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Pyrrolo[2,3-*h*]quinolinones: A new ring system with potent photoantiproliferative activity

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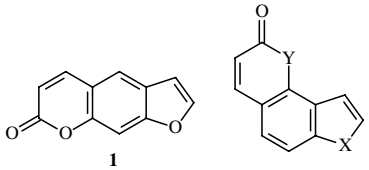
Abstract—A new class of compounds, the pyrrolo[2,3-*h*]quinolin-2-ones, nitrogen isosters of the angular furocoumarin Angelicin, was synthesized with the aim of obtaining new photochemotherapeutic agents with increased antiproliferative activity and lower undesired toxic effects than the lead compound. Two synthetic pathways were approached to allow the isolation both of the dihydroderivatives **10**–**17** and of the aromatic ring system **23**. Compounds **10**–**17** showed a remarkable phototoxicity and a great UVA dose dependence reaching IC_{50} values at submicromolar level. Intracellular localization of these compounds has been evaluated by means of fluorescence microscopy using tetramethylrhodamine methyl ester and acridine orange, which are specific fluorescent probes for mitochondria and lysosomes, respectively. A weak co-staining was observed with mitochondrial stain, whereas a specific localization in lysosomes was observed. Studies directed to elucidate the mode of action of this series of compounds revealed that they do not intercalate with DNA and do not induce photodamage to the macromolecule. On the contrary, they induce significative photodamage to lipids and proteins.

1. Introduction

Psoralen **1** (Fig. 1) belongs to a class of naturally or synthetic active substances which exhibit interesting photobiological properties.[1] They have been used in the PUVA therapy (Psoralen plus ultraviolet A radiation) for the treatment of skin diseases such as psoriasis and vitiligo or cutaneous T-cell lymphoma, in which oral or topical application of psoralens is combined with the exposure to UVA radiation. Since the introduction of extracorporeal photochemotherapy[2] (ECP, photophoresis) represents an evolution of the PUVA therapy for the treatment of cutaneous T-cell lymphoma (CTCL), it has also been applied for T-cell-mediated autoimmune disease (progressive systemic sclerosis, lupus erythematosus, pemphigo vulgaris and AIDS).[3–7]



1

2 X=Y=O
3 X=O Y=NH
4 X=O Y=S

Figure 1. Structures of Psoralen **1**, Angelicin **2** and heteroanalogues **3** and **4**.

It has also successfully been used to reverse allograft rejection after organ transplantation.[8–11]

During the last 40 years, much effort has been spent in photobiological and photochemical studies, in order to clarify the mechanism(s) of the desired phototherapeutical effects and undesired side effects of psoralens. It turned out that these heterocyclic carbonyl compounds can undergo photocycloadditions to biomolecules such as DNA, RNA, proteins and fatty acids. In particular the mechanism of photocycloaddition to DNA is now

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well understood. In the dark, psoralen molecules may form complexes with DNA via intercalation of the furocoumarin moiety between base pairs of the nucleic acid. After photoexcitation, they may form photocycloadducts to pyrimidine bases (most effectively to thymidine) involving either the C=C double bond of the furan ring (2,3-position) or that of the pyrone ring (5,6-position) or both. In this latter case "inter-strand cross links" (ISC) are formed. Moreover photoexcited psoralens may also take part in electron transfer reactions to molecular oxygen leading to the formation of superoxide anion ($O^{\cdot -}$) or hydroxyl radicals ($OH^{\cdot}$).[1] Additionally, they may also produce singlet molecular oxygen O_2 via excitation energy transfer.[1]

Unfortunately the antiproliferative effect, of this class of linear furocoumarins, is associated with undesirable side effects. In particular the induction of ISC was regarded as the main cause of short- and long-term side effects, such as skin erythemas, genotoxicity and mutagenicity,[12] and for this reason new compounds, capable of forming only monoadducts with DNA, have been synthesized. Angelicin **2**, for example, is an angular furocoumarin which, for its molecular geometry, cannot produce ISC resulting less toxic than its linear congener.[13] In this context, several angelicin isosters have been prepared and studied through the years such as furoquinolinones **3** and thioangelicin derivatives **4** (Fig. 1).[14]

Considering our interest in the chemistry of the pyrrole and the indole nucleus, in the search of new derivatives with photobiological activity, we focused our attention on the synthesis of the new ring system pyrrolo[2,3-*h*]quinolin-2-one, an angelicin isoster in which both oxygen atoms are replaced with nitrogens. This ring system incorporates the quinolin-2-one moiety, which confers many different biological properties to the nucleus to which it is condensed. In particular, some compounds show potent antitumour[14a] or cardiac stimulant activity.[15]

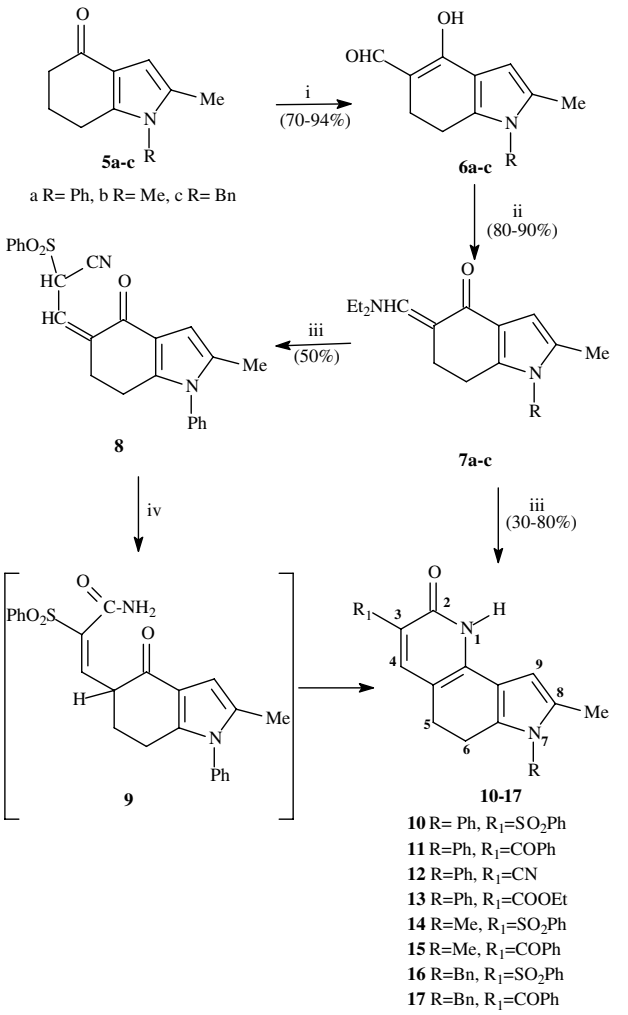
In this paper, we wish to present the two synthetic approaches and a detailed investigation on the photocytotoxicity exerted by these compounds. We have also extended our studies on the photochemical damages induced by these new derivatives on biological molecules, such as proteins, lipids and DNA, to better characterize the cellular targets involved in their phototoxic reactions. Herein, these photobiological features will be presented and discussed.

2. Results and discussion

2.1. Chemistry

We have previously reported, in a short communication, the synthetic pathway used for the synthesis of some of the compounds assessed in this study (Scheme 1).[16]

The 1,5,6,7-tetrahydro-indol-4-ones **5a–c** are versatile precursors suitable for the annelation of heterocycles



Scheme 1. Synthesis of compounds **10–17**. Reagents and conditions: (i) *t*-BuOK, HCOOEt, benzene, rt; (ii) $HNEt_2$, benzene, rt; (iii) $CNCH_2R_1$ ($R_1 = SO_2Ph$, COPh, CN, COOEt, $CONH_2$), ethanol, reflux; (iv) NEt_2, ethanol, reflux.

to the 4,5-indole position, and can be obtained by condensation of 2-acetonyl-1,4-cyclohexanedione with the proper amine in acetic acid as previously described.[17] The ketones **5a-c** were formylated to give the intermediates **6a–c** in yields from good to excellent. The formyl derivatives **6** were subjected to reaction with diethylamine to achieve in excellent yields the enamino ketones **7a–c**, key intermediates for the synthesis of the desired ring system. Considering that it was reported, that 1,3-dinucleophiles with a C–C–N sequence react with a variety of open-chain and cyclic enamino ketones to achieve the ring closure to a 2-pyridone nucleus,[18] we investigated the reactivity of our intermediates **7a–c** with cyanomethylene active compounds such as ethylcyanoacetate, benzoylacetonitrile, cyanoacetamide, malononitrile and phenylsulfonylacetonitrile. Such reactions were carried out in ethanol, and prolonged refluxing time (24–48 h) proved to be necessary to accomplish the cyclization to give compounds **11**, **12**, **14–17**. Only in the case of reaction of **7a** with phenylsulfonylacetonitrile, an intermediate of type **8** was formed, thus indicating that the reaction occurs between the nucleophilic methylene

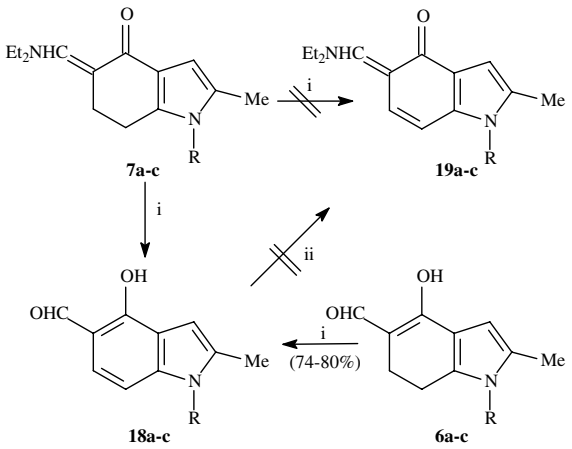
group and the α-enamino carbon atom first, which is the most electrophilic site of the enaminone. The tricyclic derivative **10** was obtained by heating under reflux the intermediate **8** with stoichiometric amount of diethylamine in ethanol. For this compound, the general procedure used for the other derivatives did not allow the isolation of the tricyclic derivative which was instead obtained in 30% yield by reacting the diethylamino methylene 1-phenyl-indolone **7a** with the anion of ethylcyanoacetate.

In general, it seems likely that, by prolonging refluxing time, the hydrolysis of the cyano group to carboxamide causes the reaction to proceed towards the direct closure to the 2-pyridone ring, in moderate to high yields without isolation of the intermediates **9**. Such a mechanism seems to be confirmed by the fact that the *N*-phenyl-substituted enaminone **7a** (R = Ph) reacts either with malononitrile or with cyanoacetamide producing the same 3-cyano-substituted pyrroloquinolinone derivative **12** thus, indicating that the carboxamido functionality is the one involved in the ring closure by nucleophilic attack of the nitrogen to the annular carbon.

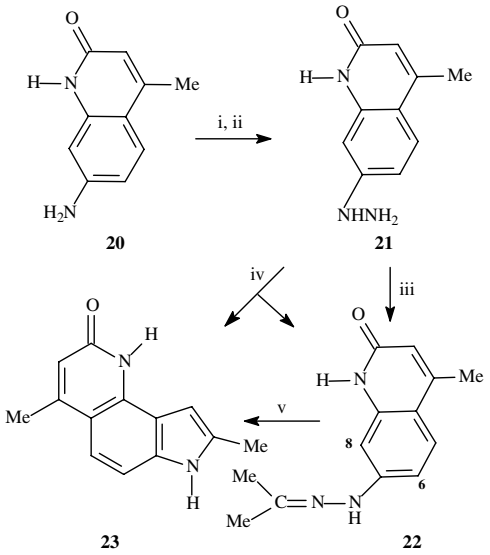
A different result was obtained when **7a–c** were reacted with the anion of the ethylcyanoacetate, obtained in situ with sodium ethoxide. In these cases in fact, the formation of the desired compounds was not observed even after many hours of heating with the sole exception of the reaction of **7a** with ethylcyanoacetate, from which the desired pyrroloquinolinone **13** was recovered from the reaction mixture in poor yield (30%).

In order to obtain flat molecules which could intercalate into double stranded DNA, aromatization of derivatives **10–17** was attempted. However, due to the poor solubility of the title compounds in most of organic solvents, reactions with many of the commonly used methods for this purpose (i.e., DDQ in refluxing benzene, toluene, dioxane, tetrahydrofurane, dichloromethane; cysteine/bubbling oxygen in refluxing ethanol; 10% Pd on charcoal/bubbling oxygen in refluxing benzene; 10% Pd on charcoal in cumene at 150 °C or 1-methylnaphtalene at 250 °C)[19a–19d] did not give the expected result even when a phase transfer catalyst was added to the reaction mixture (TDA-1).[19e] It was reasonable to try the oxidation of the diethylamino indoles **7a–c**, more soluble in most of organic solvents which could be further cyclized into the desired ring system. In this case however, due to the instability of the enaminoketones, the only products isolated from the reaction mixture were the aromatic derivatives **18a–c** in traces. However, good yields of these compounds were obtained starting from the corresponding dihydroindole derivatives **6a–c** (74–80%). Any attempt to react **18a–c** with diethylamine to give compounds **19a–c** in the same reaction conditions used for the dihydro derivatives failed, even using the secondary amine as solvent (Scheme 2).

