralen monoadducts after enzymatic digestion and acid hydrolysis of DNA irradiated in the presence of 4,4′-dimethyl-5-methoxyangelicin, in order to evidence and confirm their unusual and interesting behavior.

Results and Discussion

Chemistry. Linear furocoumarin **1**, its angular isomer **2**, and their corresponding 3-methyl derivatives

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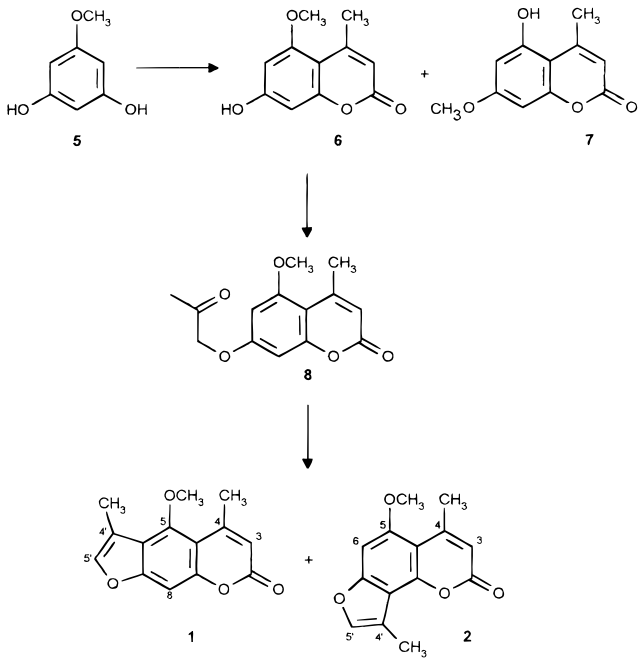
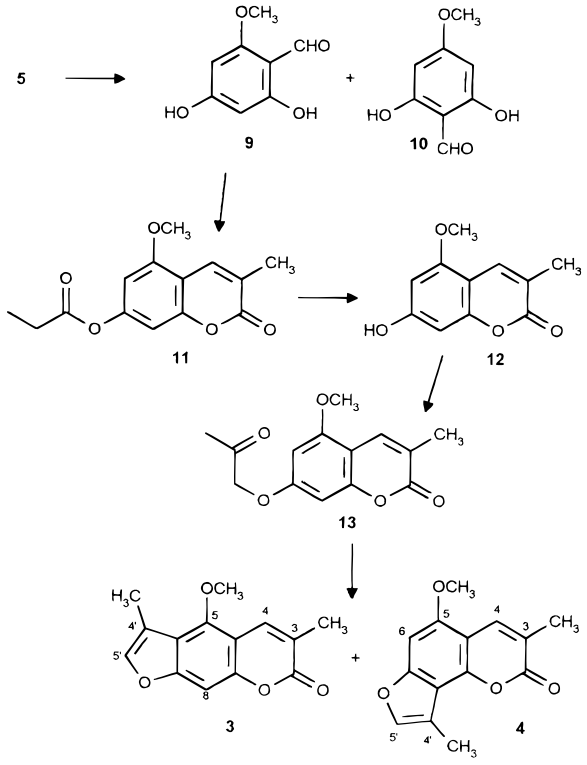
Scheme 1



Scheme 2



3 and **4** were obtained from 1,3-dihydroxy-5-methoxybenzene (**5**), as shown in Schemes 1 and 2.

The Pechmann reaction of **5** and ethyl acetoacetate in sulfuric acid gave coumarins **6** and **7** in 53:47 molar ratio and 65% combined yield. The compounds were separated by flash chromatography with 9:1 toluene/ethyl acetate as eluent and identified by NOESY experiments. The first product eluted was **7**, in which irradiation at the frequency of the methoxy signal led to enhancement of the signals due to both aromatic H(6) and H(8); for compound **6**, only the signal due to H(6) was enhanced. The 7-hydroxycoumarin **6** was converted to the β-keto ether **8** in 66% yield by reaction with chloroacetone in the presence of potassium carbonate. Heating **8** in a strong alkaline solution and then acidifying the reaction mixture afforded the isomeric cyclized products **1** and **2** in 35:65 molar ratio and 89% combined yield; these isomers were separated by flash chromatography as before. The first product eluted was unequivocally identified as **2** by NOESY experiments, since irradiation at the frequency of the methoxy signal led to enhancement of only the signal due to H(6); for **1** the signals due to both methyl groups were enhanced.

Table 1. Binding Parameteres of Complexes between Compounds and Calf Thymus DNA and Rate Constants of Their Photoreaction between Various Furocoumarins and Macromolecule

compd	K (M^{-1})[a]	$1/n$	rate constant, min^{-1}
1	630	5×10^{-3}	2.1×10^{-2}
2	3700	1.2×10^{-2}	2.2×10^{-2}
3	920	4.5×10^{-3}	0.32×10^{-2}
4	3100	0.9×10^{-2}	0.17×10^{-2}
5-MOP	1700[b]	6.5×10^{-2}	0.41×10^{-2}

[a] K = association constant to an isolated site. [b] Taken from ref 32.

For preparation of the dimethyl furocoumarins **3** and **4**, rather than HCN,[15] anisole **5** was formylated by zinc cyanide/HCl in the presence of a small amount of zinc chloride. A mixture of salicyl aldehydes **9** and **10** was thus obtained in 88:12 molar ratio and 69% combined yield. The Perkin reaction of **9** with a carefully controlled amount of propionic anhydride and sodium propionate directly gave coumarin **12** in moderate yields. However, the use of a large excess of reagents and acid hydrolysis of the intermediate 7-(propionyloxy)-coumarin **11** gave **12** in a better overall yield (57%, from **9**). The β-keto ether **13**, obtained as described above for **8**, was cyclized in a strong alkaline solution to afford furocoumarins **3** and **4** in 25:75 molar ratio and 78% overall yield from **12**. These compounds were purified by flash chromatography and identified by NOESY experiments. Since irradiation at the frequency of the methoxy signal led to enhancement of H(6), it was **4** which was eluted first.

