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Original article

1,4,8-Trimethylfuro[2,3-*H*]quinolin-2(1*H*)-one, a new furocoumarin bioisoster

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Abstract

1,4,8-Trimethylfuro[2,3-h]quinolin-2(1H)-one (compound **5a**) is the most interesting derivative among some new furoquinolinones prepared with the aim of moderating the strong toxic effects of 1,4,6,8-tetramethyl derivative (FQ), a powerful potential drug for photomedicine. Compound **5a** showed a photobiological activity lower than FQ, but considerable higher than 8-MOP, the furocoumarin used in clinical photomedicine; contrary to classic furocoumarins, **5a** induced a strong inhibition of protein synthesis in mammalian cells. Genotoxicity and skin erythema induction, the main side effects of both FQ and 8-MOP photosensitization, are virtually absent with **5a**. This behavior seems to be connected to its particular reaction mechanism: differently from furocoumarin derivatives, **5a** induced low levels of DNA-protein and no inter-strands cross-links, but formed covalent RNA-protein linkages, lesions not observed with known furocoumarins. Moreover, compound **5a** generated reactive oxygen species to a considerable extent. For these features, compound **5a** appears to be a new photosensitizing agent whose special activity deserves to be deeply investigated.

Keywords: Furoquinolinones; Reactive oxygen species; RNA-protein cross-links; Phototoxicity

1. Introduction

Linear furocoumarins, also called psoralens, are active photosensitizers used in PUVA (Psoralen plus UVA) therapy for treatment of several skin diseases [1] and in photopheresis, for preventing rejection in organ transplantation, to treat T-cell lymphoma and various autoimmune diseases [2]. The compound generally used is 8-methoxypsoralen (8-MOP) (see figure 1), but 5-methoxypsoralen (5-MOP) and 4,5′,8-trimethylpsoralen (TMP) are also employed. However, these derivatives induce some unpleasant toxic effects, such as skin erythema [1], and genotoxicity, i.e., the induction of point mutations in bacteria [3,4] and chromosomal aberrations in mammalian cells [5]. In fact, PUVA therapy shows a certain risk of skin cancer [6]. Various authors attributed these effects to the severe damage induced into DNA by furocoumarin sensitisation [7]. Upon UVA irradiation, psoralens undergo C4- cycloaddition with pyrimidine bases, forming covalent monofunctional (MA) and bifunctional adducts: inter-strand cross-links (ISC) with two pyrimidine bases on the opposite DNA strands [7], and covalent DNA-protein cross-links (DPC) [8]. Studying new potential drugs for PUVA therapy and photopheresis, we chose as a model angelicin, a natural furocoumarin, unable to induce ISC for geometrical reasons [9]. According to this research line, we have prepared and studied some angelicin bioisosters, namely furoquinolinones [10–13], with the pyrone oxygen atom replaced by a nitrogen. Among them, 1,4,6,8-tetramethylfuro[2,3-h]quinolin-2(1H)-one (FQ) (see figure 1), despite its inability of inducing ISC, is characterized by very strong antiproliferative activity. FQ also showed evident skin phototoxicity and marked genotoxicity [11,14].

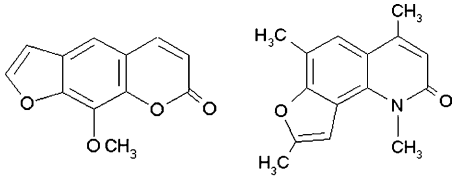

Fig. 1. Structure of 8-methoxypsoralen (8-MOP) and 1,4,6,8-tetramethylfuro[2,3-h]quinolin-2(1H)-one (FQ).

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These properties seemed to be related to FQ capacity of inducing large amounts of MA and DPC. With the aim of obtaining more effective and less toxic derivatives, we prepared some FQ analogues with methyl groups in different positions and we preliminarily studied the photobiological properties of these new bioisosters, using FQ and 8-MOP as reference compounds.

2. Chemistry

The synthetic route followed to obtain the title compounds is summarized in the figure 2. Starting materials were methyl-7-hydroxy-8-allylquinolin-2-ones **1a-b**, previously reported as key intermediates for the synthesis of other heterocyclic systems [15]. Compounds **1a-b** were submitted to cyclization in concentrated sulfuric acid, yielding the corresponding dihydrofuroquinolin-2-ones **2a-b**, which were methylated by dimethyl sulfate to give isomeric dihydro-1-methylfuroquinolin-2-ones **3a-b** and dihydro-2-methoxyfuroquinolines 4a-b with comparable yields. Dihydroderivatives **3a-b** and **4a-b** were finally aromatized in toluene solution by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) to the corresponding 1-methylfuroquinolin-2-ones **5a-b** and 2-methoxyfuroquinolines **6a-b**. The introduction of the methyl group into the tricyclic nucleus was carried out before the aromatization of the dihydroderivatives, because the methylation of the aromatic system gave only methoxyfuroquinoline and no trace of methylfuroquinolinone [10].

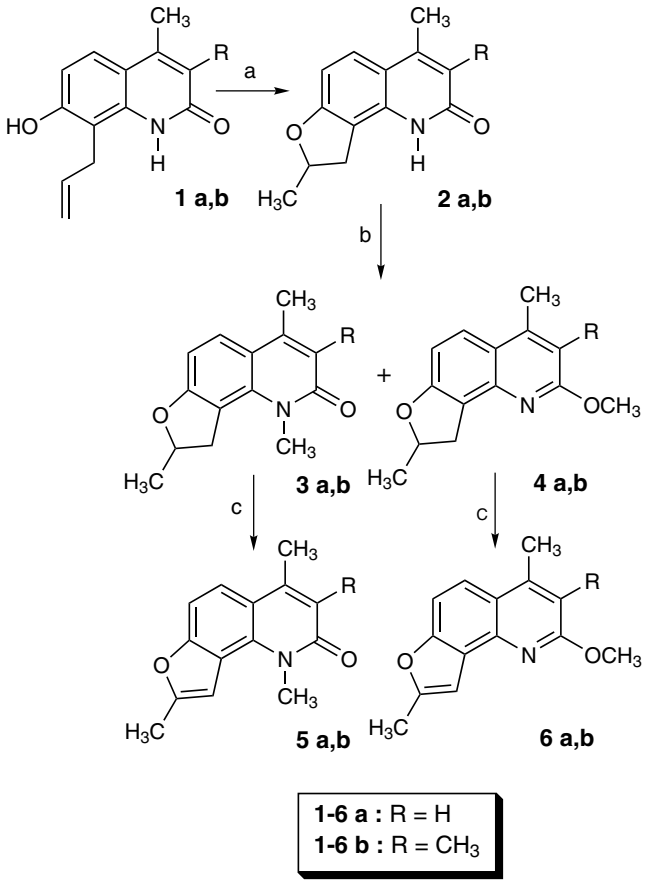

Fig. 2. Reagents and conditions: (a) conc. H_2SO_4, RT, 30 min, 52-55%; (b) Me_2SO_4, acetone, K_2CO_3, reflux, 20 h, 39-46%; (c) DDQ, toluene, reflux, 2-5 h, 49-92%.