Therefore, a different synthetic pathway was considered. It consists in the annelation of the pyrrole ring on the preformed aromatic quinoline (Scheme 3). In this light according to a procedure described in the literature,[20]



Scheme 2. Oxidation of compounds **7a–c**. Reagents and conditions: (i) DDQ, dichloromethane, rt; (ii) $HNEt_2$, benzene, rt.



Scheme 3. Synthesis of compound **23**. Reagents and conditions: (i) $NaNO_2$, hydrochloric acid, −5 °C; $SnCl_2$, hydrochloric acid, −12 °C; (ii) NaOH; (iii) acetone, ethanol, reflux; (iv) acetone, acetic acid, reflux, Na_2CO_3 10%; (v) $ZnCl_2$ 280–290 °C.

we prepared the 7-aminoquinolin-4-methyl-2-one **20**, which was used as building block for the synthesis of the desired aromatic tricyclic system. Diazotization with sodium nitrite of the amine **20** afforded the diazonium salt, which was subsequently reduced with a solution of stannous chloride in hydrochloric acid.[21] From the resulting mixture, hydrazine hydrochloride was filtered off and carefully treated with a solution of sodium hydroxide as to get the free base **21**. This latter was subsequently reacted with an excess of acetone in refluxing ethanol to give the hydrazone **22** in excellent yields (85%). When acetic acid was used as solvent, a mixture of the hydrazone **22** (60%) and the desired cyclic derivative **23** (20%) was obtained. However, attempts to convert the hydrazone into the pyrroloquinolinone **23** with polyphosphoric acid did not lead to the expected tricyclic system but produced a tarry mixture from which it

was not possible to isolate compound **23**. On the contrary when anhydrous zinc chloride was used to accomplish the cyclization of the pure hydrazone **23**, the reaction occurred in a short time avoiding undesirable byproducts and making the work-up and purification of the reaction mixture easier. Such Fisher cyclization only furnished the angular derivative **23** (65%), even if positions 6 and 8 were both available for the ring closure on the quinoline moiety (Scheme 3).

2.2. Biology

2.2.1. Physico-chemical properties. The test compounds represent isosters of the typical trycyclic system of angelicin with substituents in positions 3, 7 and 8. All compounds bear an electron-withdrawing group in position 3 so as to deactivate the photoreactive bond in positions 3–4. The only exception is compound **23** which presents an additional methyl group in position 4. The double bond in positions 8–9 results to be activated by the methyl group in position 8 lying on the trycyclic plane. Both absorption and emission maxima wavelengths of the compounds exhibit (Table 1) a remarkable bathochromic shift with respect to angelicin due to the conjugation with the substituents in positions 3 and 7. Position 3 is particularly sensitive to the nature of the substituent as demonstrated by the values of the molar extinction coefficients. In particular, the derivatives bearing the sulfonyl group (**10**, **14**, and **16**) are endowed with an higher molar extinction coefficient than those bearing the carbonyl group (**11**, **13**, **15**, and **17**). This could be due to the higher electronic density and to the increased conjugation but also to the structural rigidity of the molecule. The relative fluorescence quantum yields are measured at 298 K.[22] As it can be observed from Table 1, the 3-phenylsulfonyl derivatives show values of fluorescence quantum yields, markedly higher than the other compounds. In fact, the insertion of such substituents into the quinoline nucleus markedly affects the fluorescence both in terms of the energy and of the yields. Moreover, the partition coefficients of the test compounds were determined by a computational method.[23] The results obtained indicate that the pyrroloquinolinones are highly hydrophobic in comparison to angelicin (c log P = +2.08) taken as reference compound.

2.2.2. Phototoxicity. The phototoxicity of title compounds was investigated on three cultured cell lines of human tumour such as HL-60 (human promyelocytic leukaemia), HT-1080 (human fibrosarcoma) and LoVo (human intestinal adenocarcinoma). Table 2 shows the extent of cell survival expressed as IC_{50} which is the concentration, which induces 50% of inhibition of cell growth, after irradiation at different UVA doses. Control experiments with UVA light or drugs alone were carried out without significant cytotoxic effects (data not shown). It can be noted the compounds exhibit different values of IC_{50} depending on the substitution pattern, and a remarkable dose UVA-dependence.

All the compounds inhibit the proliferation of tumour cell lines at submicromolar/micromolar concentration. In some cases the IC_{50} values are comparable, if not lower than 8-methoxypsoralen (8-MOP) and angelicin used as reference drugs. The most interesting derivatives appear to be **10**, **11**, **17** and **15** only in the leukaemia cell line. These IC_{50} values appear particularly interesting considering the cell lines derived from solid tumours (HT-1080 and LoVo).

From a structure–activity relationship point of view the following considerations can be done: in all compounds the nature of the substituent on the pyrrole nitrogen (position 7) plays a major role in the induction of phototoxicity, in fact, with the exception of **12** (R = Ph, R′ = CN) the antiproliferative activity generally decreases following the rank order phenyl > benzyl > methyl.

Moreover, also the substituent in position 3 plays a role in the modulation of the antiproliferative activity. In fact, derivatives bearing the sulfonyl group become more active than the compounds bearing other electron-withdrawing groups.

Surprisingly compound **23**, which is devoid of the substituents in positions 3 and 7, appears to be inactive in the three cell lines at all UVA doses used.

2.2.3. Effect of scavengers on pyrroloquinolinone-induced phototoxicity. In order to evaluate the possible mechanism of action of the antiproliferative activity of these derivatives, a series of experiments were performed in which, various scavengers were included in the cell cultures during irradiation of the title compounds **10**, **11**, and **17**. The results of these studies are presented in Figure 2. The scavengers used were sodium azide

Table 1. Physico-chemical properties of Pyrroloquinolinones

Compound	λ_{max} Absorb. (nm)	λ_{max} Emiss. (nm)	ε (M^{-1} cm^{-1})	ϕ_f[a]	c log P[b]
10	421.3	451	34243	0.34	+3.93
11	441.5	460	23201	2.6×10^{-3}	+4.38
12	331.6–383.2	440	29974	1.8×10^{-4}	+3.02
13	423.0	463	24350	$1.\ 3 \times 10^{-3}$	+2.83
14	423.1	458	31307	0.33	+2.58
15	445.2	484	22139	3.3×10^{-3}	+3.29
16	421.2	464	33629	0.10	+4.26
17	442.7	484	23310	1.6×10^{-4}	+4.64
23	337.1–351.2	445	10584	2.8×10^{-2}	+2.83
Ang.	299	413	9350	1.8×10^{-2}	+2.08

[a] Relative fluorescence quantum yield determined in 0.1 N H_2SO_4 as described in Ref. 22. The estimated error is 15%.
[b] Calculated as described in Ref. 23.

Table 2. Photocytotoxicity of test compounds against three different human tumour cell lines

HL-60[a] (IC_{50}, μM)[b] Dose UVA[c] ($J\ cm^{-2}$)	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**23**	8-MOP	Ang.
2.6	1.2 ± 0.2	8.5 ± 0.3	5.4 ± 1.1	7.7 ± 3.0	>20	8.9 ± 0.1	12.4 ± 1.2	4.0 ± 0.2	>20	1.4 ± 0.1	1.5 ± 0.2
3.2	0.82 ± 0.02	3.9 ± 0.5	4.7 ± 0.5	5.5 ± 2.1	13.2 ± 2.1	6.2 ± 0.01	6.9 ± 0.2	2.2 ± 0.2	>20	1.2 ± 0.1	0.9 ± 0.1
6.5	0.45 ± 0.04	0.51 ± 0.08	1.3 ± 0.7	1.3 ± 0.5	2.8 ± 0.8	0.86 ± 0.06	1.8 ± 0.7	1.1 ± 0.1	>20	0.7 ± 0.1	0.6 ± 0.1
HT-1080[a] (IC_{50}, μM)[b] Dose UVA[c] ($J\ cm^{-2}$)	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**23**	8-MOP	Ang.
2.6	3.6 ± 0.5	6.6 ± 1.6	>20	12.2 ± 2.1	>20	>20	>20	14.7 ± 0.9	>20	7.8 ± 0.7	15.7 ± 1.9
3.2	2.3 ± 0.8	4.6 ± 0.9	13.1 ± 1.5	7.9 ± 0.9	>20	>20	15.1 ± 1.2	9.0 ± 1.1	>20	2.1 ± 0.3	2.6 ± 0.2
6.5	0.4 ± 0.04	1.7 ± 0.2	11.1 ± 1.4	2.1 ± 0.6	16.4 ± 1.4	>20	1.7 ± 0.2	1.9 ± 0.02	>20	1.5 ± 0.2	2.5 ± 0.3
LoVo[a] (IC_{50}, μM)[b] Dose UVA ($J\ cm^{-2}$)[c]	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**23**	8-MOP	Ang.
2.6	1.2 ± 0.2	4.1 ± 0.6	9.8 ± 1.1	6.9 ± 1.2	>20	>20	>20	6.5 ± 0.7	>20	1.1 ± 0.4	1.6 ± 0.2
3.2	0.6 ± 0.1	2.8 ± 0.3	8.9 ± 0.3	3.1 ± 1.3	17.5 ± 4.7	>20	10.7 ± 1.3	3.6 ± 1.3	>20	0.7 ± 0.1	0.9 ± 0.1
6.5	0.4 ± 0.02	1.3 ± 0.2	8.3 ± 1.1	1.1 ± 0.9	6.6 ± 1.1	8.9 ± 0.9	2.9 ± 0.3	0.6 ± 0.1	>20	0.4 ± 0.1	0.8 ± 0.1

[a] Human cell lines: HL-60 promyelocytic leukaemia, HT-1080 fibrosarcoma cells, LoVo intestinal adenocarcinoma.
[b] Concentration of compound required to inhibit the cell growth by 50% after 72 h of exposure as determined by MTT assay.
[c] UVA Dose expressed in $J\ cm^{-2}$ as measured at 365 nm with a Cole-Parmer radiometer.

(NaN_3) and 1,4-diazabicyclo[2,2,2]octane (DABCO), two singlet oxygen scavengers; superoxide dismutase (SOD) and catalase (CAT) which scavenge $O_2^{\cdot-}$ and H_2O_2, respectively; *N*-*N*′-dimethyl thiourea (DMTU) and Mannitol (MAN), which scavenge hydroxyl radicals ($OH^{\cdot}$); and 2,6-di-*tert*-butylhydroxyanisole (BHA) and glutathione reduced form (GSH), two free radical scavengers. The concentrations of the compounds and UVA doses were chosen on the basis of the results shown in Table 2 (dose UVA = 3.2 J cm^2). Scavengers alone did not affect the cell viability during UVA exposure (data not shown). A strong protective effect is generally observed with GSH and BHA, and MAN for the three compounds investigated and DMTU for **10** and **17**, indicating that the mechanism of action of pyrroloquinolinones is principally mediated by free radicals and in particular OH **.** radicals. A weak protective effect is generally observed with NaN_3 and DABCO, thus suggesting that singlet oxygen could be only partially involved in the cytotoxic activity of the test compounds.

2.2.4. Intracellular localization. The importance of the cellular localization for the cytotoxic action of photosensitizers has been demonstrated.[24,25] The strength of a photosensitizer is determined by the nature of the excited states or by its ability to form reactive species while, in a phototoxicological context, other factors such as subcellular localization are important parameters. Thus, it is critically important to evaluate the distribution of photosensitizers inside cells. The subcellular localization of a photosensitizer is due to its physicochemical properties such as hydrophobicity, charge, pK_a, etc. For example, lysosomes are preferential sites of two fluoroquinolones such as lomefloxacin and ciprofloxacin,[26] whereas mitochondria are the primary targets in photodynamic therapy.[27] In order to investigate the intracellular localization of pyrroloquinolinones, we used two fluorescent probes: TMRM, a lipophilic cation commonly used for the assessment of the mitochondrial potential[28,29], and acridine orange (AO), which stains lysosomes.[25] These markers fluoresce in the visible region (about 550 nm), while the test compounds emit in the blue region. Both fluorescences can be easily separated using suitable bandpass optical filters. After an incubation in HT-1080 fibrosarcoma cells, the most interesting compounds were found to incorporate and associate with subcellular structures.

