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Synthesis of pyrrolo[3,2-*h*]quinolinones with good photochemotherapeutic activity and no DNA damage

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ABSTRACT

In the search for new photochemotherapeutic agents, a series of derivatives of the ring system pyrrolo[3,2-*h*]quinoline—bioisosters of the angular furocoumarin angelicin—were synthesized through a four-step synthetic approach, in reasonable overall yields. Eight of the synthesized derivatives showed a remarkable phototoxicity against a panel of four human tumor cell lines and a great dose UV-A dependence, reaching IC_{50} values at submicromolar level. The mode of cellular death photoinduced by pyrrolo[3,2-*h*]quinolines was evaluated through a series of flow cytometric analysis and other tests were performed to clarify their mechanism of action.

1. Introduction

Psoralen **1** and its derivatives are furocoumarins, either synthetic or naturally occurring, used in the treatment of human skin diseases with hyperproliferative and/or autoimmune character in combination with UV-A light, commonly referred as PUVA therapy. The photochemotherapy of psoriasis was introduced in clinical use in the early seventies at the Harvard Medical School of the Massachusetts General Hospital by oral administration of 8-methoxypsoralen (8-MOP) and subsequent exposure of the skin to UV-A irradiation.[1] Nowadays, the PUVA therapy is commonly used in dermatology for the treatment of diseases such as vitiligo, psoriasis, plaque parapsoriasis, atopic dermatitis, generalized lichen planus, pigmentous urticaria, alopecia areata and T-cell lymphoma.[2,3]

In 1987, Edelson et al. developed the extracorporeal photochemotherapy (ECP), called photopheresis, using 8-MOP for the treatment of T-cell cutaneous lymphoma.[4] Thanks to photopheresis, which consists of the reinfusion of UV-A irradiated autologous leukocytes collected by apheresis and extracorporeally incubated with 8-MOP, the photochemotherapy of furocoumarins was used for the treatment of tumors.[5] Furocoumarins are tricyclic aromatic compounds whose planar structure allows the intercalation between nucleic acid base pairs. Upon UV-A irradiation, they can covalently bind to DNA pyrimidine bases. DNA adducts are formed by a [2+2]-photocycloaddition between one of the two photoreactive sites of psoralen (4′,5′ double bond of furan ring or 3,4 double bond of pyran ring) and the 5,6 double bond of thymine. The psoralen 4′,5′-monoadducts can further photochemically react with a pyrimidine base on the complementary strand of the DNA, thus leading to interstrand cross links (ICL).[6] Such ICL proved to be the primary cause of many side effects. Such adverse effects can limit the use of PUVA and can be divided into short-term side effects that comprise nausea, skin phototoxic reactions and immune depression and long-term ones such as premature skin aging, degeneration of the dermic collagen and elastic tissues, cataract formation and also mutagenicity and increased risk of neoplastic diseases.[7]

Many researchers managed to obtain new derivatives devoid of side effects but able to maintain the psoralen efficacy. In particular, the risk of skin neoplastic diseases could be avoided by using angelicin derivatives, since their geometrical structure makes ICL impossible.[8] The synthesis of heteroanalogues of psoralen **1** and angelicin **2** represented another promising approach against undesired side effects (Chart 1). Sulfur and nitrogen isosters, such as thioangelicins, thienocoumarins, pyrrolocoumarins and furoquinolinones, were studied and some of them showed improved interaction with DNA both in the dark and under UV-A irradiation if compared to the lead compounds.[9] More recently, we reported the synthesis of the new ring systems pyrrolo[2,3-*h*]quinolinones **3** and thiopyrano[2,3-*e*]indolones **4** (Chart 1), which are diaza

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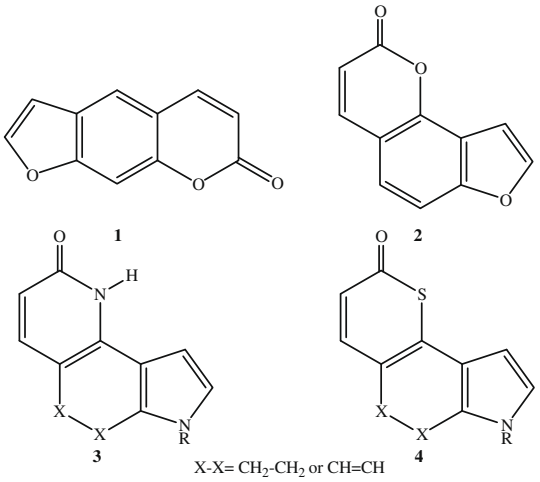


Chart 1. Structures of psoralen **1**, angelicin **2**, pyrrolo[2,3-*h*]quinolinones **3** and thiopyrano[2,3-*e*]indolones **4**.

and thio-aza analogues of angelicin **2**, respectively.[10] All derivatives of the latter ring systems showed photoantiproliferative activity in human tumor cell lines (MCF-7, Jurkat, K-562, LoVo) and keratinocytes (NCTC-2544), with IC_{50} values at micromolar and submicromolar level (**3**, IC_{50} 0.4–16.4 μM; **4**, 0.2–14.8 μM) and a significant UV-A dose-dependent cytotoxicity. Several derivatives showed a biological activity higher than that of 8-MOP, 5-methoxypsoralen (5-MOP) and angelicin, used as reference drugs. However, linear dichroism (LD) studies strongly suggested that the new derivatives did not efficaciously interact with DNA, thus indicating a different mechanism from that of the lead compound. Taking into account the previous satisfactory results on pyrroloquinolinones, and in light of the potent antitumor activity of compounds incorporating the quinoline-2-one portion,[11] it was decided to search for new angelicin heteroanalogues by focusing on the synthesis of the pyrrolo[3,2-*h*]quinoline ring system of type **9**. The aim was to evaluate the effect of the different condensation of the pyrrole nucleus to the quinoline moiety on the photobiological activity. Herein the paper discusses the synthesis of such a ring system, along with the results of its remarkable photobiological activity and the studies aimed at explaining the mode of action.

2. Results and discussion

2.1. Chemistry

The synthetic pathway optimized for pyrroloquinolinones of type **3**, starting from the corresponding tetrahydroindole-4-ones, has already pointed out the great versatility of the key enaminone intermediates which could be utilized, as reliable intermediates, for the desired cyclization due to their electronic features.[10a,10b] Tetrahydro-7*H*-indol-7-ones of type **6**, in which it is possible to introduce the suitable functionality in a position adjacent to carbonyl, would therefore represent the ideal building blocks for our synthesis. Such compounds can be prepared through known procedures. One of them, apparently convenient, led to tetrahydro-7*H*-indol-7-one **6d**, from 1,2-dioxocyclohexane and a commercially available acetal, but in low yield.[12] Instead, a more convenient multistep approach involved the annelation of the cyclohexanone moiety on the pyrrole ring.[13] Thus, compounds **5a,b** were obtained in excellent yields from the corresponding pyrrole derivative by an acylation with succinic anhydride followed by reduction. Cyclization in trifluoroacetic anhydride gave the tetrahydroindol-7-ones **6a,b** in 80–90% yield (Scheme 1).

The *N*-sulfonyl derivative **6a**, upon heating in basic medium, allowed the isolation of the *N*-unsubstituted tetrahydroindol-7-one **6c**, which, as well as the ethoxycarbonyl derivative **6b**, can undergo nucleophilic reactions with alkyl or aralkyl halides to give *N*-substituted derivatives. Thus, reaction in tetrahydrofuran or dimethylformamide with methyl iodide or benzyl or substituted benzyl chlorides in the presence of sodium hydride gave derivatives **6d,e,g–l** in 70–90% yields. Instead, the *N*-phenyl derivative **6f** was obtained in 66% yield by a modified Ulmann cross-coupling reaction.[14] The variously substituted tetrahydroindoles **6a,b,d–l** were reacted with the Bredereck reagent, *tert*-butoxy-*bis*-(dimethylamino)methane (TBDMAM),[15] to give the α-enaminoketons **7a,b,d–l** in excellent yields (80–90%). Compounds **7g,h,l** were unstable and were utilized as crude products for the successive step. Once obtained the suitable substrates **7** to undergo the final cyclization, it was planned to react them with phenylsulfonylacetonitrile or cyanoacetamide as a C–C–N 1,3-dinucleophile to get the tricyclic derivatives **9**. This choice resulted from the fact that in the pyrroloquinolinones of type **3** the presence of the phenylsulfonyl group in position 3 of the pyridone moiety was necessary to obtain compounds with good photoantiproliferative activity. Therefore, reaction of the dimethylamino substituted derivatives **7a,b,d–l** with phenylsulfonylacetonitrile in refluxing ethanol, under nitrogen atmosphere, gave the desired angelicin heteroanalogues **9a,d–m** in acceptable yields (35–65%). In the case of derivative **7a**, when the reaction was performed at room temperature, a complex mixture was formed and it was possible to isolate the uncyclized intermediate **8**, obtained in 40% yield. As the latter still possesses a cyano group, it strongly suggests that the reaction initiates with the nucleophilic attack of the methylene of the phenylsulfonylacetonitrile on the enaminone carbon, the most electrophilic site of compounds **7**. Prolonged refluxing brings about the conversion of the nitrile moiety to carboxamide, which—by nucleophilic attack to the ring carbonyl—cyclizes to the pyridone ring of the tricyclic system. Basic hydrolysis of the *N*-phenylsulfonyl derivative **9a** led, in excellent yield, to the corresponding *N*-unsubstituted derivative **9b**. The reaction of **7a** with cyanoacetamide led to a very complex reaction mixture from which the 7-cyano substituted pyrroloquinolinone **9c** was isolated in very poor yield (13%). Reaction of **7d** with the same methylene active compound did not lead to the isolation of the corresponding tricyclic derivative. This behavior was similar to that observed in the series of compounds **3** when the corresponding enaminones were reacted with cyanoacetamide.[10a,10b]

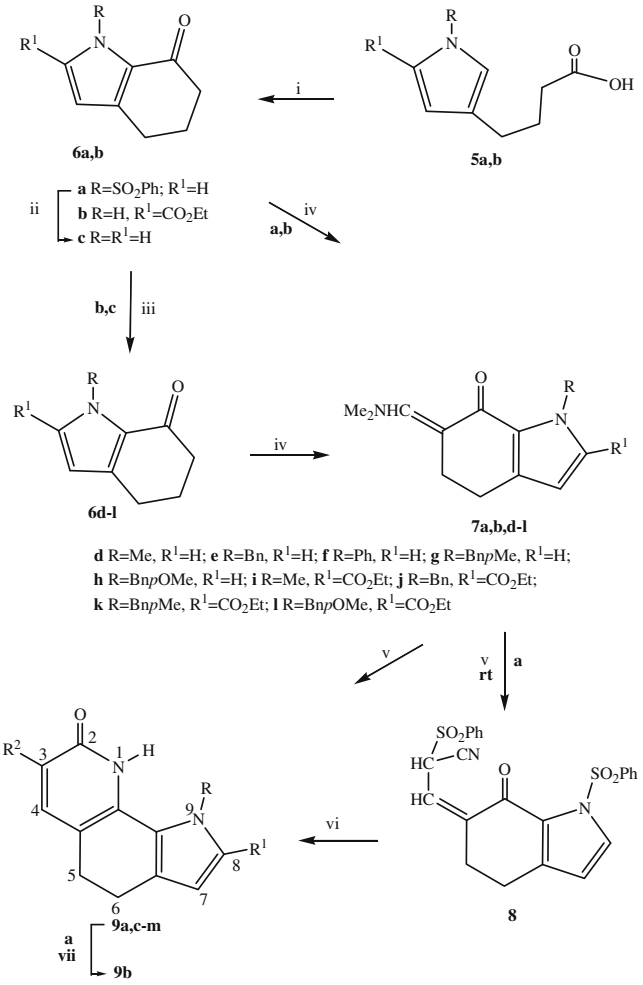
2.2. Biology

2.2.1. Physico-chemical properties

The absorption and emission spectra of pyrrolo[3,2-*h*]quinolines were measured in DMSO. All compounds absorbed solar radiation spectrum and presented bands in UV-A region; this fact is fundamental for a photosensitizer. Absorption maxima wavelengths (see Table 1) exhibited a remarkable bathochromic shift compared to angelicin (**Ang**) due to the conjugation with the substituents in 1 and 7 positions. The partition coefficients of the test compounds were calculated using the computational method as described by Ghose and Crippen.[16] Most pyrrolo[3,2-*h*]quinolines were highly hydrophobic, reaching *c* log *P* values of 6.01 in the case of **9l**.