3. Biology

The new furoquinolinones were submitted to a preliminary screening for antiproliferative activity on mammalian cells. The mechanism whereby compound **5a**, the most interesting derivative, promotes its photosenziting activity was investigated.

4. Results

4.1. Preliminary screening: antiproliferative and genotoxic activity

The antiproliferative activity of furoquinolinones **5a-b** and furoquinolines **6a-b** was tested studying the inhibition of DNA synthesis in Ehrlich cells and the clonal growth capacity in HeLa cells cultivated in vitro with or without UVA activation. 8-MOP and FQ were tested as references. The obtained results are summarized in table 1.

Among the new derivatives, compound **5a** showed the best response in both tests; by simple incubation in dark it induced an antiproliferative effect that was significantly higher than that of 8-MOP (ineffective, as expected, in these conditions) and lower than that of FQ, about ten- and five-fold in DNA synthesis inhibition and in the clonogenic test, respectively. By UVA irradiation, the picture is similar in the sense that compound **5a** exhibited a greater activity with respect to 8-MOP, in particular in the clonogenic test. Conversely, it was clearly less active than FQ that had to be tested at 10-fold lower concentration because of its extremely high photosensitising activity. Compound **5b** displayed a modest activity, while **6a** and **6b** (not shown in table 1) were practically inactive on mammalian cells.

The results obtained studying the mutagenic activity of the new compounds upon UVA irradiation in E. coli TM9, are also shown in table 1. The most genotoxic derivative appeared to be 8-MOP, followed by FQ and by the new furoquinolinones, that exhibited a reduced genotoxicity in comparison with 8-MOP (about ten times lower).

*4.2. Experiments carried out with compound **5a***

4.2.1. Experiments carried out in cell-free systems

4.2.1.1. Formation of reactive oxygen species (ROS). Compound **5a** was at first tested for its capacity of forming ROS (singlet oxygen and superoxide anion), in comparison with 8-MOP and FQ. As shown in figure 3, compound **5a** induced both ROS to a high extent. On the contrary, the two reference

Table 1
Antiproliferative and genotoxic activity

Compound	DNA synthesis in Ehrlich cells		Clonal growth in HeLa cells		Mutations in *E.Coli*
	Dark[a]	Plus UVA[b]	Dark[a]	Plus UVA[c]	TM9[d]
8-MOP	> 50	3.70 ± 0.20	> 50	0.79 ± 0.020	2.96 ± 0.21
FQ	1.70 ± 0.20	0.53 ± 0.03*	7.2 ± 0.02	0.02 ± 0.006*	1.57 ± 0.200
5a	17.60 ± 1.11	3.37 ± 0.17	34.11 ± 1.31	0.17 ± 0.020	0.21 ± 0.030
5b	33.71 ± 2.4	13.25 ± 3.40	47.51 ± 4.0	36.50 ± 2.900	0.64 ± 0.020
6a	> 50	> 50	49.3 ±3.3	> 50	0.10 ± 0.004

[a] IC_{50} ± SD, drug concentration (μM) which reduces to 50% DNA synthesis or clonal growth
[b] ID_{50} ± SD: UVA dose which reduces to 50% DNA synthesis when delivered in the presence of 20 μM drug concentration (*except FQ, tested at 2 μM).
[c] ID_{50} ± SD: UVA dose which reduces to 50% clonal growth of HeLa cells when delivered in the presence of 5 μM drug concentration (*except FQ, tested at 2 μM).
[d] Revertants per 10^6 survivors scored at 37% of the surviving fraction. SD = standard deviation

compounds appeared to be effective in producing only one reactive species (superoxide anion by 8-MOP, singlet oxygen by FQ), while they were nearly ineffective in producing the other one. Overall, compound **5a** appeared to be capable of forming a large ROS set.

4.2.1.2. DNA photobinding. An aqueous DNA solution containing compound **5a** was irradiated and then processed as described in the section Experimental Protocols. Two main fluorescent bands were separated and isolated by TLC, the bigger one (Rf 0.33) showed intense violet fluorescence, and the second one (Rf 0.72), thinner, also had strong violet fluorescence under Wood's light. By bidimensional TLC, both underwent photosplitting under 254 nm irradiation, the former yielding the parent **5a** and thymine, the latter **5a** and uracil, thus indicating the formation of **5a** photoproducts with DNA pyrimidine bases, thymine and cytosine, respectively. Only the photoproduct having Rf 0.33 was obtained in an amount sufficient for NMR spectra. 1H-NMR (200 MHz, acetone-d6), δ 1.58 (s, 3H, Me-8), 1.77 (s,3H, Me-5T), 2.39 (d, 3H, Me-4 J = 1.2 Hz), 3.87 (s, 3H, Me-1), 3.98 (dd, 1H, H-6T, J = 2.4 and 1.4 Hz,), 4.51(d, 1H, H-9, J = 1.4 Hz), 6.30 (q, 1H, H-3, J = 1.2 Hz), 6.74 (d, 1H, H-6, J = 8,7 Hz), 6.97 (broad, 1H, H-1T), 7.60 (d, 1H, H-5, J = 8.7 Hz), 8.66 (broad, 1H, H-3T). The regiochemistry of the adduct was inferred from the long range coupling (J = 1.4 Hz) between opposite cyclobutane protons H-6T and H-9, observed when NH-1T was saturated (vicinal protons in similar adducts have a J value of about 5.5 Hz) [16]. The stereochemistry of the adduct was determined by means of NOE experiments which showed, among others, significant dipolar interactions between Me-1 and Me-5T. The spatial proximity of these groups is possible only in the cis-syn configuration shown in figure 4, similar to that of the monoadducts formed by the other furoquinolinones already studied, FQ and HFQ [10].

