Bioorganic & Medicinal Chemistry 16 (2008) 9668–9683

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Thiopyrano[2,3-*e*]indol-2-ones: Angelicin heteroanalogues with potent photoantiproliferative activity

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ARTICLE INFO

Article history:
Received 17 April 2008
Revised 26 September 2008
Accepted 2 October 2008
Available online 5 October 2008

Keywords:
Thiopyrano[2,3-*e*]indol-2-ones
Angelicin
Antiproliferative activity
Photochemotherapeutic agents
Apoptosis

ABSTRACT

A new class of compounds, the thiopyrano[2,3-*e*]indol-2-ones, bioisosters of the angular furocoumarin angelicin, was synthesized with the aim of obtaining new photochemotherapeutic agents. In particular 7,8-dimethyl-thiopyranoindolone **6c** s showed a remarkable phototoxicity and a great dose UVA dependence reaching IC_{50} values at submicromolar level. This latter photoinduced a massive apoptosis and a remarkable photodamage to lipids and proteins. Although it did not intercalate DNA, it was able to cause photooxidation of DNA bases.

1. Introduction

Derivatives of psoralen **1** are naturally occurring or synthetic aromatic compounds deriving from the condensation of a coumarin nucleus with a furan ring nowadays used in the treatment of the human skin diseases such as psoriasis, T-cell lymphoma (Cutaneous T-cell lymphoma, CTCL) and vitiligo, in combination with UVA radiation, commonly referred as PUVA (psoralen plus UVA) therapy.[1] These tricyclic aromatic compounds possess a planar structure that allows their intercalation between nucleic acid base pairs. Upon UVA irradiation, the intercalated complex is activated, thus generating photoadducts with pyrimidine bases in cellular DNA.[2] The psoralen monoadducts formed can further photochemically react with a pyrimidine base on the complementary strand of the DNA thus leading to interstrand cross links (ICL), which are believed to be the primary cause of photoinduced cell killing.

To improve the photobinding capability and at the same time to decrease the phototoxicity of photochemotherapeutic agents were synthesized derivatives of angelicin **2**, a natural compound, angular isomer of psoralen, whose geometry does not permit the simultaneous alignment of the two photoreactive sites with pyrimidine bases, thus allowing only monofunctional photobinding.[3]

Another promising approach, mainly aimed to dissociate undesiderable side effects from the therapeutic ones, was the synthesis of heteroanalogues of angelicin. Thus, sulphur or nitrogen isosters such as thioangelicins, thienocoumarins, pyrrolocoumarins, and furoquinolinones were studied demonstrating in some cases improved interaction with DNA both in the dark and under UVA light in comparison to angelicin itself.[4] We have recently reported the synthesis and the photochemotherapeutic activity of the new ring system pyrrolo[2,3-*h*]quinolin-2-one of type **3** or **4**, an angelicin heteroanalogue, in which nitrogen atoms replaced both oxygens on the furan and the pyrone ring.[5]

These compounds were highly phototoxic on a panel of human tumor cell lines with GI_{50} in the micromolar and submicromolar level, being in some instances more cytotoxic than 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP) and angelicin used as reference drugs. However, studies of linear dichroism (LD) strongly suggested that the new derivatives did not efficaciously interact with DNA, thus indicating a different mechanism from that of furocoumarins.[5]

Continuing our studies in the search for new angelicin heteroanalogues and considering our previous satisfactory results on pyrroloquinolinones, we focused our attention on the synthesis on the thiopyrano[2,3-*e*]indol-2-one ring system of type **5** or **6**, respectively (Chart 1). Now, we wish to report the synthesis of such ring system along with the results of their photobiological activity and studies directed to elucidate the mode of action.

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doi:10.1016/j.bmc.2008.10.002

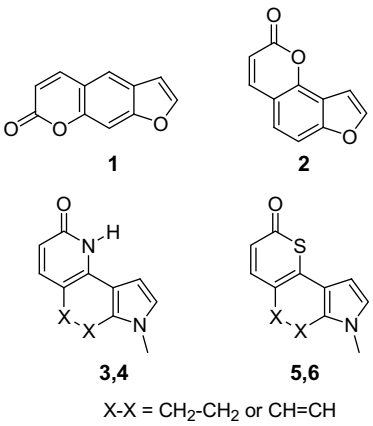
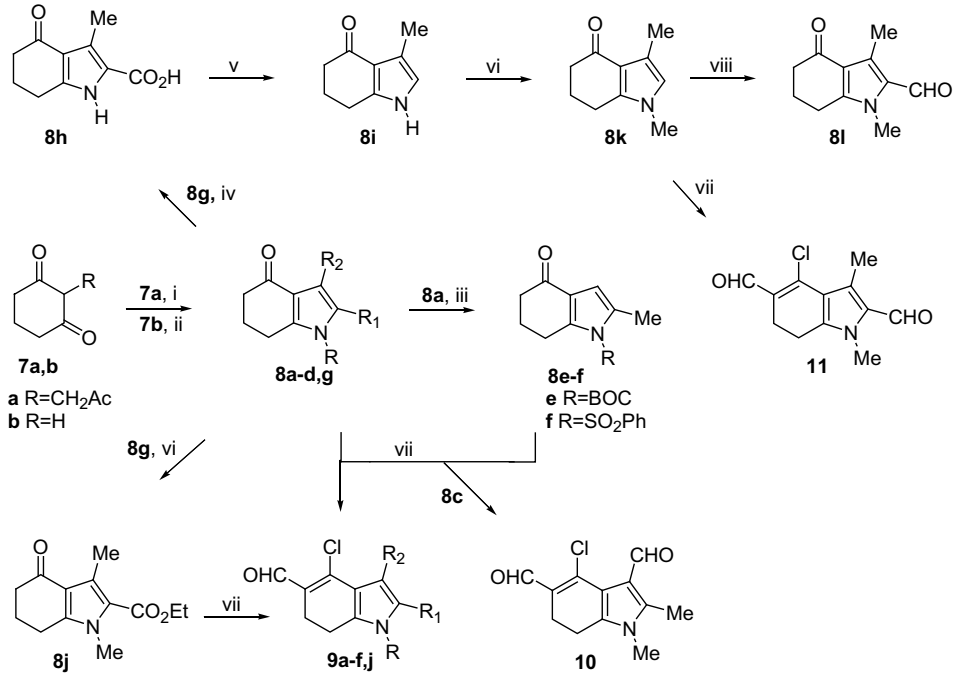


Chart 1.