It can be observed from Figure 3 that compound **10**, chosen as representative, diffuses in the cytoplasm and only partially co-localizes with TMRM. On the contrary, a more evident accumulation in lysosomes can be observed suggesting that lysosomes may be a major site of photosensitization for this class of compounds.

2.2.5. Photodegradation/photostability. All derivatives underwent a remarkable photodegradation, as shown by the changes in absorption spectra upon UVA irradiation in buffered aqueous solution (see Section 5). Distinct isosbestic points were observed, suggesting that photoproducts are most likely formed. Interestingly, the only exception is represented by compound **23** which, in comparison with the other derivatives, does not show any variation of the absorption spectrum even after a long time of irradiation (see Section 5).

In order to obtain precise informations about the photodegradation kinetics, at appropriate time intervals the irradiated solutions were analysed by HPLC. Kinetic analysis of the photodegradation process reveals an apparent first order kinetics for the test compounds. The rate constants (k) were obtained from the slopes

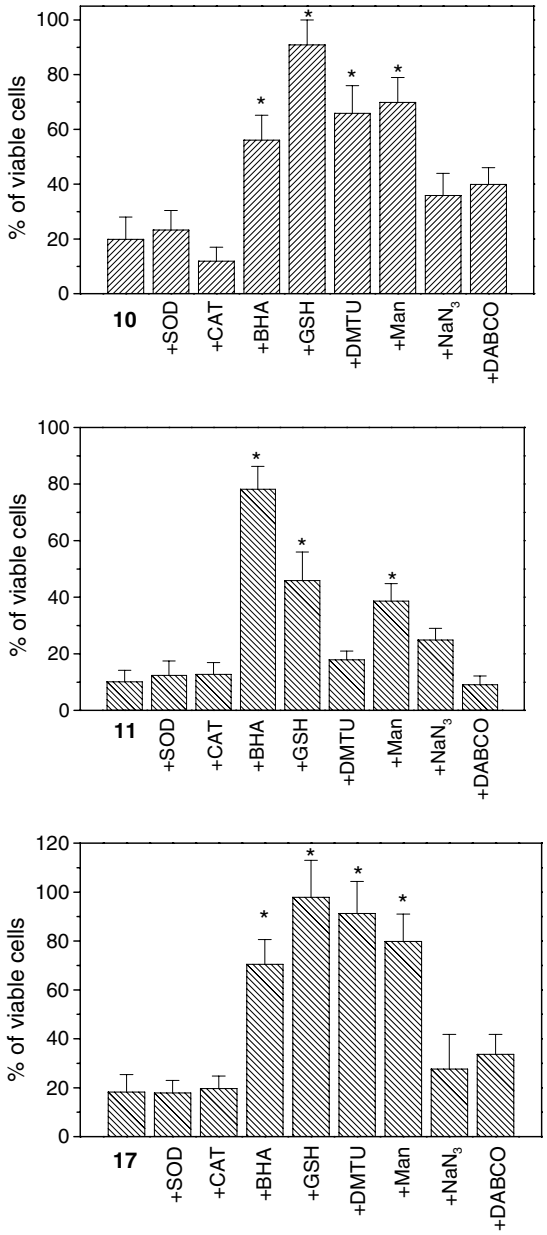


Figure 2. Effect of different scavengers on the phototoxicity induced by compounds **11** (upper panel) **10** (middle panel) and **17** (lower panel) in HT-1080 fibrosarcoma. Cell viability was assayed by MTT test after 72 h from the irradiation in the presence of compounds **11**, **10** and **17** and SOD (2000 U.I./ml), CAT (2000 U.I./ml), BHA (10^{-5} M), Man (10 mM), GSH (1 mM) DMTU (1 mM) NaN3 (10 mM) and DABCO (1 mM).

of the plots by linear regression analysis and the results are presented in Table 3. It can be noted that pyrroloquinolinones **10**, **11**, **12**, **16**, and **17** underwent a very rapid degradation, their half life being in the range of 13–63 min. The potential involvement of photoproducts generated upon UVA irradiation has been studied. Furthermore, a partial characterization of the photoproducts was performed by LC–MS. In Figures 4–6 are reported the TIC (total ion current) of the most active compounds (**10**, **11**, and **17**) and the mass values of the principal peaks are reported in Table 4. As it can be observed, irradiation of **11** produces three photoproducts in high yields. The other two compounds **10** and **17** produce many photoproducts among which those with mass value of −10, −2, and +32 in respect to the mass of the starting compound can be observed as for compound **11**.

These values indicate that an oxidative pathway takes place and the fact that the differences of mass values are always the same indicates that the tricyclic system is not involved in the photodegradation process. The potential involvement of photoproducts generated upon UVA irradiation has also been studied. HL-60 cells and LoVo cells were incubated for 72 h in the presence of a solution of pyrroloquinolinones which were previously irradiated for 30 min. The cell viability was assayed as described before. The obtained results (data not shown) have revealed no cytotoxic effects, suggesting that the events that lead to cellular death are solely due to a phototoxic damage and not to a production of toxic photoproducts.

2.2.6. Lipid peroxidation. Having hydrophobic character, as their partition coefficients indicate, (see Table 1) the pyrroloquinolinones may be expected to be localized mainly in plasma and/or in subcellular membranes, as confirmed by fluorescence microscopy studies, making these structures particularly sensitive to photodamage.[30] The thiobarbituric assay (TBA test) was used in order to determine whether lipid peroxidation occurs upon irradiation of HT-1080 cells incubated in the presence of test compounds. The TBA test was performed on the supernatants medium and in the cell homogenates as described in the experimental section.[31] Figure 7 shows the results obtained for compounds **10** and **11** as a function of light doses. Thiobarbituric reactive substances (TBARS) are significantly produced when the cells are exposed to the compounds and UVA both in the supernatant and in the cell extract. A good correlation is observed between the extent of lipid peroxidation monitored by the TBA test and the degree of phototoxicity.

2.2.7. Protein photodamage. The photosensitization capacity of test compounds (**10**, **11**) towards other components of cellular membranes, such as proteins, was estimated by measuring the photoinduced cross-linking in erythrocyte ghost proteins.[32] Light-induced cross-linking of spectrin, a protein associated with the cytoplasmic side of the RBC membrane, in the presence of these compounds was detected by the partial or total disappearance of the two spectrin bands, (220,000 and 245,000 Da) on SDS–PAGE, while cross-linked aggregates cannot run inside the gel and remain at the top.

Figure 8(A and B) shows the pictures of the SDS–PAGE of ghosts irradiated in the presence of compounds **11** and **10**. Under aerobic conditions the two compounds cause an UVA dose-dependent disappearance of the two spectrin subunit bands whereas in the presence of nitrogen this effect is reduced indicating the involvement of reactive oxygen species. To further investigate the photosensitizing properties of the title compounds towards proteins, solutions containing bovine serum albu-

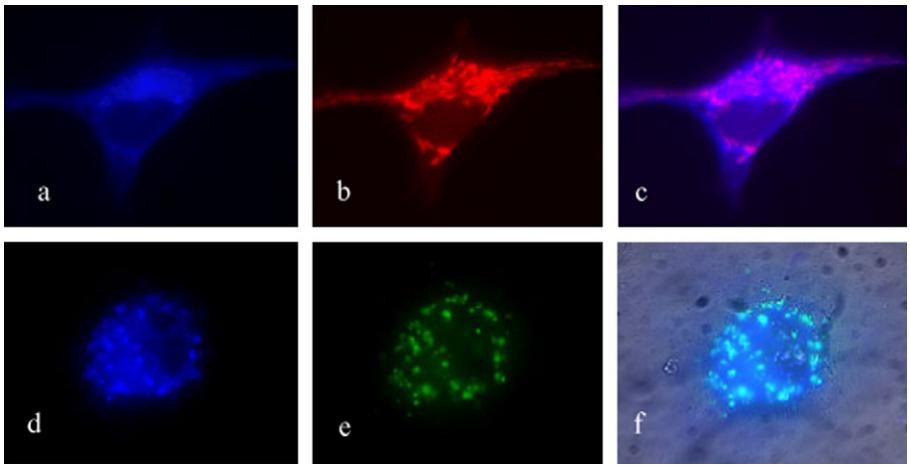


Figure 3. Fluorescence microphotographs showing the intracellular localization of **10** in HT-1080 fibrosarcoma cells in the presence of TMRM (panels b and c) and AO (panels e and f). The cells were treated as described in Material and Methods. a, fluorescence image of **10**; b, fluorescence image of TMRM; c, Overlay image of **10** localization generated by transferring the blue color of the compounds seen in panel a, onto the corresponding fluorescence image of panel b. Pink is used as a false color to indicate the localization of **10**. d, fluorescence image of the **10**; e, fluorescence image of AO; f, Overlay image of **10** localization generated by transferring the blue color of the compounds seen in panel d onto the corresponding fluorescence image panel e. Clear green is used as a false color to indicate the localization of **10**.

Table 3. Photodegradation kinetic parameters of the title compounds

Compound[a]	k (min^{-1}) $\times 10^{-2}$	$t_{1/2}$ (min)
10	5.31	13.0
11	4.66	13.7
12	1.10	63.0
16	4.81	14.4
17	2.17	31.9

[a] The rate constant and the half-life of photodegradation were determined in phosphate buffer 10 mM, pH-7.2.

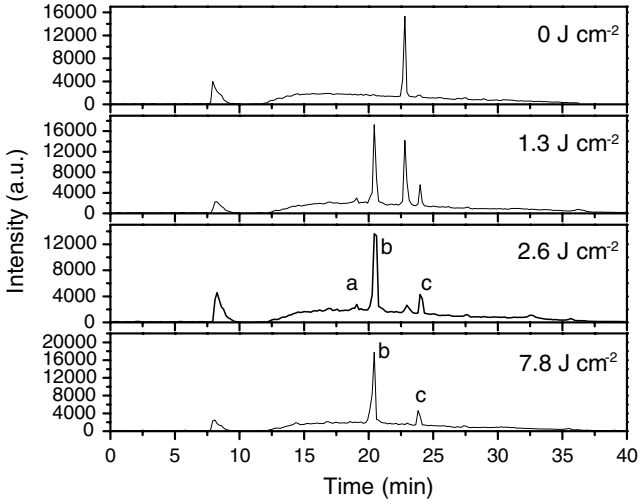


Figure 4. LC-MS profile of upon UVA irradiation in phosphate buffer under aerobic conditions. Compound **11**.

min (BSA), as a model[33], and the drugs in phosphate buffer were irradiated for different times. Tryptophane (Trp) content was directly analysed by monitoring the characteristic fluorescence of Trp residues.[34] As depicted in Figure 9, a rapid decrease of the emission fluorescence was observed under aerobic conditions for **11**, **10**, and

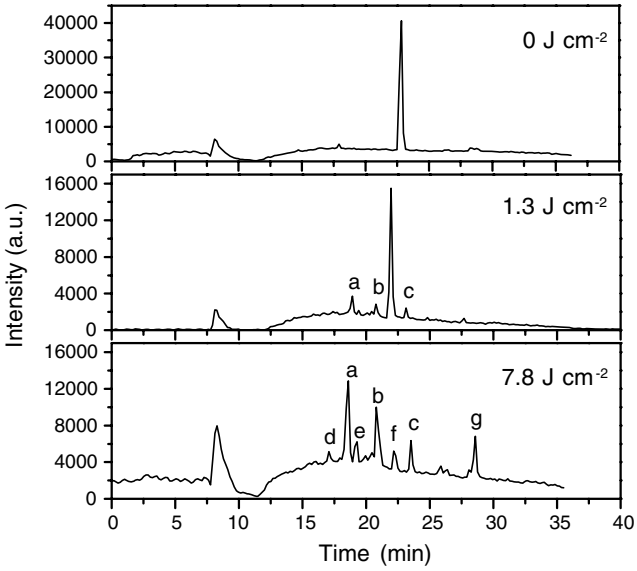


Figure 5. LC-MS profile of upon UVA irradiation in phosphate buffer under aerobic conditions. Compound **10**.

17. No effects were observed when BSA alone was irradiated in the same conditions. When N_2 was bubbled through the solution prior and during the irradiation in the presence of test compounds, a significant protection for the protein photodamage was observed. No significant effects were observed when the protein was irradiated in the presence of drugs and superoxide dismutase (SOD) or in the deuterated phosphate buffer indicating that neither superoxide anion nor singlet oxygen is involved in the protein photodamage.