Noncovalent Binding to DNA. It is well-known that furocoumarins first form a reversible intercalated complex with the nucleic acid and that this step is a preliminary necessary event for the successive photoaddition.[4] The binding process was evaluated by equilibrium dialysis experiments using labeled furocoumarins.[16] The experimental data, analyzed using the neighbor exclusion model,[17] allowed computation of the binding parameters. The complexation ability of the new derivatives (see Table 1) is similar to the reference drug, the values of these parameters being of the same order of magnitude as those of 5-MOP.

Photobinding to DNA. Irradiation of DNA solutions in the presence of the tritiated new compounds allowed determination of the amount of each furocoumarin covalently linked to the macromolecule; 5-MOP was also tested as reference. Figure 1 reports the photobinding of these compounds as a function of irradiation time: both angular and linear 4,4′-dimethyl derivatives appear to be remarkably more effective than their nonmethylated congener (3 times more reactive than 5-MOP). The two 3,4′-dimethyl isomers also bind efficiently to nucleic acid, although to a lower extent than the reference drug. These photoreactions behave like pseudo-first-order reactions with respect to the

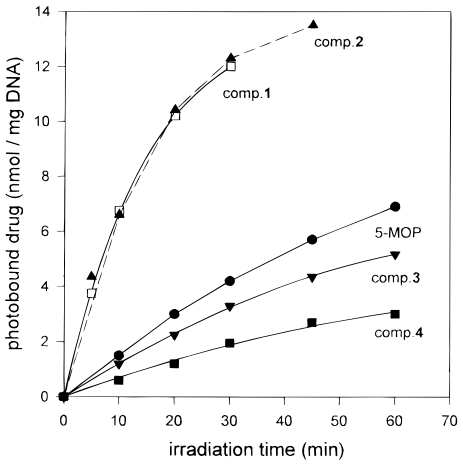


Figure 1. Photobinding of compounds **1**−**4** and 5-MOP to double-stranded DNA from calf thymus (nucleotide−drug ratio = 75) as a function of irradiation time.

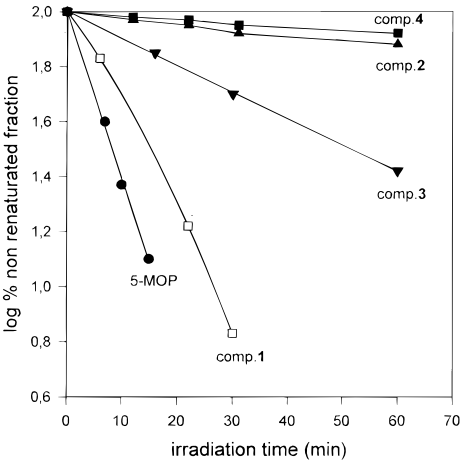


Figure 2. Cross-linking of compounds **1**−**4** and 5-MOP to double-stranded DNA from calf thymus (nucleotide−drug ratio = 75) as a function of irradiation time.

furocoumarin complexed to the macromolecule.[12] The rate constant values, reported in Table 1, reveal the good activity of the 4,4′-dimethyl derivatives.

Cross-Linking. Figure 2 shows the cross-linking ability of the dimethyl compounds; as expected, by virtue of their angular structure, compounds **2** and **4** are monofunctional; compound **3** is less efficient in inducing cross-links than reference drug 5-MOP, and compound **1** appears as efficient as its unsubstituted congener in similar experimental conditions. Following Lown and Sim (1978),[18] we calculated the average number of cross-links per DNA molecule (at 10 min irradiation, the figures were 1.38, 0.69, and 0.23 for 5-MOP, **1**, and **3**, respectively) and compared them with the photobinding data (Figure 1). The mono-/cross-link ratio was then 0.10, 0.19, and 0.92 for compounds **1**, **3**, and 5-MOP. In other words, in terms of ability to photobind macromolecule vs ability to form diadducts in the double helix of DNA, both linear derivatives have a lower ratio, so that lower toxicity may be expected.

Photobinding to Bacterial DNA and Synthetic Polynucleotides. Further information on drug interaction with its own receptor (nucleic acid) can be gained easily by identification of site specificity in the photobinding process. Thus we followed the photobinding time course of compounds **1** to **4** and 5-MOP to nucleic acids having different base pair compositions. Since

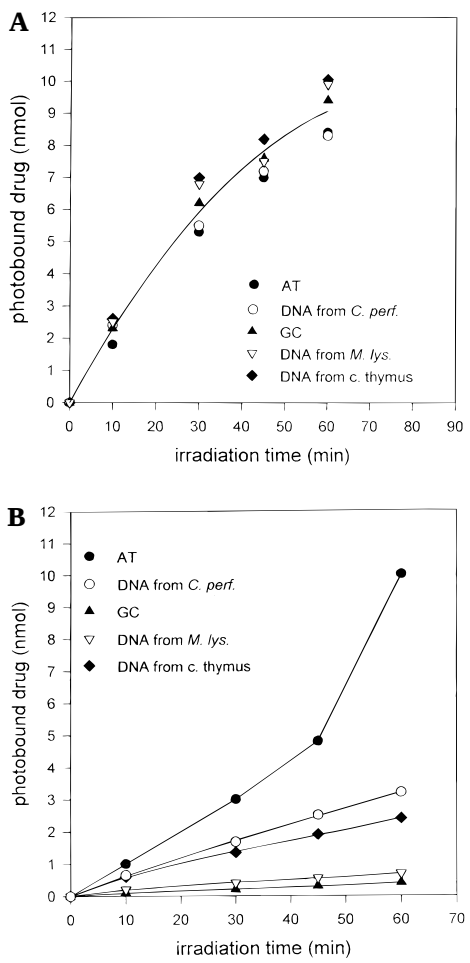


Figure 3. (A) Photobinding of compound **2** to synthetic polydeoxyribonucleotides and DNA with various A-T contents, as a function of irradiation time. (B) Photobinding of 5-MOP to synthetic polydeoxyribonucleotides and DNA with various A-T contents, as a function of irradiation time.