2. Results and discussion

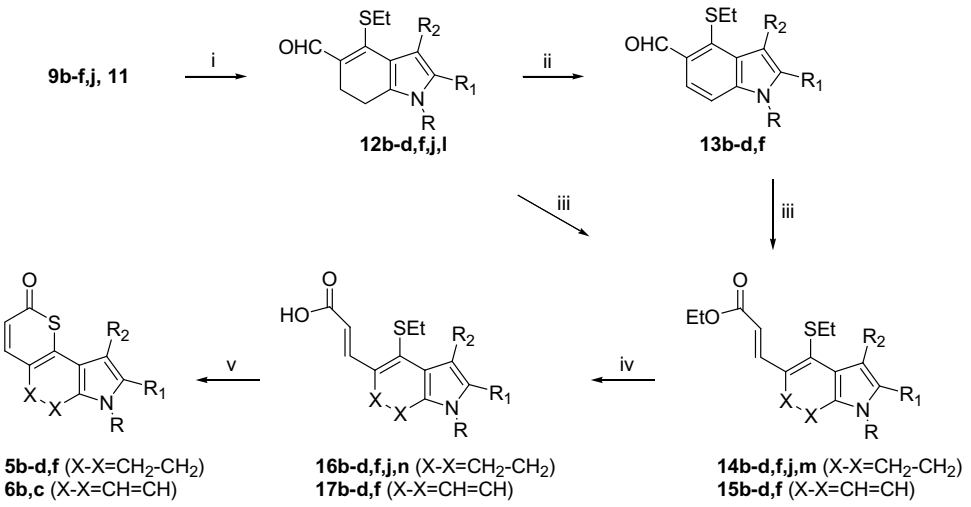
2.1. Chemistry

The synthetic route to thiopyrano[2,3-*e*]indol-2-ones started from 2-acetonyl-1,3-cyclohexanedione **7a** which was reacted, in acetic acid, with ammonium acetate to get the 1,5,6,7-tetrahydroindol-4-one **8a** or with suitable primary amines to obtain the N-substituted tetrahydroindolones **8b–d**.[5b] Derivatives **8e** and **8f** were obtained by reaction of the *N*-unsubstituted derivative **8a** with *t*-butyl dicarbonate or benzensulfonyl chloride, respectively. To investigate the influence on the biological activity of the methyl group in the eight position of the tricyclic ring we thought to switch it to the nine position. Thus, 1,3-cyclohexanedione **7b** was subjected to Knorr reductive condensation by reaction with diethyl hydroxyl iminomalonate in the presence of zinc dust to give the tetrahydroindole **8g**.[6] Alkaline hydrolysis of the ester functionality of this latter afforded **8h** which underwent decarboxylation to give the 3-methyl substituted tetrahydroindole **8i**. Alkylation of the indole nitrogen of derivatives **8g** and **8i** with iodomethane allowed the isolation of **8j** and **8k**, respectively. The synthetic pathway proceeded through the acylation of the position five of the tetrahydroindoles **8** which was performed by using the Vilsmeier–Haack reagent ($DMF/POCl_3$). This step proceeded very easily for tetrahydroindoles **8b–d,f,j** provided the strict control of the temperature (0 °C) and the very short reaction time (3 min) to avoid tarry insoluble materials as the corresponding chloroformylated derivatives **9** are unstable and, once isolated (yields 60–95%), have to be stored at low temperatures (−18 °C). *N*-Unsubstituted tetrahydroindole **8a**, due to the poor solubility in the solvent mixture used for the chloroformylation, did not succeed in reacting with the Vilsmeier–Haack reagent, producing the corresponding chloro-aldehyde **9a** in very low yields (3%), and the starting material was recovered almost quantitatively from the reaction mixture even after prolonged reaction time. Although the BOC protective group of **8e** increased the solubility of the compound, the corresponding chloroformylated product **9e** was isolated only in 10% yield since it was very unstable and difficult to handle. In the case of the *N*-methyl tetrahydroindole **8c**, along with the desired chloroformylated compound **9c**, was isolated the chloro-bisformylated derivative **10** in 20% yield. Instead, in the case of derivative **8k**, the bisformylated compound **11** was the only reaction product isolated in 90% yield. The formation of this product could not be avoided by decreasing the reaction temperature (−40 °C) and/or the reaction time (1–2 min). When stoichiometric amount of Vilsmeier–Haack reagent was used, the reaction did not proceed and when prolonging the heating, tarry material was formed and only the 2-formyl derivative **8l** was isolated from the reaction mixture (40%) (scheme 1). The next step in the synthesis was the nucleophilic substitution of the chlorine atom of the chloroaldehydes **9b–d,f,j**



a $R=R_2=H$, $R_1=Me$; **b** R=Ph, $R_1=Me$, $R_2=H$; **c** $R=R_1=Me$, $R_2=H$; **d** R=Bn, $R_1=Me$, $R_2=H$; **e** R=BOC, $R_1=Me$, $R_2=H$; **f** $R=SO_2Ph$, $R_1=Me$, $R_2=H$; **g** R=H, $R_1=CO_2Et$, $R_2=Me$; **j** $R=R_2=Me$, $R_1=CO_2Et$.

Scheme 1. Reagents and conditions: (i) AcONH4 or RNH2, AcOH, 60 °C; (ii) $(CO_2Et)_2C{=}NOH/Zn$, AcOH, reflux; (iii) compound **8e:** *t*-Butyl dicarbonate, DMAP, benzene/DMF, reflux; compound **8f** benzensulfonylchloride, NaH, DMF, r.t.;(iv) NaOH, EtOH, reflux; (v) HCl, EtOH, r.t.; (vi) NaH, THF, MeI, rt; (vii) $DMF/POCl_3$, DCM, 0 °C then reflux; (viii) $DMF/POCl_3$, DCM, −40 °C.



a R=R_2=H, R_1=Me; **b** R=Ph, R_1=Me, R_2=H; **c** R=R_1=Me, R_2=H; **d** R=Bn, R_1=Me, R_2=H; **e** R=BOC, R_1=Me, R_2=H; **f** R=SO_2Ph, R_1=Me, R_2=H; **j** R=R_2=Me, R_1=CO_2Et; **l** R=R_2=Me, R_1=CHO; **m** R=R_2=Me, R_1=CH=CHCO$_2$Et; **n** R=R_2=Me, R_1=CHCHCO$_2$H.

Scheme 2. Reagents and conditions: (i) HSEt, K_2CO_3, DMF, r.t.; (ii) DDQ, benzene, reflux; (iii) $(EtO)_2P(O)CH_2O_2Et$/*t*-BuOK, DMF, r.t then 50 °C; (iv) KOH, EtOH 50%, reflux; (v) PPA, Δ.

and **11** which afforded the thioethers **12b**–**d**,**f**,**j**,**l** in excellent yields (80–95%), upon reaction with the ethanthiolate anion, generated in situ by using ethanthiol and potassium carbonate as the base. Oxidation with DDQ of thioethers **12b**–**d**,**f** gave good yields (65–85%) of the corresponding stable aromatic aldehydes **13b**–**d**,**f**. Transformation of the thioethers **12b**–**d**,**f**,**j**,**l** and **13b**–**d**,**f** into the vinylogous esters was attempted, using the Wittig–Horner reaction with triethyl phosphonoacetate and barium hydroxide catalyst (C-200) under solid–liquid phase-transfer conditions.[7] However, under these conditions, our substrates reacted very slowly and reactions did not reach completeness. Best results were achieved with the Wittig–Horner reaction, whose anion was generated in situ in the presence of *t*-BuOK as base in anhydrous dimethylformamide. Cooling the reaction mixture during the addition of triethyl phosphonoacetate was crucial to avoid self-condensation products of the generated anion. The esters **14b**–**d**,**f**,**j**,**m** (75–92%) and **15b**–**d**,**f** (70–96%) were obtained in good to excellent yields and easily hydrolyzed to the corresponding acids **16b**–**d**,**f**,**j**,**n** (72–93%) and **17b**–**d**,**f** (65–93%), respectively. The first attempt to cyclize the α–β unsaturated acids **16** and **17** was performed using trimethylsilyl polyphosphate prepared from P_2O_5 and hesamethyldisiloxane in chloroform according to the Yamamoto procedure.[8] However, such reagent was unsuitable for our purpose giving tarry reaction mixtures from which we were not able to isolate the desired tricyclic compounds. When the dihydro acids **16b**–**d**,**f**,**j**,**n** were heated at temperatures ranging from 70 to 130 °C in PPA, cyclization occurred, with the exception of derivative **16j**,**n** allowing thus isolation of the corresponding tetrahydro thiopyrano indoles **5b**–**d**,**f** (30–40%). Instead, in the case of the aromatic acids **17** only thiopyrano indoles **6b**,**c** (30–32%) were obtained (Scheme 2). Derivatives **6d**,**f** could not be isolated also using $ZnCl_2$ as Lewis acid to promote the desired ring closure. Attempts of direct oxidation of the polycondensed system **5** with DDQ in dichloromethane or benzene to get derivatives **6** were not successful.

2.2. Biology

2.2.1. Physico-chemical properties

The test compounds represent isosters of the typical tricyclic system of angelicin. Both absorption and emission maxima wavelengths of the compounds exhibited (Table 1) a remarkable bathochromic shift with respect to angelicin (299 nm) as reference compound. The relative fluorescence quantum yields were measured at 298 K.[9] As it can be observed from Table 1, all derivatives showed fluorescence quantum yields values similar to that of angelicin. Partition coefficients of the test compounds were determined by a computational method.[10] The results indicated that the thiopyrano were highly hydrophobic in comparison to angelicin.

2.2.2. Phototoxicity

The phototoxicity of test compounds was investigated on a panel of cultured cell lines of human tumor such as HL-60 (promyelocityc leukaemia), Jurkat (T cells leukaemia), HT-1080 (fibrosarcoma) and LoVo (intestinal adenocarcinoma). We also used an immortalized cell line of human keratinocytes NCTC-2544 for comparison. Table 2 shows the extent of cell survival expressed as IC_{50}, which is the concentration (μM) that induces 50% of the inhibition of cell growth, after irradiation at different UVA doses (2.5 and 3.75 J cm^{-2}). Control experiments with UVA light or drugs alone were carried out without significant cytotoxic effects (data not shown). It can be noted that the compounds exhibited different values of IC_{50}, according to the substitution pattern, and generally a remarkable UVA dose -dependence.

Almost all the compounds inhibited the proliferation of tumor cells at submicromolar/micromolar concentration. The most interesting compound was **6c** whose IC_{50} values were comparable, if not lower, than 8-methoxypsoralen (8-MOP) and Angelicin (ANG), taken as reference compounds. The most active compound **6c** is a fully aromatic derivative and this characteristic seems to be important for the photoactivity since the corresponding dehydroderivative **5c** is devoid of any effect. It is interesting to note that among the thiopyrano indoles the most active compounds were the aromatic derivatives at variance with the pyrroloquinolinones series in which the aromatic derivative was devoid of activity.