2.2.2. Photodegradation/photostability

When irradiated in solution, furocoumarins are subjected to photolysis.[17] Thus, absorption spectra of 20 μM pyrrolo[3,2-*h*]quinolinones in DMSO were recorded after increasing UV-A doses. All spectra were significantly modified by increasing UV-A doses, indicating that all compounds underwent a certain degree of photodegradation (see Fig. 1 of Supplementary data). Figure 1 reports the



9: a R=R^2=SO_2Ph, R^1=H; **b** R=R^1=H, R^2=SO_2Ph; **c** R=SO_2Ph, R^1=H, R^2=CN; **d** R=Me, R^1=H, R^2=SO_2Ph; **e** R=Bn, R^1=H, R^2=SO_2Ph; **f** R=Ph, R^1=H, R^2=SO_2Ph; **g** R=Bn*p*Me, R^1=H, R^2=SO_2Ph; **h** R=Bn*p*OMe, R^1=H, R^2=SO_2Ph; **i** R=H, R^1=CO_2Et, R^2=SO_2Ph; **j** R=Me, R^1=CO_2Et, R^2=SO_2Ph; **k** R=Bn, R^1=CO_2Et, R^2=SO_2Ph; **l** R= Bn*p*Me, R^1=CO_2Et, R^2=SO_2Ph; **m** R= Bn*p*OMe, R^1=CO_2Et, R^2=SO_2Ph

Scheme 1. Reagents and conditions: (i) trifluoroacetic anhydride, rt, 24 h, 80–90%; (ii) KOH (10%), EtOH, reflux, 2 h, 88%; (iii) for **6d**, **e**, **g–l**: NaH, THF or DMF, 0 °C to rt, 1 h then alkyl or aralkylhalide at 0 °C to rt or reflux 2–3 h, 70–90%, for **6f**: K_2CO_3, *N*-methylpyrrolidone, N_2, rt, 1 h, then CuBr, rt 1 h, then PhI, reflux 4 h, 66%; (iv) TBDMAM, toluene, N_2, reflux, 1–48 h, 80–90%; (v) $PhSO_2CH_2CN$ or H_2NCOCH_2CN (for **8**, yield 40%), EtOH, N_2, reflux 1–48 h, 13–65%; (vi) EtOH, reflux, 4 h, 45%; (vii) KOH, EtOH, reflux, 1 h, 90%.

photodegradation of **9c** and **9e** as an example. The changes are due to a simple reduction of maxima absorbance, as well as the onset of new bands and the shift of peak in wavelength. In most spectra, the presence of isosbestic points can be detected. An isosbestic point indicates that two absorbing species, the non-degraded compound and a photoproduct, are in equilibrium in solution.

2.2.3. Cellular phototoxicity

The phototoxicity tests were carried out in different human tumor cell lines: K-562 (chronic myeloid leukemia), Jurkat (T-cell leukemia), LoVo (intestinal adenocarcinoma) and MCF-7 (breast adenocarcinoma). The same tests were performed in an immortalized cell line of human keratinocytes, NCTC-2544. Cellular survival experiments were carried out without irradiation to evaluate a possible cytotoxicity, and with two different UV-A doses (2.5 and 3.75 J/cm^2), using Wood's lamps emitting principally at 365 nm. UV-A doses are compatible with those used in PUVA therapy[3a] and were chosen to avoid cellular death in irradiated controls. For phototoxicity experiments, cells were incubated with compounds **9** or **Ang** for 30 min prior to irradiation. Cellular survival was monitored by MTT (3-[4,5-dimethylthiazol-2yl] 2,5-diphenyl tetrazolium bromide) reduction test[18] after 72 h of incubation with compounds in the case of cytotoxicity tests or after 72 h from irradiation in the phototoxicity ones. The incubation of these cells with compounds **9** without irradiation did not induce any change in cellular survival in respect to control (not treated cells). Table 2 shows IC_{50} 72 h after irradiation. Among pyrrolo[3,2-*h*]quinolinones, some compounds were non-phototoxic at the employed concen-

Table 1
Physico-chemical properties of pyrrolo[3,2-*h*]quinolinones

Compound	λ_{max} abs[a] (nm)	ε (M^{-1} cm^{-1})[a]	λ_{max} emiss[a] (nm)	*c* log *P*[b]
9a	375	42,432	415	2.98 ± 0.66
9b	430	37,138	456	1.42 ± 0.40
9c	442	13,565	479	1.97 ± 0.66
9d	375	37,148	416	1.92 ± 0.40
9e	375	33,182	415	3.44 ± 0.45
9f	430	26,707	413	3,79 ± 0.42
9g	375	40,531	413	3.90 ± 0.45
9h	376	48,050	415	3.36 ± 0.47
9i	439	44,091	461	3.43 ± 0.64
9j	381	41,576	406	4.03 ± 0.64
9k	381	38,202	406	5.55 ± 0.68
9l	381	37,088	405	6.01 ± 0.68
9m	382	35,577	406	5.47 ± 0.69
Ang	306	14,602	413	2.01 ± 0.34

abs, absorbance; emiss, emission; Ang, angelicin.
[a] Measured in dimethyl sulfoxide.
[b] Calculated as described in Ref. 16.

trations (**9a, 9b, 9c, 9d** and **9f**), whereas others showed high phototoxicity(**9e, 9g, 9h, 9i, 9j, 9k, 9l** and **9m**) with IC_{50} values lower than 10 μM When detected, the photoantiproliferative activity of pyrrolo[3,2-*h*]quinolinones was dependent on UV-A dose and on compound concentration. The most phototoxic compounds, **9j, 9k** and **9m**, presented micromolar–submicromolar IC_{50} values that were similar or even lower (in cell lines derived from solid tumors) than those of the reference compound, angelicin. A relationship between structure and activity can be hypothesized starting from the structure of the most phototoxic compounds **9j, 9k, 9m**. The latter compounds present lipophilic groups (Me; Bn; Bn*p*OMe) in position 1 and an ethoxycarbonyl moiety in position 2. The presence of more lipophilic groups (Bn*p*Me, **9l**) or the absence of a substituent at position 1 (**9i**) reduces the activity. The comparison between the most active compounds and their analogues devoid of the ester group in position 2 (**9a–h**) is very interesting: indeed, the latter molecules showed a partial or total inactivity. The presence of the ethoxycarbonyl substituent in position 2 is therefore extremely important for the phototoxicity of compounds **9**.

2.2.4. Externalization of phosphatidylserine

The apoptotic loss of plasma membrane asymmetry with the exposure of phosphatidylserine (PS) was used to evaluate the mode of cell death. In fact, this early event of apoptosis was monitored by flow cytometry, using a PS-binding protein, Annexin V,

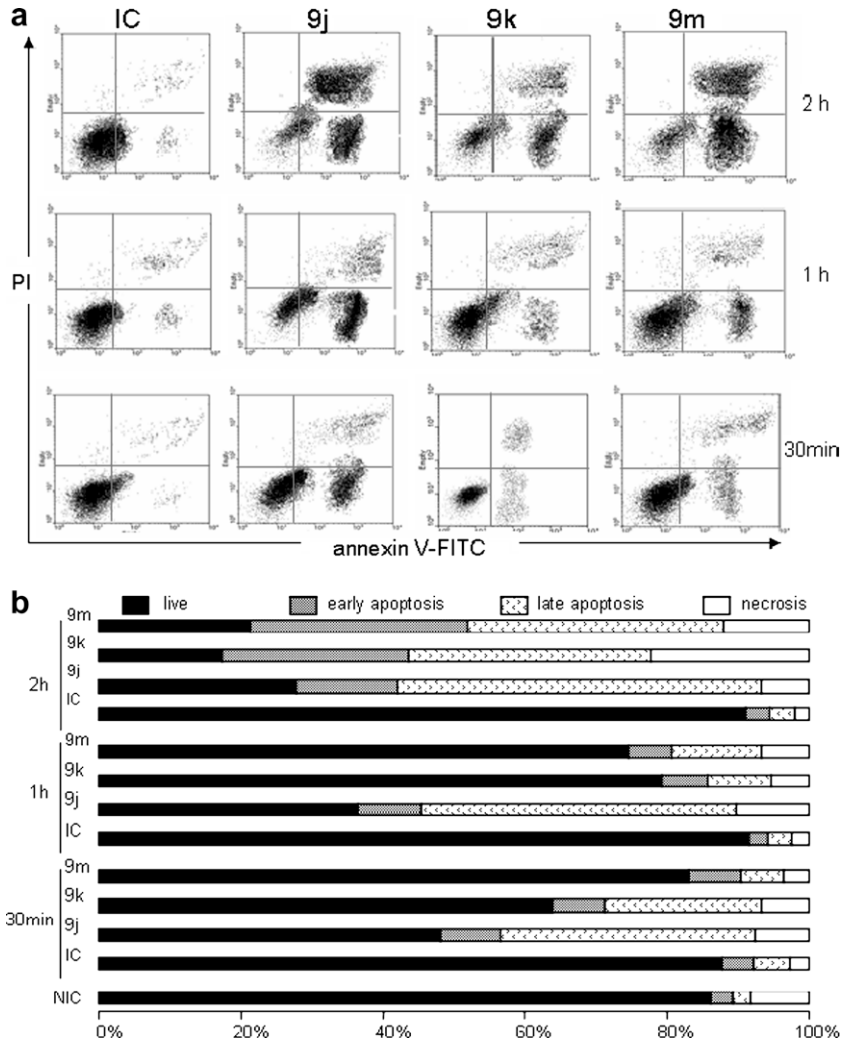


Figure 1. Assessment of phosphatidylserine surface exposure. (a) Expression of phosphatidylserine and analysis of propidium iodide staining after 30 min, 1 and 2 h from the irradiation (2.5 J/cm^2) of Jurkat cells in the presence of 1 μM **9j**, **9k** and **9m**. Cells were stained with Annexin V-FITC (FL1) and of PI (FL3) and analyzed by flow cytometry. IC, irradiated control; NIC, non-irradiated control. (b) Percentages of cells detected by Annexin V/PI staining as mean of three different experiments.

Table 2
Photocytotoxicity of pyrrolo[3,2-*h*]quinolinones in a panel of human cell lines

Compound	IC_{50}[a] (μM)									
	Jurkat		K-562		LoVo		MCF-7		NCTC	
	2.5[b]	3.75	2.5	3.75	2.5	3.75	2.5	3.75	2.5	3.75
9a	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
9b	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
9c	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
9d	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
9e	1.3 ± 0.2	1.1 ± 0.1	4.8 ± 0.5	3.3 ± 0.5	2.6 ± 0.2	2.2 ± 0.2	4.0 ± 0.6	1.7 ± 0.7	7.5 ± 0.8	5.5 ± 0.6
9f	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
9g	1.6 ± 0.1	1.2 ± 0.2	2.7 ± 0.2	2.4 ± 0.1	2.7 ± 0.1	2.3 ± 0.2	3.8 ± 0.5	2.4 ± 0.2	3.3 ± 0.3	2.6 ± 0.5
9h	1.6 ± 0.2	1.2 ± 0.1	3.1 ± 0.3	2.5 ± 0.2	2.8 ± 0.3	2.0 ± 0.2	4.2 ± 0.3	1.8 ± 0.1	4.3 ± 0.3	2.7 ± 0.2
9i	3.5 ± 0.2	2.5 ± 0.2	6.1 ± 0.2	5.3 ± 0.3	5.5 ± 0.2	4.6 ± 0.4	6.1 ± 0.2	4.3 ± 0.3	9.3 ± 0.6	7.2 ± 0.7
9j	0.7 ± 0.1	0.5 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.1
9k	0.8 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
9l	1.5 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	1.6 ± 0.2	1.0 ± 0.1	1.7 ± 0.1	1.4 ± 0.1	2.7 ± 0.2	2.2 ± 0.2
9m	0.8 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.6 ± 0.2	1.3 ± 0.1	1.9 ± 0.1	1.6 ± 0.1
Ang[c]	1.0 ± 0.2	0.9 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	3.6 ± 0.4	1.5 ± 0.3	4.4 ± 0.5	1.5 ± 0.2	4.2 ± 0.5	0.9 ± 0.1

[a] Values are expressed as means ± SEM of at least three independent experiments.
[b] UV-A doses expressed as J/cm^2.
[c] **Ang**, angelicin reference drug.

conjugated with fluorescein isothiocyanate (FITC). Another probe, propidium iodide (PI), was added during the analysis for detecting the integrity of plasmatic membrane. PI penetrated the cell only with a collapsed plasmatic membrane, intercalated into DNA and gave the typical red fluorescence. Thus, the cytofluorimetric measure discriminated between intact (FITC−/PI−), early apoptotic (FITC+/PI−), late apoptotic/necrotic (FITC+/PI+) or necrotic cells (PI+).[19] As reported in Figure 1, treated cells demonstrated a time-dependent apoptotic features of cell death. Among the pyrrolo[3,2-*h*]quinolinones tested, compound **9j** was the most active, showing important apoptotic effects 30 min after incubation, whereas the apoptotic effects of compounds **9k** and **9m** became evident 2 h after treatment. A small percentage of necrotic cells was also detected but the main mode of cell death seemed to be apoptosis.

2.2.5. Cell cycle analysis

This test is based on the fact that DNA content is different in each phase of the cell cycle. Thus, after fixation in ethanol, cells were stained with PI which made it possible to do rapid determinations of DNA per cell.[20] From the data in Table 3, the most important modification was the onset of a subG1 peak, 24 h after treatment. The subG1 phase represents apoptotic cells and the rise in fluorescence is a consequence of the ordered degradation of DNA during cell death. This phenomenon is considered to be a later event than PS externalization, thus producing results that are consistent with the findings of the previous experiment. In contrast with **Ang**, which induced a S phase block, the other compounds produced a decrease of all phase percentages (G1, S and G2-M) as most cells were detected in subG1 phase.