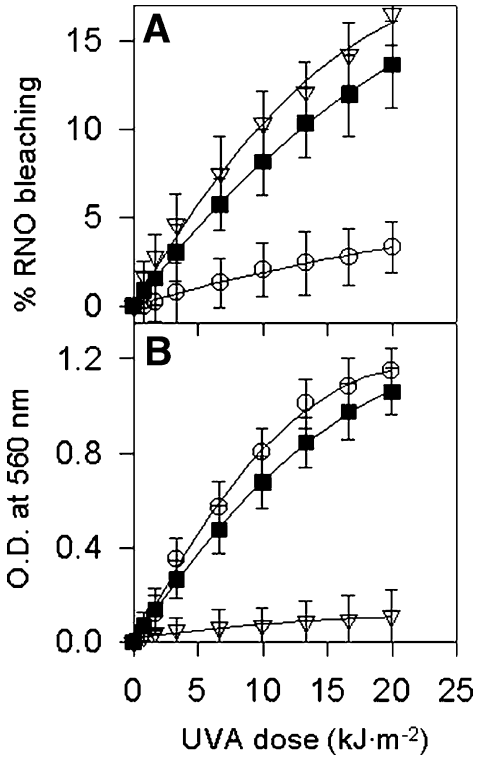

Fig. 3. ROS formation by **5a** photosensitization. All compounds were tested at a 20 μM concentration. Panel A: singlet oxygen detection; Panel B: Superoxyde anion detection. Symbols: **5a**, ■; 8-MOP, ○; FQ, ▽. Each point represents the mean ± S.D. of three independent experiments.

Figure 5 shows the chromatogram obtained by HPLC analysis of the hydrolysed mixture obtained from the DNA-**5a** photoreaction. Two photoadducts were seen at 2.8 and 3.3 min, when the detector was set at 320 nm (where only furan-side monoadducts absorb), corresponding to **5a**-cytosine and **5a**-thymine C4-monoadducts, respectively.

Due to the lack of radioactively labelled **5a** compounds, an exact quantification was impossible. However, because the UV absorption (ε= 6055 at 334 nm) of **5a** is close to that of FQ (ε = 6500 at 338 nm), the absorption of the two monoadducts with thymine should be similar, too. The HPLC

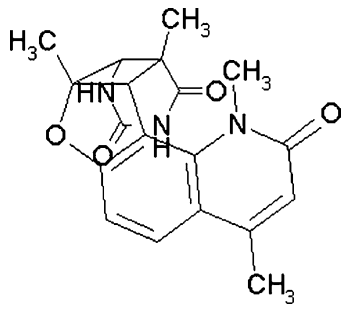

Fig. 4. Molecular structure of furan side monoadduct formed between compound **5a** and thymine.

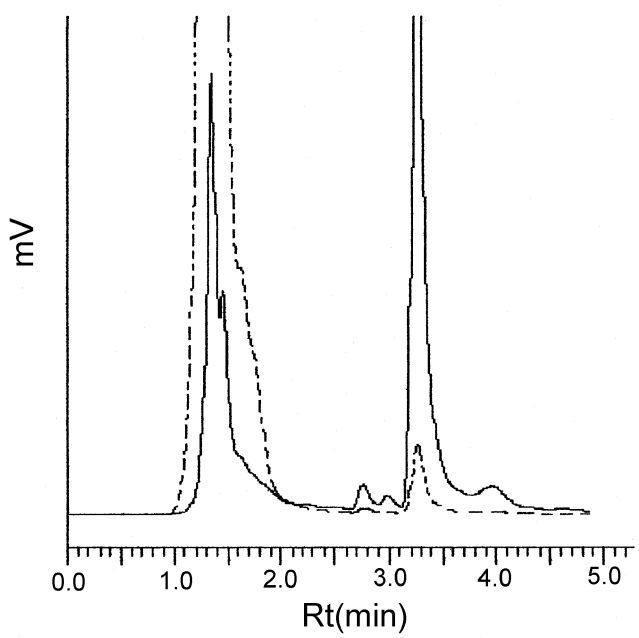

Fig. 5. HPLC profiles of chloroform-extracted .acid hydrolysate of **5a** photomodified DNA-detected at 250 nm (dotted line) and 320 nm (full line).

peak areas corresponding to the photoproducts formed by irradiating equimolar solutions of the compounds were measured, and allowed us to conclude that **5a** possesses DNA photoreactivity similar or even higher than FQ, an analogue already studied by the aid of a radioactive sample [10].

4.2.1.3. Detection of ISC. The formation of ISC by UVA irradiation was studied in vitro using PM2-linearized DNA and gel-electrophoretic separation of single-stranded, not cross-linked DNA, from double-stranded, cross-linked DNA. The results are shown in figure 6. Compound **5a**, like FQ [11,12], was unable of inducing significant amounts of ISC; as expected, 8-MOP induced a large amount of ISC. A quantitative approach detecting ethidium bromide fluorescence before and after denaturating DNA according to Brent [17] performed in the most severe experimental conditions, gave for compound **5a** a fraction of cross-linked DNA lower than 0.1 and about 0.95 for 8-MOP. This is an expected result, as the low capacity of ISC formation by angular furocoumarins is well known [9].