2.2.3. Effect of scavengers on thiopyrano indole 6c induced phototoxicity

In an attempt to evaluate the possible mechanism of action of these derivatives, we carried out a series of experiments, in which various scavengers were included in the LoVo cell cultures during

Table 1
Physico-chemical parameters for thiopyrano derivatives **5b–d,f** and **6b,c**.

Cpd	λ_{max}.absorb[a]	ε ($M^{-1}{}^{*}cm^{-1}$)[a]	λ_{max}.absorb[b] (nm)	λ_{exc}(nm)	λ_{max} emiss[b] (nm)	Φ_F[c]	*c*Log*P*[d]
4b	401	19,263	407	367	479	3.5×10^{-2}	+4.69
4c	382	11,450	414	407	458	n.d.[e]	+2.82
4d	411	15,182	414	370	477	3.2×10^{-2}	+4.33
4f	395	13,744	392	345	417	n.d.	+3.88
6b	348	9693	354	390	424, 447, 484	$<10^{-3}$	+5.48
6c	353	7849	362	300	447	3.1×10^{-2}	+3.26
ANG	306	14,602	299	343	413	1.8×10^{-2}	+2.08

Cpd, compound; absorb, absorbance; emiss, emission.
[a] Measured in dimethyl sulfoxide in a concentration range of 5–20 μM.
[b] Measured in phosphate buffer 10 mM pH 7.2.
[c] Relative fluorescence quantum yield determined in 0.1 N H_2SO_4 as described in Ref. 9.
[d] Calculated as described in Ref. 10.
[e] n.d., not determined.

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

Compound	(IC_{50}, μM)[a]									
	HL-60[b]		Jurkat		HT-1080		LoVo		NCTC-2544	
	2.5 J/cm^2[c]	3.75 J/cm^2	2.5 J/cm^2	3.75 J/cm^2	2.5 J/cm^2	3.75 J/cm^2	2.5 J/cm^2	3.75 J/cm^2	2.5 J/cm^2	3.75 J/cm^2
5b	6.2 ± 0.6	2.7 ± 0.3	4.2 ± 0.4	3.5 ± 0.4	11.0 ± 0.1	10.5 ± 0.1	>20	12.3 ± 0.2	>20	>20
5c	>10	>10	>10	>10	>20	>20	>20	>20	>20	>20
5d	4.0 ± 0.4	2.7 ± 0.3	4.0 ± 0.4	2.2 ± 0.3	11.2 ± 0.2	9.8 ± 0.7	>20	7.2 ± 0.8	>20	>20
5f	>10	>10	7.3 ± 0.7	5.0 ± 0.4	>20	>20	>20	>20	>20	>20
6b	2.6 ± 0.3	1.3 ± 0.2	2.5 ± 0.3	3.5 ± 0.4	5.4 ± 0.5	2.0 ± 0.2	5.1 ± 0.5	4.2 ± 0.4	17.0 ± 1.7	14.8 ± 1.5
6c	0.6 ± 0.06	0.3 ± 0.1	0.5 ± 0.03	0.2 ± 0.01	0.9 ± 0.1	0.6 ± 0.03	1.0 ± 0.1	0.8 ± 0.2	2.1 ± 0.2	1.6 ± 0.2
ANG	1.2 ± 0.1	0.9 ± 0.2	0.9 ± 0.2	n.d.[d]	15.7 ± 1.9	2.6 ± 0.2	1.6 ± 0.2	0.9 ± 0.1	4.2 ± 0.5	1.5 ± 0.3
8-MOP	1.4 ± 0.2	1.2 ± 0.4	1.2 ± 0.3	n.d.	7.8 ± 0.7	1.9 ± 0.2	1.1 ± 0.4	0.7 ± 0.1	5.5 ± 0.6	0.9 ± 0.1

[a] Concentration of compound required to inhibit the cell growth by 50% after 72 h of exposure as determined by MTT assay.
[b] Human cell lines: HL-60 human promyelocityc leukaemia, Jurkat human T-cell leukaemia, HT-1080 fibrosarcoma cells. LoVo intestinal adenocarcinoma, NCTC-2544 human keratynocytes.
[c] Dose UVA expressed in J cm^{-2} as measured at 365 nm with a Cole–Parmer radiometer.
[d] n.d., not determined.

irradiation using the most active compound **6c**. The results of these studies are presented in Figure 1. We added sodium azide, a triplet excited state quencher (thus avoiding the formation of singlet oxygen via triplet excited state); superoxide dismutase (SOD), catalase (CAT) which scavenge $O^{\bullet -}$ and H_2O_2, respectively; *N*-*N*′-dimethylthiourea (DMTU) and Mannitol (MAN), which scavenge hydroxyl radicals ($OH^{?}$) and the free radical scavengers 2,6-di-*t*-butylhydroxyanisole (BHA), tocopherol (TOC) and reduced glutathion (GSH). Scavengers on their own did not have any effect on cell viability during UVA exposure (data not shown). A strong protective effect was observed with GSH and BHA, DMTU suggesting that the mechanism of action of thiopyrano indole **6c** was mainly mediated by free radicals and in particular OH**.** radicals. No protective effects were observed with NaN_3 indicating that singlet oxygen could not been involved in the photocytotoxic activity of the test compound. Moreover, a modest protection was observed with SOD, CAT and TOC.

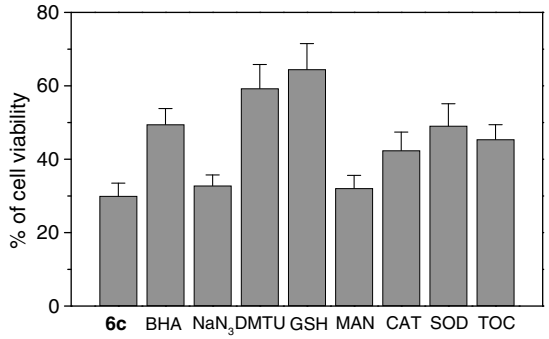


Figure. 1. Effect of different scavengers on the phototoxicity induced by compounds **6c** in LoVo cells. Cell viability was assayed by MTT test after 72 h from the irradiation (2.5 J cm^{-2}) in the presence of 1.25 μM **6c** and SOD (2000 U I/ml), CAT (2000 U I/ml), BHA (10^{-5} M), Man (10 mM), GSH (1 mM), DMTU (1 mM), NaN_3 (10 mM) and Tocopherol (60 μM).

2.2.4. Externalization of phosphatidylserine

With the purpose to investigate the mode of cell death (apoptosis or necrosis), we carried out various experiments irradiating the cells in the presence of **6c** since this compound alone in dark has showed no activity. Apoptosis is characterized by a variety of morphological characteristics. Changes in the plasma membrane are one of the earliest of these features. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment.[11] Annexin-V is a 35–36 kDa Ca^{2+}-dependent phospholipid binding protein which possesses high affinity for PS, and binds to cells with exposed PS. We performed a biparametric cytofluorimetric analysis using propidium iodide (PI) and AnnexinV-FITC, which stain DNA and phosphatidylserine (PS) residues, respectively.[12] Figure 2 shows the percentage of Annexin-V positive Jurkat cells after 24 h from UVA irradiation (2.5 J cm^{-2}) in the presence of **6c** at different concentrations. A dose-dependent induction of apoptotic cells was observed in good agreement with MTT experiments. A small percentage of necrotic cells (2–3%) was detected only at the highest employed concentration (data not shown).

2.2.5. Mitochondrial depolarization

As demonstrated above, irradiation of Jurkat cells in the presence of **6c** induced apoptosis, thus this observation prompted us

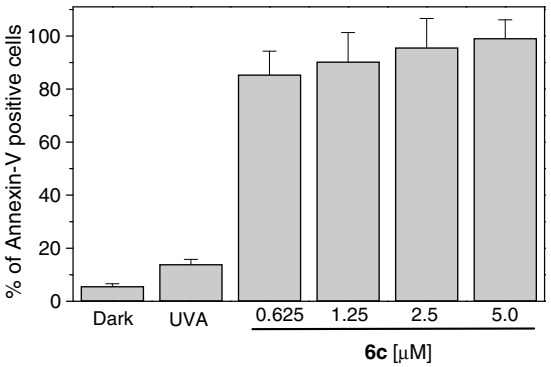


Figure 2. Percentage of Annexin-V positive cells for **6c** after 24 h from the irradiation. Jurkat cells were irradiated (2.5 J cm^{-2}) in the presence of **6c** at the indicated concentrations and analysed after 24 h by flow cytometry as described in Section 4.

to investigate the activation of apoptotic process after the treatment with **6c**. Impairment in mitochondrial function is an early event in the executive phase of programmed cell death in different cell types and appears as the consequence of a preliminary reduction of the mitochondrial transmembrane potential.[13,14] The early $\Delta\psi$mt disruption would be the result of an opening of mitochondrial transition pores, and this permeability transition would trigger the release of apoptogenic factors such as Apoptosis Inducing Factor (AIF) or cytochrome *c*.