Table 3
Cell percentages in each cell cycle phase after 24 h from irradiation (25 J/cm^2)

	G_1	S	G_2-M	subG_1
NIC	50.0	16.5	25.0	8.5
9j	16.1	6.6	14.0	63.3
9k	16.1	8.2	7.2	68.5
9m	12.6	6.1	6.6	74.7
Ang	12.3	46.7	10.2	30.8

NIC, non-irradiated control.

2.2.6. Membrane potential

The collapse of mitochondrial membrane potential ($\Delta\psi_{mt}$) is one of the events of mitochondrial dysfunction. The determination of $\Delta\psi_{mt}$ was carried out through a flow cytometric analysis using JC-1 as a probe. This test was performed 2 and 4 h after irradiation of Jurkat cells in the presence of **Ang**, **9j, 9k** and **9m**. In normal cells (high $\Delta\psi_{mt}$), JC-1 accumulates selectively in mitochondria and, as a consequence of the electrochemical gradient, forms red fluorescence aggregates which are associated with emission at 590 nm, whereas JC-1 forms monomers which emit at 530 nm if the mitochondrial membrane is depolarized (low $\Delta\psi_{mt}$).[21] As reported in Figure 2, a time-dependent increase of percentage of cells with collapsed mitochondrial potential was detected after irradiation in the presence of **Ang**, and **9k**. For **9m** a rapid involvement of mitochondria in cell death induction can be hypothesized as a high percentage of cells JC-1 monomers can be detected even after only 2 h from irradiation.

Conversely, compound **9j** did not induce mitochondrial dysfunction. Thus, since the loss of $\Delta\psi_{mt}$ precedes the cell death, compounds **9k** and **9m** induced apoptosis through mitochondrial depolarization.

2.2.7. ROS production

The mitochondrial membrane depolarization was associated with mitochondrial production of reactive oxygen species (ROS).[22] The determination of ROS generation during apoptosis was performed using the fluorescence indicators hydroethidine (HE) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), whose fluorescence appeared if reactive oxygen species were produced.[23] HE was oxidized by superoxide anion into ethidium bromide, which emitted red fluorescence. DCFDA was oxidized to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) by a variety of peroxides including hydrogen peroxide. Results are shown in Figure 3 and in Figure 2 of Supplementary data. There was a significant production of ROS for compounds **Ang**, **9k** and **9m**, as evidenced by the high percentage of HE- and DCF-positive cells, while there was minor ROS production in the case of **9j**. The data are consistent with mitochondrial membrane depolarization.

2.2.8. Lysosomal stability assessment

Flow cytometric analysis was performed, by using the fluorescent dye acridine orange (AO), in order to investigate the integrity of lysosomes after irradiation with pyrroloquinolinones. AO is a

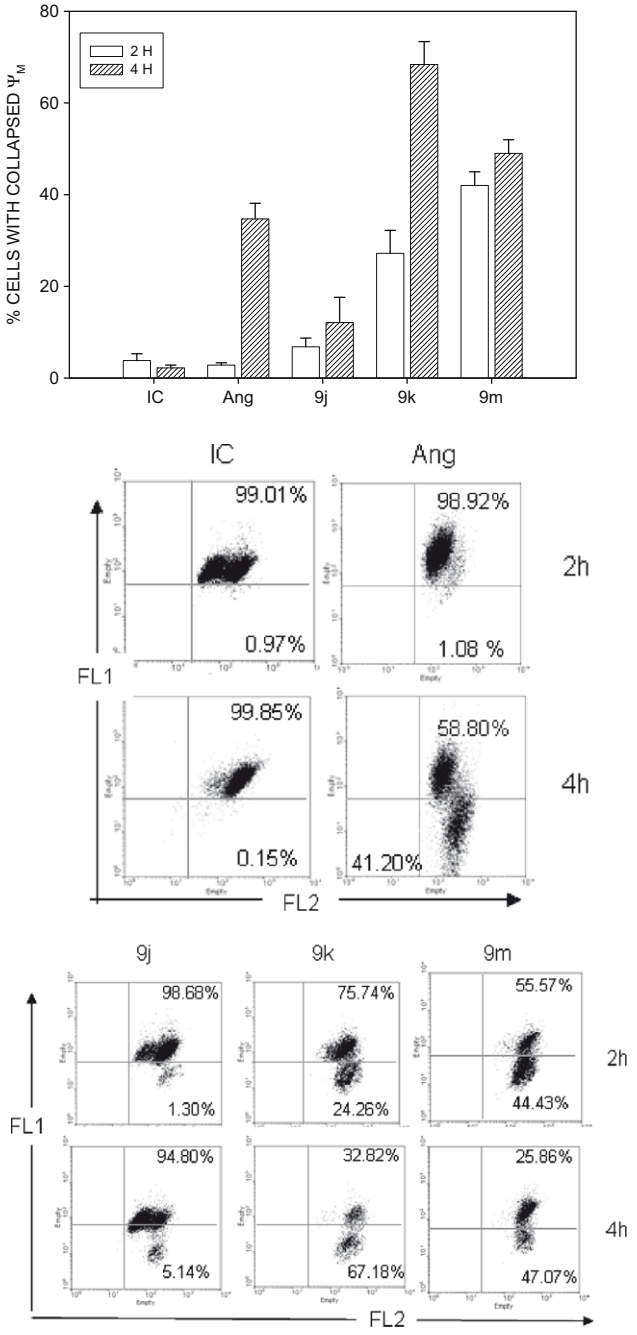


Figure 2. Assessment of mitochondrial dysfunction. Upper panel: induction of loss of mitochondrial membrane potential 2 and 4 h after irradiation (2.5 J/cm^2) of Jurkat cells in the presence of 2.5 μM **Ang** or 1 μM **9j**, **9k** and **9m**. Cells were stained with JC-1 and analyzed by FACS. Values are expressed as means ± SD of at least three independent experiments. Lower panels: examples of flow cytometry analysis of mitochondrial membrane potential.

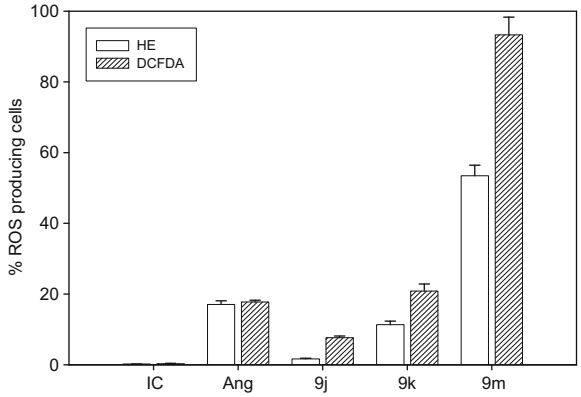


Figure 3. Assessment of mitochondrial dysfunction. Production of ROS in Jurkat cells 6 h after irradiation (2.5 J/cm^2) with 2.5 μM **Ang** or 1 μM **9j**, **9k** and **9m**. Cells were stained with HE or DCFDA and analyzed by FACS. Values are expressed as means ± SD of three independent experiments.

lysosomotropic base as well as a metachromatic fluorochrome exhibiting red fluorescence when highly concentrated, as in the case of intact lysosomes in which it is retained its protonated form. When lysosomes are damaged, AO relocates to the cytosol where it is predominantly in the deprotonated form and it shows green fluorescence.[24] The percentage of cells with intact lysosomes was evaluated by assessing red fluorescence after AO staining. A time-dependent increase of lysosomal disruption was assessed after irradiation in the presence of **Ang**, **9j, 9k** and **9m** (Fig. 4), indicating that lysosomes were significantly involved in cell death.

2.2.9. Linear dichroism

A potential interaction with DNA was investigated, as this biomolecule represents an important target for antiproliferative activity of PUVA. In order to understand the binding nature between the test compounds and DNA, linear dichroism (LD) measurements were performed on solutions with salmon testes DNA (st-DNA) and the title compounds at various molar ratios. Indeed, linear dichroism proved to be an efficient tool to evaluate the binding mode between a dye and nucleic acids.[25] The sign and the extent of LD signal is related to the orientation of the compound with respect to DNA helix axis. **Ang** is a DNA intercalant as can be observed by its LD spectrum: a significant increase of the values of LD of the DNA band at 260 nm for these drug–DNA complexes was observed, indicating that the DNA became better oriented due to a stiffening of the helix upon binding of the drug. The negative LD signals in the long-wavelength absorption (300–360 nm) of **Ang** revealed that it was oriented perpendicularly to the flow field. The investigation of the LD spectra of three derivatives (**9j, 9k** and **9m**) in the presence of st-DNA revealed that they gave no LD bands in the 300–450 nm region despite a strong absorption (Fig. 5). These observations strongly suggested that the new derivatives did not interact efficaciously without irradiation with the macromolecule, as indeed demonstrated with natural furocoumarins.

2.2.10. pBR322 DNA strand breaks

Even if pyrrolo[3,2-*h*]quinolinones did not display a high affinity for DNA, further experiments were carried out to determine whether the new derivatives were able to photosensitize DNA strand break activity. Their ability to photodamage DNA was evaluated by using supercoiled circular DNA, as it allowed the detection of structural alterations such as strand breaks. Double-stranded supercoiled (SC or I form) plasmid is sensitive to damage by a variety of photosensitizers.[26,27] Cleavage of one strand produces a relaxed, but still double-stranded, open circular (OC or II form) DNA. Further cleavage of the other strand within a short distance of the first site of cleavage generates a linear (L or III form) DNA. In addition to frank strand breaks, the oxidative damage of purine and/or pyrimidine bases was also evaluated by using the base excision repair enzymes Formamido pyrimidine glycosilase (Fpg) and Endonuclease III (Endo III), respectively. Damaged base recognition was followed by an elimination step, resulting in DNA breakage. The irradiation of supercoiled plasmid pBR322 was conducted in the presence of **Ang**, **9j, 9k** and **9m** ([DNA]/[Compound] = 1:3). After the irradiation, pBR322 aliquots were incubated with Fpg or Endo III at 37 °C for 30 min. DNA samples were resolved by an electrophoretic run in agarose 1% gel thanks

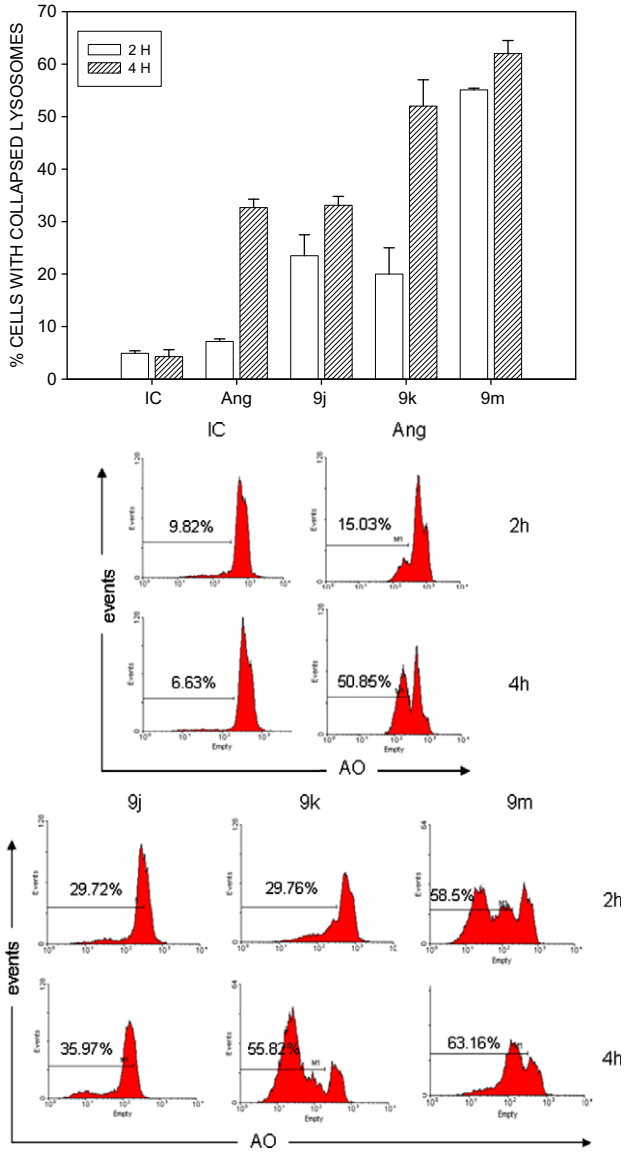


Figure 4. Assessment of lysosomial dysfunction. Upper panel: percentages of cells stained with AO (acridine orange) were reported and analyzed by flow cytometry 2 and 4 h after irradiation (2.5 J/cm^2) in the presence of 2.5 μM **Ang** or 1 μM **9j**, **9k** and **9m**. Values are expressed as means ± SD of at least three independent experiments. Lower panels: examples of flow cytometry analysis of Jurkat cells stained with AO.