4.2.2. Experiments carried out in mammalian cells

4.2.2.1. Inhibition of RNA and protein synthesis in Ehrlich cells. To investigate the mechanism of photosensitizing activity of **5a** on biological systems, its effect on RNA and protein syntheses in Ehrlich cells was studied. Table 2 shows the results, expressed as ID50 values, i.e., the UVA dose delivered in the presence of the selected drug concentration that induced a 50% inhibition of the biological function studied, calculated by probit analysis. 8-MOP, and in particular FQ, appeared to be very effective on RNA synthesis, whereas **5a** induced only a moderate inhibition. In spite of

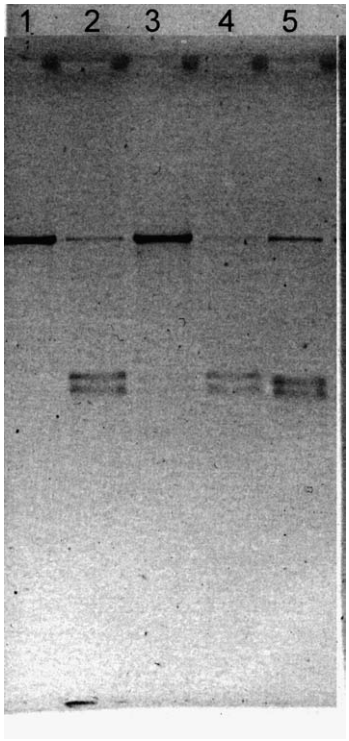

Fig. 6. ISC formation in linearized PM2 DNA detected by electrophoresis. Drug concentration was 2 molecules per base pair of DNA. For comparison, 8-MOP was also studied in the same experimental conditions. Lane 1: double-strand DNA; lane 2: denatured single-strand DNA: lane 3: DNA irradiated with 4 kJ m^{-2} in the presence of 8-MOP; lanes 4 and 5: DNA treated with compound **5a** and exposed to 2 and 4 kJ m^{-2} of UVA light, respectively.

this result, compound **5a** demonstrated a strong inhibition of protein synthesis, while both the reference compounds were practically inactive. Moreover, as macromolecular synthesis was assayed by a short term test, the effect on protein synthesis may be due to direct damage on ribosomes rather than to a shortage of m-RNA.

4.2.2.2. Detection of DNA-protein cross-links (DPC) and RNA-protein cross-links (RPC). The formation of covalent linkages between proteins and DNA or RNA by **5a** photosensitization was studied in HeLa cells by alkaline elution (table 3). Compound **5a** induced a low amount of DPC, about one third of that formed by 8-MOP, and less than one order of magnitude of that by FQ. The experiments on RPC formation gave a quite different picture, as shown in figure 7. Compound **5a** induced covalent linkages between RNA and proteins, and their amount increased according to the UVA dose (see the insert in figure 7). It should be noted

Table 2
Inhibition of RNA and protein syntheses

Compound	ID_{50} (kJ·m^{-2}) ±SD	
	RNA	Proteins
8-MOP	3.62 ± 0.18 [a)]	>100[a)]
FQ	0.43 ± 0.01 [a)]	>100[a)]
5a	12.7 ± 0.3 [a)]	0.12 ± 0.03[b)]

Drug concentration: a) 20 μM; b) 5 μM

Table 3
Detection of DPC

Compound[a)]	DPC[b)] ± SD	Relative activity
8-MOP	0.22 ± 0.05	1.0
FQ	2.1 ± 0.3	9.5
5a	0.063 ± 0.004	0.3

[a] All compounds were tested at 20 μM concentration and 5 kJ·m^{-2} UVA dose; the results are the average of at least three independent experiments.
[b] DPC were detected by alkaline elution; the results are expressed as the number of DPC induced every 10^6 nucleotides DNA segment, according to Kohn [27]. SD: standard deviation.

that irradiation in the presence of compound **5a** and under nitrogen atmosphere failed to induce RPC.

4.2.3. Experiments on guinea pig skin

Compound **5a** was also assayed for its capacity of inducing skin erythema on albino guinea pig skin, as shown in table 4. 8-MOP and FQ showed their known phototoxicity even in mild experimental conditions, while compound **5a** appeared to be unable to induce skin erythemas in similar conditions. Only under very severe conditions (at relatively high drug concentrations) a light erythema was observed.

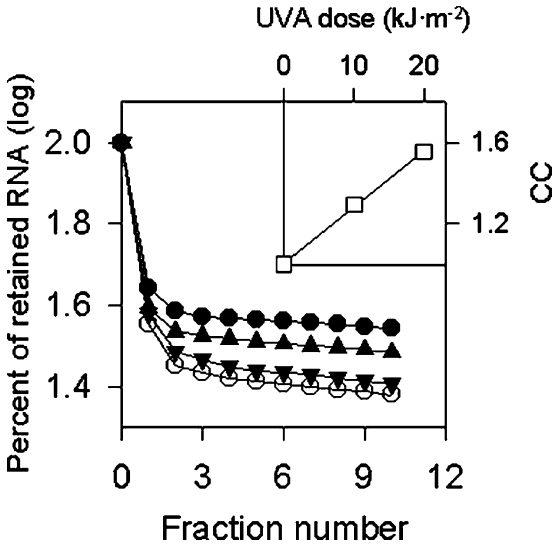

Fig. 7. RPC formation by **5a** sensitization. HeLa cells were exposed to UVA light in the presence of **5a** (20 μM) and then RPC were detected by alkaline elution. Symbols: controls, ○; cells treated with **5a** in air at 10 kJ·m^{-2}, ▲; at 20 kJ·m^{-2}, ●; cells treated with **5a** in a nitrogen atmosphere at 20 kJ·m^{-2}, ▼ Insert: results obtained calculating the cross-linking coefficient (CC) in air, as described in the Experimental section.

Table 4
Phototoxicity on guinea pig skin

Compound	Drug concentration (μM·cm^{-2})	UVA dose (KJ·m^{-2})	Erythema intensity
Controls	0	20	- - - -
8-MOP	4.6	5	+ - - -
	30	5	+ + + +
FQ	3.3	5	+ - - -
5a	30	20	- - - -
	100	20	+ - - -
	200	30	+ + - -

Simbols: - - - -: no erythema; + - - -: barely visible erythema; + + - -: light erythema; + + + -: medium erythema; + + + +: strong erythema.