We used the lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine (JC-1)[15] to monitor the changes in $\Delta\psi$mt induced by the thiopyrano indole **6c** in combination with UVA irradiation. Cells were irradiated with the UVA dose of 2.5 J cm^{-2} and **6c** at the concentration of 5 μM. Cells were harvested and analyzed at different times from the irradiation. The results are presented in Figure 3.

A rapid increase of the percentage of cells with a collapsed $\Delta\psi$mt, can be observed, and this may be a signal of the opening of the mitochondrial megachannels also called the permeability transition pores (PTP). These results suggested that mitochondria were strongly involved in the induction of apotosis photoinduced by derivative **6c**.

2.2.6. Mitochondrial reactive oxygen species production

The mitochondrial membrane depolarization has been associated with mitochondrial production of reactive oxygen species (ROS).[16] Therefore, we investigated whether ROS production increased after irradiation with the test compounds. To analyze the effects of **6c** on the production of ROS during apoptosis, we utilized the fluorescence indicator hydroethidine (HE), whose fluorescence appeared if reactive oxygen species were generated.[17] HE was oxidized by superoxide anion into ethidium bromide, which emitted red fluorescence. Superoxide is produced by mitochondria due to a shift from the normal 4-electron reduction of O_2 to a 1-electron reduction when cytochrome *c* is released from mitochondria. ROS generation was also measured by the dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), which was oxidized to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) by a variety of peroxides including hydrogen peroxide.[17] The results are shown in Figure 4. It can be noticed that there was a remarkable production of ROS, as evidenced by the high percentage of HE- and DCFDA-positive cells, in excellent agreement with mitochondrial membrane depolarization.

In parallel, we evaluated the damage produced by ROS in mitochondria by assessing the oxidation state of cardiolipin, a phospholipid restricted to the inner mitochondrial membrane. We used 10-*N*-nonyl acridine orange (NAO), a probe whose fluorescence is independent from mitochondrial permeability transition.[18] The dye interacts stoechiometrically with intact non-oxidized cardiolipin. In good agreement with the production of ROS, treated cells showed a dramatic increase in the oxidized cardiolipin.

2.2.7. Oxidative stress

With the aim of assessing the effect of ROS accumulation on cellular redox potential we measured the rate of redox modulator glutathione (GSH) in Jurkat cells irradiated in the presence of **6c** at different concentrations. For this purpose, flow cytometric analysis was carried out using monobromobimane (mBBr) as specific probe.

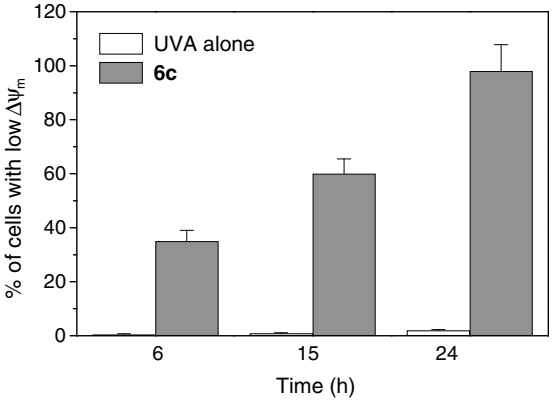


Figure 3. Induction of mitochondrial depolarization by **6c** (5 μM) at different times from the irradiation (2.5 J cm^{-2}). Data are represented as means SEM of three experiments.

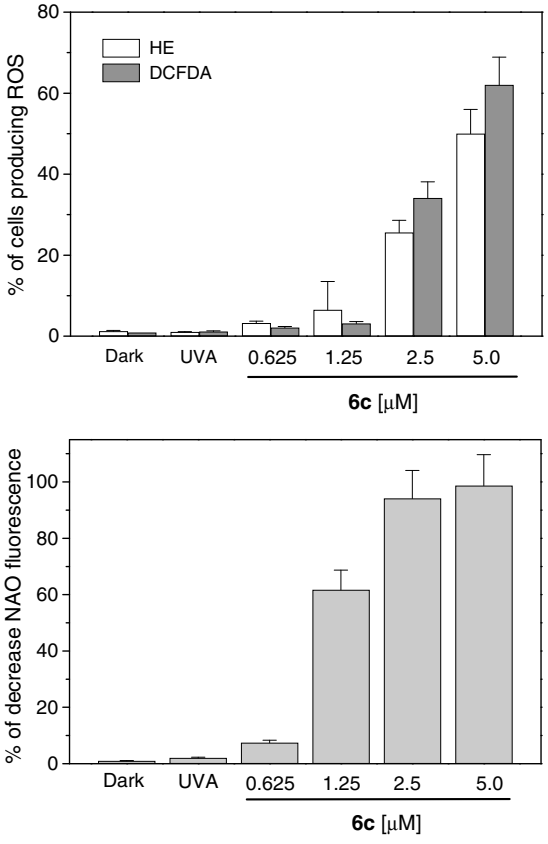


Figure 4. Production of reactive oxygen species (upper panel) and cardiolipin oxidation (lower panel) in Jurkat cells after 24 h from the irradiation (2.5 J cm^{-2}) at the indicated concentrations of **6c**. Data are represented as means SEM of three experiments.

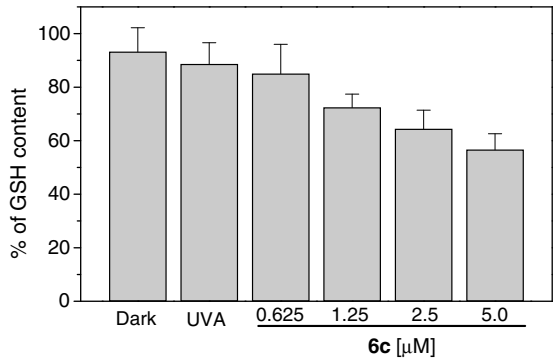


Figure 5. Decreased levels of intracellular GSH after 24 h from the irradiation (2.5 J cm^{-2}) of Jurkat cells in the presence of **6c** at the indicated concentrations. Data are represented as means SEM of three experiments.

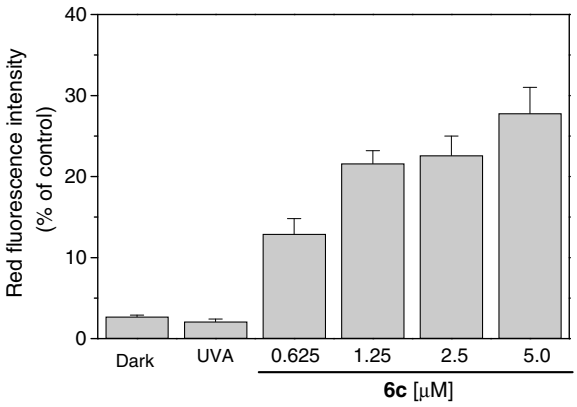


Figure 6. Percentages of cells stained with AO (acridine orange) were reported and analysed by flow cytometry, after 24 h from the irradiation in the presence of **6c** at the indicated concentrations.

The cell permeable mBBr probe is non fluorescent but forms a fluorescent adduct with GSH (GS-mBBr) in a non enzymatic reaction.[19] Cellular appearance of GS-mBBr fluorescence was monitored after 24 h, and results are depicted in Figure 5. The intracellular content of GSH of Jurkat cells was observed to fall after irradiation in the presence of **6c** in a concentration dependent manner. The reduction in GSH content is well correlated with the appearance of HE and DCFDA positive cells after treatment with **6c**.

2.2.8. Assessment of lysosomal stability

It has been previously shown that mitochondrial and/or lysosomial alterations are involved in cell death caused by many photosensitisers including fluoroquinolones[20] and porphyrins.[21] Furthermore, in a previous paper, we found out the preferential disposition of pyrroloquinolinones in lysosomes.[5b] To investigate the integrity of lysosomes after irradiation with **6c**, we performed flow cytometric analysis using the fluorescent dye acridine orange (AO). AO is a lysosomotropic base and a metachromatic fluorochrome exhibiting red fluorescence when highly concentrated, as in the case of intact lysosomes where AO is retained in its charged protonated form, and green fluorescence at low concentration as in damaged lysosomes. In this case, AO relocates to the cytosol where it is predominantly in the deprotonated form.[22,23] The number of intact lysosomes can be evaluated by assaying red fluorescence after the AO staining of the cells exposed to the photosensitizer. As can be observed in Figure 6, a significant extent of lysosomal damage was photoinduced by **6c** 24 h after the irradiation, although it was lower than the mitochondrial damage described above. Altogether the results suggest that lysosomes are not preferential sites of photosensitization for this derivative.