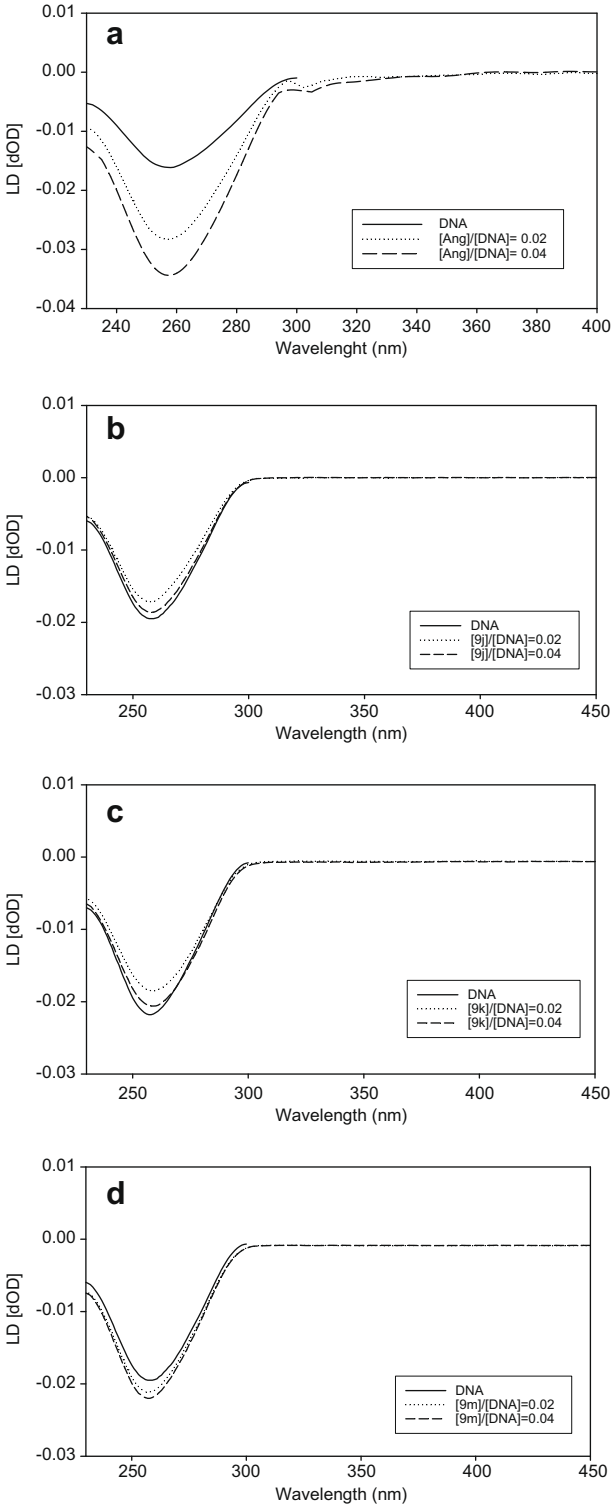


Figure 5. Linear dichroism (LD) spectra of mixtures of salmon testes DNA and compounds **Ang** (a), **9j** (b), **9k** (c) and **9m** (d) at different [Drug]/[DNA] ratio (0, 0.02 and 0.04).

to the different hydrodynamic properties of the plasmid forms (Fig. 3 of Supplementary data). The percentages of OC form were calculated as suggested by Ciulla et al.[27] and are summarized in Figure 6. The results pointed out that **Ang** and the new compounds did not generate any formation of open circular or linear bands, indicating that they did not sensitize single strand breaks. Even after the treatment with the two base excision enzymes, no DNA modification was detected for compounds **9** whereas a significant increase of OC band was detected in the case of **Ang** thus indicating an important induced DNA oxidative damage.

2.2.11. Protein photodamage

In order to investigate the photosensitizing properties of the title compounds towards proteins, solutions containing bovine serum albumin (BSA) or ribonuclease A (RNAseA) as models and the drug in phosphate buffer were irradiated several times. The degree of oxidative modifications was measured by monitoring the carbonyl content, an index of photodamage of proteins.[28] Results are reported in Figure 4 of Supplementary data and demonstrate that the carbonyl content of BSA and RNAse significantly increased after irradiation in all three test compounds and with a greater extent than the **Ang** induced one.

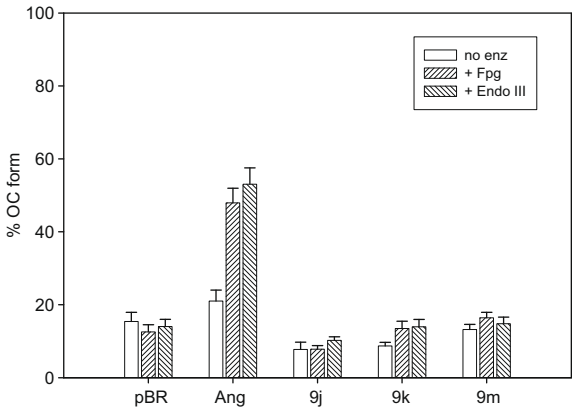


Figure 6. DNA base modifications photoinduced by pyrrolo[3,2-*h*]quinolinones. Percentage of open circular form (OC) after irradiation (3.75 J/cm^2) of supercoiled pBR322 form (SC) in the presence of **Ang** (b), **9j** (a), **9k** (a) and **9m** (a) ([DNA]/[Drug] = 1:3) and treated with base excision repair enzymes Formamido pyrimidine glycosilase (Fpg) and Endonuclease III (Endo III). Values are expressed as means ± SD of at least three independent experiments.

2.2.12. Lipid peroxidation

Membrane lipids may be a central site of photodamage if sensitizing agents localize in the membrane bilayer.[29] Being hydrophobic, as their partition coefficients indicated (see Table 1), pyrrolo[3,2-*h*]quinolinones may be expected to be localized mainly in plasma and/or in subcellular membranes, making these structures particularly sensitive to photodamage. The thiobarbituric assay (TBA test) was used in order to determine a potential lipid peroxidation after irradiation of Jurkat cells in the presence of test compounds. The TBA test was performed as described in Section 4. Briefly, this assay used the reaction of one molecule of malondialdehyde (MDA), which is a secondary product of lipid peroxidation, with two molecules of TBA, forming a pink chromogen, which was monitored by fluorescence at 553 nm.[30] Figure 5 of Supplementary data shows the results for compounds **Ang**, **9j, 9k** and **9m**. Thiobarbituric reactive substances (TBARS) were significantly produced in a concentration-dependent manner when cells were exposed to the compounds and UV-A. A good correlation was observed between the extent of lipid peroxidation and the hydrophobic character of pyrrolo[3,2-*h*]quinolinones.

3. Conclusion

In this report, the synthesis of pyrrolo[3,2-*h*]quinolinones **9** new photochemotherapeutic derivatives for the treatment of hyperproliferative diseases is described. These compounds were synthesised with the aim of modulating the long-term side effects related to the PUVA therapy, in particular the DNA photodamage. Most of the pyrrolo[3,2-*h*]quinolinones were phototoxic and from a structure–activity relationship point of view, the presence of a lipophilic group in position 1 seemed to be important as well as an ethoxycarbonyl moiety in position 2, a group needed for the phototoxicity of other analogues of angelicin.[31] It is worthy to observe that all these compounds are not fully unsaturated as in the case of angelicin; on the contrary, they are dihydro-derivatives, such as the most phototoxic compounds of series **3**.[10a,10b]

The evaluation of the photoinduced cellular death by pyrroloquinolinones was performed. Cell cycle analysis of cells irradiated in the presence of compounds **9** were carried out. It is reported that significant modifications on cell cycle after PUVA treatment depends on the type of cells. For example, a G1 phase arrest was observed in NCTC,[32] whereas a G2-M phase block was detected in Karpas 299 T-lymphoma cells.[33] The main change in cell cycle profile after UV-A irradiation in the presence of pyrroloquinolinones is due to the onset of a subG1 peak, indicating a distribution of cells with a low content in DNA: this is a typical feature of apoptotic cells in which a nuclear DNA fragmentation occurs. As mitochondrion was found to be a key effector of apoptosis and many reports evidenced a mitochondrial dysfunction brought about by PUVA therapy,[34] a possible involvement of this organelle in photoinduced cell death was studied. A clear and rapid involvement of mitochondria was observed in **9k** and **9m** apoptosis but a scarce one in **9j** cell death. The involvement of another organelle, such as lysosome, in inducing apoptosis was examined. Indeed, in addition to necrosis, the lysosomal destabilization can be associated with apoptosis: for example, the release of lysosomal hydrolytic enzymes was found to lead the activation of caspase cascade or of other lytic cytosol enzymes.[24] Lysosomes proved to be involved in pyrroloquinolinones cell death.

Studies directed to elucidate the molecular target, demonstrated that pyrroloquinolinones photoinduce oxidation in model proteins and lipid peroxidation in cell membrane significantly involved in their phototoxicity. Moreover, a potential interaction with DNA was investigated, as this biomolecule represents an important target for the antiproliferative activity of PUVA. Pyrroloquinolinones did not interact efficiently with DNA (no intercalation); and above all these latter did not induce DNA photodamage (no DNA strand breaks and no DNA oxidative damages). This finding represents a significant advantage for a possible introduction in therapy of pyrroloquinolinones, as the main side effects of classical psoralen administration are genotoxicity, mutagenicity and skin cancer.

4. Experimental

4.1. Chemistry

All melting points were taken on a Buchi-Tottoli capillary apparatus and were uncorrected; IR spectra were determined, in $CHBr_3$, 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 Avance II series 200 MHz spectrometer. Column chromatography was performed with Merck silica gel 230–400 Mesh ASTM or with a SEPACORE chromatography apparatus BÜCHI. Elemental analyses (C, H, N) were within ±0.4% of the theoretical values.

1-(Phenylsulfonyl)-1,4,5,6-tetrahydro-7*H*-indole-7-one (**6a**), ethyl 1,4,5,6,-tetrahydro-7*H*-indole-7-one-2-carboxylate (**6b**) and 1,4,5,6-tetrahydro-7*H*-indole-7-one (**6c**) were prepared as previously described and the white solids obtained showed spectroscopic data identical to those reported in the literature (**6a**[13a] yield 85%; mp: 115–116 °C; 6b[13b] yield 82%; mp: 96–97 °C; 6c[13a] yield 85%; mp: 90–92 °C).

4.1.1. Preparation of 1-substituted 1,4,5,6-tetrahydro-7*H*-indole-7-ones (6d,e,g–l)

To a solution of the suitable ketone **6b** or **6c** (15 mmol) dissolved in anhydrous THF or DMF (20 mL), NaH (0.64 g, 16 mmol) was added at 0 °C and the reaction mixture was stirred at rt. After 1 h the suitable alkyl or aralkyl halide (16 mmol) was added at 0 °C and the reaction mixture was stirred at rt or refluxed for 2–4 h. Then, the reaction mixture was poured onto crushed ice and the precipitate was filtered off. In absence of precipitate the aqueous solution was extracted with dichloromethane (DCM, 3× 50 mL) and the organic layers were separated, dried over sodium sulfate and the solvent removed in vacuo. Column chromatography of the residue, using DCM as eluent, gave the expected product.

4.1.2. 1-Methyl-1,4,5,6-tetrahydro-7*H*-indole-7-one (6d)

This compound was obtained from the reaction of **6c** with iodomethane in THF after 3 h at rt: brown oil; yield 90%; IR: υ 1635 (CO) cm^{-1}; ^1H NMR: δ 1.89–2.01 (2H, m, CH_2), 2.35 (2H, t, J = 6.0 Hz, CH_2), 2.65 (2H, t, J = 6.0 Hz, CH_2), 3.82 (3H, s, CH_3), 5.96 (1H, d, J = 2.5 Hz, H-3), 7.03 (1H, d, J = 2.5 Hz, H-2); ^{13}C NMR: δ 23.3 (t), 24.8 (t), 35.9 (q), 38.7 (t), 106.3 (d), 126.2 (s), 130.7 (d), 136.8 (s), 187.7 (CO). Anal calcd for $C_9H_{11}NO$: C, 72.46; H, 7.43; N, 9.39. Found: C, 72.56; H, 7.69; N, 9.30.

4.1.3. 1-Benzyl-1,4,5,6-tetrahydro-7*H*-indole-7-one (6e)

This compound was obtained from the reaction of **6c** with benzylchloride in DMF after 2 h at rt: brown oil; yield 90%; IR: υ 1637 (CO) cm^{-1}; ^1H NMR: δ 1.88–2.01 (2H, m, CH_2), 2.35 (2H, t, J = 6.0 Hz, CH_2), 2.68 (2H, t, J = 6.0 Hz, CH_2), 5.49 (2H, s, CH_2), 6.05 (1H, d, J = 2.5, H-3), 7.12–7.34 (6H, m, Ar and H-2); ^{13}C NMR: δ 23.4 (t), 24.8 (t), 38.8 (t), 50.8 (t), 107.2 (d), 125.5 (s), 126.9 (d), 127.2 (d), 128.4 (d), 130.4 (d), 137.5 (s), 138.9 (s), 187.7 (CO). Anal calcd for $C_{15}H_{15}NO$: C, 79.97; H, 6.71; N, 6.22. Found: C, 80.30; H, 7.00; N, 6.03.