Another protein model used in this study was RNAseA which is a protein that does not possess Trp residues but has tyrosine (Tyr) residues in its sequence.[35,36] Its emission band centred at about 350 nm significantly

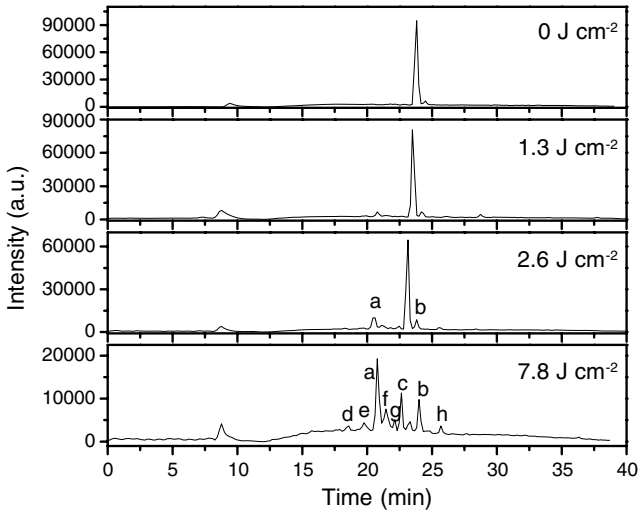


Figure 6. LC-MS profile of upon UVA irradiation in phosphate buffer under aerobic conditions. Compound **17**.

Table 4. MS spectral data of photodegradation products of test compounds

Compound/photoproducts	Mass values[a]
10	417.14
a	407.11 (−10)
b	495.17 (+78)
c	415.12 (−2)
d	449.13 (+32)
e	421.14 (+4)
f	451.13 (+34)
11	381.17
a	413.17 (+32)
b	371.14 (−10)
c	379.15 (−2)
17	395.18
a	385.18 (−10)
b	393.19 (−2)
c	409.15 (+14)
d	427.17 (+32)
e	427.17 (+32)
f	322.11 (−73)
g	427.17 (+32)

[a] In parentheses is indicated the variation of mass in comparison to the parent compound.

decreases after irradiation in the presence of compounds **11**, **10**, and **17**. Again, this effect is markedly reduced in anaerobic conditions. Deuterated phosphate buffer, which increases singlet oxygen lifetime or SOD, did not induce any variation of the degradation profile, as described above for BSA.

2.2.8. DNA interactions and DNA-photodamage. In order to understand the binding nature between the title compounds and DNA, linear dichroism (LD) measurements[37,38] were performed on solutions with salmon testes DNA (st-DNA) and the title compounds at various molar ratios. Inspection of the LD spectra of two derivatives (**10** and **23**) chosen as representative (Fig. 10) in the presence of st-DNA reveals that despite a strong absorption in the 300–500 nm region, the two compounds give no LD bands in this region. Furthermore,

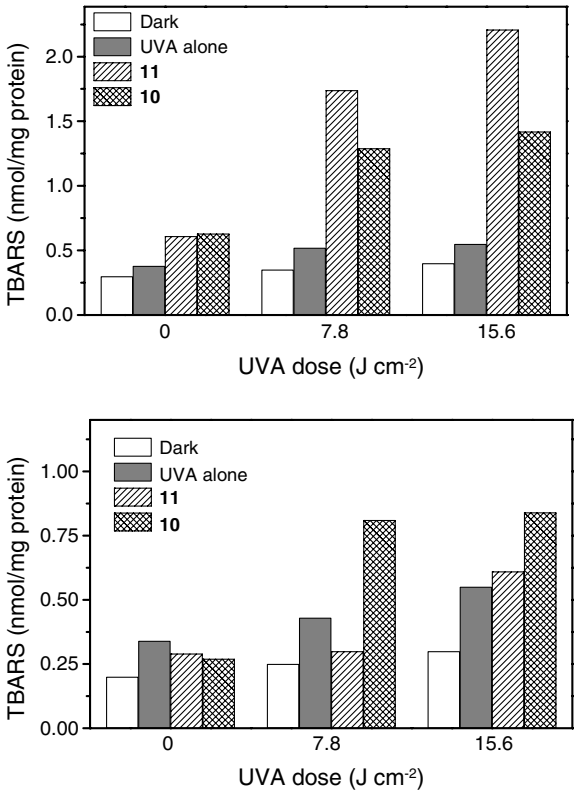


Figure 7. Lipid peroxidation induced in HT-1080 fibrosarcoma cells 24 h after the irradiation in the presence of **11** and **10** at the concentration of 20 μM. The TBA test was performed on the supernatants medium (upper panel) and in the cell homogenates (lower panel) as described in the experimental section.

fluorescence titrations carried out with the same derivatives (data not shown) showed a poor fluorescence quenching confirming that the title compounds are loosely bound to DNA. These facts strongly suggest that the new derivatives do not interact efficaciously, without irradiation, with the macromolecule, as indeed demonstrated with natural furocoumarins.[1]

Further experiments were carried out in order to determine whether the new derivatives were able to photosensitize DNA strand break activity. Their ability to photodamage DNA was evaluated using supercoiled circular DNA because it is a very sensitive tool for damage detection.[39] In addition to frank strand break we have also evaluated if purine and/or pyrimidine bases were involved in the oxidative damage to DNA using base excision repair enzymes Formamido pyrimidin glycosilase (Fpg) and Endonuclease III (Endo III), respectively.

Supercoiled circular DNA allows the detection of structural alterations such as strand breaks or damaged bases. DNA strand break can be induced either directly (frank strand breaks) or indirectly using DNA repair enzymes, in this case we determined the number of DNA modification sensitive to the following repair enzymes (i) Fpg protein which has been demonstrated to recognize 8-hydroxy guanine, purines whose imidazole ring is open (Fapy residues) and sites of base loss (apurinic sites). (ii) Endonuclease III, which is known to recog-

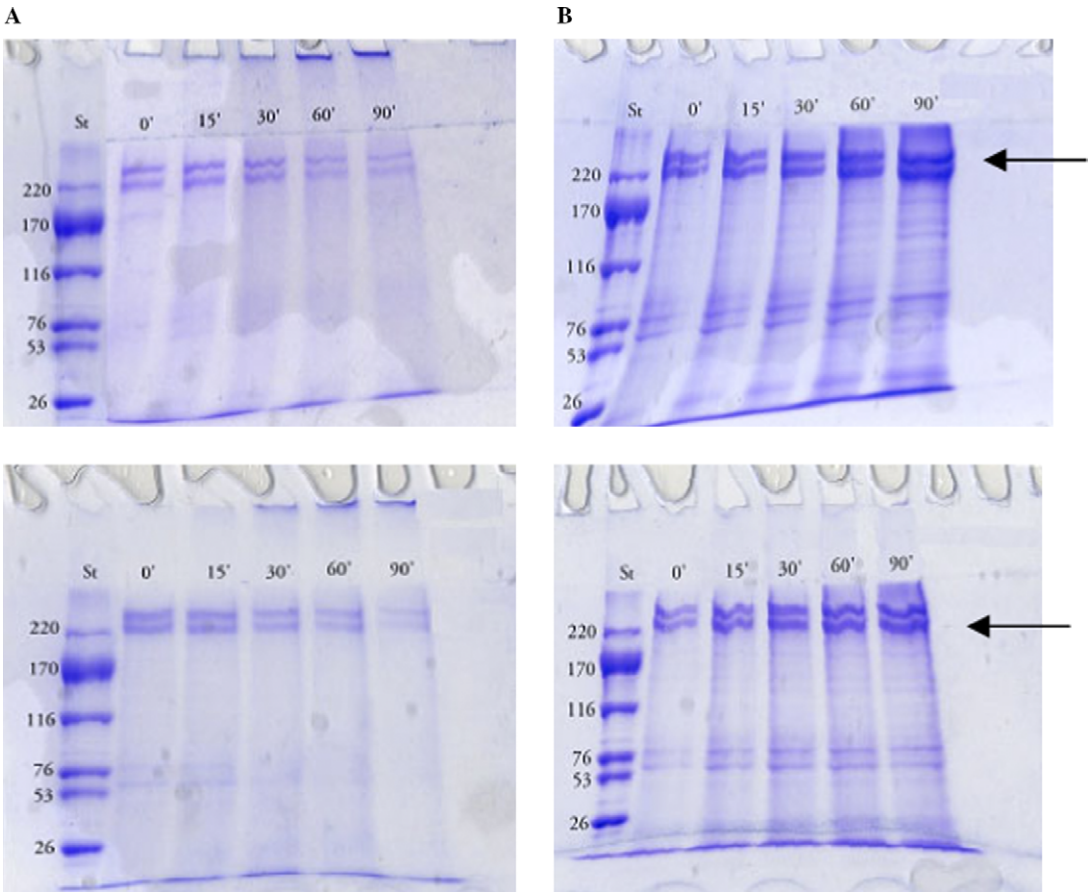


Figure 8. Electrophoretic pattern of the photoinduced cross-link of spectrin in RBC ghosts irradiated at the indicates times (UVA dose = 0.24 J cm^{-2} min^{-1}) in the presence of **11** (upper panels) and **10** (lower panels) at the concentration of 20 μM. Column A, aerobic condition; Column B, N_2 purged solution. Spectrin band is indicated by an arrow. St is a mixture of molecular weight standard, which value in KDa is depicted in the left of the figure.

nize, in addition to apurinic sites, 5,6-dihydropyrimidine derivatives. Damaged base release is followed by a β–δ reaction and a β-elimination step, respectively, resulting in DNA breakage.[40]

The obtained results (see Section 5) indicate that the new compounds do not sensitize the formation of single strand breaks at any of the UVA dose used. Furthermore, the treatment with the two base excision enzymes reveals that they are not able to photooxidize DNA bases. Altogether these results indicate that the new pyrroloquinolinones differently from psoralens do not efficaciously interact with DNA and other mechanisms are operating for this new class of compounds.

3. Conclusions

The data reported herein indicate that pyrroloquinolinones represent a new interesting class of potentially useful compounds in photochemotherapy. They represent isosters of the tricyclic system of the angelicin with the additional substituents in positions 3 and 7 that increase the conjugation of the system. In fact, the tricyclic compound **23** surprisingly is completely inactive. This seems to be in contrast with previous studies. It was reported, in fact, that the introduction of a nitrogen atom on the furane ring decreases the photobiological activity, as in the case of linear and angular pyrrolocoumarines. Moreover, 5,6-dihydro furo and thienoquinolinones bearing an electron-withdrawing substituent, such as an ester functionality, are devoid of activity.[19b]

Interestingly, the conjugation of the tricyclic ring with phenylsulfonyl groups strongly increases their photoactivity as demonstrated by the rapid photodegradation and the remarkable induction of phototoxicity. Regarding the mechanism of action, the lack of affinity of the new compounds with DNA upon UVA irradiation could be of great relevance in modulating the long-term toxic effect such as skin cancer or mutagenesis exhibited by psoralen. Indeed, it seems that lysosomes and/or mitochondria could be target for pyrroloquinolinones inducing photodamage to lipids and proteins. A first indication about their mechanism of action comes from the experiments of cytotoxicity carried out in the presence of different scavengers. Results demonstrate the participation of free radicals and in particular the involvement of the hydroxyl radicals. It will be necessary to carry out further investigation on the photochemical and photophysical aspect to better clarify their mechanism of action.