2.2.9. Caspase-3 assay

Many studies demonstrated that programmed cell death is associated with activation of the large family of cysteine proteases (caspases), as key elements involved in the sequence of events that lead to cell death.[24] Caspase-3, in particular, is essential for the propagation of the apoptotic signal after exposure to many DNA-damaging agents, such as anticancer drugs, and in most cases of photodynamic therapies it is activated by the photosensitisers used.[25,26] Therefore, it was interesting to evaluate the effect of **6c** on the activity of caspase-3. Lysates from Jurkat cells were prepared and incubated for 24 h after the irradiation in the presence of **6c** at different concentrations. Then, they were assayed for activity of caspase-3, using an assay solution in which the tetrapeptide substrate Ac-DEVD (*N*-acetyl-Asp-Glu-Val-Asp) was conjugated with a chromophore (*p*-NA, *p*-nitroanilina). The results (Fig. 7) indicated that 24 h after irradiation, **6c** was able to activate caspase-3 in a dose dependent manner, suggesting that the mitochondrial apoptotic pathway plays a major role in the cellular death photoinduced by **6c**.

2.2.10. Lipid peroxidation

Being hydrophobic as demonstrated by their partition coefficients, the thiopyrano indole derivatives may be expected to localize mainly in plasma and/or subcellular membranes, making these structures particularly sensitive to photodamage.[27] The thiobarbituric acid (TBA) assay was used in order to determine whether lipid peroxidation occurred upon irradiation of Jurkat cells incubated in the presence of test compound.[28] The TBA test was performed on the supernatant medium and in the cell homogenates 24 h after irradiation.[28] Figure 8 presents the results for **6c** as a function of light doses. Thiobarbituric reactive substances (TBARS) were significantly produced when the cells were exposed to the compound and UVA both in the supernatant and in the cell extract.

2.2.11. Protein photodamage

The photosensitization capacity of **6c** towards other components of cellular membranes, such as proteins, was estimated by measuring the photoinduced cross-linking in erythrocyte ghost proteins.[29] Light-induced cross-linking of spectrin, a protein associated with the cytoplasmic side of the RBC membrane, was detected by the partial or total disappearance of the two spectrin bands (220.000 and 245.000 Da) on SDS–PAGE as cross-linked aggregates were not able to run inside the gel and remained at the top.

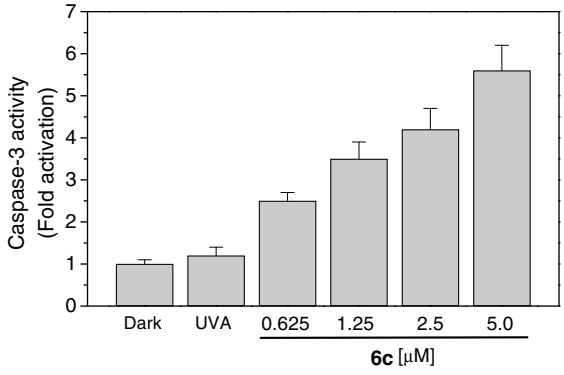


Figure 7. Caspase-3 induced activity by **6c**. Jurkat cells were exposed to 2.5 J cm^{-2} of UVA in the presence of increasing concentrations of **6c**. After 24 h, cells were harvested and the lysates were assayed for caspase-3 activity using the caspase-3 substrate Ac-DEVD-pNA. Data are represented as fold increase of activity of the enzyme in comparison to the non-irradiated control.

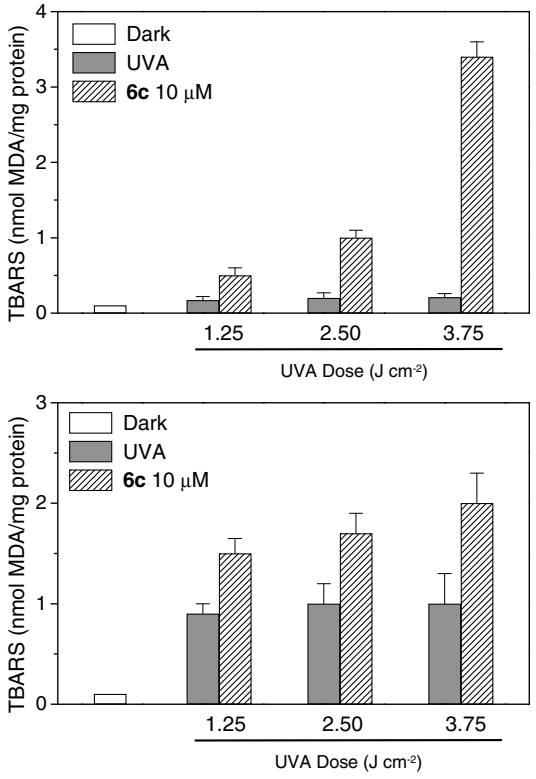


Figure 8. Lipid peroxidation induced in NCTC-2544 cells after 24 h from the irradiation at the indicated UVA doses in the presence of **6c** at the concentration of 10 μM. The TBA test was performed on the supernatant medium and on the cell homogenates as described in Section 4. Upper panel: supernatant medium, lower panel: cell homogenates.

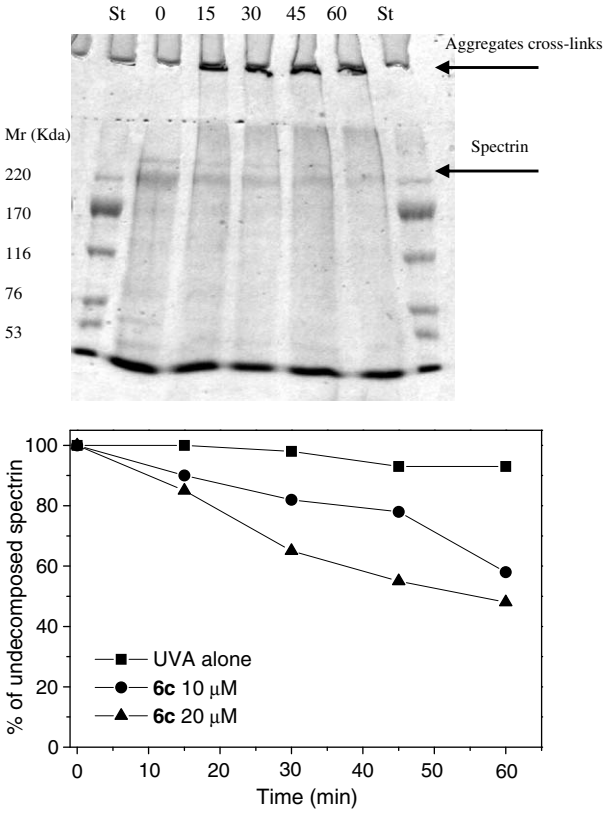


Figure 9. Upper panel: Electrophoretic pattern of the photoinduced cross-link of spectrin in RBC ghosts irradiated at the indicates times in the presence of **6c** at the concentration of 10 μM. Spectrin band and cross-link aggregates are indicated by an arrow. St is a mixture of molecular weight standard, whose values are depicted in the left of the figure. Lower panel: Quantitation by gel densitometry of the relative abundance of spectrin after irradiation at various UVA doses in the presence of **6c** at different concentrations.

Figure 9 shows the pictures of the SDS–PAGE gels of ghosts irradiated in the presence of **6c**. The compound caused an UVA dose-dependent disappearance of the two spectrin subunit bands.

In order to investigate more deeply photosensitizing properties of the title compounds towards proteins, solutions containing bovine serum albumin (BSA) or ribonuclease A (RNAseA) as models[30] 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.[31] The results are reported in Figure 10 (upper panels) and demonstrate that **6c** significantly increased the carbonyl content of BSA and RNAse after irradiation.

Moreover, the amount of the aromatic aminoacid tryptophan (Trp) was directly analysed by monitoring the characteristic fluorescence of Trp residues.[32] As depicted in Figure 10 (lower panels), a rapid decrease of the emission fluorescence was observed for **6c**. No effects were observed when BSA was irradiated in the same conditions without **6c**. Another protein model used in this study was RNAseA, which is devoid of Trp but has Tyr residues in its sequence.[33] As a consequence, its fluorescence emission band, centred at about 350 nm, was totally due to Tyr residues. RNAseA emission band decreases less than BSA after irradiation in the presence of compounds **6c**.