thymine is by far the preferred pyrimidine base in psoralen photobinding, generally increasing the A-T content causes a linear increase in the amount of the drug covalently bound to the polynucleotide.[19,20] We noted that the new linear derivatives show behavior similar to that of classical furocoumarins (data not shown), whereas the photoreactivity of angular furocoumarins does not depend on A-T content. To further investigate this unusual behavior, the photobinding of these compounds to poly[dA-dT]−poly[dA-dT] and poly[dG-dC]−poly[dG-dC] was studied. The results are plotted in Figure 3 in terms of the photoreactivity of compound **2** and 5-MOP to three DNA samples with different base compositions and two polynucleotides, as a function of irradiation time. Figure 4 shows the extent of covalent photobinding plotted against the A-T content (28% in *Micrococcus lysodeikticus*, 60% in calf thymus, 72% in *Clostridium perfringens*). The photoreactivity of compound **2** and of its congener **4** was substantially constant for all DNA samples, indicating much more widespread photoaddition along the polynucleotide and therefore an identical ability to react with cytosine and thymine.

Preparation of Adducts. To isolate and characterize the adducts between furocoumarin **2** and the nucleic acid, an aqueous DNA solution was irradiated (365 nm) in the presence of the examined furocoumarin. After

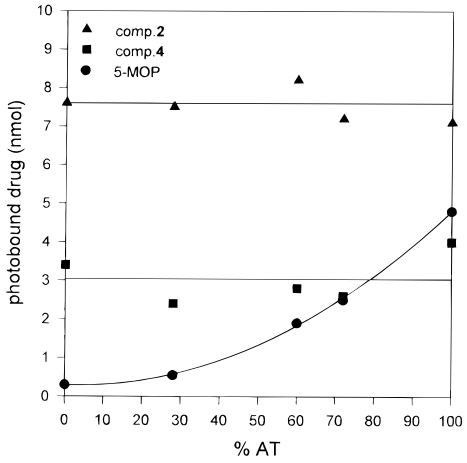


Figure 4. Photobinding of compounds **2**, **4**, and 5-MOP as a function of % A-T content in nucleic acids (irradiation time 45 min).

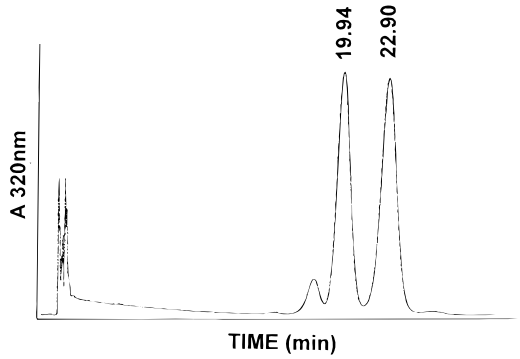


Figure 5. HPLC elution profile of photoadducts forming between calf thymus DNA and compound **2**, obtained after enzymatic digestion of macromolecule. C-18 column eluted with a methanol/water mixture (40:60), at a flow rate of 1 mL/min.

irradiation and precipitation, the photomodified DNA was enzymatically digested (see the Experimental Section) and analyzed by HPLC. The chromatographic elution profile is shown in Figure 5: two distinct photocompounds are evident at retention times of 19.94 (Add I) and 22.9 (Add II) min, respectively. Both UV absorption spectra showed evident absorption at about 330 nm, suggesting cycloaddition at the furan side. The isolated photocompounds were photosplit with 254 nm light (see the Experimental Section), and the process was followed spectrophotometrically (Figure 6 for Add I). Gradual decrease of the absorption band at 330 nm accompanied by increase of the bands at 310 and 250−260 nm, typical of parent compound **3** (pyrimidine bases also absorb at 260 nm). Add I was photosplit into 4,4′-dimethyl-5-methoxyangelicin and uridine; Add II gave 4,4′-dimethyl-5-methoxyangelicin and thymidine, as shown by TLC comparison with authentic samples. It is well-known that DNA base cytosine easily undergoes hydrolysis when its 5,6 bond is saturated: thus the presence of uracil indicated that our compound had been photoadded to cytosine in the nucleic acid.[21]

To confirm the chromatographic data, the HPLC-isolated fractions were submitted to FAB analysis in a glycerol matrix. The most important peaks are shown in Table 2: the prominent ion at *m*/*z* 473 corresponds to the uridine photoadduct, and that at *m*/*z* 487 to that of thymidine. Furthermore, uridine and thymidine are seen in the relative spectra in the lower mass region.

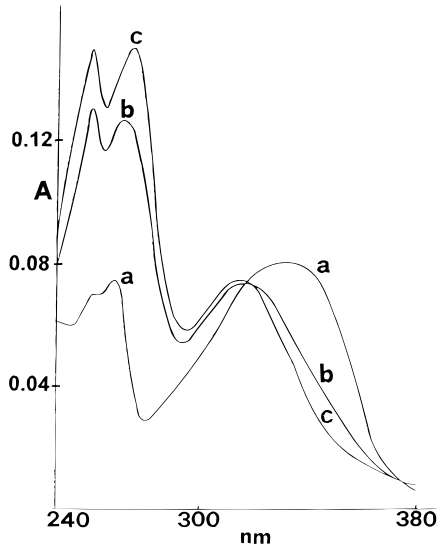


Figure 6. UV absorption spectra of an ethanol solution of photoadduct I before (a) and after irradiation at 254 nm for 5 (b) and 10 min (c).