5. Discussion

The tetramethylfuroquinolinone FQ is a powerful photosensitizing drug, able to induce marked antiproliferative and genotoxic effects in mammalian cells, together with marked skin phototoxicity [11,14]. FQ photobinds to DNA forming large amounts of monofunctional and bifunctional adducts; however, the latter are not ISC but DPC, in particular DPC at length greater than zero (DPCL > 0) in which the sensitizer moiety is a physical part of the bridge between DNA and proteins [10–12]. With the aim of obtaining new drugs having better features than earlier derivatives, we prepared some FQ analogues. By introducing a methyl group at 3 position (compounds **5b** and **6b**) we obtained derivatives showing a very low photobiological activity. A similar result, already obtained with linear furocoumarins [18], was attributed to a critical steric hindrance at the level of 3,4 double bond, that is one of the two reactive sites involved in ISC formation [7]. Also in angelicins, in spite of their inability of forming ISC [9], the presence of a methyl group at 3 position interfered with their photochemical and photobiological properties [19]. Therefore, we can suppose that a similar type of interference may account for the moderate photobiological activity of **5b** and **6b**. Moreover, the insertion of a methoxy group at 2 position (replacing the carbonyl one, compounds **6a-b**) completely changed the pyridonic nucleus into a pyridinic one, i.e., a full aromatic system devoid of any photoreactivity. Compound **5a** (lacking the methyl group at 6 position in comparison with FQ) is certainly the best among the new derivatives. Studying angelicin derivatives, we already observed that a methyl at 6 position increased antiproliferative activity but side effects become very large [9,20]. Unfortunately, a radioactive sample of compound **5a** is at present not available, so we could not quantify its DNA covalent photobinding; however, in some experiments, carried out with DNA in vitro and HPLC analysis, **5a** photosensitization seemed to produce C4-cycloadducts (mainly thymine furan-side monoadducts showing cis-syn configuration) in amounts very close to or even higher than those formed by FQ in similar experimental conditions. By analogy to the previously determined photoreactive properties of FQ and 8-MOP [10], it can be inferred that **5a** covalently links to DNA about ten times stronger than 8-MOP. On the other hand, we do show here that compound **5a** does not form ISC and induces DPC in low amounts only, i.e. approximately one third compared to 8-MOP.

Compound **5a** appears to be very effective in inducing both singlet oxygen and superoxide anion, while 8-MOP and FQ formed significant amounts of only one of these ROS. Contrary to the two reference derivatives, **5a** selectively affects protein synthesis in Ehrlich cells. Using alkaline elution, we were able to detect the formation of RPC solely in

the presence of oxygen but not in the presence of nitrogen. This damage, when induced in ribosomes, could be responsible for protein synthesis inhibition. Compound **5a** appears to be more active than 8-MOP, especially in the clonogenic test on HeLa cells; moreover, it shows very low genotoxic potential as shown in the mutation test in E. coli TM9, a bacterial strain very sensitive to mutagens. Compound **5a** shows a very low capacity of inducing skin erythema. At present we do not know the mechanism of phototoxicity induced by furocoumarins or their isosters, although various hypothesis have been suggested, for example, ISC formation [21] and singlet oxygen generation [22]. More recently, studying various furocoumarins and bioisosters, we observed a certain correlation between the formation of skin erythema and their capacity of forming DPC at length greater than zero (DPCL > 0, as formed by 8-MOP and FQ [10–12]) rather than DPC formed between DNA and proteins without any intermediate [12,23] (DPC at zero length, DPCL=0) [8,14].

Lastly, compound **5a** shows a molecular mechanism somewhat different from that of classic furocoumarins and a good photosensitizing activity together with low side effects. The mechanism of action of this new furocoumarin bioisoster may deserve further studies in view of possible therapeutic applications in photomedicine.

6. Experimental protocols

6.1. Chemistry

Melting points were determined on a Gallenkamp MFB-595-010M melting point apparatus and are uncorrected. Analytical TLC was performed on pre-coated 60 F254 silica gel plates (0.25 mm; Merck) developing with a CHCl3/MeOH mixture (9:1) unless overwise indicated. Preparative column chromatography was performed using silica gel 60 (0.063-0.100 mm; Merck), eluting with CHCl3. 1H NMR spectra were recorded at 200 MHz on a Varian Gemini-200 spectrometer with TMS as internal standard. Elemental analyses were obtained on all intermediates and are within ±0.4% of theoretical values. Starting 4-methyl-7-hydroxy-8-allyl-quinolin-2-one **1a** and 3,4-dimethyl-7-hydroxy- 8-allyl-quinolin-2-one **1b** were prepared according to published methods [15].

*6.1.1. 8,9-Dihydro-2*H*-furo[2,3-h]quinolin-2-ones (**2a-b**). General procedure*

Compound **1** (10.0 mmol) was dissolved in conc. H2SO4 (25 mL) and the solution was kept at room temperature for 0.5 h. The mixture was poured into cold water (250 mL). The precipitate obtained was filtered, washed with water and crystallized from MeOH to give **2**.

*6.1.2. 8,9-Dihydro-4,8-dimethyl-2*H*-furo[2,3-h]quinolin-2-one (**2a**)*

yield 55%; m.p. 272°C; 1H NMR (CDCl3) δ = 7.48 (d, J = 8.6 Hz, 1H, 5-H), 6.72 (d, J = 8.6 Hz, 1H, 6-H), 6.37 (q, J = 1.1 Hz, 1H, 3-H), 5.13 (qdd, Jq = 6.3 Hz, Jd = 8.9, 7.4 Hz, 1H, 8-H), 3.64 (dd, J = 15.7, 8.9 Hz, 1H, 9-H), 3.06 (dd, J = 15.7, 7.4 Hz, 1H, 9-H), 2.45 (d, J = 1.1 Hz, 3H, 4-Me), 1.55 (d, J = 6.3 Hz, 3H, 8-Me); elemental analysis calcd.for C13H13NO2 (215.3): C 72.54, H 6.09, N 6.51; found C 72.32, H 6.19, N 6.34.

*6.1.3. 8,9-Dihydro-3,4,8-trimethyl-2*H*-furo[2,3-h]quinolin-2-one (**2b**)*

yield 52%; m.p. 274°C; 1H NMR (CDCl3) δ = 7.51 (d, J = 8.8 Hz, 1H, 5-H), 6.71 (d, J = 8.8 Hz, 1H, 6-H), 5.15 (qdd, Jq = 6.3 Hz, Jd = 9.1, 7.4 Hz, 1H, 8-H), 3.60 (dd, J = 15.4, 9.1 Hz, 1H, 9-H), 3.04 (dd, J = 15.4, 7.4 Hz, 1H, 9-H), 2.44 (s, 3H, 4-Me), 2.24 (s, 3H, 3-Me), 1.55 (d, J = 6.3 Hz, 3H, 8-Me); elemental analysis calcd.for C14H15NO2 (229.3): C 73.34, H 6.59, N 6.11; found C 73.20, H 6.49, N 6.09.