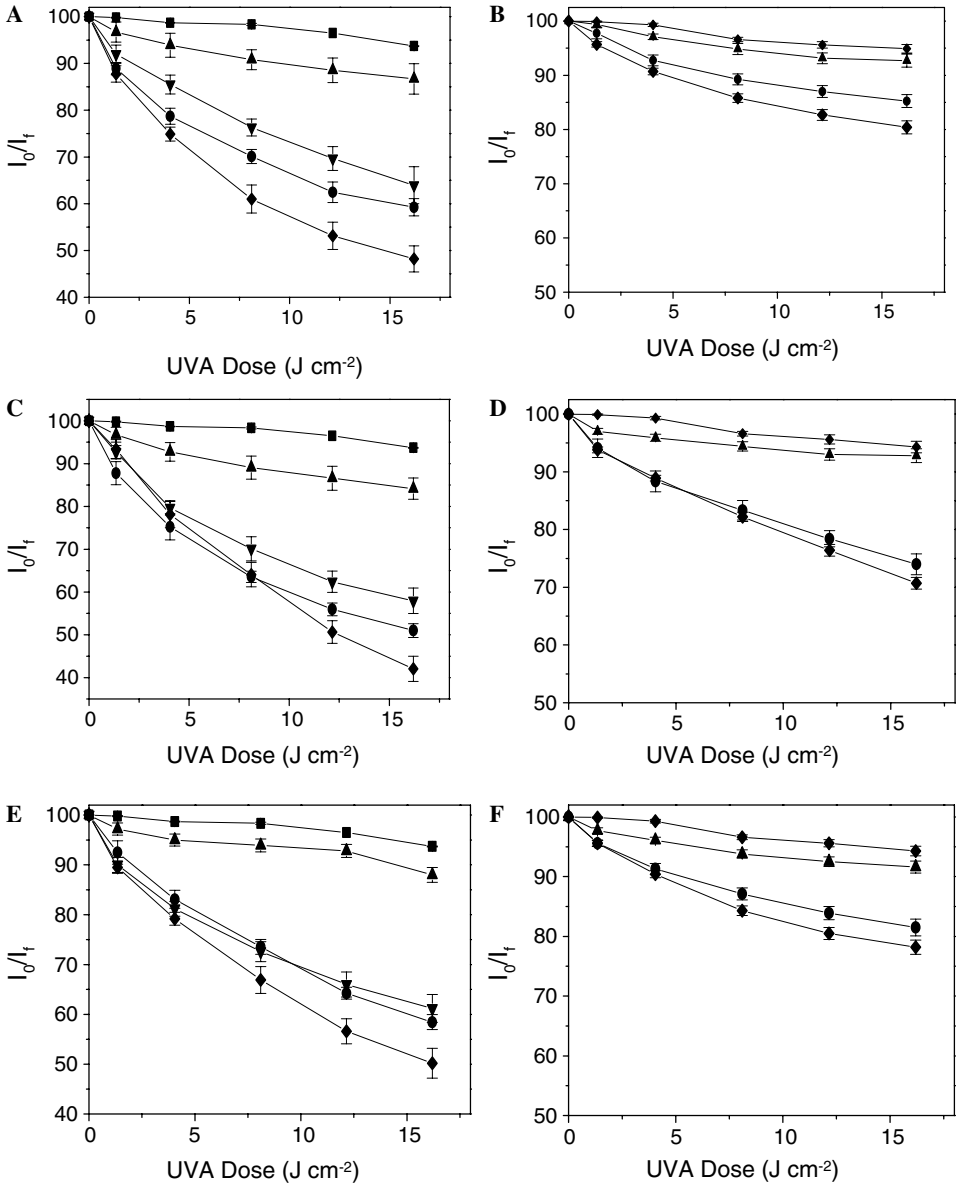


Figure 9. Photosensitising effects on BSA (Panels A, C and E) and RNAseA (panels B, D and F) of compounds **11** (A,B), **10** (C,D), and **17** (E,F), at the dose of 10 μM after irradiation, in different conditions: in aerated (●) and deaerated (▲) solutions; plus SOD 2000 UI/ml(▼); in deuterated phosphate buffer (♦); (■) BSA alone.

4. Experimental

4.1. Chemistry

All melting points were taken on a Buchi–Tottoli capillary apparatus and are uncorrected; IR spectra were determined with a Jasco FT/IR 5300 spectrophotometer; ^{1}H and ^{13}C NMR spectra were measured in DMSO-d_6 solutions, unless otherwise specified (TMS as internal reference), at 200 and 50.3 MHz, respectively, using a Bruker AC series 200 MHz spectrometer. Column chromatography was performed with Merck silica gel 230–400 Mesh ASTM or with a SEPACORE chromatography apparatus BUCHI. Elemental analyses (C, H, and N) were within ±0.4% of the theoretical values.

4.1.1. General procedure for the 2-methyl-1-substituted-1,5,6,7-tetrahydro-indole-4*H*-4-ones (5a–c). These compounds were prepared by reacting the commercially available 1,3-dihydroresorcin, according to the Stetter and Lauterbach procedure,[17] with chloroacetone and potassium hydroxide in methanol at room temperature for 48 h to afford the 1,3-acetonylcyclohexandione as a mixture of the two tautomers which was used without any further purification. Condensation with the suitable amine in acetic acid at 60 °C for 2 h yielded the 2-methyl-1-substituted-1,5,6,7-tetrahydro-indole-4-ones **5a–c** (60–90%).

4.1.2. 2-Methyl-1-phenyl-1,5,6,7-tetrahydro-4*H*-indole-4-one (5a). This product was obtained as white solid (60%) by reaction of 1,3-acetonylcyclohexandione with

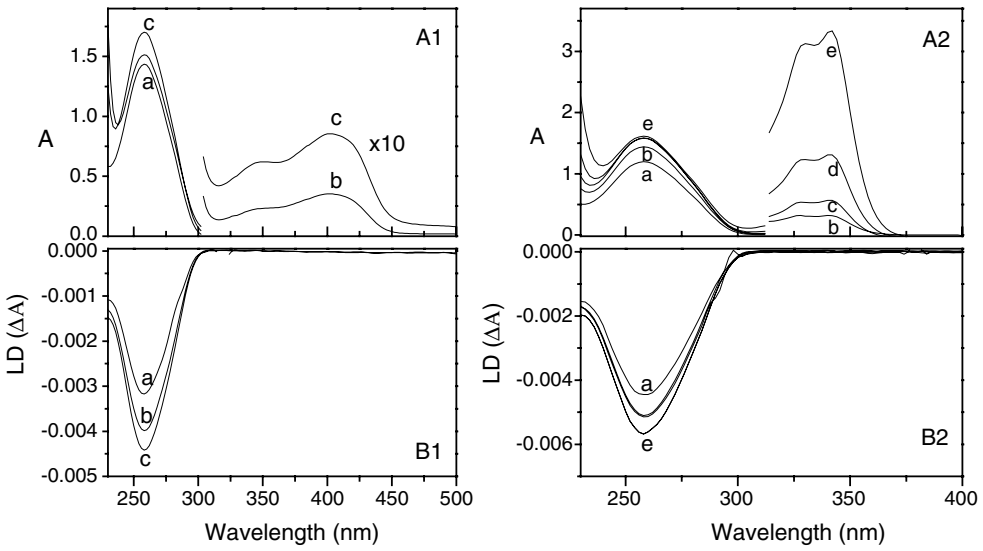


Figure 10. Absorbance (A), and Linear dichroism (LD) spectra of mixtures of salmon testes DNA and compounds **10** (A1,B1) and **23** (A2,B2) at different [Drug]/[DNA] ratio (a = 0.00, b = 0.02, c = 0.04, d = 0.08, e = 0.2).

phenylamine: mp 153–155 °C; IR: 1648 (CO) cm^{-1}; ^1H NMR: σ 1.94 (q, J = 6.2 Hz, 2H, CH_2), 2.01 (s, 3H, CH_3), 2.33 (t, J = 6.2 Hz, 2H, CH_2), 2.50 (t, J = 6.2 Hz, 2H, CH_2), 6.22 (s, 1H, H-3), 7.38 (d, J = 6.9 Hz, 2H, H-2′, H-6′), 7.47–7.57 (m, 3H, H-3′, H-4′, H-5′,). ^{13}C NMR: σ 12.3 (CH_3), 22.1 (CH_2), 23.4 (CH_2), 37.5 (CH_2), 103.1 (CH), 119.5 (C), 127.4 (2× CH), 128.5 (CH), 129.5 (2× CH), 130.5 (C), 136.5 (C), 143.9 (C), 192.5 (CO). Anal. calcd for $C_{15}H_{15}NO$: C, 79.97; H, 6.71; N, 6.22. Found: C, 80.01; H, 7.06; N, 6.36.

4.1.3. 1,2-Dimethyl-1,5,6,7-tetrahydro-4*H*-indole-4-one (5b). This product was obtained as a white solid (80%) by reaction of 1,3-acetonylcyclohexandione with methylamine: mp 100–102 °C; IR: 1637 (CO) cm^{-1}; ^1H NMR: σ 2.10 (q, J = 6.1 Hz, 2H, CH_2), 2.24 (s, 3H, CH_3), 2.32 (t, J = 6.1 Hz, 2H, CH_2), 2.77 (t, J = 6.1 Hz, 2H, CH_2), 3.48 (s, 3H, CH_3), 6.11 (s, 1H, H-3). ^{13}C NMR: σ 11.6 (CH_3), 21.3 (CH_2), 23.3 (CH_2), 30.1 (CH_3), 37.4 (CH_2), 102.1 (CH), 118.7 (C), 130.2 (C), 143.5 (C), 192.0 (CO). Anal. calcd for $C_{10}H_{13}NO$: C, 73.59; H, 8.03; N, 8.58. Found: C, 73.72; H, 8.14; N, 8.62.

4.1.4. 1-Benzyl-2-methyl-1,5,6,7-tetrahydro-4*H*-indole-4-one (5c). This product was obtained as white solid (90%) by reaction of 1,3-acetonylcyclohexandione with benzylamine: mp 140–142 °C; IR: 1638 (CO) cm^{-1}; ^1H NMR: σ 1.94–1.98 (m, 2H, CH_2), 2.10 (s, 3H, CH_3), 2.29 (t, J = 5.9 Hz, 2H, CH_2), 2.67 (t, J = 5.9 Hz, 2H, CH_2), 5.13 (s, 2H, CH_2), 6.11 (s, 1H, H-3), 6.99 (dd, J = 7.5, 1.5 Hz, 2H, H-2′, H-6′), 7.22–7.39 (m, 3H, H-3′, H-4′, H-5′). ^{13}C NMR: σ 11.7 (CH_3), 21.4 (CH_2), 23.4 (CH_2), 37.4 (CH_2), 46.4 (CH_2), 102.9 (CH), 119.1 (C), 125.9 (2× CH), 127.2 (CH), 128.7 (2× CH), 130.2 (C), 137.4 (C), 143.7 (C), 192.2 (CO). Anal. calcd for $C_{16}H_{17}NO$: C, 80.30; H, 7.16; N, 5.85. Found: C, 80.60; H, 7.06; N, 5.80.

4.2. General procedure for the 5-carbaldehyde-4-hydroxy-2-methyl-1-substituted-6,7-dihydro-1*H*-indoles (6a–c)

To a suspension of *t*-BuOK (8 g, 36 mmol) in anhydrous benzene (45 mL), a solution of the suitable tetrahydroindole (**5a**–**c**) (36 mmol) in the same solvent (70 mL) was added with stirring under nitrogen atmosphere at room temperature. After 1 h stirring, the solution was ice-cooled and treated dropwise with a solution of ethyl formate (2.6 mL, 36 mmol) in anhydrous benzene (30 mL) under stirring. The solution was allowed to reach room temperature and after 24 h, the solvent then was removed in vacuo. To the crude residue diethyl ether was added and extracted with water. The aqueous extracts were combined, washed with diethyl ether and acidified at 0 °C with 6 M hydrochloric acid. The solid which separated was filtered off, air-dried recrystallized from cyclohexane to give:

4.2.1. 5-Carbaldehyde-4-hydroxy-2-methyl-1-phenyl-6,7-dihydro-1*H*-indole (6a). This product was obtained as white solid (80%) by formylation of tetrahydroindole **5a**: mp 84–86 °C; IR: 3020 (OH), 1637 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): σ 2.00 (s, 3H, CH_3), 2.40–2.50 (m, 4H, 2× CH_2), 6.40 (s, 1H, H-3), 7.22 (d, J = 7.2 Hz, 2H, H-2′, H-6′), 7.45–7.51 (m, 3H, H-3′, H-4′, H-5′), 9.88 (s, 1H, *CH*O), 14.01 (bs, 1H, OH). ^{13}C NMR ($CDCl_3$): σ 12.5 (CH_3), 22.3 (CH_2), 24.7 (CH_2), 103.7 (CH), 108.1 (C), 119.8 (C), 127.4 (2 × CH), 128.7 (CH), 129.4 (2× CH), 132.2 (C), 136.8 (C), 143.1(C), 160.4 (C), 187.9 (CHO). Anal. calcd for $C_{16}H_{15}NO_2$: C, 75.87; H, 5.97; N, 5.53. Found: C, 75.65; H, 5.80; N, 5.42.

4.2.2. 5-Carbaldehyde-1,2-dimethyl-4-hydroxy-6,7-dihydro-1*H*-indole (6b). This product was obtained as white solid (70%) by formylation of tetrahydroindole **5b**: mp 80–82 °C; IR: 3024 (OH), 1632 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): σ 2.19 (s, 3H, CH_3), 2.57 (t, J = 6.3 Hz, 2H, CH_2), 2.69 (t, J = 6.3 Hz, 2H, CH_2), 3.41 (s, 3H, CH_3), 6.26 (s, 1H, H-3), 9.82 (s, 1H, *CH*O), 14.12 (bs, 1H,

OH). ^{13}C NMR ($CDCl_3$): σ 12.1 (CH_3), 21.7 (CH_2), 24.6 (CH_2), 30.3 (CH_3), 103.1 (CH), 107.9 (C), 119.1 (C), 131.6 (C), 142.6 (C), 160.1 (C), 200.3 (CHO). Anal. calcd for $C_{11}H_{13}NO_2$: C, 69.09; H, 6.85; N, 7.32. Found: C, 69.13; H, 6.70; N, 7.12.