Table 2. Mass Spectrometry Data

adduct I			adduct II		
m/*z*	%	assignment	*m*/*z*	%	assignment
473	3	$[M + H]^+$	487	5	$[M + H]^+$
355	3.8	$[M - C_5O_3H_9]^+$	413	11	$[MH - C_3H_6O_2]^+$
244	14	$[comp]^+$	270	5	$[C_{16}H_{14}O_4]^+$
227	24	$[C_9H_{11}O_5N_2]^+$	244	7	$[comp]^+$
207	31	$[C_{10}H_{10}O_4 + H]^+$	242	10	$[C_{10}H_{14}O_5]^+$
198	20	$[comp - OC_2H_6]^+$	226	10	$[C_9H_{12}O_5N_2]^+$
177	10	$[C_{10}H_8O_3 + H]^+$	128	25	$[thymine + H]^+$
117	28	[deoxyribose]	117	15	[deoxyribose]
113	40	$[uracil + H]^+$			

Several irradiation procedures followed by acid hydrolysis, which also removes the sugar moiety, and TLC purification allowed us to obtain sufficient amounts of the base−angelicin photoadducts for NMR experiments. The silica gel plates showed two violet-fluorescent bands with R_f values of 0.43 (Add I H^+) and 0.56 (Add II H^+), respectively, when the plates were eluted with ethyl acetate−ethanol, 9:1. The silica was extracted with ethanol, which was then removed *in vacuo*, and the residue dissolved in acetone-d_6.

Add I H^+:^{1}H-NMR (acetone-d_6) δ 1.86 (1s, 3H, 4′-Me), 2.50 (d, 3H, 4-Me, J = 1.2 Hz), 3.54 (d, 5-H_U, J = 9.9 Hz), 3.92 (s, 3H, OMe), 4.52 (ddd, 6-H_U, J = 9.9, 5.1, 2.0 Hz), 5.02 (d, 5′-H, J = 5.1 Hz), 5.86 (q, 1H, 3-H, J = 1.2 Hz), 6.46 (s, 1H, 6-H), 6.68 (br, 1H, 1-H_T), 8.50 (br, 1H, 3-H_T).

Add II H^+ differs from I H^+ only in the cyclobutane signals: two methyl groups are present at 1.58 and 1.73 ppm (2s, 3H each, 5-Me_T, 4′-Me) while two protons resonate at 4.17 (dd, 1H, 6-H_T, J = 5.3, 2.6 Hz) and 5.01 (d, 1H, 5′-H, J = 5.3 Hz), respectively.

The NMR data thus confirm that the compounds are C_4 cycloadducts between the furocoumarin and either thymine or uracil. The coupling pattern of the cyclobutyl protons shows that, for them, the reaction took place with *syn* regiochemistry: in both adducts 6-H of the base and 5′-H of angelicin are vicinal in the cyclobutane ring. Moreover, the coupling constant values are in agreement with those found in several furan side adducts having *cis* stereochemistry.[22,23] The structure shown in Figure 7 was thus assigned to the adducts.

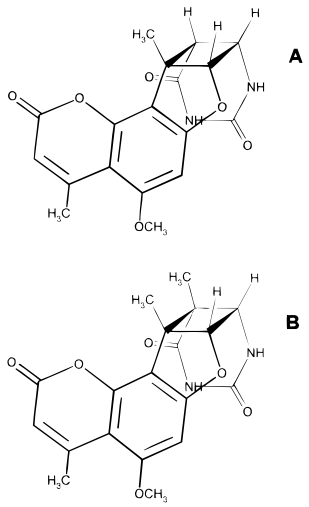


Figure 7. Molecular structure of *cis-syn* furan side adduct beetwen 4,4′-dimethyl-5-MOA and uracil (Add I H^+) or thymine (Add II H^+).

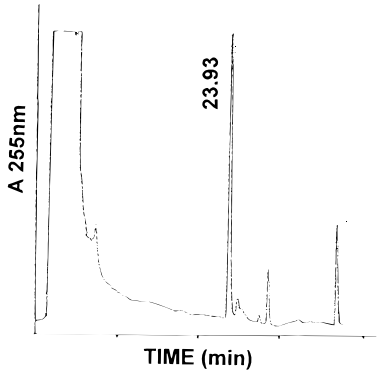


Figure 8. HPLC elution profile of photoadducts forming between calf thymus DNA and compound **1**, obtained after acid hydrolysis of macromolecule.

Compound **1** was also submitted to acid hydrolysis and HPLC analysis (see Figure 8); only one important peak appears at 23.93 min, whose absorption spectrum revealed that this compound undergoes photoaddition to thymine in the nucleic acid on the furan side. NMR experiments were performed and confirmed this assumption: ^{1}H-NMR (acetone-d_6) δ 1.62 (s, 3H, 4′-Me), 1.77 (s, 3H, 5-Me$_T$), 2.58 (d, 3H, 4-Me, J = 1.3 Hz), 4.02 (s, 3H, OMe), 4.80 (d, 1H, 6-H$_T$, J = 5.0 Hz), 4.95 (d, 1H, 5′-H), 6.00 (q, 1H, 3-H), 6.55 (s, 1H, 8-H). These findings can be explained by the adduct with *cis-syn* configuration. No evidence of photobinding to cytosine was obtained with compound **1**.

Formation of Singlet Oxygen. For all compounds, 40 min irradiation induced bleaching of *N*,*N*-dimethyl-*p*-nitrosoaniline only by 1.2−1.5%, showing that they are poorly efficient in generating this reactive species of oxygen. These results could indeed represent a desired property as it may result in lower skin phototoxicity.

Photobiological Activity. The results of cell growth inhibition studies are shown in Table 3, expressed in terms of IC_{50}. Besides 5-MOP, we took as reference compounds 8-MOP (the drug most pregnantly used in photochemotherapy) and 5-MOA, although the latter was known to be practically inactive.[15] Toward HL60 cells, both 4,4′-dimethyl derivatives and the angular 3,4′-dimethyl derivative had almost the same activity as 8-MOP, while the linear 3,4′-dimethyl derivative was much less active.