*6.1.4. 8,9-Dihydro-1-methyl-2*H*-furo[2,3-h]quinolin-2-ones (**3a-b**) and 8,9-dihydro-2-methoxyfuro[2,3-h]quinolines (**4a-b**). General procedure*

A mixture of **2** (5.0 mmol), dimethyl sulfate (6.0 mmol) and anhydrous K_2CO_3 (10.0 g) in acetone (250 mL) was refluxed until **2** disappeared (20 h, TLC). After cooling, the solid was filtered off and washed with fresh acetone. The solvent was evaporated under reduced pressure from the combined filtrate and washings. The residue was purified by column chromatography to give **4**, followed by **3**.

*6.1.5. 8,9-Dihydro-1,4,8-trimethyl-2*H*-furo[2,3-h]quinolin-2-one (**3a**)*

yield 46%; m.p. 185°C (MeOH); 1H NMR (CDCl3) δ = 7.50 (d, J = 8.7 Hz, 1H, 5-H), 6.73 (d, J = 8.7 Hz, 1H, 6-H), 6.40 (q, J = 1.1 Hz, 1H, 3-H), 4.97 (qdd, Jq = 6.2 Hz, Jd = 9.0, 7.9 Hz, 1H, 8-H), 3.93 (dd, J = 14.7, 9.0 Hz, 1H, 9-H), 3.85 (s, 3H, 1-Me), 3.39 (dd, J = 14.7, 7.9 Hz, 1H, 9-H), 2.39 (d, J = 1.1 Hz, 3H, 4-Me), 1.52 (d, J = 6.2 Hz, 3H, 8-Me); elemental analysis calcd.for C14H15NO2 (229.3): C 73.34, H 6.59, N 6.11; found C 73.31, H 6.50, N 6.10.

*6.1.6. 8,9-Dihydro-1,3,4,8-tetramethyl-2*H*-furo[2,3-h]quinolin-2-one (**3b**)*

yield 42%; m.p. 195°C (MeOH); 1H NMR (CDCl3) δ = 7.55 (d, J = 8.7 Hz, 1H, 5-H), 7.72 (d, J = 8.7 Hz, 1H, 6-H), 4.95 (qdd, Jq = 6.2 Hz, Jd = 8.7, 7.7 Hz, 1H, 8-H), 3.92 (dd, J = 15.0, 8.7 Hz, 1H, 9-H), 3.87 (s, 3H, 1-Me), 3.37 (dd, J = 15.0, 7.7 Hz, 1H, 9-H), 2.39 (s, 3H, 4-Me), 2.24 (s, 3H, 3-Me), 1.51 (d, J = 6.2 Hz, 3H, 8-Me); elemental analysis calcd.for C15H17NO2 (243.3): C 74.05, H 7.04, N 5.76; found C 74.01, H 6.98, N 5.95.

*6.1.7. 8,9-Dihydro-4,8-dimethyl-2-methoxyfuro[2,3-h]quinoline (**4a**)*

yield 41%; m.p. 92°C (MeOH); 1H NMR (CDCl3) δ = 7.69 (d, J = 8.8 Hz, 1H, 5-H), 6.95 (d, J = 8.8 Hz, 1H, 6-H), 6.57 (q, J = 1.0 Hz, 1H, 3-H), 5.14 (qdd, Jq = 6.3 Hz, Jd = 9.2, 7.3 Hz, 1H, 8-H), 4.02 (s, 3H, 2-OMe), 3.68 (dd,

J = 15.8, 9.2 Hz, 9-H), 3.14 (dd, 1H, J = 15.8, 7.3 Hz, 9-H), 2.57 (d, 3H, J = 1.0 Hz, 4- Me), 1.55 (d, 3H, J = 6.3 Hz, 1H, 8-Me); elemental analysis calcd. for C14H15NO2 (229.3): C 73.34, H 6.59, N 6.11; found C 73.29, H 6.52, N 6.05.

*6.1.8.8,9-Dihydro-2-methoxy-3,4,8-trimethylfuro[2,3-h]quinoline (**4b**)*

yield 39%; m.p. 122°C (MeOH); 1H NMR (CDCl3) δ = 7.69 (d, J = 8.9 Hz, 1H, 5-H), 6.93 (d, J = 8.9 Hz, 1H, 6-H), 5.10 (qdd, Jq = 6.3 Hz, Jd = 9.1, 7.8 Hz, 1H, 8-H), 4.04 (s, 3H, 2-OMe), 3.68 (dd, J = 15.7, 9.1 Hz, 1H, 9-H), 3.13 (dd, J = 15.7, 7.8 Hz, 1H, 9-H), 2.52 (s, 3H, 4-Me), 2.23 (s, 3H, 3-Me), 1.55 (d, J = 6.3 Hz, 3H, 8-Me); elemental analysis calcd.for C15H17NO2 (243.3): C 74.05, H 7.04, N 5.76; found C 74.00, H 6.98, N 5.75.

*6.1.9. 1-Methyl-2*H*-furo[2,3-h]quinolin-2-ones (**5a-b**) and 2- methoxyfuro[2,3-h]quinolines (**6a-b**). General procedure*

A mixture of **3** or **4** (2 mmol) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (5 mmol) in anhydrous toluene (100 mL) was refluxed until starting product disappeared (2-5 h, TLC). After cooling, the solid was filtered off and the solvent evaporated under reduced pressure. The residue was purified by column chromatography and crystallized from MeOH to give **5** or **6**.

*6.1.10. 1,4,8-Trimethyl-2*H*-furo[2,3-h]quinolin-2-one (**5a**)*

yield 92%; m.p. 216°C; 1H NMR (CDCl3) δ = 7.57 (d, J = 8.8 Hz, 1H, 5-H), 7.35 (d, J = 8.8 Hz, 1H, 6-H), 6.94 (q, J = 1.1 Hz, 1H, 3-H), 6.59 (q, J = 1.1 Hz, 1H, 3-H), 4.04 (s, 3H, 1-Me), 2.52 (d, J = 1.1 Hz, 6H, 4-Me and 8-Me); elemental analysis calcd.for C14H13NO2 (227.3): C 73.99, H 5.77, N 6.16; found C 73.71, H 5.72, N 6.03.