4.2.3. 1-Benzyl-5-carbaldehyde-4-hydroxy-2-methyl-6,7-dihydro-1*H*-indole (6c). This product was obtained as white solid (94%) by formylation of tetrahydroindole **5c**: mp 93–95 °C; IR: 3023 (OH), 1635 (CO) cm^{-1}; ^{1}H NMR ($CDCl_3$): σ 2.14 (s, 3H, CH_3), 2.53 (t, J = 5.1 Hz, 2H, CH_2), 2.62 (t, J = 5.1 Hz, 2H, CH_2), 5.03 (s, 2H, CH_2), 6.35 (s, 1H, H-3), 6.91 (d, J = 7.2 Hz, 2H, H-2′, H-6′), 7.18–7.36 (m, 3H, H-3′, H-4′, H-5′), 9.94 (s, 1H, *CH*O), 14.04 (bs, 1H, OH). ^{13}C NMR ($CDCl_3$): σ 11.9 (CH_3), 21.7 (CH_2), 24.5 (CH_2), 47.0 (CH_2), 103.7 (CH), 107.8 (C), 119.5 (C), 125.5 (2× CH), 127.6 (CH), 128.8 (2× CH), 131.6 (C), 136.4 (C), 142.7 (C), 160.2 (C), 187.6 (CHO). Anal. calcd for $C_{17}H_{17}NO_2$: C, 76.38; H, 6.41; N, 5.24. Found: C, 76.18; H, 6.20; N, 5.12.

4.3. General procedure for the synthesis of 5-[(diethylamino)methylene]-2-methyl-1-substituted-1,5,6,7-tetrahydro-4*H*-indol-4-ones (7a–c)

A solution of the suitable hydroxy-methyl-1-substituted-dihydroindole **6a**–**c** (10 mmol) in benzene (40 mL) was added dropwise at room temperature and under nitrogen atmosphere to a stirred solution diethylamine (20 mmol) in benzene (30 mL). The mixture was stirred at room temperature for 24 h and evaporated to dryness. To the crude residue, Et_2O was added and the insoluble material was filtered off to afford the desired compounds as white solids.

4.3.1. 5-[(Diethylamino)methylene]-2-methyl-1-phenyl-1,5,6,7-tetrahydro-4*H*-indol-4-one (7a). This product was obtained (80%) by reacting compound **6a** with diethylamine: mp 146–148 °C; IR: 1637 (CO) cm^{-1}; ^{1}H NMR: σ 1.13 (t, J = 6.7 Hz, 6H, 2× CH_2 CH_3), 2.03 (s, 3H, CH_3), 2.48 (t, J = 6.7 Hz, 2H, CH_2), 2.78 (t, J = 6.7 Hz, 2H, CH_2), 3.27 (q, J = 6.7 Hz, 4H, 2× CH_2CH_3), 6.17 (s, 1H, H-3), 7.27 (s, 1H, CH), 7.36 (d, J = 6.2 Hz, 2H, H-2′, H-6′), 7.43–7.58 (m, 3H, H-3′, H-4′, H-5′). ^{13}C NMR: σ 12.4 (CH_3), 14.5 (2× CH_3), 21.9 (CH_2), 23.5 (CH_2), 47.1 (2× CH_2), 101.1 (CH), 104.3 (CH), 120.5 (C), 127.3 (2× CH), 128.1 (CH), 129.3 (2× CH), 129.3 (C), 129.4 (C), 136.7 (C), 143.8 (C), 182.5 (CO). Anal. calcd for $C_{20}H_{24}N_2O$: C, 77.89; H, 7.84; N, 9.08. Found: C, 77.50; H, 7.64; N, 8.86.

4.3.2. 5-[(Diethylamino)methylene]-1,2-dimethyl-1,5,6,7-tetrahydro-4*H*-indol-4-one (7b). This product was obtained (90%) by reacting compound **6b** with diethylamine: mp 107–108 °C; IR: 1637 (CO) cm^{-1}; ^{1}H NMR: σ 1.13 (t, J = 6.9 Hz, 6H, 2× CH_2 CH_3), 2.14 (s, 3H, CH_3), 2.66 (t, J = 6.9 Hz, 2H, CH_2), 2.80 (t, J = 6.9 Hz, 2H, CH_2), 3.26 (q, J = 6.9 Hz, 4H, 2× CH_2), 3.36 (s, 3H, CH_3), 6.00 (s, 1H, H-3), 7.20 (s, 1H, CH). ^{13}C NMR: σ: 11.8 (CH_3), 14.5 (2× CH_3), 21.1 (CH_2), 23.5 (CH_2), 29.7 (CH_3), 47.1 (2× CH_2), 101.4 (C), 103.1 (CH), 119.6 (C), 129.1 (C), 139.6 (C), 143.5 (CH), 182.4 (CO). Anal. calcd for $C_{15}H_{22}N_2O$: C, 73.13; H, 9.00; N, 11.37. Found: C, 73.00; H, 8.72; N, 11.10.

4.3.3. 5-[(Diethylamino)methylene]-1-benzyl-2-methyl-1,5,6,7-tetrahydro-4*H*-indol-4-one (7c). This product was obtained (85%) by reacting compound **6c** with diethylamine: mp 68–70 °C; IR: 1633 (CO) cm^{-1}; ^{1}H NMR: σ 1.11 (t, J = 6.7 Hz, 6H, 2× CH_2 CH_3), 2.10 (s, 3H, CH_3), 2.61 (t, J = 5.8 Hz, 2H, CH_2), 2.79 (t, J = 5.8 Hz, 2H, CH_2), 3.25 (q, J = 6.7 Hz, 4H, 2× CH_2CH_3), 5.09 (s, 2H, CH_2), 6.08 (s, 1H, H-3), 6.96 (d, J = 7.2 Hz, 2H, H-2′, H-6′), 7.21–7.36 (m, 3H, H-3′, H-4′, H-5′). ^{13}C NMR: σ 11.7 (CH_3), 14.5 (2× CH_3), 21.3 (CH_2), 23.6 (CH_2), 46.1 (CH_2), 47.1 (2× CH_2), 101.2 (C), 103.9 (CH), 120.1 (C), 125.9 (2× CH), 127.1 (CH), 128.7 (2× CH), 129.1 (C), 137.9 (C), 139.7 (C), 143.7 (CH), 182.5 (CO). Anal. calcd for $C_{21}H_{26}N_2O$: C, 78.22; H, 8.13; N, 8.69. Found: C, 78.00; H, 7.92; N, 8.58.

4.4. General procedure for the synthesis of 3,7-disubstituted-8-methyl-1,5,6,7-tetrahydro-2*H*-pyrrolo[2,3-*h*]quinolin-2-ones (10–17)

To a solution of the enaminones **7a**–**c** (20 mmol) in anhydrous ethanol (10 mL), the suitable cyanomethylene compound (22 mmol) was added and heated under reflux for 24–48 h. The solid material which separated from the solution was filtered off, washed with fresh ethanol and dried. Recrystallization from methanol gave the desired compounds as yellow solids. In the case of the reaction of **7a** with phenylsulfonylacetonitrile, was isolated the intermediate **8**.

4.4.1. 2-Methyl-1-phenyl-5-(2′-phenylsulfonyl)-propanenitrile-1,5,6,7- tetrahydro-4*H*-indol-4-one 8. This product was obtained as a yellow solid (50%): mp 234–235 °C; IR: 1637 (CO), 2216 (CN) cm^{-1}; ^{1}H NMR: σ 2.01 (s, 3H, CH_3), 2.09–2.62 (m, 4H, 2× CH_2), 5.29 (d, J = 10.8 Hz, 1H, CH), 6.30 (s, 1H, H-3), 6.46 (d, J = 10.8 Hz, 1H, CH), 7.38 (d, J = 8.1 Hz, 2H, Ar), 7.54–7.80 (m, 8H, Ar). ^{13}C NMR: σ 12.3 (CH_3), 21.6 (CH_2), 25.7 (CH_2), 68.4 (CH), 103.9 (CH), 119.8 (C), 122.1 (CH), 127.4 (2× CH), 128.7 (CH), 129.0 (2× CH), 129.1 (2× CH), 129.6 (2× CH), 132.0 (C), 134.2 (CH), 136.0 (C), 137.5 (C), 142.5 (C), 143.8 (C), 164.0 (C), 180.8 (CO). Anal. calcd for $C_{24}H_{20}N_2SO_3$: C, 69.21; H, 4.84; N, 6.73. Found: C, 69.00; H, 4.52; N, 6.68.

4.4.2. 8-Methyl-7-phenyl-3-phenylsulfonyl-1,5,6,7-tetrahydro-2*H*-pyrrolo[2,3-*h*]quinolin-2-one (10). This product was obtained (80%) by reacting the intermediate **8** in refluxing ethanol and stoichiometric amount of diethylamine: mp > 300 °C; IR: 2921 (NH), 1635 (CO) cm^{-1}; ^{1}H NMR: σ 2.02 (s, 3H, CH_3), 2.56 (t, J = 7.6 Hz, 2H, CH_2), 2.80 (t, J = 7.6 Hz, 2H, CH_2), 6.71 (s, 1H, H-9), 7.38 (d, J = 6.8 Hz, 2H, Ar), 7.50–7.69 (m, 6H, Ar), 7.96 (d, J = 6.8 Hz, 2H, Ar), 8.10 (s, 1H, H-4), 12.24 (s, 1H, NH). ^{13}C NMR: 12.5 (CH_3), 20.8 (CH_2), 25.4 (CH_2), 103.3 (CH), 107.0 (C), 111.8 (C), 118.1 (C), 127.2 (2× CH), 127.5 (2× CH), 128.4 (2× CH), 128.5

(CH), 129.4 (2× CH), 130.8 (C), 132.5 (CH), 136.2 (C), 137.7 (C), 141.5(C), 142.0 (CH), 148.6 (C), 158.2 (CO). Anal. calcd for $C_{24}H_{20}N_2O_3S$: C, 69.21; H, 4.84; N, 6.73. Found: C, 69.11; H, 4.69; N, 6.53.

4.4.3. 8-Methyl-7-phenyl-3-benzoyl-1,5,6,7-tetrahydro-2*H*-pyrrolo[2,3-*h*]quinolin-2-one (11). This product was obtained (50%) by reacting the diethylamino methylene 1-phenyl-indolone **7a** with benzoylacetonitrile: mp > 300 °C; IR: 2904 (b, NH), 1631 (b, CO) cm^{-1}; ^1H NMR: σ 2.11 (s, 3H, CH_3), 2.63 (t, J = 7.6 Hz, 2H, CH_2), 2.82 (t, J = 7.6 Hz, 2H, CH_2), 6.81 (s, 1H, H-9), 7.40–7.64 (m, 8H, Ar), 7.74 (s, 1H, H-4), 7.76 (d, J = 7.6 Hz, 2H, Ar), 12.1 (s, 1H, NH). ^{13}C NMR: σ 12.6 (CH_3), 20.9 (CH_2), 25.4 (CH_2), 103.2 (CH), 107.6 (C), 111.8 (C), 119.8 (C), 127.3 (2× CH), 127.8 (2× CH), 128.5 (CH), 128.8 (2× CH), 129.4 (2× CH), 130.6 (C), 131.8 (CH), 136.3 (C), 136.9 (C), 138.7 (C), 144.1 (CH), 145.8 (C), 160.7 (CO), 193.8 (CO). Anal. calcd for $C_{25}H_{20}N_2O_2$: C, 78.93; H, 5.30; N, 7.36. Found: C, 78.80; H, 5.00; N, 6.98.

4.4.4. 3-Cyano-8-methyl-7-phenyl-1,5,6,7-tetrahydro-2*H*-pyrrolo[2,3-*h*]quinolin-2-one (12). This product was obtained (30%) by reacting the diethylamino methylene 1-phenyl-indolone **7a** with malononitrile or cyanoacetamide: mp > 300 °C; IR: 2948 (b, NH), 2210 (CN), 1627 (CO) cm^{-1}; ^1H NMR: σ 2.06 (s, 3H, CH_3), 2.50 (t, J = 6.6 Hz, 2H, CH_2), 2.64 (t, J = 6.6 Hz, 2H, CH_2), 6.87 (s, 1H, H-9), 7.39 (d, J = 6.6 Hz, 2H, H-2′, H-6′), 7.41 (s, 1H, H-4), 7.50–7.61 (m, 3H, H-3′, H-4′, H-5′), 11.6 (s, 1H, NH). ^{13}C NMR: σ 12.4 (CH_3), 20.8 (CH_2), 24.2 (CH_2), 91.5 (C), 104.2 (CH), 111.4 (C), 113.5 (C), 118.0 (C), 127.4 (2× CH), 128.6 (CH), 129.5 (2× CH), 131.0 (C), 134.6 (CH), 136.1 (C), 138.5 (C), 150.3 (C), 161.2 (CO). Anal. calcd for $C_{19}H_{15}N_3O$: C, 75.73; H, 5.02; N, 13.94. Found: C, 75.33; H, 4.88; N, 13.74.