2.2.12. DNA interactions and DNA-photodamage

In order to understand the binding nature between compound **6c** and DNA, linear (LD) and circular dichroism (CD) measurements[34,35] were performed on solutions with salmon testes DNA (st-DNA) and the title compound at various molar ratios. Inspection of the LD spectra (Fig. 11, upper panel) in the presence of st-DNA reveals that despite a strong absorption in the 300–400 nm region, the compound give no LD bands in this region indicating that the compound is not intercalated into DNA. Furthermore CD spectra carried out with the same derivatives, showed that increasing concentrations of **6c** does not lead to significant changes of the CD spectrum of st DNA confirming that the title compound are loosely bound to DNA. These facts strongly suggest that compound **6c** do not interact efficaciously, without irradiation, with the macromolecule, as indeed demonstrated with natural furocoumarins.[2]

Moreover, further experiments were performed in order to determine whether the new derivatives were able to photosensitize DNA strand breakage. Supercoiled circular DNA allowed the detection of structural alterations such as strand breaks or oxidatively damaged bases. We determined the degree of oxidative base damage using two base excision repair enzymes: Formamidopyrimidine DNA glycosilase (Fpg) and Endonuclease III (Endo III) that cleave oxidized DNA at the sites of modified purine and pyrimidine bases, respectively. The excision of damaged base is followed by a DNA breakage.[36]

In addition to endonuclease sensitive modifications, the number of single strand breaks generated by the excited photosensitizer was quantified. Figure 12 (upper panel) shows the results obtained for **6c** tested at different [DNA]/[drug] ratios. The test compound was not able to induce DNA damage without UVA irradiation. For compound **6c**, the formation of single strand breaks, expressed as the percentage of form II (Fig. 11), is not significantly different from the irradiated control, even at the highest ratio employed. On the contrary, it was able to photooxidize nucleotidic bases, as assessed after enzymatic digestion with Fpg. and Endo

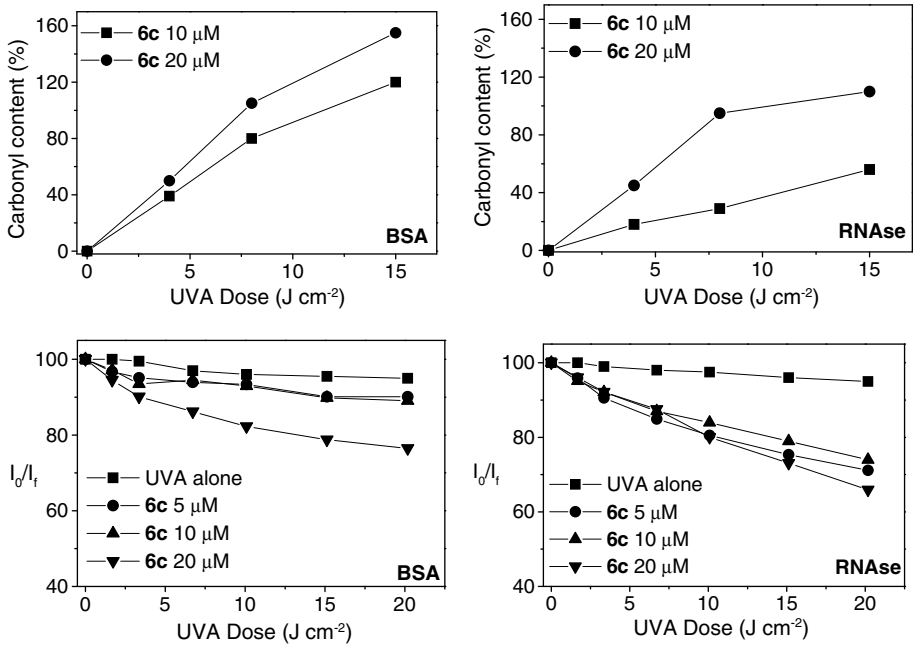


Figure 10. Photosensitized protein damage by **6c**. BSA and RNAse were irradiated at different UVA dose in the presence of **6c** in phosphate buffer at the indicated concentration. Protein oxidation was evaluated by monitoring the carbonyl content (upper panels) and the loss of emission intensity of the proteins (lower panels).

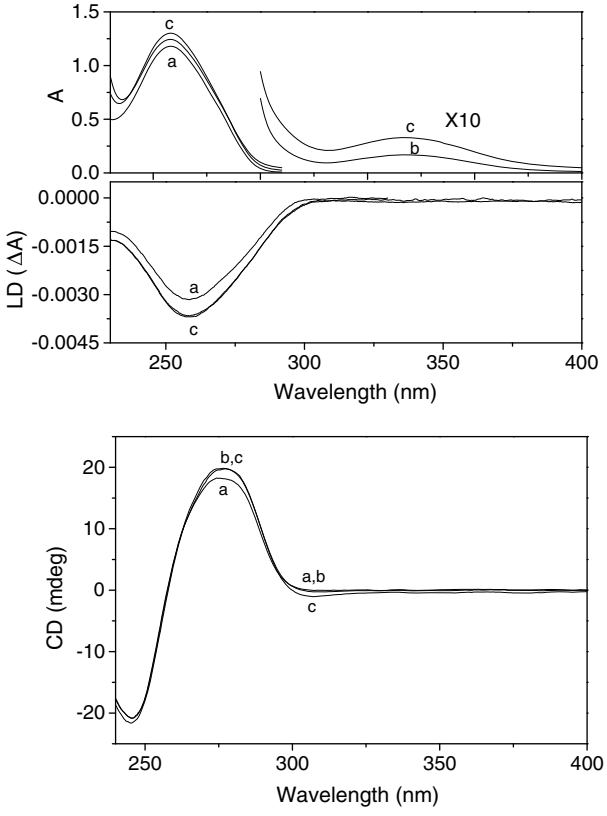


Figure 11. Upper panel: Absorbance (A), and Linear dichroism (LD) spectra of mixtures of salmon testes DNA and compounds **6c** at different [Drug]/[DNA] ratio (*a* = 0.00, *b* = 0.02, *c* = 0.04). Lower panel: Circular dichroism spectra of of mixtures of salmon testes DNA and compounds **6c** at different [Drug]/[DNA] ratio (*a* = 0.00, *b* = 0.02, *c* = 0.04).

III. In fact, high levels of single strand breaks were detected after enzyme digestion and in particular after Endo III treatment suggesting a preferential oxidation of pyrimidine bases.

In order to investigate the possible mechanism of action of the photosensitized DNA damage, the same experiments were performed in the presence of Mannitol, sodium azide and GSH. It can be observed (Fig. 12 lower panel) that a significative reduction of the photoinduced DNA damage was observed with mannitol and GSH indicating the formation of free radicals species and/or as intermediates. No protection was observed in the presence of NaN_3 suggesting that singlet oxygen is not involved in the mechanism of action.

3. Conclusions

The data reported herein indicate that thiopyrano[2,3-*e*]indol-2-ones are endowed with a potent photoantiproliferative activity, and they could represent a new interesting class of potentially useful compounds in photochemotherapy. The most active compound, **6c**, presented a double bond in five-six position and a methyl group in seven position. Less active were the dihydro derivatives at variance with the pyrroloquinolinones series in which the dihydroderivatives showed potent photoantiproliferative activity, and the aromatic compound was not active. An important feature of these new compounds seems to be their incapability to bind and form covalent adduct with DNA upon UVA irradiation although, as demonstrated in this paper, they are able to photooxidize DNA bases in particular pyrimidine ones. This could be important in view of the long term toxic effect such as skin cancer or mutagenesis exhibited by psoralen.

A preliminary indication about their mechanism of action derives from the experiments of photocytotoxicity carried out in the presence of different scavengers. The results demonstrated the participation of free radicals and in particular the involvement of the hydroxyl radical.

We have demonstrated using the most phototoxic compound **6c** that after UVA irradiation the most prevalent mode of cell death is apoptosis. The apoptotic process, involves principally a mitochondrial damage as clearly demonstrated by the loss of mitochondrial potential and cardiolipin oxidation after irradiation of Jurkat cells in the presence of **6c**. In addition the fundamental role played by