*6.1.11. 1,3,4,8-Tetramethyl-2*H*-furo[2,3-h]quinolin-2-one (**5b**)*

yield 49%; m.p. 199°C; 1H NMR (CDCl3) δ = 7.62 (d, J = 8.9 Hz, 1H, 5-H), 7.32 (d, J = 8.9 Hz, 1H, 6-H), 6.91 (q, J = 1.0 Hz, 1H, 9-H), 4.05 (s, 3H, 1-Me), 2.51 (q, J = 0.7 Hz, 3H, 4-Me), 2.50 (d, J = 1.0 Hz, 3H, 8-Me), 2.31 (q, J = 0.7 Hz, 3H, 3-Me); elemental analysis calcd.for C15H15NO2 (241.3): C 74.66, H 6.27, N 5.81; found C 74.51, H 6.40, N 5.99.

*6.1.12. 4,8-Dimethyl-2-methoxyfuro[2,3-h]quinoline (**6a**)*

yield 66%; m.p. 113°C; 1H NMR (CDCl3) δ = 7.68 (d, J = 8.9 Hz, 1H, 5-H), 7.48 (d, J = 8.9 Hz, 1H, 6-H), 7.02 (q, J = 1.1 Hz, 1H, 9-H), 6.74 (q, J = 1.0 Hz, 1H, 3-H), 4.10 (s, 3H, 2-OMe), 2.66 (d, J = 1.0 Hz, 3H, 4-Me), 2.56 (d, J = 1.1 Hz, 3H, 8-Me); elemental analysis calcd.for C14H13NO2 (227.3): C 73.99, H 5.77, N 6.16; found C 73.89, H 5.82, N 6.05.

*6.1.13. 2-Methoxy-3,4,8-trimethylfuro[2,3-h]quinoline (**6b**)*

yield 60%; m.p. 165°C; 1H NMR (CDCl3) δ = 7.69 (d, J = 9.0 Hz, 1H, 5-H), 7.47 (d, J = 9.0 Hz, 1H, 6-H), 7.02 (q, J = 1.0 Hz, 1H, 9-H), 4.13 (s, 3H, 2-OMe), 2.61 (q, J = 0.6 Hz, 3H, 4-Me), 2.55 (d, J = 1.0 Hz, 3H, 8-Me), 2.33 (q, J = 0.6 Hz, 3H, 3-Me); elemental analysis calcd.for C15H15NO2 (241.3): C 74.66, H 6.27, N 5.81; found C 74.41, H 6.11, N 5.65.

6.2. Biology

The test compounds were dissolved in dimethyl sulfoxide (DMSO; 4.5 mM) and the solutions kept at –20 °C in the dark. Just before the experiment, a calculated amount of compound solution was added in the dark to PBS or to the growth medium containing cells, to a final solvent concentration of 0.5%. All procedures involving animals and their care were conducted in conformity with the institutional guidelines, that are in compliance with National and European Economic Community Council Directives.

6.2.1. UVA irradiation

DNA solutions and bacterial or mammalian cell suspensions containing the test compound were incubated at room temperature for 15 min in the dark, put into Petri dishes (5 cm in diameter; 3 mL), and exposed to UVA light. Samples for alkaline elution were kept in an ice-bath also during the entire treatment, including gamma irradiation, until the lysis step. UVA exposures were performed with Philips HPW 125 lamps, provided with a built-in Philips filter; emission was in the 320-400 nm range, with a maximum, over 90% of the total, at 365 nm. Irradiation intensities were determined by a radiometer (model 97503, Cole-Parmer Instrument Co., Niles, IL, USA), and set in two different ways, according to compound activity: lamp placed at 20 cm of distance (giving rise to 0.5·10-5 W m^{-2}) or lamp placed at 45 cm (giving rise to 0.9·10-6 W m^{-2}). In every experiment, controls exposed to 10 kJ m^{-2} of UVA light alone were included showing no significant difference as compared to the untreated controls (data not shown). In all experiments, we used higher 8-MOP concentrations for its lower activity, thus obtaining significant and well detectable responses, at appropriate UVA doses. With regard to the spectroscopic features of the tested compounds, the extinction coefficients at the absorption peak in the near UV range, in absolute ethanol, are as follows: at 338 nm, FQ: 6500, at 334 nm, **5a**: 6055, **5b**: 6900, at 297 nm, **6a**: 7470.

6.2.2. Detection of reactive oxygen species (ROS)

Formation of singlet oxygen was measured according to the method of Kraljic and El Mohsni [24]. Aqueous solutions, containing phosphate buffer (0.02 M, pH 7.3), p-nitrosodimethylaniline (RNO) ($4{\cdot}10^{-5}$ M), L-histidine ($1{\cdot}10^{-3}$ M) and the test compound at a concentration of 20 μM were irradiated for increasing times in quartz cuvettes. RNO bleaching was determined by reading the optical density at 440 nm. To detect the formation of superoxide anion, the method described by Pathak and Joshi was used [22]. Aqueous solutions containing carbonate buffer (pH 10), nitro

blue tetrazolium (NBT) ($1.6 \cdot 10^{-4}$) and the test compound at a concentration of 20 μM were irradiated as before, and the optical density at 560 nm was determined.

6.2.3. Interaction with DNA in vitro

6.2.3.1. DNA photobinding. Isolation of the products An aqueous solution of DNA (2.3 mM, 100 ml) containing 2 mM NaCl, 1mM EDTA and 30 μM of test compound was exposed to UVA light (30 J cm^{-2}). The ethanol precipitated DNA was collected with a glass rod, washed with 80% ethanol and dissolved in 30 ml of 0.5 N HCl, heated at 100°C for 1 h, neutralized, and extracted three times with chloroform. The organic phases were collected, dried over Na_2SO_4, evaporated, and the residue was analyzed by TLC (silica gel plates, 0.25 mm thick, from E. Merck, developed with ethyl acetate-ethanol, 9:1).

6.2.3.2. Photoreactivity. Aliquots (4 ml) of the same DNA solution were irradiated in the presence of test compound (0.03 mM) with UVA light (20 J cm^{-2}). After precipitation, hydrolysis and neutralisation, 50-μl samples were analyzed by HPLC (RP18 column, 250 x 4 mm, 5 μm, from E. Merck, Darmstadt, Germany, isocratically eluted with methanol-water, 1:1 (v/v); UV detection at 250 and 320).