4.1.4. 1-(*p*-Methyl-benzyl)-1,4,5,6-tetrahydro-7*H*-indole-7-one (6g)

This compound was obtained from the reaction of **6c** with *p*-methyl-benzylchloride in DMF after 2 h at rt: white solid; yield 75%; mp: 80–82 °C; IR: υ 1637 (CO) cm^{-1}; ^1H NMR: δ 1.88–2.00 (2H, m, CH_2), 2.24 (3H, s, CH_3), 2.35 (2H, t, J = 6.3 Hz, CH_2), 2.67 (2H, t, J = 6.3 Hz, CH_2), 5.43 (2H, s, CH_2), 6.03 (1H, d, J = 2.5 Hz, H-3), 7.05 (2H, d, J = 7.5 Hz, H-3″ and H-5″), 7.10 (2H, d, J = 7.5 Hz, H-2″ and H-6″), 7.20 (1H, d, J = 2.5 Hz, H-2); ^{13}C NMR: δ 20.6 (q), 23.4 (t), 24.8 (t), 38.8 (t), 50.6 (t), 107.1 (d), 125.4 (s), 127.1 (d), 128.9 (d), 130.2 (d), 135.8 (s), 136.4 (s), 137.4 (s), 187.7 (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, 5.50.

4.1.5. 1-(*p*-Methoxy-benzyl)-1,4,5,6-tetrahydro-7*H*-indole-7-one (6h)

This compound was obtained from the reaction of **6c** with *p*-methoxy-benzylchloride in DMF after 2 h at rt: white solid; yield 75%; mp: 82–84 °C; IR: υ 1637 (CO) cm^{-1}; ^1H NMR: δ 1.90–2.00 (2H, m, CH_2), 2.36 (2H, t, J = 6.0 Hz, CH_2), 2.66 (2H, t, J = 6.0 Hz, CH_2), 3.70 (3H, s, CH_3), 5.40 (2H, s, CH_2), 6.02 (1H, d, J = 2.5 Hz, H-3), 6.85 (2H, d, J = 7.5 Hz, H-3″ and H-5″), 7.16 (2H, d, J = 7.5 Hz, H-2″ and H-6″), 7.20 (1H, d, J = 2.5 Hz, H-2); ^{13}C NMR: δ 23.4 (t), 24.8 (t), 38.8 (t), 50.2 (t), 54.9 (q), 107.1 (d), 113.7 (d), 125.4 (s), 128.6 (d), 130.1 (d), 130.8 (s), 137.4 (s), 158.5 (s), 187.7 (CO). Anal calcd for $C_{16}H_{17}NO_2$: C, 75.27; H, 6.71; N, 5.49. Found: C, 75.18; H, 7.02; N, 5.68.

4.1.6. Ethyl 1-methyl-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (6i)

This compound was obtained from the reaction of **6b** with iodomethane in DMF after 2 h under reflux: brown solid; yield 88%; mp: 51–52 °C; IR: υ 1707 (CO), 1651 (CO) cm^{-1}; ^1H NMR: δ 1.29 (3H, t, J = 7.1 Hz, CH_3), 1.91–2.03 (2H, m, CH_2), 2.47 (2H, t, J = 6.1 Hz, CH_2), 2.68 (2H, t, J = 6.1 Hz, CH_2), 4.13 (3H, s, CH_3), 4.26 (2H, q, J = 7.1 Hz, CH_2), 6.71 (1H, s, H-3); ^{13}C NMR: δ 14.1 (q), 22.8 (t), 24.1 (t), 34.1 (q), 39.6 (t), 60.4 (t), 114.0 (d), 127.5 (s), 129.9 (s), 134.6 (s), 160.4 (CO), 190.1 (CO). Anal calcd for $C_{12}H_{15}NO_3$: C, 65.14; H, 6.83; N, 6.33. Found: C, 64.90; H, 7.10; N, 6.68.

4.1.7. Ethyl 1-benzyl-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (6j)

This compound was obtained from the reaction of **6b** with benzylchloride in DMF after 3 h at rt: brown solid; yield 70%; mp: 84–85 °C; IR: υ 1709 (CO), 1655 (CO) cm^{-1}; ^1H NMR: δ 1.21 (3H, t, J = 7.1 Hz, CH_3), 1.93–2.05 (2H, m, CH_2), 2.48 (2H, t, J = 6.0 Hz, CH_2), 2.74 (2H, t, J = 6.0 Hz, CH_2), 4.20 (2H, q, J = 7.1 Hz, CH_2), 6.01 (2H, s, CH_2), 6.84 (1H, s, H-3), 6.93–7.31 (5H, m, Ar); ^{13}C NMR: δ 13.9 (q), 22.9 (t), 24.1 (t), 39.6 (t), 48.7 (t), 60.5 (t), 99.4 (s), 115.1 (d), 126.0 (d), 126.8 (d), 128.3 (d), 130.6 (s), 135.9 (s), 139.5 (s), 160.5 (CO), 190.0 (CO). Anal calcd for $C_{18}H_{19}NO_3$: C, 72.71; H, 6.44; N, 4.71. Found: C, 72.45; H, 6.69; N, 4.75.

4.1.8. Ethyl 1-(*p*-methyl-benzyl)-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (6k)

This compound was obtained from the reaction of **6b** with *p*-methyl-benzylchloride in DMF after 2 h at rt: white solid; yield 73%; mp: 65–66 °C; IR: υ 1710 (CO), 1651 (CO) cm^{-1}; ^1H NMR: δ 1.22 (3H, t, J = 7.1 Hz, CH_3), 1.92–2.04 (2H, m, CH_2), 2.22 (3H, s, CH_3), 2.47 (2H, t, J = 6.0 Hz, CH_2), 2.72 (2H, t, J = 6.0 Hz, CH_2), 4.20 (2H, q, J = 7.1 Hz, CH_2), 5.96 (2H, s, CH_2), 6.82 (1H, s, H-3), 6.85 (2H, d, J = 8.0 Hz, H-3″ and H-5″), 7.06 (2H, d, J = 8.0 Hz, H-2″ and H-6″); ^{13}C NMR: δ 14.0 (q), 20.5 (q), 22.9 (t), 24.1 (t), 39.6 (t), 48.4 (t), 60.5 (t), 115.0 (d), 126.0 (d), 127.1 (s), 128.8 (d), 129.7 (s), 135.4 (s), 135.8 (s), 135.9 (s), 160.2 (CO), 190.0 (CO). Anal calcd for $C_{19}H_{21}NO_3$: C, 73.29; H, 6.80; N, 4.50. Found: C, 73.45; H, 6.70; N, 4.24.

4.1.9. Ethyl 1-(*p*-methoxy-benzyl)-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (6l)

This compound was obtained from the reaction of **6b** with *p*-methoxy-benzylchloride in DMF after 2 h at rt: yellow oil; yield 75%; IR: υ 1711 (CO), 1651 (CO) cm^{-1}; ^1H NMR: δ 1.23 (3H, t, J = 7.1 Hz, CH_3), 1.95–2.04 (2H, m, CH_2), 2.47 (2H, t, J = 5.9 Hz, CH_2), 2.72 (2H, t, J = 5.9 Hz, CH_2), 3.69 (1H, s, CH_3), 4.22 (2H, q, J = 7.1 Hz, CH_2), 5.92 (2H, s, CH_2), 6.77–6.97 (5H, m, Ar and H-3); ^{13}C NMR: δ 14.0 (q), 22.9 (t), 24.1 (t), 39.6 (t), 47.9 (t), 54.9 (q), 60.5 (t), 113.7 (d), 115.1 (d), 127.1 (s), 127.6 (d), 129.6 (s), 130.7 (s), 135.4 (s), 158.2 (s), 160.3 (CO), 190.0 (CO). Anal calcd for $C_{19}H_{21}NO_4$: C, 69.71; H, 6.47; N, 4.28. Found: C, 70.02; H, 6.70; N, 4.14.

4.1.10. Preparation of 1-phenyl-1,4,5,6-tetrahydro-7*H*-indole-7-one (6f)

To a solution of **6c** (1.3 g, 9.63 mmol) in *N*-methyl-pyrrolidone (19 mL), K_2CO_3 (2.0 g, 14.45 mmol) was added under nitrogen atmosphere and the reaction mixture was stirred at rt for 1 h. Then CuBr (2.8 g, 19.26 mmol) was added and the reaction stirred for 1 h and finally iodobenzene (4.0 mL, 35.63 mmol) was added and the reaction refluxed for 4 h. After cooling, HCl (5%, 17 mL) was added to the reaction mixture and stirred for 1 h, then AcOEt (25 mL) was added and stirred for further 30 min. The reaction mixture was filtered through celite and washed with AcOEt (25 mL). The organic layer was shaken for 1 h with ice and NaCl, separated, dried over sodium sulfate and the solvent removed in vacuo to give a brown solid; yield 66%; mp: 66–68 °C; IR: υ 1650 (CO) cm^{-1}; ^1H NMR: δ 1.96–2.09 (2H, m, CH_2), 2.39 (2H, t, J = 6.1 Hz, CH_2), 2.76 (2H, t, J = 6.1 Hz, CH_2), 6.23 (1H, d, J = 2.6 Hz, H-3), 7.23–7.45 (6H, m, Ar and H-2); ^{13}C NMR: δ 23.5 (t), 24.5 (t), 38.9 (t), 108.6 (d), 125.5 (d), 126.1 (s), 127.0 (d), 128.4 (d), 131.1 (d), 130.1 (s), 139.7 (s), 186.3 (CO). Anal calcd for $C_{14}H_{13}NO$: C, 79.59; H, 6.20; N, 6.63. Found: C, 79.30; H, 6.00; N, 6.80.

4.1.11. Preparation of 1-substituted 6-[(dimethylamino) methylene]-1,4,5,6-tetrahydro-7*H*-indole-7-one (7a,b,d–l)

To a solution of **6a,b,d–l** (5.3 mmol) in anhydrous toluene (10 mL) TBDMAM (3.31 mL, 16 mmol) was added under nitrogen

atmosphere and the reaction mixture was refluxed. After cooling, the precipitate was filtered and shaken in diethyl ether (25 mL), filtered and air dried.

4.1.11.1. 6-[(Dimethylamino)methylene]-1-(phenylsulfonyl)-1,4,5,6-tetrahydro-7*H*-indole-7-one (7a). This product was obtained from **6a** after 4 h reflux and precipitated as yellow solid; yield 90%; mp: 116–118 °C; IR: υ 1637 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.58 (2H, t, *J* = 6.5 Hz, CH_2), 2.84 (2H, t, *J* = 6.5 Hz, CH_2), 3.00 (6H, s, 2× CH_3), 6.16 (1H, d, *J* = 2.7 Hz, H-3), 7.27 (1H, s, CH), 7.37–7.52 (3H, m, Ar), 7.61 (1H, d, *J* = 2.7 Hz, H-2), 8.05 (2H, d, *J* = 7.2 Hz, H-2′ and H-6′); ^{13}C NMR ($CDCl_3$): δ 23.5 (t), 24.2 (t), 43.4 (q), 102.9 (s), 109.8 (d), 127.8 (d), 127.9 (d), 128.5 (d), 130.3 (s), 133.1 (d), 138.1 (s), 139.6 (s), 148.6 (d), 176.6 (CO). Anal calcd for $C_{17}H_{18}N_2O_3S$: C, 61.80; H, 5.49; N, 8.48. Found: C, 62.10; H, 5.26; N, 8.20.

4.1.11.2. Ethyl 6-[(dimethylamino)methylene]-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (7b). This product was obtained from **6b** after 2 h reflux and precipitated as yellow solid; yield 80%; mp: 209–211 °C; IR: υ 3421 (NH), 1703 (CO), 1635 (CO) cm^{-1}; ^1H NMR: δ 1.27 (3H, t, *J* = 7.1 Hz, CH_3), 2.58 (2H, t, *J* = 6.7 Hz, CH_2), 2.88 (2H, t, *J* = 6.7 Hz, CH_2), 3.07 (6H, s, 2× CH_3), 4.21 (2H, q, *J* = 7.1 Hz, CH_2), 6.60 (1H, s, H-3), 7.36 (1H, s, CH), 12.01 (1H, s, NH); ^{13}C NMR: δ 14.2 (q), 22.1 (t), 24.2 (t), 43.2 (q), 59.9 (t), 102.7 (s), 112.6 (d), 125.1 (s), 129.8 (s), 132.8 (s), 148.2 (d), 160.3 (CO), 177.7 (CO). Anal calcd for $C_{14}H_{18}N_2O_3$: C, 64.10; H, 6.92; N, 10.68. Found: C, 64.46; H, 7.26; N, 10.30.