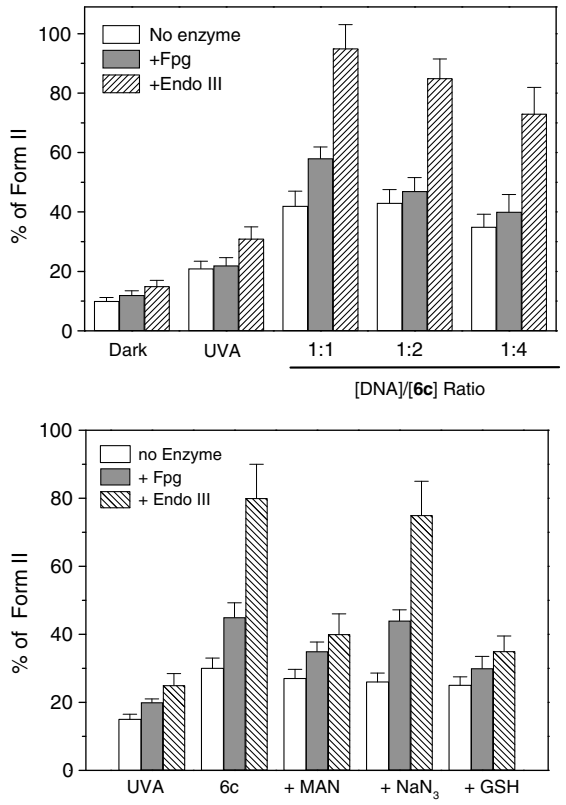


Figure 12. Upper panel: DNA strand breaks photoinduced by the compounds **6c**, expressed as percentage of form II obtained after densitometric analysis of the gel. pBR322 supercoiled circular DNA was irradiated at the doses of 3.5 J cm^{-2} at the DNA]/[**6c**] ratios of 1, 2 and 4, and then treated with the base excision repair enzymes Fpg and Endo III, as described in Section 4. Lower panel: Induction of strand breaks in supercoiled pBR322 plasmid DNA, by **6c** at molar [DNA]/[**6c**] ratio of 1 after UVA irradiation (3.5 J cm^{-2}) in the presence of different conditions as indicated in the figure. MAN (10 mM), GSH (1 mM), NaN_3 (10 mM). After irradiation the photosensitised DNA was treated with the enzymes Fpg and Endo III as described in experimental section. Data are expressed as percentage of form II obtained after densitometric analysis of the digital picture of the agarose gel, and represent means ± SEM of three independent experiments.

mitochondria, was further confirmed by the activation of caspase-3. Other subcellular organelles such as lysosomes appear less susceptibile to the phototoxic action of compound **6c**.

At molecular level the effects of **6c** seems to be mediated by a photodamage to lipid, proteins and DNA. In this context it will be necessary to carry out further investigation on the photochemical and photophysical aspect to better clarify their mechanism of action.

4. Experimental

4.1. Chemistry

All melting points were taken on a Buchi–Tottoli capillary apparatus and were uncorrected; IR spectra were determined with a Jasco FT/IR 5300 spectrophotometer; ^1H 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 using a BÜCHI apparatus. Elemental analyses (C, H, N) were within ±0.4% of the theoretical values.

4.1.1. Preparation of 1,5,6,7-tetrahydro-4H-indole-4-ones (8a–k)

4.1.1.1. 2-Methyl-1,5,6,7-tetrahydro-4H-indole-4-one (8a). Derivative **8**a was prepared by reaction of 2-acetonyl-1,3-cyclohexanedione **7a** with ammonium acetate in acetic acid at 60 °C for 2 h.[5b] After cooling the reaction mixture was poured onto crushed ice and the precipitate formed was filtered, air dried and purified by recrystallization from ethanol to give **8a** (88%): mp 198–200 °C; IR: 3212–2939 (NH), 1624 (CO) cm^{-1}; ^1H NMR: δ 1.94 (quint, J = 5.9 Hz, 2H, CH_2), 2.09 (s, 3H, CH_3), 2.21 (t, J = 5.9 Hz, 2H, CH_2), 2.65 (t, J = 5.9 Hz, 2H, CH_2), 5.86 (s, 1H, H-3), 11.01 (s, 1H, NH). ^{13}C NMR: δ 12.4 (CH_3), 22.1 (CH_2), 23.7 (CH_2), 37.6 (CH_2), 101.7 (CH), 119.6 (C), 128.2 (C), 142.8 (C), 192.3 (CO). Anal. $C_9H_{11}NO$: C, 72.46; H, 7.43; N, 9.39. Found: C, 72.68; H, 7.10; N, 9.08.

Derivatives **8b–d** were prepared according to previous procedures.[5b]

4.1.1.2. 1-*t*-Butoxycarbonyl-2-methyl-1,5,6,7-tetrahydro-4H-indole-4-one (8e). To a solution of **8a** (3.5 g, 23 mmol) in anhydrous benzene (100 mL) and DMF (8 mL), dimethylaminopyridine (DMAP) (0.32 g, 3 mmol) and *t*-butyl dicarbonate (10.3 g, 46 mmol) were added. The mixture was heated under reflux for 24 h, then cooled and treated with a saturated solution of sodium hydrogen carbonate. After 30 min stirring, the organic layer was separated and dried. The solvent was removed and the crude material was purified by cromatography column (DCM: EtOAc 95:5) to give **8e** (85%): mp 87–88 °C; IR: 1739 (CO), 1674 (CO) cm^{-1}; ^1H NMR: δ 1.58 (s, 9H, 3× CH_3), 1.95–2.08 (m, 2H, CH_2), 2.34 (t, J = 6.2 Hz, 2H, CH_2), 2.35 (s, 3H, CH_3), 2.99 (t, J = 6.2 Hz, 2H, CH_2), 6.18 (s, 1H, H-3). ^{13}C NMR: δ 15.4 (CH_3), 23.2 (CH_2), 24.6 (CH_2), 27.4 (3× CH_3), 37.0 (CH_2), 84.9 (C), 106.5 (CH), 121.6 (C), 131.9 (C), 144.9 (C), 148.9 (CO), 193.4 (CO). Anal. $C_{14}H_{19}NO_3$: C, 67.45; H, 7.68; N, 5.62. Found: C, 67.20; H, 7.52; N, 5.80.

4.1.1.3. 2-Methyl-1-(phenylsulfonyl)-1,5,6,7-tetrahydro-4H-indole-4-one (8f). To a solution of **8a** (2 g, 13 mmol) in anhydrous DMF (15 mL) sodium hydride (0.36 g, 15 mmol) was added. After 1 h stirring, the solution was ice-cooled and treated with benzensulfonylchloride (2 mL, 15 mmol) under stirring (1 h). The reaction mixture was allowed to reach room temperature then it was poured onto crushed ice and filtered. The solid which separated was filtered off, air dried and and recrystallized from ethanol to give **8f** (98%): mp 82–83 °C; IR: 1664 (CO) cm^{-1}; ^1H NMR: δ 2.05 (q, J = 5.9 Hz, 2 H, CH_2), 2.35 (s, 3H, CH_3), 2.38 (t, J = 5.9 Hz, 2H, CH_2), 3.11 (t, J = 5.9 Hz, 2H, CH_2), 6.31 (s, 1H, H-3), 7.67–7.87 (m, 3H, H-3', H-4', H-5'), 7.93 (d, J = 7.9 Hz, 2H, H-2', H-6'). ^{13}C NMR: δ 14.3 (CH_3), 23.0 (CH_2), 23.7 (CH_2), 36.8 (CH_2), 108.1 (CH), 122.5 (C), 126.5 (2× CH), 130.2 (CH), 132.5 (C), 134.9 (2 × CH), 138.0 (C), 145.3 (C), 193.3 (CO). Anal. $C_{15}H_{15}NO_3S$: C, 62.27; H, 5.23; N, 4.84. Found: C, 62.40; H, 5.53; N, 4.74.

4.1.1.4. 2-Ethoxycarbonyl-3-methyl-1,5,6,7-tetrahydro-4H-indole-4-one (8g). This compound was prepared according to a known procedure.[6] A solution of the commercially available 1,3-cyclohexanedione **7b** (8.8 g, 80 mmol) in acqueous acetic acid (90%, 100 mL) containing sodium acetate (20.5 g, 250 mmol) was heated at 90 °C. Zinc powder (17.6 g, 270 mmol) was added followed by dropwise addition of a freshly prepared solution of diethyl hydroxyl iminomalonate. This latter was prepared by stirring at room temperature for 3 h a solution of ethylacetoacetate (11 g, 80 mmol) in acetic acid (60 mL) with a solution of sodium nitrite (6 g, 90 mmol) dissolved in water. When the addition of diethyl hydroxyl iminomalonate was finished, the reaction mixture was heated at 100 °C for 30 min. After 24 h stirring at room temperature, the mixture was poured onto crushed ice and the solid collected by filtration. Recrystallization with ethanol furnished derivative **8g** (60%): mp 165–167 °C; IR: 3425 (NH),

1685 (CO), 1649 (CO) cm^{-1}; ^1H NMR: δ 1.29 (t, J = 7.4 Hz, 3H, CH_3), 1.98 (quint, J = 5.9 Hz, 2H, CH_2), 2.33 (t, J = 5.9 Hz, 2H, CH_2), 2.48 (s, 3H, CH_3), 2.75 (t, J = 5.9 Hz, 2H, CH_2), 4.21 (q, J = 7.4 Hz, 2H, CH_2), 11.88 (s, 1H, NH). ^{13}C NMR: δ 11.2 (CH_3), 14.3 (CH_3), 22.3 (CH_2), 23.0 (CH_2), 38.6 (CH_2), 59.5 (CH_2), 118.8 (C), 119.0 (C), 126.7 (C), 146.3 (C), 160.9 (CO), 194.3 (CO). Anal. $C_{12}H_{15}NO_3$: C, 65.14; H, 6.83; N, 6.33. Found: C, 64.92; H, 7.00; N, 6.60.