4.4.5. 3-Ethoxycarbonyl-8-methyl-7-phenyl-1,5,6,7-tetrahydro-2*H*-pyrrolo[2,3-*h*]quinolin-2-one (13). This product was obtained (30%) by reacting the diethylamino methylene 1-phenyl-indolone **7a** with the anion of ethylcyanoacetate generated in situ by the addition of stoichiometric sodium ethylate and by heating 72 h under reflux: mp > 300 °C; IR: 2930 (b, NH), 1714 (CO), 1620 (CO) cm^{-1}; ^1H NMR: σ 1.22 (t, J = 7.0 Hz, 3H, CH_3), 2.01 (s, 3H, CH_3), 2.47 (t, J = 7.4 Hz, 2H, CH_2), 2.70 (t, J = 7.4 Hz, 2H, CH_2), 4.14 (q, J = 7.0 Hz, 2H, CH_2), 6.71 (s, 1H, H-9), 7.33–7.54 (m, 5H, Ar), 7.89 (s, 1H, H-4), 11.91 (s, 1H, NH). ^{13}C NMR: σ 12.5 (CH_3), 14.3 (CH_3), 20.9 (CH_2), 25.4 (CH_2), 59.3 (CH_2), 87.9 (C), 103.2 (CH), 116.3 (C), 127.3 (2× CH), 128.5 (CH), 129.4 (2× CH), 131.0 (C), 134.6 (C), 136.1 (C), 138.7 (CH), 138.5 (C), 143.5 (C), 157.1 (CO), 169.7 (CO). $C_{23}H_{20}N_2O_3$: C, 72.40; H, 5.79; N, 8.04. Found: C, 72.00; H, 5.45; N, 7.92.

4.4.6. 7,8-Dimethyl-3-phenylsulfonyl-1,5,6,7-tetrahydro-2*H*-pyrrolo[2,3-*h*]quinolin-2-one (14). This product was obtained (80%) by reacting the diethylamino methylene 1-methyl-indolone **7b** with phenylsulfonylacetonitrile: mp > 300 °C; IR 2925 (b, NH), 1624 (CO) cm^{-1}; ^1H NMR: σ 2.07 (s, 3H, CH_3), 2.40 (t, J = 6.6 Hz, 2H, CH_2), 2.70 (t, J = 6.6 Hz, 2H, CH_2), 3.22 (s, 3H, CH_3), 6.41 (s, 1H, H-9), 7.41–7.57 (m, 3H, H-3′, H-5′, H-4′), 7.86 (dd, J = 8.1, 1.5 Hz, 2H, H-2′ e H-6′), 7.91 (s, 1H, H-4), 11.84 (s, 1H, NH). ^{13}C NMR: σ 11.9 (CH_3), 20.0 (CH_2), 25.2 (CH_2), 30.3 (CH_3), 102.3 (CH), 106.4 (C), 117.5 (C), 127.5 (2× CH), 128.1 (C), 128.5 (2× CH), 128.7 (C), 131.1 (C), 132.6 (CH), 138.3 (C), 142.3 (CH), 148.3 (C), 157.5 (CO). Anal. calcd for $C_{19}H_{18}N_2O_3S$: C, 64.39; H, 5.12; N, 7.90. Found: C, 64.00; H, 5.04; N, 7.58.

4.4.7. 3-Benzoyl-7,8-dimethyl-1,5,6,7-tetrahydro-2*H*-pyrrolo[2,3-*h*]quinolin-2-one (15). This product was obtained (40%) by reacting the diethylamino methylene-1-methyl-indolone **7b** with benzoylacetonitrile: mp > 300 °C; IR: 2915 (b, NH), 1624 (b, CO) cm^{-1}; ^1H NMR: σ 2.19 (s, 3H, CH_3), 2.75–2.78 (m, 4H, 2× CH_2), 3.44 (s, 3H, CH_3), 6.55 (s, 1H, H-9), 7.38–7.57 (m, 3H, H-3′, H-5′, H-4′), 7.67 (s, 1H, H-4), 7.68 (d, J = 6.2 Hz, 2H, H-2′, H-6′), 11.87 (s, 1H, NH). ^{13}C NMR: σ 11.9 (CH_3), 20.10 (CH_2), 25.3 (CH_2), 30.2 (CH_3), 102.1 (CH), 107.1 (C), 110.6 (C), 118.8 (C), 127.7 (2× CH), 128.8 (2× CH), 130.6 (C), 131.6 (CH), 137.1 (C), 139.0 (C), 144.1 (CH), 146.6 (C), 160.7 (CO), 193.7 (CO). Anal. calcd for $C_{20}H_{18}N_2O_2$: C, 75.45; H, 5.70; N, 8.80. Found: C, 75.30; H, 5.30; N, 8.66.

4.4.8. 7-Benzyl-8-methyl-3-phenylsulfonyl-1,5,6,7-tetrahydro-2*H*-pyrrolo[2,3-*h*]quinolin-2-one (16). This product was obtained (80%) by reacting the diethylamino methylene 1-benzyl-indolone **7c** with benzylsulfonylacetonitrile: mp > 300 °C; IR: 2942 (b, NH), 1635 (CO) cm^{-1}; ^1H NMR: σ 2.10 (s, 3H, CH_3), 2.73–2.81 (m, 4H, 2× CH_2), 5.17 (s, 2H, CH_2), 6.62 (s, 1H, H-9), 7.98 (dd, J = 7.5, 1.5 Hz, 2H, Ar), 7.25–7.38 (m, 3H, Ar), 7.51–7.64 (m, 3H, Ar), 7.96 (dd, J = 6.9, 1.6 Hz, 2H, Ar), 8.02 (1H, s, H-4), 12.14 (1H, s, NH). ^{13}C NMR: σ 11.9 (CH_3), 20.2 (CH_2), 25.3 (CH_2), 46.4 (CH_2), 103.0 (CH), 106.6 (C), 110.7 (C), 118.0 (C), 125.9 (2× CH), 127.2 (CH), 127.5 (2× CH), 128.5 (2× CH), 128.7 (2× CH), 130.9 (C), 132.6 (CH), 137.3 (C), 138.2 (C), 141.4 (C), 142.4 (CH), 148.1 (C), 157.5 (CO). Anal. calcd for $C_{25}H_{22}N_2O_3S$: C, 69.75; H, 5.15; N, 6.51. Found: C, 69.35; H, 4.96; N, 6.30.

4.4.9. 3-Benzoyl-7-benzyl-1,5,6,7-tetrahydro-2*H*-pyrrolo[2,3-*h*]quinolin-2-one (17). This product was obtained (50%) by reacting the diethylamino methylene 1-benzyl-indolone **7c** with benzoylacetonitrile: mp > 300 °C; IR: 2892 (b, NH), 1635 (b, CO) cm^{-1}; ^1H NMR: σ 2.12 (s, 3H, CH_3), 2.74–2.78 (m, 4H, 2× CH_2), 5.17 (s, 2H, CH_2), 6.67 (s, 1H, H-9), 7.00 (d, J = 7.3, 2H, Ar), 7.23–7.57 (m, 6H, Ar), 7.68 (s, 1H, H-4), 6.70 (d, J = 7.3 Hz, 2H, Ar), 12.0 (s, 1H, NH). ^{13}C NMR: σ 11.9 (CH_3), 20.3 (CH_2), 25.3 (CH_2), 46.4 (CH_2), 102.9 (CH), 111.3 (C), 119.2 (C), 125.9 (2× CH), 127.2 (CH), 127.8 (2× CH), 128.7 (2× CH), 128.7 (C), 128.8 (2× CH), 130.4 (C), 131.6 (CH), 137.0 (C), 137.4 (C), 138.9 (C), 144.0 (CH), 1146.2 (C), 160.7 (CO), 193.7 (CO). Anal. calcd for $C_{26}H_{22}N_2O_2$: C, 79.17; H, 5.62; N, 7.10. Found: C, 78.87; H, 5.32; N, 6.90.

4.5. Oxidation of 5-carbaldehyde-4-hydroxy-2-methyl-1-substituted-6,7-dihydro-1*H*-indoles (6a–c)

A warm solution of DDQ (5 mmol) in anhydrous dichloromethane (30 mL) was added dropwise to a warm and stirred solution of the suitable indole derivative **6a**–**c** (5 mmol) in the same solvent (20–40 mL). After the addition was completed, the reaction mixture was stirred at room temperature for 3–4 h and then filtered. The filtrate was washed with 1 M NaOH (30 mL) and once with water (30 mL). The organic layer was dried over sodium sulfate and evaporated under reduced pressure. The residue was recrystallized from ethanol to give the aromatic derivatives **18a**–**c**.

4.5.1. 5-Carbaldehyde-4-hydroxy-2-methyl-1-phenyl-1*H*-indole (18a). This product was obtained as a white solid (80%): mp 174–176 °C; IR: 3150 (b, OH), 1633 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): σ 2.00 (s, 3H, CH_3), 6.60 (d, $J = 9.2$ Hz, 1H, H-7), 6.71 (s, 1H, H-3), 7.35 (d, $J = 9.2$ Hz, 1H, H-6), 7.40–7.47 (d, $J = 7.2$ Hz, 2H, H-2′, H-6′), 7.55–7.64 (m, 3H, H-3′, H-4′, H-5′), 8.50 (s, 1H, *CH*O), 10.05 (s, 1H, OH). ^{13}C NMR ($CDCl_3$): σ 12.9 (CH_3), 100.0 (CH), 103.1 (CH), 113.6 (C), 115.8 (C), 124.4 (CH), 127.6 (2× CH), 128.5 (CH), 129.7 (2× CH), 132.2 (C), 136.8 (C), 142.3(C), 151.8 (C), 193.9 (CHO). Anal. calcd for $C_{16}H_{13}NO_2$: C, 76.48; H, 5.21; N, 5.57. Found: C, 76.40; H, 4.86; N, 5.42.

4.5.2. 5-Carbaldehyde-1,2-dimethyl-4-hydroxy-1*H*-indole (18b). This product was obtained as white solid (75%): mp 152–154 °C; IR: 3230 (OH), 1629 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): σ 2.37 (s, 3H, CH_3), 3.61 (s, 3H, CH_3), 6.48 (s, 1H, H-3), 6.84 (d, $J = 8.8$ Hz, 1H, H-7), 7.16 (d, $J = 8.8$ Hz, 1H, H-6), 9.80 (s, 1H, *CH*O), 12.14 (bs, 1H, OH). ^{13}C NMR ($CDCl_3$): σ 12.2 (CH_3), 29.7 (CH_3), 98.5 (CH), 102.9 (CH), 112.8 (C), 115.9 (C), 123.8 (CH), 137.2 (C), 142.0 (C), 155.9 (C), 195.3 (CHO). Anal. calcd for $C_{11}H_{11}NO_2$: C, 69.83; H, 5.86; N, 7.40. Found: C, 69.60; H, 5.76; N, 7.30.

4.5.3. 1-Benzyl-5-carbaldehyde-4-hydroxy-2-methyl-1*H*-indole (18c). This product was obtained as white solid (74%): mp 120–121 °C; IR: 3288 (OH), 1635 (CO) cm^{-1}; ^1H NMR: σ 2.33 (s, 3H, CH_3), 5.43 (s, 2H, CH_2), 6.60 (s, 1H, H-3,), 6.98 (d, $J = 7.2$ Hz, 2H, H-2′, H-6′), 7.08 (d, $J = 8.0$ Hz, 1H, H-7), 7.19–7.31 (m, 3H, H-3′, H-4′, H-5′), 7.36 (d, $J = 8.0$ Hz, 1H, H-6), 10.02 (s, 1H, *CH*O), 11.71 (s, 1H, OH). ^{13}C NMR: σ 12.3 (CH_3), 46.1 (CH_2), 99.4 (CH), 103.3 (CH), 113.1 (C), 116.3 (C), 124.2 (CH), 126.1 (2× CH), 127.2 (CH), 128.6 (2× CH), 136.9 (C), 137.6 (C), 142.0 (C), 156.0 (C), 194.2 (CHO). Anal. calcd for $C_{17}H_{15}NO_2$: C, 76.96; H, 5.70; N, 5.28. Found: C, 76.79; H, 5.56; N, 5.10.