4.1.11.3. 6-[(Dimethylamino)methylene]-1-methyl-1,4,5,6-tetrahydro-7*H*-indole-7-one (7d). This product was obtained from **6d** after 4 h reflux and precipitated as brown solid; yield 90%; mp: 49–51 °C; IR: υ 1633 (CO) cm^{-1}; ^1H NMR: δ 2.55 (2H, t, *J* = 6.4 Hz, CH_2), 2.83 (2H, t, *J* = 6.4 Hz, CH_2), 3.02 (6H, s, 2× CH_3), 3.83 (1H, s, CH_3), 5.87 (1H, d, *J* = 2.5 Hz, H-3), 6.86 (1H, d, *J* = 2.5 Hz, H-2), 7.23 (1H, s, CH); ^{13}C NMR: δ 23.0 (t), 24.7 (t), 35.8 (q), 43.1 (q), 103.5 (s), 105.3 (d), 127.3 (s), 128.6 (d), 131.8 (s), 146.6 (d), 178.4 (CO). Anal calcd for $C_{12}H_{16}N_2O$: C, 70.56; H, 7.90; N, 13.71. Found: C, 70.30; H, 7.66; N, 13.42.

4.1.11.4. 1-Benzyl-6-[(dimethylamino)methylene]-1,4,5,6-tetrahydro-7*H*-indole-7-one (7e). This product was obtained from **6e** after 48 h reflux and precipitated as brown solid; yield 80%; mp: 116–118 °C; IR: υ 1633 (CO) cm^{-1}; ^1H NMR: δ 2.57 (2H, t, *J* = 6.8 Hz, CH_2), 2.83 (2H, t, *J* = 6.8 Hz, CH_2), 3.02 (6H, s, 2× CH_3), 5.56 (2H, s, CH_2), 5.94 (1H, d, *J* = 2.5 Hz, H-3), 7.02 (1H, d, *J* = 2.5 Hz, H-2), 7.12–7.28 (6H, m, Ar and CH); ^{13}C NMR: δ 23.0 (t), 24.6 (t), 43.1 (q), 50.5 (t), 103.4 (s), 106.2 (d), 126.7 (s), 126.9 (d), 127.0 (d), 128.0 (d), 128.2 (d), 132.3 (s), 139.6 (s), 147.0 (d), 178.3 (CO). Anal calcd for $C_{18}H_{20}N_2O$: C, 77.11; H, 7.19; N, 9.99. Found: C, 77.00; H, 6.94; N, 10.15.

4.1.11.5. 6-[(Dimethylamino)methylene]-1-phenyl-1,4,5,6-tetrahydro-7*H*-indole-7-one (7f). This product was obtained from **6f** after 24 h reflux and precipitated as brown solid; yield 80%; mp: 129–131 °C; IR: υ 1637 (CO) cm^{-1}; ^1H NMR: δ 2.63 (2H, t, *J* = 6.4 Hz, CH_2), 2.91 (2H, t, *J* = 6.4 Hz, CH_2), 3.02 (6H, s, 2× CH_3), 6.13 (1H, d, *J* = 2.7 Hz, H-3), 7.08 (1H, d, *J* = 2.7 Hz, H-2), 7.18 (1H, s, CH), 7.24–7.42 (5H, m, Ar); ^{13}C NMR: δ 23.2 (t), 24.2 (t), 43.1 (q), 102.9 (s), 107.6 (d), 125.0 (d), 126.3 (d), 127.7 (s), 128.2 (d), 128.9 (d), 134.2 (s), 140.3 (s), 147.0 (d), 176.6 (CO). Anal calcd for $C_{17}H_{18}N_2O$: C, 76.66; H, 6.81; N, 10.52. Found: C, 77.00; H, 7.05; N, 10.17.

4.1.11.6. 6-[(Dimethylamino)methylene]-1-(*p*-methylbenzyl)-1,4,5,6-tetrahydro-7*H*-indole-7-one (7g), 6-[(dimethylamino) methylene]-1-(*p*-methoxybenzyl)-1,4,5,6-tetrahydro-7*H*-indole-7-one (7h), ethyl 1-(*p*-methoxybenzyl)-6-[(dimethylamino)methylene]-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (7l). These compounds, obtained from **6g, 6h, 6l**, respectively, after 3 h reflux as brown oils, were unstable and were utilized for the successive step without purification.

4.1.11.7. Ethyl 6-[(dimethylamino)methylene]-1-methyl-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (7i). This product was obtained from **6i** after 3 h reflux and precipitated as brown solid; yield 80%; mp: 50–52 °C; IR: υ 1707 (CO), 1631 (CO) cm^{-1}; ^1H NMR: δ 1.27 (3H, t, *J* = 7.1 Hz, CH_3), 2.54 (2H, t, *J* = 7.3 Hz, CH_2), 2.76 (2H, t, *J* = 7.3 Hz, CH_2), 3.08 (6H, s, 2× CH_3), 4.17 (3H, s, CH_3), 4.23 (2H, q, *J* = 7.1 Hz, CH_2), 6.66 (1H, s, H-3), 7.39 (1H, s, CH); ^{13}C NMR: δ 14.2 (q), 22.4 (t), 24.1 (t), 33.9 (q), 43.3 (q), 59.9 (t), 103.7 (s), 113.6 (d), 125.4 (s), 129.6 (s), 132.2 (s), 148.8 (d), 160.5 (CO), 178.8 (CO). Anal calcd for $C_{15}H_{20}N_2O_3$: C, 65.20; H, 7.30; N, 10.14. Found: C, 65.54; H, 7.12; N, 10.05.

4.1.11.8. Ethyl 1-benzyl-6-[(dimethylamino)methylene]-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (7j). This product was obtained from **6j** after 1 h reflux and precipitated as yellow solid; yield 85%; mp: 101–103 °C; IR: υ 1703 (CO), 1635 (CO) cm^{-1}; ^1H NMR: δ 1.20 (3H, t, *J* = 7.0 Hz, CH_3), 2.61 (2H, t, *J* = 6.1 Hz, CH_2), 2.83 (2H, t, *J* = 6.1 Hz, CH_2), 3.06 (6H, s, 2× CH_3), 4.16 (2H, q, *J* = 7.0 Hz, CH_2), 6.13 (2H, s, CH_2), 6.78 (1H, s, H-3), 6.92–7.25 (5H, m, Ar), 7.38 (1H, s, CH); ^{13}C NMR: δ 14.1 (q), 22.3 (t), 24.0 (t), 43.3 (q), 48.2 (t), 59.9 (t), 103.5 (s), 114.7 (d), 125.0 (s), 126.0 (d), 126.5 (d), 128.2 (d), 130.2 (s), 131.9 (s), 139.7 (s), 149.1 (d), 160.3 (CO), 178.6 (CO). Anal calcd for $C_{21}H_{24}N_2O_3$: C, 71.57; H, 6.86; N, 7.95. Found: C, 71.84; H, 7.01; N, 7.63.

4.1.11.9. Ethyl 1-(*p*-methyl-benzyl)-6-[(dimethylamino)methylene]-1,4,5,6-tetrahydro-7*H*-indole-7-one-2-carboxylate (7k). This product was obtained from **6k** after 4 h reflux and precipitated as yellow solid and purified on a silica pad (DCM); yield 80%; mp: 84–86 °C; IR: υ 1709 (CO), 1631 (CO) cm^{-1}; ^1H NMR: δ 1.22 (3H, t, *J* = 7.1 Hz, CH_3), 2.56–2.68 (4H, m, 2× CH_2), 2.64 (3H, s, CH_3), 3.04 (6H, s, 2× CH_3), 4.22 (2H, q, *J* = 7.1 Hz, CH_2), 6.04 (2H, s, CH_2), 6.82 (2H, d, *J* = 7.9 Hz, Ar), 6.87 (1H, s, H-3), 7.05 (2H, d, *J* = 7.9 Hz, Ar), 7.61 (1H, s, CH); ^{13}C NMR: δ 14.0 (q), 21.6 (q), 21.8 (t), 43.2 (q), 48.2 (t), 60.3 (t), 113.5 (s), 115.0 (d), 126.0 (d), 128.8 (d), 131.0 (s), 133.1 (s), 135.8 (s), 136.2 (s), 152.6 (d), 160.2 (CO), 180.01 (CO). Anal calcd for $C_{22}H_{26}N_2O_3$: C, 72.11; H, 7.15; N, 7.64. Found: C, 72.40; H, 7.38; N, 7.30.

4.1.12. Preparation of 1,7-disubstituted 1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*]quinoline-8-one (9a–m)

To a suspension of **7a,b,d–l** (4 mmol) in anhydrous ethanol (50 mL), the suitable cyanomethylene compound (6 mmol) in anhydrous ethanol (60 mL) was added dropwise under nitrogen atmosphere. After the addition the reaction mixture was refluxed. Upon cooling, the precipitate formed was filtered and purified by recrystallization or by column chromatography (Sepacore Büchi) using DCM/AcOEt 9:1 as eluent.

4.1.12.1. 1,7-bis(Phenylsulfonyl)-1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*]quinolin-8-one (9a). This product was obtained from the reaction of **7a** with phenylsulfonylacetonitrile after 48 h reflux. The yellow solid precipitated was recrystallized from ethanol; yield 65%; mp: 349–351 °C; IR: υ 3218 (NH), 1635 (CO) cm^{-1}; ^1H NMR: δ 2.70–2.86 (4H, m, 2× CH_2), 6.83 (1H, d, *J* = 2.0 Hz, H-3), 7.54–7.71 (7H, m, Ar and H-2), 7.91–7.98 (4H, m, Ar), 8.21 (1H, s, H-6), 11.90 (1H, s, NH); ^{13}C NMR: δ 20.4 (t), 25.1 (t), 99.5 (d), 111.1 (s), 113.5 (d), 123.6 (s), 124.9 (s), 126.6 (d), 127.9 (d), 128.4 (s), 128.8 (d), 129.8 (d), 131.8 (s), 133.2 (d), 133.7 (d), 140.4 (s), 141.3 (s), 143.9 (d), 156.3 (CO). Anal calcd for

$C_{23}H_{18}N_2O_5S_2$: C, 59.21; H, 3.89; N, 6.00. Found: C, 59.00; H, 4.01; N, 5.89. When the same reaction was carried out at rt was obtained a complex mixture which, after column chromatography (eluent DCM/EtOAc 9:1), furnished intermediate **8** as brown oil.

4.1.12.2. 1-Phenylsulfonyl-6-[2-(phenylsulfonyl)-propanenitrile]-1,4,5,6-tetrahydro-7*H*-indole-7-one (8). Yield 40%; IR: υ 2362 (CN), 1693 (CO) cm^{-1} ; ^1H NMR: δ 2.53–2.72 (4H, m, 2× CH_2), 5.86 (1H, d, *J* = 10.7 Hz, CH), 6.02 (1H, d, *J* = 10.7 Hz, CH), 6.36 (1H, d, *J* = 2.6 Hz, H-3), 7.15–8.08 (11H, m, Ar and H-2); ^{13}C NMR: δ 23.7 (t), 34.3 (t), 111.0 (d), 125.5 (d), 128.1 (d), 128.4 (d), 128.5 (d), 128.8 (s), 129.2 (s), 129.3 (d), 131.6 (d), 133.3 (d), 134.4 (s), 134.6 (d), 136.8 (d), 137.6 (s), 147.7 (s), 143.7 (s), 162.8 (CO). Anal calcd for $C_{23}H_{18}N_2O_5S_2$: C, 59.21; H, 3.89; N, 6.00. Found: C, 59.04; H, 4.12; N, 6.12.

By refluxing in ethanol this intermediate, the cyclized compound **9a**, in 45% yield, was obtained.

4.1.12.3. 1-(Phenylsulfonyl)-1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*]quinolin-7-carbonitrile-8-one (9c). This product was obtained from the reaction of **7a** with cyanoacetamide after 32 h reflux. The yellow solid precipitated was recrystallized from ethanol; yield 13%; mp: 309–311 °C; IR: υ 3309 (NH), 2220 (CN), 1653 (CO) cm^{-1}; ^1H NMR: δ 2.69–2.92 (4H, m, 2× CH_2), 6.85 (1H, s, H-3), 7.61–7.77 (4H, m, Ar and H-2), 7.96 (2H, d, *J* = 8.0 Hz, H-2′ and H-6′), 8.04 (1H, s, H-6), 12.06 (1H, s, NH); ^{13}C NMR: δ 20.3 (t), 25.0 (t), 99.5 (d), 112.2 (s), 113.5 (d), 117.0 (s), 125.0 (s), 126.7 (d), 128.3 (s), 129.8 (d), 131.9 (s), 133.8 (d), 138.8 (s), 141.3 (s), 148.4 (d), 159.5 (CO). Anal calcd for $C_{18}H_{13}N_3O_3S$: C, 61.53; H, 3.73; N, 11.96. Found: C, 61.32; H, 3.60; N, 12.20.