6.2.3.3. Inter-strand cross-links detection. Inter-strand cross-links were detected using linearized supercoiled circular DNA of PM2 bacteriophage (Boehringer Mannheim GmbH, Mannheim, Germany) as already described [12]. Briefly, PM2-linearized DNA (2 μL for each sample) was added to the test compound (2 μL; 4x in DMSO) at the appropriate concentration, so that the final molar ratio drug/base pairs was 2 (final drug concentration: $6.25 \cdot 10^{-5}$ M). Two samples for each compound were incubated at room temperature in the dark for 15 min and then exposed to UVA light. DNA was precipitated, recovered and analyzed by agarose gel (0.7%) electrophoresis, as previously described [12], to obtain good separation of denatured and non denatured (cross-linked) DNA. The gel was photographed by a Polaroid camera placed over a UV TM36 transilluminator (UVP Inc., San Gabriel, CA, USA).

6.2.4. Mutagenesis Tests

The *E. coli* strain WP2 TM9, defective in excision repair (*uvrA*$^-$), carrying the R46 plasmid and a nonsense mutation in the trpE gene, which is reverted by UV light and base pair substitution mutagens [25], was used. All these experiments were performed as already described [10]. The mutation frequency was expressed as mutants per 10^6 survivors, computed by dividing the number of revertants observed per plate by the number of surviving bacteria at the same treatment and subtracting from the result the number of revertant colonies per million survivors observed in controls. In this test, all manipulations were done under red light.

6.2.5. Skin phototoxicity

Skin phototoxicity was tested on depilated albino guinea-pigs (outbred Dunkin-Hartley strain), as described [14]. Compounds were applied topically as 4 mM methanol solutions and the skin exposed to UVA light. The animals were observed for 48 hrs, checking the minimal drug amount capable of inducing a barely visible erythema.

6.2.6. Macromolecular synthesis in Ehrlich cells

DNA, RNA and protein synthesis were assayed in Ehrlich ascites tumor cells (Lettrè strain, weekly transferred by transplant into NCL mice) as already described [26]. Just after UVA irradiation, 10^6 cells were incubated in growth medium for 30 min in the presence of 40 kBq mL^{-1} of [^{3}H]thymidine (4.77 TBq mM^{-1}), [^{3}H]uridine (1.1 TBq mM^{-1}) or [^{3}H]leucine (2.37 TBq mM^{-1}) (Amersham International Inc., UK). The results were calculated as the percentage of radioactivity incorporated into untreated cells (approximately 3-6 kBq). All determinations were carried out on a Packard 4430 spectrometer. Every experiment was carried out at least three times and the data were computed by probit analysis.

6.2.7. Experiments with human cells cultivated in vitro

HeLa cells were grown in Nutrient Mixture F-12 Ham medium (Sigma Chemical Co, St Louis, MO, USA), containing 5% foetal calf serum (increased to 10% in the clonal growth), and supplemented with antibiotics. Cell growth was accomplished at 37 °C in a 5% carbon dioxide atmosphere. Every experiment was carried out at least three times. Clonal growth HeLa cells ($1.5\text{-}2 \times 10^5$) were seeded in Petri dishes in growth medium (4 mL). After 24 h, the medium was replaced with a fresh one containing the compound to be studied for 15 min and the cells were exposed to the UVA light. Aliquots of 200 cells were seeded in growth medium, incubated for 7 days, and the colonies were stained and counted, discarding those with less than 50 cells. The efficiency of the clonal growth, that is, the ratio between the number of the formed colonies and the number of the seeded cells, was about 0.85.

6.2.7.1. Detection of DNA-protein cross-links (DPC). DPC were detected by alkaline elution performed according to Kohn [27]; each experiment was carried out using an internal standard, i.e. untreated cells labeled with [^{3}H]thymidine and submitted only to a well defined dose of gamma rays, while treated cells were labeled with [^{14}C]thymidine. Cells in exponential growth were labeled by overnight incubation in the presence of 7.4 KBq mL^{-1} [^{3}H]thymidine or 3.7 KBq mL^{-1} [^{14}C]thymidine (2.2 GBq mM^{-1}; Amersham International Inc., UK). The radioactive medium was removed and replaced by a fresh one containing the test compound for [^{14}C]cells, and by a fresh one containing only 0.5% DMSO for [^{3}H]cells. The cells were exposed to UVA, washed and submitted to alkaline elution as previously described [26]. Elution was carried out using a Gilson Minipuls peristaltic pump at a flow of 0.03-0.04 mL min^{-1}. The radioactivity of

both isotopes was determined in the fractions collected with a Gilson fraction collector (approximately, 3.5 mL per fraction). The data were expressed as number of DPC induced per million of nucleotides according to Kohn [27].

6.2.7.2. Detection of RNA-protein cross-links (RPC). RPC were detected by alkaline elution [27] with slight modifications. Cells were labeled overnight in the presence of [^{3}H]uridine (7.4 KBq mL^{-1}) and then treated as described for DPC determination. Before submission to alkaline elution, the samples were not exposed to gamma rays; all other experimental steps were carried out similarly to DPC method. The data thus obtained were expressed in terms of cross-linking coefficient (CC), a parameter which is proportional to the number of RPC. It is defined as the ratio between the fraction of the radioactivity retained on the filters observed with the treated cells (Ft) and that of the controls (Fc): CC = Ft / Fc. These fractions were calculated using the method of the extrapolation at the zero fraction, as described by Kohn [27] for DPC.

6.2.8. Gamma ray irradiation

Gamma irradiation were always performed on ice using a ^{60}Co source working at the Reparto Applicazioni, Legnaro, Padova, Istituto di Fotochimica e Radiazioni d'Alta Energia (FRAE), C.N.R., with a dose-rate of 2.5 Gy min^{-1}, as determined by Fricke solution [28].

6.2.9. Radioactivity determination

Fractions from alkaline elution were counted using Ultima Gold XR (Packard Instruments, Meriden, CT, USA) and a Packard Tri-Carb 1900 TR spectrometer. Double-isotopes counting was accomplished automatically on the bases of quenching curves obtained using [^{3}H] and [^{14}C] radioactivity standards. Filters from macromolecular synthesis determinations were counted using a toluene-based scintillator (PPO 5 g, dimethyl-POPOP 0.25 g, toluene up to 1 L of solution).

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