Table 3. Cell Growth Inhibition by Irradiation (365 nm) in the Presence of Examined Compounds and 5- and 8-MOP as Reference Drugs

	cell lines IC_{50} (μM)		
compd	HL60	HeLa	A 431
8-MOP	0.75 ± 0.09	3.16 ± 0.34	2.48 ± 0.18
5-MOP	1.30 ± 0.07	4.23 ± 0.21	3.55 ± 0.23
5-MOA	>10	>10	7.72 ± 0.41
1	0.67 ± 0.02	1.76 ± 0.25	1.35 ± 0.05
2	0.69 ± 0.03	1.26 ± 0.14	1.32 ± 0.02
3	2.72 ± 0.11	6.83 ± 0.52	7.75 ± 0.61
4	0.59 ± 0.04	1.06 ± 0.12	1.37 ± 0.07

Table 4. Skin Phototoxicity in Guinea Pigs after Exposure to 50 μg cm^{-2} of Tested Compounds and 20 kJ m^{-2} of UVA

compd	formation of erythema[a]
8-MOP	+++ (with edema)
5-MOP	+++ (without edema)
5-MOA[b]	− − −
1	− − −
2	− − −
3	++−
4	− − −

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

On HeLa cells, the concentration needed to inhibit growth was lowest for the two angular derivatives and slightly higher for compound **1**, all three being more active than 8-MOP and 5-MOP; compound **3** again shows little efficacy. Finally, also on A431 cells, cytotoxicity data confirmed the significant activity of compounds **1**, **2**, and **4.**

Tests of skin photosensitizing potency on guinea pigs performed with the new compounds revealed that the two angular and the linear 4,4′-derivatives were completely devoid of phototoxicity. Instead, the linear 3,4′-derivative caused erythema, although to a substantially lower extent than that induced by the two parent compounds 5-MOP and 8-MOP (see Table 4).

Conclusions

The effects of methyl groups on 5-methoxypsoralen and 5-methoxyangelicin were evaluated in terms of photointeraction with DNA and antiproliferative activity. DNA photobinding of the 4,4′-dimethyl derivatives, as shown in Figure 1, is higher than that of 5-MOP, while the 3,4′ derivatives show lower photoreactivity. As expected, both linear derivatives are cross-linking agents, although less effective than 5-MOP, and the angular compounds behave as monofunctional photoreagents. Furthermore, all of the compounds photoreact almost exclusively through the double bond at the furan ring. However, when photobinding was studied in DNA samples having different base content, a different behavior appeared between the linear and angular compounds. The former, like 5-MOP and all furocoumarins studied until now, show higher photobinding with increasing A-T content. Instead, the photoreactivity of the latter is independent of base composition. This was further proven with compound **2** by isolating and characterizing the photoproducts forming in calf thymus DNA: after enzymatic hydrolysis, a similar yield of cycloadducts with thymidine and cytidine was found. In previous studies carried out with several

angular derivatives, thymine has always been shown to be strongly preferred over cytosine for photobinding to DNA:[5] in particular, this is the behavior of 4′-methylangelicin.[24] Moreover, we performed a photoreaction with 5-MOA, using the acid hydrolysis described for compound **1** in the Experimental Section; a single fluorescent photoadduct was evidenced by both HPLC and TLC. The unusually high photobinding to cytosine should thus be attributed to the simultaneous presence of both 5-methoxy and 4'-methyl substituents. Compound **1** also reacts only with thymine. The behavior of the angular derivatives may stimulate research about their possible biological importance.

Antiproliferative activity, measured on three different cell lines, parallels in vitro photobinding, being **1** ≈ **2** > 5-MOP > **3**.

Surprisingly, 3,4′-dimethyl-5-MOA (**4**) was as active as **1** and **2**, even showing lower photoreactivity than **3** itself and being a pure monofunctional agent.

Of the new compounds, only 3,4′-dimethyl-5-MOP (**3**) is able to cause skin erythema in guinea pigs, but it is less severe than that caused by 5-MOP. While a lack of phototoxicity was expected for the two angular derivatives, it was noteworthy for linear compound **1**, although other bifunctional psoralens (3,4′-dimethyl-8-MOP,[7] 4-(hydroxymethyl)-4′-methylpsoralen, and 4-(hydroxymethyl)-4′-methyl-8-MOP[25]) are devoid of phototoxic side effects.

Both the antiproliferative and erythemogenic activities of this series of compounds also show that the classical scheme DNA photobinding → antiproliferative effect and cross-link formation → skin phototoxicity[10] never occurs, and that interpretation of the structure−activity relationship is still unclear.

Since cell damage may also be induced by the photodynamic action of furocoumarins,[26] we investigated the possible production of singlet oxygen by the new four compounds. All of them were practically unable to induce this active species of oxygen, being less active than 5-MOP itself, which is known to be a poor $^1O_2$ producer.[26]

Compounds **1** and **2** are new analogs of 5-MOP which show higher antiproliferative activity without inducing skin erythema. For compound **4**, the lack of correlation between biological activity and DNA photobinding capacity suggests that different mechanisms and/or targets are involved.

The mutagenic and toxicological aspects of new 4′-methyl derivatives of 5-MOP and 5-MOA will also be evaluated in view of a possible therapeutic approach.

Experimental Section

Melting points are uncorrected and were determined in a Reichert Kofler thermopan or in capillary tubes in a Büchi 510 apparatus. IR spectra were recorded in a Perkin-Elmer 1640FT spectrometer (KBr disks, ν in cm^{-1}). ^1H NMR spectra were recorded in Varian Gemini (200 MHz) or Bruker AMX (300 MHz) spectrometers (chemical shift in ppm from TMS, *J* in hertz). Mass spectrometry was carried out on a Hewlett-Packard 5988A spectrometer. Elemental analyses were performed by a Perkin-Elmer 240B microanalyser 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).