4.1.12.4. 1-Methyl-7-(phenylsulfonyl)-1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*]quinolin-8-one (9d). This product was obtained from the reaction of **7d** with phenylsulfonylacetonitrile after 24 h reflux. The yellow solid precipitated was recrystallized from ethanol; yield 55%; mp: 208–209 °C; IR: υ 3320 (NH), 1635 (CO) cm^{-1}; ^1H NMR: δ 2.51 (2H, t, *J* = 6.9 Hz, CH_2), 2.82 (2H, t, *J* = 6.9 Hz, CH_2), 3.94 (3H, s, CH_3), 6.00 (1H, d, *J* = 2.4 Hz, H-3), 6.93 (1H, d, *J* = 2.4 Hz, H-2), 7.54–7.72 (3H, m, Ar), 7.92 (2H, d, *J* = 8.0 Hz, H-2′ and H-6′), 8.03 (1H, s, H-6), 11.47 (1H, s, NH); ^{13}C NMR: δ 21.6 (t), 27.4 (t), 36.2 (q), 99.5 (d), 102.3 (s), 106.6 (d), 117.4 (s), 124.6 (s), 127.6 (d), 128.0 (d), 128.8 (d), 129.3 (s), 133.0 (d), 141.2, (s), 141.3 (s), 157.9 (CO). Anal calcd for $C_{18}H_{16}N_2O_3S$: C, 63.51; H, 4.74; N, 8.23. Found: C, 63.72; H, 4.95; N, 8.02.

4.1.12.5. 1-Benzyl-7-(phenylsulfonyl)-1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*]quinolin-8-one (9e). This product was obtained from the reaction of **7e** with phenylsulfonylacetonitrile after 24 h reflux. The solid precipitated was purified by column chromatography (Sepacore Büchi) using DCM/AcOEt 9:1 as eluent. Brown solid; yield 45%; mp: 213–214 °C; IR: υ: 3214 (NH), 1631 (CO) cm^{-1}; ^1H NMR: δ 2.65 (2H, t, *J* = 7.0 Hz, CH_2), 2.83 (2H, t, *J* = 7.0 Hz, CH_2), 5.79 (2H, s, CH_2), 6.06 (1H, d, *J* = 2.5 Hz, H-3), 7.09 (1H, d, *J* = 2.5 Hz, H-2), 7.12–7.28 (5H, m, Ar), 7.54–7.71 (3H, m, Ar), 7.90–7.95 (2H, m, Ar), 8.00 (1H, s, H-6), 11.61 (1H, s, NH); ^{13}C NMR: δ 21.6 (t), 27.5 (t), 50.8 (t), 99.5 (d), 107.3 (d), 114.7 (s), 120.9 (s), 124.4 (s), 126.9 (d), 127.1 (d), 127.7 (d), 128.3 (d), 128.9 (d), 129.7 (s), 133.1 (d), 137.2 (s), 139.3 (s), 141.2 (d), 150.9 (s), 158.1 (CO). Anal calcd for $C_{24}H_{20}N_2O_3S$: C, 69.21; H, 4.84; N, 6.73. Found: C, 69.46; H, 4.63; N, 6.50.

4.1.12.6. 1-Phenyl-7-(phenylsulfonyl)-1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*]quinolin-8-one (9f). This product was obtained from the reaction of **7f** with phenylsulfonylacetonitrile after 32 h reflux. The crude precipitate was purified by column chromatography (Sepacore Büchi) using DCM/AcOEt 9:1 as eluent. Yellow solid; yield 55%; mp: 251–252 °C; IR: υ 3359 (NH), 1654 (CO) cm^{-1}; ^1H NMR: δ 2.68 (2H, t, *J* = 6.7 Hz, CH_2), 2.79 (2H, t, *J* = 6.7 Hz, CH_2), 6.34 (1H, d, *J* = 2.7 Hz, H-3), 7.29 (1H, d, *J* = 2.7 Hz, H-2), 7.32–7.67 (8H, m, Ar), 7.90–7.96 (2H, d, *J* = 8.0 Hz, H-2′ and H-6′), 8.15 (1H, s, H-6), 10.10 (1H, s, NH); ^{13}C NMR: δ 22.0 (t), 26.5 (t), 99.5 (d), 108.6 (s), 109.5 (d), 112.1 (s), 119.0 (s), 121.6 (s), 124.5 (d), 127.4 (d), 127.9 (d), 128.7 (d), 129.3 (d), 130.9 (d), 133.0 (d), 133.6 (s), 138.6 (s), 140.9 (s), 156.4 (CO). Anal calcd for $C_{23}H_{18}N_2O_3S$: C, 68.64; H, 4.51; N, 6.96. Found: C, 68.40; H, 4.80; N, 7.06.

4.1.12.7. 1-(*p*-Methyl-benzyl)-7-(phenylsulfonyl)-1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*]quinolin-8-one (9g). This product was obtained from the reaction of **7g** with phenylsulfonylacetonitrile after 32 h reflux. The crude precipitate was purified by column chromatography (Sepacore Büchi) using DCM/AcOEt 9:1 as eluent. Yellow solid; yield 40%; mp: 178–180 °C; IR: υ 3217 (NH), 1626 (CO) cm^{-1}; ^1H NMR: δ 2.19 (3H, s, CH_3), 2.64 (2H, t, *J* = 7.5 Hz, CH_2), 2.80 (2H, t, *J* = 7.5 Hz, CH_2), 5.71 (2H, s, CH_2), 6.04 (1H, d, *J* = 2.5 Hz, H-3), 7.02–7.10 (5H, m, Ar and H-2), 7.55–7.67 (3H, m, Ar), 7.90–7.95 (2H, m, Ar), 8.00 (1H, s, H-6), 11.56 (1H, s, NH); ^{13}C NMR: δ 20.6 (q), 21.6 (t), 50.7 (t), 99.5 (d), 105.2 (d), 107.2 (d), 124.6 (s), 126.9 (d), 127.7 (d), 128.8 (d), 128.9 (d), 129.8 (s), 133.1 (d), 136.2 (s), 137.2 (s), 140.5 (s), 141.2 (s), 152.5 (s), 158.0 (s), 174.0 (CO). Anal calcd for $C_{25}H_{22}N_2O_3S$: C, 69.75; H, 5.15; N, 6.51. Found: C, 69.55; H, 5.02; N, 6.64.

4.1.12.8. 1-(*p*-Methoxy-benzyl)-7-(phenylsulfonyl)-1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*]quinolin-8-one (9h). This product was obtained from the reaction of **7h** with phenylsulfonylacetonitrile after 32 h reflux. The crude precipitate was purified by column chromatography (Sepacore Büchi) using DCM/AcOEt 9:1 as eluent. Yellow solid; yield 45%; mp: 154–156 °C; IR: υ 3315 (NH), 1635 (CO) cm^{-1}; ^1H NMR: δ 2.64 (2H, t, *J* = 7.5 Hz, CH_2), 2.78 (2H, t, *J* = 7.5 Hz, CH_2), 3.34 (3H, s, CH_3), 5.68 (2H, s, CH_2), 6.04 (1H, d, *J* = 2.5 Hz, H-3), 6.80 (2H, d, *J* = 8.0 Hz, H-3″ and H-5″), 7.08 (1H, d, *J* = 2.5 Hz, H-2), 7.12 (2H, d, *J* = 8.0 Hz, H-2″ and H-6″), 7.56–7.68 (3H, m, Ar), 7.93 (2H, d, *J* = 8.6 Hz, H-2′ and H-6′), 8.00 (1H, s, H-6), 11.59 (1H, s, NH); ^{13}C NMR: δ 21.6 (t), 27.5 (t), 50.3 (t), 54.9 (q), 99.5 (d), 107.2 (d), 113.6 (d), 114.4 (s), 121.1 (s), 126.7 (d), 128.4 (d), 128.9 (d), 129.6 (s), 131.1 (s), 133.1 (d), 133.7 (d), 136.8 (s), 141.2 (s), 145.0 (s), 158.0 (s), 158.4 (CO). Anal calcd for $C_{25}H_{22}N_2O_4S$: C, 67.25; H, 4.97; N, 6.27. Found: C, 67.05; H, 5.18; N, 6.00.

4.1.12.9. Ethyl 7-(phenylsulfonyl)-1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*]quinolin-8-one-2-carboxylate (9i). This product was obtained from the reaction of **7b** with phenylsulfonylacetonitrile after 32 h reflux. The yellow solid precipitated was recrystallized from ethanol; yield 60%; mp: 322–323 °C; IR: υ 3280 (NH), 3246 (NH), 1711 (CO), 1651 (CO) cm^{-1}; ^1H NMR: δ 1.30 (3H, t, *J* = 7.1 Hz, CH_3), 2.67–2.88 (4H, m, 2× CH_2), 4.29 (2H, q, *J* = 7.1 Hz, CH_2), 6.73 (1H, s, H-3), 7.55–7.72 (3H, m, Ar), 7.98 (2H, d, *J* = 8.0 Hz, H-2′ and H-6′), 8.21 (1H, s, H-6), 12.06 (2H, bs, 2× NH); ^{13}C NMR: δ 14.2 (q), 20.5 (t), 25.4 (t), 60.3 (t), 110.8 (s), 113.4 (d), 122.8 (s), 123.9 (s), 126.1 (s), 127.9 (d), 128.4 (s), 128.7 (d), 133.1 (d), 140.6 (2× s), 143.8 (d), 156.5 (CO), 159.8 (CO). Anal calcd for $C_{20}H_{18}N_2O_5S$: C, 60.29; H, 4.55; N, 7.03. Found: C, 59.98; H, 4.68; N, 6.85.

4.1.12.10. Ethyl 1-methyl-7-(phenylsulfonyl)-1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*]quinolin-8-one-2-carboxylate (9j). This product was obtained from the reaction of **7i** with phenylsulfonylacetonitrile after 28 h reflux. The crude precipitate was purified by column chromatography (Sepacore Büchi) using DCM/AcOEt 9:1 as eluent. Yellow solid; yield 52%; mp: 214–216 °C; IR: υ

3120 (NH), 1703 (CO), 1643 (CO) cm^{-1}; ^1H NMR: δ 1.27 (3H, t, J = 7.1 Hz, CH_3), 2.66 (2H, t, J = 6.5 Hz, CH_2), 2.87 (2H, t, J = 6.5 Hz, CH_2), 4.22 (2H, q, J = 7.1 Hz, CH_2), 4.28 (1H, s, CH_3), 6.78 (1H, s, H-3), 7.57–7.74 (3H, m, Ar), 7.98 (2H, d, J = 8.0 Hz, H-2′ and H-6′), 8.17 (1H, s, H-6), 11.96 (1H, s, NH); ^{13}C NMR: δ 14.2 (q), 20.9 (t), 27.2 (t), 34.8 (q), 59.8 (t), 99.9 (d), 114.7 (d), 117.4 (s), 125.7 (s), 126.8 (s), 127.8 (d), 129.0 (d), 130.8 (s), 133.3 (d), 138.3 (s), 140.7 (s), 157.9 (s), 160.3 (CO), 170.3 (CO). Anal calcd for $C_{21}H_{20}N_2O_5S$: C, 61.15; H, 4.89; N, 6.79. Found: C, 61.52; H, 4.62; N, 6.85.

4.1.12.11. Ethyl 1-benzyl-7-(phenylsulfonyl)-1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*]quinolin-8-one-2-carboxylate (9k). This product was obtained from the reaction of **7j** with phenylsulfonylacetonitrile after 24 h reflux. The crude precipitate was purified by column chromatography (Sepacore Büchi) using DCM/AcOEt 9:1 as eluent. Brown solid; yield 42%; mp: 167–169 °C; IR: υ 3309 (NH), 1701 (CO), 1637 (CO) cm^{-1}; ^1H NMR: δ 1.21 (3H, t, J = 7.1 Hz, CH_3), 2.70 (2H, t, J = 7.3 Hz, CH_2), 2.90 (2H, t, J = 7.3 Hz, CH_2), 4.17 (2H, q, J = 7.1 Hz, CH_2), 6.43 (2H, s, CH_2), 6.88 (1H, s, H-3), 6.91 (2H, d, J = 7.2 Hz, H-2″ and H-6″), 7.09–7.23 (3H, m, Ar), 7.55–7.72 (3H, m, Ar), 7.95 (2H, d, J = 7.8 Hz, H-2′ and H-6′), 8.14 (1H, s, H-6), 11.93 (1H, s, NH); ^{13}C NMR: δ 14.1 (q), 20.8 (t), 27.2 (t), 48.4 (t), 59.9 (t), 115.9 (d), 117.3 (s), 123.9 (s), 125.6 (s), 126.0 (d), 126.6 (d), 127.5 (s), 127.8 (d), 128.2 (d), 129.0 (d), 130.7 (s), 133.4 (d), 138.1 (d), 139.4 (s), 140.7 (s), 150.4 (s), 157.9 (CO), 160.1 (CO). Anal calcd for $C_{27}H_{24}N_2O_5S$: C, 66.38; H, 4.95; N, 5.73. Found: C, 66.18; H, 5.12; N, 5.60.