Compounds **18a**–**c** were obtained in traces from the oxidation of derivatives **7a**–**c** under the same reaction conditions used for the oxidation of compounds **6a**–**c**.

4.6. 7-Amino-4-methylquinolin-2(1*H*)-one (20)

This compound was prepared according to the procedure reported.[19] A mixture of the commercially available 1,3-phenylendiamine (17.3 g, 164 mmol) and ethylacetoacetate (21.2 mL, 164 mmol) was heated at 130 °C for 48 h. The white solid which separated upon cooling was filtered off and recrystallized from methanol to provide **20** (60%): mp 168–170 °C; IR: 3407 (NH), 3300 and 3184 (NH_2), 1662 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): σ 2.29 (s, 1H, CH_3), 5.73 (br s, 2H, NH_2), 6.00 (s, 1H, H-3), 6.42 (d, $J = 2.2$ Hz, 1H, H-8), 6.50 (dd, $J = 8.7$, 2.2 Hz, 1H, H-6), 7.36 (d, $J = 8.7$ Hz, 1H, H-5), 11.25 (s, 1H, NH). ^{13}C NMR ($CDCl_3$): σ 18.4 (CH_3), 96.8 (CH), 110.4 (C), 110.5 (CH), 114.6 (CH), 125.5 (CH), 140.7 (C), 148.0 (C), 151.0 (C), 162.5 (CO). Anal. calcd for $C_{10}H_{10}N_2O$: C, 68.95; H, 5.79; N, 16.08. Found: C, 68.70; H, 5.68; N, 15.96.

4.7. 7-Hydrazino-4-methylquinolin-2(1*H*)-one (21)

This compound was prepared according to the procedure reported.[20] A solution of the 7-aminoquinoline **20** (1.74 g, 10 mmol) in hydrochloric acid (10 mL) was diazotized at −5 °C with sodium nitrite (0.75 g, 11 mmol) dissolved in water (3 mL). The mixture was stirred at that temperature for 4 h and the diazonium salt was reduced by pouring it into a cold solution of stannous chloride (5 g, 22 mmol) dissolved in hydrochloric acid (5 mL). After stirring at −12 °C for 1.5 h, the mixture of the hydrazine hydrochloride and stannic chloride was filtered off, dissolved in water and the solution basified with 1 N NaOH. The solid which separated was filtered, dried to afford the free base (1.5 g, 80%) which was used in the next step without any further purification: mp 162–164 °C; IR: 3450 (NH), 3264 and 3145 (NH_2), 1648 (CO) cm^{-1}; ^1H NMR: σ 2.41 (s, 1H, CH_3), 4.14 (br s, 2H, NH_2), 5.97 (s, 1H, H-3), 6.59 (d, $J = 2.0$ Hz, 1H, H-8), 6.64 (dd, $J = 7.6$, 2.0 Hz, 1H, H-6), 7.35 (s, 1H, NH), 7.37 (d, $J = 7.6$ Hz, 1H, H-5), 11.18 (s, 1H, NH). ^{13}C NMR: σ 18.4 (CH_3), 94.2 (CH), 107.9 (CH), 110.7 (C), 114.8 (CH), 125.0 (CH), 140.7 (C), 147.8 (C), 154.1 (C), 162.3 (CO). Anal. calcd for $C_{10}H_{11}N_3O$: C, 63.48; H, 5.86; N, 22.21. Found: C, 63.28; H, 5.76; N, 22.10.

4.8. 4-Methyl-7-[2-(1-methylethylidene)hydrazine]quinolin-2(1*H*)-one (22)

To a solution of the hydrazine **21** (1.3 g, 6.9 mmol) in ethanol (20 mL), acetone (0.8 mL, 7.5 mmol) was added dropwise and the reaction mixture was heated under reflux for 1 h. Upon cooling a white solid separated which was filtered off and dried in the dessiccator. Recrystallization from ethanol produced the hydrazone **22** (85%): mp 240–241 °C; IR: 3511 (NH), 3282 (NH), 1631 (CO) cm^{-1}; ^1H NMR: σ 1.91 (s, 1H, CH_3), 1.97 (s, 1H, CH_3), 2.32 (s, 1H, CH_3), 6.04 (s, 1H, H-3), 6.91 (dd, $J = 8.7$, 2.4 Hz, 1H, H-6), 6.98 (d, $J = 2.4$ Hz, 1H, H-8), 7.46 (d, $J = 8.7$ Hz, 1H, H-5), 9.09 (s, 1H, NH), 11.26 (s, 1H, NH). ^{13}C NMR: σ 16.8 (CH_3), 18.4 (CH_3), 25.0 (CH_3), 96.0 (CH), 108.5 (CH), 112.0 (C), 115.7 (CH), 125.2 (CH), 140.4 (C), 145.5 (C), 147.7 (C), 148.2 (C), 162.2 (CO). Anal. calcd for $C_{13}H_{15}N_3O$: C, 68.10; H, 6.59; N, 18.33. Found: C, 67.84; H, 6.30; N, 18.00.

When the reaction was carried out in refluxing acetic acid (12 mL), after the separation of the solid the acetic filtrate was poured into crushed ice, and upon neutralization with 10% sodium carbonate an insoluble material formed which was filtered off, recrystallized from ethanol to afford a mixture of the hydrazone **22** (60%) and of **23**.

4.9. 4,8-Dimethyl-1,7-dihydro-2*H*-pyrrolo[2,3-*h*]quinolin-2-one (23)

(20%): mp 198–200 °C; IR: 3451 (NH), 3245 (NH), 1651 (CO) cm^{-1}; ^1H NMR: σ 1.94 (s, 1H, CH_3), 2.41 (s, 1H, CH_3), 6.31 (s, 1H, H-9), 6.67 (s, 1H, H-3), 6.72 (d, $J = 9.3$ Hz, 1H, H-6), 7.58 (d, $J = 9.3$ Hz, 1 H, H-5), 9.80 (s, 1H, NH), 12.1 (s, 1H, NH). ^{13}C NMR: σ 18.7 (CH_3), 20.6 (CH_3), 95.6 (CH), 110.5 (CH), 112.1 (CH), 111.1 (C), 113.6 (C), 125.9 (CH), 139.5 (C), 151.6 (C), 152.2 (C), 161.6 (C),169.1 (CO). Anal. calcd for $C_{13}H_{12}N_2O$: C, 73.57; H, 5.70; N, 13.20. Found: C, 73.27; H, 5.38; N, 13.00.

Compound **23** was also obtained upon reaction of the hydrazone **22** (0.3 g, 1.3 mmol) with anhydrous zinc chloride (1.5 g, 10 mmol). The reaction mixture was heated to melting (280–290 °C) for 1 h. After cooling, the solid mass was suspended in water, filtered, dried and purified by SEPACORE chromatography using a BÜCHI apparatus (ethyl acetate/methanol, 95:5). Recrystallization from ethanol afforded the cyclic derivative **23** (0.18 g, 65%).

4.10. Biology

4.10.1. Irradiation procedure. HPW 125 Philips lamps, mainly emitting at 365 nm, were used for irradiation experiments. The spectral irradiance of the source was 4.0 mW cm^{-2} as measured, at the sample level, by a Cole-Parmer Instrument Company radiometer (Niles, IL), equipped with a 365-CX sensor.

4.10.2. Spectrophotometric measurement. UV–vis absorption spectra were recorded on a Perkin-Elmer Lambda 15 spectrophotometer and emission spectra were recorded on a Perkin-Elmer LS-50B luminescence spectrophotometer.

4.10.3. Cellular phototoxicity. Human promyelocytic leukaemia cells (HL-60) were grown in RPMI-1640 medium, (Sigma Co, MO, USA) human fibrosarcoma cells (HT-1080) were grown in DMEM (Sigma Co, MO, USA), and intestinal adenocarcinoma cells (LoVo) were grown in Ham's F12 medium (Sigma Co, MO, USA) all supplemented with 115 U/mL of penicillin G (Invitrogen, Milano, Italy), 115 μg/mL streptomycin (Invitrogen, Milano, Italy) and 10% fetal calf serum (Invitrogen, Milano, Italy). Individual wells of a 96-well tissue culture microtitre plate (Falcon BD) were inoculated with 100 μl of complete medium containing 8×10^3 HL-60 cells or 5×10^3 HT-1080 and LoVo cells. The plates were incubated at 37 °C in a humidified 5% incubator for 18 h prior to the experiments. After medium removal, 100 μl of the drug solution, dissolved in DMSO and diluted with Hanks' Balanced Salt Solution (HBSS, pH 7.2), was added to each well and incubated at 37 °C for 30 min and then irradiated. After irradiation, the solution was replaced with the medium, and the plates were incubated for 72 h. Cell viability was assayed by the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide)] test, as described previously.[41] Analogous experiments were performed in the presence of various additives.

4.10.4. Fluorescence microscopy. HT-1080 fibrosarcoma cells were grown in sterile microscope slides and treated with the test compounds at the concentration of 20 μM for 30 min, then washed with HBSS. Cellular fluorescence images were acquired with an Olympus IMT-2 inverted microscope, as previously described.[42] For subcellular co-localization studies tetramethyl rhodamine methyl ester (TMRM) and acridine orange (AO) were used as fluorescent probes which stain mitochondria and lysosomes, respectively. Pyrroloquinolones' excitation was performed at 330 nm and emission was read at 450 nm. Long pass emission filter settings were used to separate the emission of the probes from that of the test compounds. Data were acquired and analysed using the Metamorph software (Universal imaging).

4.10.5. Lipid peroxidation. For these experiments, HT-1080 cells were seeded at the concentration of 50,000 cells/mL in 35-mm Petri dishes grown for two days to reach confluency and then were irradiated in HBSS in the presence of the test compounds. At different times of irradiation supernatant was collected and frozen after addition of 2,6-di-*tert*-butyl-*p*-cresol (BHT, 2% in ethanol). Cells were removed with the aid of a cell scraper after addition of 500 μl water. Lipid peroxidation both in the supernatants and cell extracts was measured using a thiobarbituric acid assay as described previously.[29] A standard curve of 1,1,3,3-tetraethoxypropane was used to quantitate the amount of malonaldehyde produced. Data are expressed in terms of nanomoles of TBARS normalized to the total protein content measured as described,[42] in an aliquot of the cell extract.

4.10.6. Protein photo-cross-link. Erythrocytes' plasmatic membranes (ghosts) were prepared as described.[43] The test compounds were added to the membrane suspension (1.0 mg/mL protein concentration) and irradiated for different times. The membrane samples were reduced and denatured by addition of β-mercaptoethanol and SDS at 90 °C for 3 min, and bromophenol blue (BPB) was added before polyacrylamide gel electrophoresis analysis (5% running gel, 3% stacking gel). The quantitation of the bands, stained with Coomassie brilliant blue R-250 was achieved by image analyzer software Quantity One (Bio-Rad, Milano, Italy).

4.10.7. Studies on isolated proteins. Solutions of Bovine serum albumin (BSA) (0.5 mg/mL) in phosphate buffer 10 mM were irradiated in the presence of the test compounds for various times in a quartz cuvette. At each time the tryptophan (Trp) content was followed by monitoring the characteristic Trp fluorescence as described by Balasubramanian et al.[34] Solutions of Ribonuclease

A (RNAseA), 0.5 mg/mL in phosphate buffer 10 mM, were irradiated in the presence of the test compounds for various times in a quartz cuvette. The fluorescence was followed, as described above. Analogous experiments were performed by bubbling nitrogen prior to and during the irradiation and also in the presence of superoxide dismutase. Further experiments were performed in deuterated phosphate buffer.

4.10.8. Flow linear dichroism. Linear-dichroism (LD) spectra were recorded in a 'flow cell' on a Jasco J500A spectropolarimeter equipped with an IBM PC and a Jasco J interface. The determination and interpretation of the data was performed as previously described.[44] DNA concentration was 2.27 mM and the measurements were performed at different [DNA]/[dye].

4.10.9. HPLC separation and photoproducts characterization. LC-MS was performed using a Perkin-Elmer Series 200 (Norwalk, CT, USA), equipped with Waters 'X-Terra' column (3.5 μm; 3.5× 150 mm i.d.) chromatographic separations. The mobile phase consisted of: eluent A, water/formic acid (99:1); eluent B, acetonitrile/formic acid (99:1). Gradient: 5% B (0 min), 95% B (30 min); flow rate 60 μl/min. ESI/MS measurements were performed with an API-TOF. Mariner 5220 (Perseptive Biosystem Stratford, Texas, USA).

5. Supplementary material

Agarose gel electrophoresis, UV–vis spectra of photodegradation and flow linear dichroism spectra of the compounds. This material is available free of charge via the Internet at www.sciencedirect.com.

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