7-Hydroxy-5-methoxy-4-methylcoumarin (6) and 5-Hydroxy-7-methoxy-4-methylcoumarin (7). Ethyl acetoacetate (10 mL, 78.5 mmol) was added to 1,3-dihydroxy-5-methoxybenzene (**5**; 10 g, 71.4 mmol) in concentrated H_2SO_4 (80 mL) and stirred under reflux for 2 h. The reaction mixture was then poured into cold water (500 mL) and left to stand overnight. The resulting precipitate was filtered out, washed, and purified by FC with 9:1 toluene/ethyl acetate as eluent. In order of elution, unreacted **5** (2.8 g), **7** (3.6 g, 30.2%), and **6** (3.6 g, 34%) were isolated; analytical samples of **6** and **7** were obtained by recrystallizing the chromatographed materials from 1:1 toluene/ethyl acetate. Compound **6**: mp 256−258 °C; ^1H NMR (DMSO-d_6) 10.60 (s, 1H, OH), 6.36 (d, 1H, 6-H, J = 2.3), 6.32 (d, 1H, 8-H, J = 2.3), 5.93 (q, 1H, 3-H, J = 1.2), 3.83 (s, 3H, OMe), 2.46 (d, 3H, 4-Me, J = 1.2); IR 3190, 1695, 1635, 1475, 1362, 1202, 1122, 822. Anal. ($C_{11}H_{10}O_4$) C, H. Compound **7**: mp 263−264 °C; ^1H NMR (DMSO-d_6) 10.74 (s, 1H, OH), 6.44 (d, 1H, 6-H, J = 2.4), 6.33 (d, 1H, 8-H, J = 2.4), 5.95 (q, 1H, 3-H, J = 1.2), 3.78 (s, 3H, OMe), 2.51 (d, 3H, 4-Me, J = 1.2); IR 3162, 1683, 1633, 1600, 1349. Anal. ($C_{11}H_{10}O_4$) C, H.

7-(Acetonyloxy)-5-methoxy-4-methylcoumarin (8). To a solution of **6** (2 g, 9.71 mmol) in anhydrous acetone (1 L) were added chloroacetone (2 mL, 25 mmol) and potassium carbonate (10 g). The mixture was stirred under reflux for 12 h, the resulting precipitate was filtered out, and the filtrate was retained. The solvent was evaporated *in vacuo* to leave a residue, which was purified by FC with 9:1 toluene/ethyl acetate as eluent to give **8** (1.6 g, 66%): mp 198−200 °C; ^1H NMR ($CDCl_3$) 6.40 (d, 1H, 6-H, J = 2.4), 6.32 (d, 1H, 8-H, J = 2.4), 5.99 (q, 1H, 3-H, J = 1.2), 4.63 (s, 2H, CH_2), 3.88 (s, 3H, OMe), 2.54 (d, 3H, 4-Me, J = 1.2), 2.30 (s, 3H, MeCO); IR 1717, 1620, 1600, 1358, 1165, 1125. Anal. ($C_{14}H_{14}O_5$) C, H.

4,4′-Dimethyl-5-methoxyfuro[3,2-*g*]coumarin (1) and 4,4′-Dimethyl-5-methoxyfuro[2,3-*h*]coumarin (2). To a solution of **8** (1g, 3.8 mmol) in ethanol (200 mL) was added 0.1 M NaOH (200 mL). The mixture was refluxed for 3 h and then acidified with HCl; this acidic solution was evaporated to half its volume before it was extracted with chloroform. The extract was washed and dried with Na_2SO_4, and the solvent was removed under reduced pressure to afford a residue, which was purified by FC with 95:5 hexane/ethyl acetate as eluent. Compound **2** (543 mg, 58%) was eluted first, and then **1** (291 mg, 31%). Analytical samples of both compounds were obtained by recrystallizing the chromatographed materials from ethanol/hexane. Compound **1**: mp 190−192 °C; ^1H NMR ($CDCl_3$) 7.38 (q, 1H, 5′-H, J = 1.4), 7.24 (s, 1H, 8-H), 6.15 (q, 1H, 3-H, J = 1.3), 3.90 (s, 3H, OMe), 2.69 (d, 3H, 4-Me, J = 1.3), 2.42 (d, 3H, 4′-Me, J = 1.4); IR 3097, 2928, 1715, 1621, 1573, 1148, 1062, 837; MS m/z 244 (M^+, 100), 229 (15), 201 ($(M + 1)^+ - CO_2$, 57), 115 (14), 58 (42). Anal. ($C_{14}H_{12}O_4$) C, H.

Compound **2**: mp 210−211 °C; ^1H NMR ($CDCl_3$) 7.29 (q, 1H, 5′-H, J = 1.4), 6.82 (s, 1H, 6-H), 6.08 (q, 1H, 3-H, J = 1.2), 3.91 (s, 3H, OMe), 2.61 (d, 3H, 4-Me, J = 1.2), 2.47 (d, 3H, 4′-Me, J = 1.4); IR 3122, 1725, 1605, 1590, 1108; MS m/z 244 (M^+, 100), 216 (29), 201 ($(M + 1)^+ - CO_2$, 69), 115 (12). Anal. ($C_{14}H_{12}O_4$) C, H.

2,4-Dihydroxy-6-methoxybenzaldehyde (9). To a solution of compound **5** (10 g, 71.5 mmol) in anhydrous diethyl ether (200 mL) were added zinc cyanide (8.3 g, 71 mmol) and zinc chloride (2 g). Hydrogen chloride was bubbled for 2 h through the mixture, which was then left to stand overnight. The resulting precipitate was filtered out, redissolved in water (200 mL), and boiled for 5 min. The cooled aqueous solution afforded a second precipitate which was purified by FC with 1:1 hexane/ethyl acetate as eluent. Subsequent recrystallization from toluene yielded 7.5 g (51%) of **9**: mp 201−202 °C; ^1H NMR (DMSO-d_6) 12.35 (s, 1H, OH), 10.96 (s, 1H, OH), 9.95 (s, 1H, CHO), 5.99 (d, 1H, 5-H, J = 1.9), 5.87 (d, 1H, 3-H, J = 1.9), 3.82 (s, 3H, OMe); IR 3228, 1643, 1602, 1506, 1303, 1209, 1164. Anal. ($C_8H_8O_4$) C, H.