4.1.12.12. Ethyl 1-(*p*-methyl-benzyl)-7-(phenylsulfonyl)-1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*] quinolin-8-one-2-carboxylate (9l). This product was obtained from the reaction of **7k** with phenylsulfonylacetonitrile after 24 h reflux. The crude precipitate was purified by column chromatography (Sepacore Büchi) using DCM/AcOEt 9:1 as eluent. Brown solid; yield 35%; mp: 98–100 °C; IR: υ 3299 (NH), 1701 (CO), 1664 (CO) cm^{-1}; ^1H NMR: δ 1.22 (3H, t, J = 7.1 Hz, CH_3), 2.16 (3H, s, CH_3), 2.69 (2H, t, J = 7.4 Hz, CH_2), 2.85 (2H, t, J = 7.4.Hz, CH_2), 4.19 (2H, q, J = 7.1 Hz, CH_2), 6.37 (2H, s, CH_2), 6.78 (2H, d, J = 7.9 Hz, H-3″ and H-5″), 6.87 (1H, s, H-3), 6.98 (2H, d, J = 7.9 Hz, H-2″ and H-6″), 7.59–7.69 (3H, m, Ar), 7.94 (2H, d, J = 7.5 Hz, H-2′ and H-6′), 8.12 (1H, s, H-6), 11.92 (1H, s, NH); ^{13}C NMR: δ 14.1 (q), 20.5 (q), 20.8 (t), 27.2 (t), 59.9 (t), 64.9 (t), 99.5 (d), 115.9 (d), 117.0 (s), 123.7 (s), 125.9 (d), 126.3 (s), 127.5 (s), 127.9 (d), 128.8 (d), 128.9 (s), 129.0 (d), 130.7 (s), 133.4 (d), 135.7 (s), 136.4 (s), 140.6 (s), 157.9 (CO), 160.1 (CO). Anal calcd for $C_{28}H_{26}N_2O_5S$: C, 66.91; H, 5.21; N, 5.57. Found: C, 66.85; H, 5.54; N, 5.42.

4.1.12.13. Ethyl 1-(*p*-methoxy-benzyl)-7-(phenylsulfonyl)-1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*] quinolin-8-one-2-carboxylate (9m). This product was obtained from the reaction of **7l** with phenylsulfonylacetonitrile after 22 h reflux. The crude precipitate was purified by column chromatography (Sepacore Büchi) using DCM/AcOEt 9:1 as eluent. Brown solid; yield 37%; mp: 98–100 °C; IR: υ 3297 (NH), 1701 (CO), 1664 (CO) cm^{-1}; ^1H NMR: δ 1.23 (3H, t, J = 7.1 Hz, CH_3), 2.69 (2H, t, J = 7.2 Hz, CH_2), 2.85 (2H, t, J = 7.2 Hz, CH_2), 3.40 (3H, s, CH_3), 4.21 (2H, q, J = 7.1 Hz, CH_2), 6.34 (2H, s, CH_2), 6.74 (2H, d, J=7.2 Hz, H-3″ and H-5″), 6.86 (1H, s, H-3), 6.90 (2H, d, J = 7.2 Hz, H-2″ and H-6″), 7.57–7.69 (3H, m, Ar), 7.95 (2H, d, J = 6.8 Hz, H-2′ and H-6′), 8.14 (1H, s, H-6), 11.95 (1H, s, NH); ^{13}C NMR: δ 14.1 (q), 20.8 (t), 27.2 (t), 47.7 (t), 54.9 (q), 64.9 (t), 99.5 (d), 113.6 (d), 115.9 (d), 117.2 (s), 124.1 (s), 127.5 (d), 127.6 (s), 127.9 (d), 129.0 (d), 129.7 (s), 130.6 (s), 131.3 (s), 131.6 (s), 133.4 (d), 138.1 (s), 140.6 (s), 159.1 (CO), 160.2 (CO). Anal calcd for $C_{28}H_{26}N_2O_6S$: C, 64.85; H, 5.05; N, 5.40. Found: C, 64.70; H, 5.42; N, 5.53.

4.1.12.14. 7-(Phenylsulfonyl)-1,4,5,9-tetrahydro-8*H*-pyrrolo[3,2-*h*]quinolin-8-one (9b). To a suspension of **9a** (70 mg, 0.145 mmol) in ethanol (20 mL), KOH (0.06 g, 1 mmol) was added and the reaction mixture was refluxed for 1 h. After cooling, the reaction mixture was made acid with 6 N HCl. The yellow solid formed was filtered, air dried and purified by column chromatography (Sepacore Büchi) using DCM/AcOEt 9:1 as eluent. Yield 90%; mp: 410 °C; IR: υ 3365 (NH), 3255 (NH), 1647 (CO) cm^{-1}; ^1H NMR: δ 2.64–2.83 (4H, m, 2× CH_2), 6.13 (1H, t, J = 2.4 Hz, H-3), 7.15 (1H, d, J = 2.4 Hz, H-2), 7.52–7.69 (3H, m, Ar), 7.96 (2H, d, J = 8.0 Hz, H-2′ and H-6′), 8.08 (1H, s, H-6), 11.28 (1H, s, NH), 11.97 (1H, s, NH); ^{13}C NMR: δ 21.2 (t), 25.9 (t), 99.5 (d), 108.8 (d), 118.8 (s), 119.9 (s), 125.6 (d), 127.6 (d), 128.6 (d), 129.6 (s), 132.8 (d), 141.2 (s), 142.4 (s), 142.9 (s), 156.8 (CO). Anal calcd for $C_{17}H_{14}N_2O_3S$: C, 62.56; H, 4.32; N, 8.58. Found: C, 62.74; H, 4.15; N, 8.40.

4.2. Biology

4.2.1. Chemicals

Plasmid pBR322 was bought from Fermentas (Burlington, Canada). Base excision repair enzymes (BER), Formamido pyrimidine glycosilase (Fpg) and Endonuclease III (Endo III), were a generous gift from Dr. S. Boiteux (CEA, Fontenay aux Roses, France). If not otherwise indicated, all the chemicals were purchased from Sigma–Aldrich (Milano, Italy).

Irradiation procedure. HPW 125 Philips lamps (Wood's 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.

4.2.2. Spectrophotometric measurement

All spectrophotometric measures were performed using a Perkin-Elmer Lambda 15 spectrophotometer and emission spectra were recorded on a Perkin-Elmer LS-50B fluorimeter.

4.2.3. Cellular phototoxicity

Human chronic myeloid leukemia cells (K-562) and human T-cell leukemia cells (Jurkat) were grown in RPMI-1640 medium, human breast adenocarcinoma cells (MCF-7) and human keratinocytes (NCTC-2544) were grown in DMEM medium (Dulbecco's modified Eagle's medium) and intestinal adenocarcinoma cells (LoVo) were grown in Ham's F12 medium. All cellular media were supplemented with 115 U/mL of penicillin G, 115 μg/mL streptomycin and 10% fetal bovine serum (Invitrogen, Milano, Italy). Individual wells of a 96-well tissue culture microtiter plate were inoculated with 100 μL of complete medium containing 8×10^3 K-562, Jurkat cells or 5×10^3 MCF-7, LoVo and NCTC-2544 cells. Plates were incubated at 37 °C in a humidified 5% CO_2 incubator for 18 h before starting the experiments. Drugs were dissolved in DMSO and then diluted with Hank's Balanced Salt Solution (HBSS, pH 7.2) for phototoxicity test (concentration range: 20–0.31 μM). DMSO never exceeded 1%. After removal of the medium, 100 μL of the drug solution were added to each well and incubated at 37 °C for 30 min and then irradiated (2.5 or 3.75 J/cm^2). After irradiation, drug solutions were replaced with medium and plates were incubated for 72 h. Cell viability was assayed by MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide)] test, as previously described.[18]

4.2.4. Externalization of phosphatidylserine

For the assessment of phosphatidylserine surface exposure by apoptotic cells, 100,000 Jurkat cells were irradiated with 2 μM solutions of test compounds. After 30 min, 1 and 2 h from the incubation, samples were centrifuged and 100 μL of Annexin

V-FITC/PI solution were added, according to the kit instructions (Annexin V-FLUOS Staining kit, Roche, Penzberg Germany). Samples were incubated for 15 min in the dark and then analyzed by BD FACS Calibur (Becton Dickinson, New York, USA) flow cytometer which measures the fluorescence of FITC (green, FL1) and of PI (red, FL3).[19] At least 10,000 events for each sample were acquired.

4.2.5. Cell cycle analysis

The flow cytometric analysis of DNA content, was executed irradiating 5×10^5 Jurkat cells, in exponentially growth, in the presence of 1 μM of pyrroloquinolinones. After the removal of drug solutions, cells were incubated for 24 h in RPMI-1640 medium, then fixed with ice-cooled ethanol (70%), treated overnight with phosphate-buffer saline (PBS) containing RNAse (10 KU/mL) and finally stained with propidium iodide (PI, 10 μg/mL). Samples were analyzed on a BD FACS Calibur flow cytometer. Results of cell cycle analysis, were examined using WinMDI 2.9 (Windows Multiple Document Interface for Flow Cytometry). Data were expressed as fractions of cells in the different cycle phases.

4.2.6. Mitochondrial membrane potential

The mitochondrial membrane potential was assessed using the lipophilic cation JC-1. Jurkat cells were collected by centrifugation 2 and 4 h after the irradiation, and resuspended in HBSS containing the JC-1 at the concentration of 1 μg/mL. The cytofluorimetric analysis (BD FACS Calibur) was performed collecting green (FL1) and orange (FL2) fluorescence.[21] At least 10,000 events for each sample were acquired.

4.2.7. Reactive oxygen species assessment

ROS production was assessed by flow cytometry using two different probes: hydroethidine (HE), which reveals superoxide anion, and 2′,7′-dichlorodihydrofluorescin diacetate (DCFDA), which reveals the presence of hydrogen peroxide. After 6 h from irradiation, cells were collected by centrifugation and resuspended in HBSS (Hank's balanced salt solution) containing the fluorescence probes HE, DCFDA at the concentration of 2.5 and 5 μM, respectively. Cells were then incubated at 37 °C for 15 or 30 min. The fluorescence was recorded with the flow cytometer (BD FACS Calibur) using the 488 nm wavelength as excitation and the emission at 620 nm for HE and at 530 nm for DCFDA.[23] At least 10,000 events for each sample were acquired.

4.2.8. Lysosomal stability assessment

Jurkat cells were assessed for lysosomal stability using the acridine orange (AO) uptake method.[24] After 2 and 4 h from irradiation, cells were collected by centrifugation and resuspended in RPMI-1640 containing AO at the concentration of 1 μM. The fluorescence was directly recorded with the flow cytometer (BD FACS Calibur) using the 488 nm wavelength as excitation and the emission in FL3 channel. At least 10,000 events for each sample were acquired.

4.2.9. Linear dichroism

LD measurements were performed with a Jasco J500A circular dichroism spectropolarimeter (Jasco, Cremella, Italy), converted for LD and equipped with an IBM PC and Jasco J interface. For these analyses, the sample orientation was obtained using a flow device designed by Wada and Kozawa,[35] which presents a cylindrical rotating cuvette, a 0.14 cm optical path and a constant flow of 800 rpm. LD spectra were performed using different [Drug]/[DNA] ratios, dissolved in phosphate buffer (10 mM, pH 7.2).

4.2.10. pBR322 DNA stand breaks

Each pBR322 DNA sample (100 ng) dissolved in phosphate buffer (10 mM, pH 7.2) was irradiated with UV-A in the presence of the compounds. After irradiation, two aliquots of sample were incubated at 37 °C with Fpg (Formamido pyrimidine glycosilase) and Endo III (Endonuclease III), respectively, as described by Epe et al.[36] Before starting the run, buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) was added to DNA. Samples were loaded on 1% agarose gel and the run was carried out in TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0) at 80 V for 2 h. After staining with ethidium bromide solution, gel was washed with water and the DNA bands were detected under UV irradiation with a UV transilluminator. Photographs were taken by a digital photocamera Kodak DC256 and the quantification of the bands was achieved by image analyzer software Quantity One (Bio Rad, Milano, Italy). The fractions of supercoiled (Form I) and open circular DNA (Form II) were calculated as described by others.[27]

4.2.11. Studies on isolated proteins

Solutions of BSA and RNAse A (2 mg/mL) in phosphate buffer were irradiated in the presence of the test compounds for various times in a quartz cuvette. The degree of protein oxidation was spectrophotometrically monitored through the methods of Levine et al.[28] by derivatization with 2,4-dinitrophenylhydrazine.

4.2.12. Lipid peroxidation

Jurkat cells (500,000) were irradiated (2.5 J/cm^2) in the presence of the most active compounds and then incubated for 24 h. To verify lipidic peroxidation, after cell centrifugation, 900 μL of surnatant were collected and put at 253 K after having added 90 μL of 2,6-di-*tert*-butyl-*p*-cresol (BHT, 2% in absolute ethanol). Cells were washed, resuspended in 500 μL of water, and 400 μL of cells were lysed with 400 μL of SDS (Sodium dodecyl sulfate, 1% in water). This suspension was divided into two aliquots: 50 μL of BHT were added in 500 μL; 300 μL were used for protein quantification with Peterson method.[37] Lipid peroxidation was measured using a thiobarbituric acid assay as described by Morliere et al.[30] A standard curve of 1,1,3,3 tetraethoxypropane was used to quantify the amount of produced malondialdehyde. Data were expressed in terms of nanomoles of TBARS normalized to the total protein content in an aliquot of the cell extract.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.080.

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