5-Methoxy-3-methyl-7-(propionyloxy)coumarin (11). A mixture of **9** (1.5 g, 8.9 mmol), propionic anhydride (12 mL, 93.7 mmol), sodium propionate (2 g, 2.1 mmol), and pyridine (0.75 mL) was refluxed for 7 h. The reaction mixture was then

poured into 3 M HCl (500 mL) and left to stand overnight. The resulting precipitate was filtered out, washed, and purified by FC with 4:1 toluene/ethyl acetate as eluent to afford **11** (1.4 g, 59%): mp 117−119 °C; ^{1}H NMR ($CDCl_3$) 7.84 (q, 1H, 4-H, J = 1.2), 6.69 (d, 1H, 6-H, J = 1.9), 6.48 (d, 1H, 8-H, J = 1.9), 3.90 (s, 3H, OMe), 2.62 (q, 2H, CH_2, J = 7.5), 2.19 (d, 3H, 3-Me, J = 1.2), 1.27 (t, 3H, CH_3CH_2, J = 7.5); IR 2984, 2944, 1762, 1731, 1616, 1453, 1149, 1113, 1066, 822. Anal. ($C_{14}H_{14}O_5$) C, H.

7-Hydroxy-5-methoxy-3-methylcoumarin (12). To coumarin **11** (1.7 g, 6.5 mmol) in methyl alcohol (150 mL) was added 3 M HCl (75 mL), and the mixture was refluxed for 30 min. After cooling, the resulting precipitate was filtered out to yield 1.25 g (93.5%) of **12**: mp 260−261 °C; ^{1}H NMR (DMSO-d_6) 10.30 (s, 1H, OH), 7.76 (q, 1H, 4-H, J = 1.1), 6.32 (m, 2H, 6-H, 8-H), 3.85 (s, 3H, OMe), 2.02 (d, 3H, 3-Me, J = 1.2); IR 3346, 1685, 1597, 1260, 1192. Anal. ($C_{11}H_{10}O_4$) C, H.

7-(Acetonyloxy)-5-methoxy-3-methylcoumarin (13). Prepared from **12** as described for compound **8** to yield 88% of **13**: mp 148−151 °C. ^{1}H NMR ($CDCl_3$): 7.79 (q, 1H, 4-H, J = 1.2), 6.38 (d,1H, 6-H, J = 2.3), 6.29 (d, 1H, 8-H, J = 2.3), 4.61 (s, 2H, CH_2), 3.90 (s, 3H, OMe), 2.30 (s, 3H, MeCO), 2.17 (d, 3H, 3-Me, J = 1.2). IR: 1706, 1626, 1205, 1162, 1117. Anal. ($C_{14}H_{14}O_5$) C, H.

3,4′-Dimethyl-5-methoxyfuro[3,2-*g*]coumarin (3) and 3,4′-Dimethyl-5-methoxyfuro[2,3-*h*]coumarin (4). Prepared from **13** as described for compounds **1** and **2** to yield 66.5% of **4** and 22.5% of **3**. Compound **3**: mp 193−194 °C; ^{1}H NMR ($CDCl_3$) 7.86 (q, 1H, 4-H, J = 1.3), 7.36 (q, 1H, 5′-H, J = 1.4), 7.21 (s, 1H, 8-H), 3.98 (s, 3H, OMe), 2.40 (d, 3H, 4′-Me, J = 1.4), 2.25 (d, 3H, 3-Me, J = 1.3); IR 1712, 1618, 1139, 1090; MS m/z 244 (M^+, 100), 229 (88), 201 ($(M + 1)^+ - CO_2$, 60), 115 (15). Anal. ($C_{14}H_{12}O_4$) C, H. Compound **4**: mp 219−220 °C; ^{1}H NMR ($CDCl_3$) 7.94 (q, 1H, 4-H, J = 1.3), 7.29 (q, 1H, 5′-H, J = 1.4), 6.79 (s, 1H, 6-H), 3.94 (s, 3H, OMe), 2.47 (d, 3H, 4′-Me, J = 1.4), 2,21 (d, 3H, 3-Me, J = 1.3); IR 1707, 1618, 1116, 1066; MS m/z 244 (M^+, 100), 229 (43), 201 ($(M + 1)^+ - CO_2$, 66), 115 (12). Anal. ($C_{14}H_{12}O_4$) C, H.

5-MOA was synthesized according to the method Rodighiero and Antonello (1955).[15]

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

Nucleic Acids. Calf thymus DNA was purchased from Sigma Chemical Co. (St. Louis, MO). Its hypochromicity, determined according to Marmur and Doty,[27] was over 35%. DNA from *Micrococcus lysodeikticus* (Cat. D-8259) and *Clostridium perfringens* (Cat. D-1760), poly[dA-dT]*poly[dA-dT] (Cat. P-0883) and poly[dG-dC]*poly[dG-dC] (Cat. P-9389) also came from Sigma.

Radiochemical Determinations. Radioactivity measurements were made by means of a Packard Model TRI-CARB 4000 liquid scintillation spectrometer: the efficiency of the apparatus for counting tritium was within 35−40%.

All compounds were tritium-labeled by Amersham plc (Amersham, Buckinghamshire, U.K.) and purified on thin layer chromatography plates (silica gel plates Merck art. 5717, 2 mm). Plates were developed with $CHCl_3$; purified products showed a specific activity in the range 7.7−13.2 Ci/mol.

Equilibrium Dialysis. Equilibrium dialysis experiments were essentially performed following published procedures.[28] To avoid binding of the drug to the membrane, all membranes were previously saturated by putting them in a buffer solution containing an excess of furocoumarin to reach equilibrium. DNA concentrations expressed in phosphates ranged between 0.3 and 3 mM; initial drug concentrations were in the range 6.5−9.14 μM. In these conditions all compounds were soluble in the buffer medium.

Irradiation Procedure. Irradiation were performed by means of two Philips HPW 125 lamps equipped with a Philips filter emitting over 90% at 365 nm; the DNA irradiation intensity, determined by a potassium ferrioxalate chemical actinometer,[29] was 5.5 J s^{-1} m^{-2}.

Photobinding to DNA *in Vitro*. Small measured volumes of concentrated ethanol solutions of the labeled compounds were added to aqueous 0.05% calf thymus DNA (containing 10 mM TRIS, 10 mM NaCl, and 0.5 mM EDTA, pH = 7) to achieve a DNA/compound molar ratio of about 75. Aliquots of these solutions were introduced into calibrated glass tubes, immersed in a thermostatically controlled bath and then irradiated for various periods of time. After irradiation, the DNA was precipitated with NaCl and ethanol and washed with ethanol 80%, and the pellets were dissolved in the initial volume of buffer before radiochemical measurements were performed.

In the experiments on bacterial DNA and synthetic polynucleotides, buffer solutions of nucleic acids (1.5×10^{-4} M) were added to the labeled furocoumarins (1.85×10^{-5} M). Irradiations were performed as above; after irradiation, the solutions were extracted with a mixture of chloroform/isoamyl alcohol (19:1) in order to remove the non-photobound drug and other low molecular weight products. Afterward, the aqueous solutions were used for radiochemical measurements.

Evaluation of Interstrand Cross-Links *in Vitro*. After irradiation in the presence of the tritiated furocoumarins, the DNA solutions were thermally denatured (95 °C for 15 min) and quickly cooled in ice. One milliliter of DNA solution was introduced into the top of a column (0.7 cm × 4 cm) of hydroxylapatite (Biogel type, Cat. N. 130-0420, Bio Rad Laboratories, CA) developing with a linear gradient of a 0.05−0.3 M phosphate buffer, pH = 7.00. Fractions of 3.6 mL were collected and the absorbance at 260 nm was recorded.

Preparation of Adducts. Two and half milligrams of the furocoumarin were dissolved in 1 mL of ethanol, and this solution was mixed with 15 mL of a solution of DNA (15 mg) in Tris-NaCl 5 mM buffer pH 7.5. 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 2.5 h at room temperature. After irradiation, solid NaCl (up to 1 M concentration) and 2 volumes of ethanol were added to the solution; the precipitated DNA was collected on a glass rod, washed with 80% ethanol, and dried under high vacuum. In the case of compound **2**, the pellet was dissolved in hydrolytic buffer (CH_3COONa 20 mM, NaCl 50 mM, $ZnSO_4$ 1 mM, $MgCl_2$ 10 mM, pH 4.6). Enzymatic digestion was performed according to published procedures.[28] After treatment with alkaline phosphatase, the solution was filtered through Millipore Teflon filters (2 μm) and directly injected into a Perkin-Elmer Series 410 LC pump equipped with a LC 235 diode array detector, using a C18 column (Merck): the eluent methanol/water (40:60 v/v) was used in an isocratic mode for 20 min, followed by 20 min 100% methanol, always at a flow rate of 1 mL min^{-1}. The fractions were collected with an automatic LKB apparatus: those corresponding to chromatographic peaks were submitted to spectrophotometric and FAB analyses. Instead, DNA irradiated with compound **1** was hydrolyzed in 0.5 N HCl at 100 °C for 1 h, neutralized, and analyzed by HPLC using the same apparatus, as shown in Figure 8. Larger DNA samples irradiated in the presence of compounds **1** and **2** were hydrolyzed with HCl and extracted with $CHCl_3$, and the adducts were separated on TLC plates and eluted with ethyl acetate/ethanol, 9:1.[22]

Photoreversal of Adducts. Fractions of the eluted HPLC solutions were concentrated and irradiated in quartz cuvettes with a mineral lamp (model UVGL 15, Ultra-Violet Products Inc. San Gabriel, CA) placed at a 1 cm distance. The photosplitting reaction was followed spectrophotometrically; the reverted solutions were concentrated and chromatographed on silica gel plates (Merck 5715) developed with ethyl acetate/ethanol, 8:2 (v/v).

Mass Spectrometry. Mass spectrometric measurements were performed on a VG ZAB 2F double-focused, reverse-geometry instrument, operating in FAB (Fast Atom Bombardment) conditions. Glycerol solutions of the samples were bombarded with 8 keV Xe atoms.

Production of Singlet Oxygen. The production of $^1O_2$ by various compounds (4.38 μM) was determined following the spectrophotometric method proposed by Kraljic and El Mohsni[30] based on the bleaching of *N*,*N*-dimethyl-*p*-nitrosoaniline (50 μM) by $^1O_2$ in the presence of histidine (0.01 M) and recording the absorbance changes at 440 nm.

Photobiological Methods. Cell Cultures. Materials. HL60, HeLa, and A431 cells were grown respectively in RPMI

1640, Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., Poole, Dorset, U.K.) supplemented with 10% fetal clone II (Hy Clone Laboratories Inc., Logan, UT) and antibiotics (Sigma). The cells were cultured in a moist atmosphere of 5% carbon dioxide in air.

Inhibition of Growth Assays. Cells (10^5) were seeded into each well of a 24-well microtitex plate. After incubation for 24 h, various concentrations of the test agent were added in complete medium without phenol red. Four hours later, the cells were irradiated with a UVA dose of 0.083 J cm^{-2} and incubated for 20 h. The trypan blue assay was performed to determine cell viability. Cytotoxicity data were expressed as IC_{50} values, i.e., the concentrations of the agent (μM) inducing 50% reduction in cell numbers compared with control cultures.

All irradiations were performed at 365 nm using a Spectroline Camp model ENF-260 C/F (Spectronics Corporation, Westbury, NY). Irradiation intensity was checked on a UV-X radiometer (Ultraviolet Products Inc., Cambridge, U.K.).

Skin Phototoxicity. Skin phototoxicity was tested on depilated albino guinea pigs (outbred Dunkin-Hartley strain), as described by Carlassare et al.[31] Compounds were applied topically on the skin as a 0.1% methanol solution up to 0.05 mg cm^{-2}. The animals were kept in the dark for 45 min, and then the treated skin was irradiated with 20 kJ m^{-2} of UVA; erythema was scored after 48 h.

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Supporting Information Available: The ^1H NMR spectrum of Add I H^+ (1 page). Ordering information is given on any current masthead page.

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