4.1.1.5. 3-Methyl-1,5,6,7-tetrahydro-4H-indole-4-one-2-carboxylic acid (8h). To a suspension of **8g** (7.6 g, 34 mmol) in ethanol (38 mL), 30% NaOH (10 mL) was added. The mixture was heated under reflux for 1 h, then cooled and washed with ethyl ether. The acqueous extract was acidified with 6 N HCl and the solid precipitated was filtered off and dried to furnish derivative **8h** (97%): mp 273–274 °C; IR: 3442 (OH), 3255 (NH), 1670 (CO), 1601 (CO) cm^{-1}; ^1H NMR: δ 1.98 (q, J = 5.8 Hz, 2H, CH_2), 2.32 (t, J = 5.8 Hz, 2H, CH_2), 2.47 (s, 1H, CH_3), 2.74 (t, J = 5.8 Hz, 2H, CH_2), 11.80 (1H, s, NH), 12.52 (1H, s, OH). ^{13}C NMR: δ 10.9 (CH_3), 21.9 (CH_2), 22.7 (CH_2), 38.2 (CH_2), 118.6 (C), 119.2 (C), 125.8 (C), 145.5 (C), 162.1 (CO), 194.0 (CO). Anal. $C_{10}H_{11}NO_3$: C, 62.17; H, 5.74; N, 7.25. Found: C, 62.41; H, 5.50; N, 7.10.

4.1.1.6. 3-Methyl-1,5,6,7-tetrahydro-4H-indole-4-one (8i). To a warm suspension of the carboxylic acid **8h** (4.8 g, 24 mmol) in ethanol (55 mL), 6 N HCl was added dropwise. Stirring was kept until bubbling ceased (20 min). The solvent was removed in vacuo and the crude residue was treated with brine. The solid precipitated was filtered off and dried to furnish derivative **8i** (60%): mp 194–196 °C; IR: 3213 (NH), 1639 (CO) cm^{-1}; ^1H NMR: δ 1.97 (q, J = 6.1 Hz, 2H, CH_2), 2.13 (s, 1H, CH_3), 2.26 (t, J = 6.1 Hz, 2H, CH_2), 2.69 (t, J = 6.1 Hz, 2H, CH_2), 6.44 (s, 1H, H-2), 10.96 (s, 1H, NH). ^{13}C NMR: δ 11.5 (CH_3), 22.4 (CH_2), 23.8 (CH_2), 38.3 (CH_2), 116.1 (CH), 116.7 (C), 117.7 (C), 143.3 (C), 193.7 (CO). Anal. $C_9H_{11}NO$: C, 72.46; H, 7.43; N, 9.39. Found: C, 72.20; H, 7.68; N, 9.55.

4.1.1.7. Methylation of derivatives 8g and 8i. To a suspension of **8g** or **8i** (20 mmol) in anhydrous THF (40 mL) sodium hydride (0.5 g, 20 mmol) was added. After 1 h stirring, iodomethane (1.12 mL, 20 mmol) was added. The mixture was stirred for 5 h for **8g** or 1 h for **8i** at room temperature, then poured onto crushed ice. The solid which separated was filtered off and dried to furnish derivative **8j** or **8k**.

4.1.1.8. 1,3-Dimethyl-2-ethoxycarbonyl-1,5,6,7-tetrahydro-4H-indole-4-one (8j). The solid which separated was derivative **8j** (88%): mp 216–217 °C; IR: 1680 (CO), 1653 (CO) cm^{-1}; ^1H NMR: δ 1.30 (t, J = 7.1 Hz, 3H, CH_3), 2.02 (quint, J = 6.2 Hz, 2H, CH_2), 2.34 (t, J = 6.2 Hz, 2H, CH_2), 2.49 (s, 3H, CH_3), 2.78 (t, J = 6.2 Hz, 2H, CH_2), 3.71 (s, 3H, CH_3), 4.24 (q, J = 7.1 Hz, 2H, CH_2). ^{13}C NMR: δ 12.3 (CH_3), 14.6 (CH_3), 21.9 (CH_2), 22.7 (CH_2), 33.6 (CH_3), 38.9 (CH_2), 60.2 (CH_2), 118.2 (C), 121.0 (C), 128.4 (C), 148.5 (C), 161.7 (CO), 194.7 (CO). Anal. $C_{13}H_{17}NO_3$: C, 66.36; H, 7.28; N, 5.95. Found: C, 66.00; H, 7.46; N, 6.14.

4.1.1.9. 1,3-Dimethyl-1,5,6,7-tetrahydro-4H-indole-4-one (8k). The solid which separated was derivative **8k** (92%): mp 127–128 °C; IR: 1639 (CO) cm^{-1}; ^1H NMR: δ 2.01 (q, J = 6.0 Hz, 2H, CH_2), 2.12 (s, 1H, CH_3), 2.25 (t, J = 6.0 Hz, 2H, CH_2), 2.68 (t, J = 6.0 Hz, 2H, CH_2), 3.46 (s, 1H, CH_3), 6.46 (s, 1H, H-2). ^{13}C NMR: δ 11.3 (CH_3), 20.9 (CH_2), 23.2 (CH_2), 32.7 (CH_3), 37.9 (CH_2), 116.6 (C), 118.0 (C), 121.1 (CH), 143.5 (C), 193.3 (CO). Anal. $C_{10}H_{13}NO$: C, 73.59; H, 8.03; N, 8.58. Found: C, 73.90; H, 7.73; N, 8.92.

4.1.2. General procedure for the chloroformylation of derivatives 8a–f,j,k

A solution of anhydrous DMF (4 mL, 50 mmol) in anhydrous DCM (17 mL) was cooled at 0 °C. Phosphorous oxychloride (4 mL, 43 mmol) was dissolved in anhydrous DCM (4 mL) and added dropwise as to keep the temperature at 0 °C. The mixture was stirred for 10 min and then rapidly heated to reflux. Immediately after the boiling began, a solution of **8a–f,j,k** (13 mmol) in anhydrous DCM (5 mL) was added all at once. The mixture was stirred under reflux for 3 min and then cooled to 10 °C. A 4 N sodium acetate solution (40 mL) and DCM (14 mL) were added to the mixture under stirring. The organic layer was decanted, evaporated under reduced pressure. Upon adding ice to the oily residue a solid separated which was filtered, dried and purified by chromatography column (DCM).

4.1.2.1. From 8a, 4-chloro-2-methyl-6,7-dihydro-1*H*-indole-5-carbaldehyde (9a). Compound **9a** was obtained as white solid (3%): mp 124–125 °C; IR: 3276 (NH), 1633 (CO) cm^{-1}; ^1H NMR: δ 2.17 (s, 3H, CH_3), 2.52–2.66 (m, 4H, 2× CH_2), 5.96 (s, 1H, H-3), 9.95 (s, 1H, CHO), 11.36 (s, 1H, NH). ^{13}C NMR: δ 12.5 (CH_3), 20.2 (CH_2), 22.5 (CH_2), 102.8 (CH), 117.3 (C), 121.0 (C), 130.0 (C), 135.3 (C), 145.2 (C), 187.3 (CHO). Anal. $C_{10}H_{10}ClNO$: C, 61.39; H, 5.15; N, 7.16. Found: C, 61.00; H, 5.48; N, 6.96.

4.1.2.2. From 8b, 4-chloro-2-methyl-1-phenyl-6,7-dihydro-1*H*-indole-5-carbaldehyde (9b). Compound **9b** was obtained as white solid (70%): mp 113–115 °C; IR: 1643 (CO) cm^{-1}; ^1H NMR: δ 2.06 (s, 3H, CH_3), 2.48–2.69 (m, 4H, 2 × CH_2), 6.28 (s, 1H, H-3), 7.39 (d, J = 6.4 Hz, 2H, H-2', H-6'), 7.48–7.62 (m, 3H, H-3', H-4' e H-5'), 10.01 (s, 1H, CHO). ^{13}C NMR: δ 12.3 (CH_3), 20.1 (CH_2), 22.3 (CH_2), 104.3 (CH), 117.2 (C), 122.2 (C), 127.1 (2× CH), 128.6 (CH), 129.5 (2× CH), 131.2 (C), 136.1 (C), 136.2 (C), 144.2 (C), 187.6 (CHO). Anal. $C_{16}H_{14}ClNO$: C, 70.72; H, 5.19; N, 5.15. Found: C, 70.52; H, 5.00; N, 5.45.

4.1.2.3. From 8c, 4-chloro-1,2-dimethyl-6,7-dihydro-1*H*-indole-5-carbaldehyde (9c). Compound **9c** was obtained as white solid (60%): mp 108–110 °C; IR: 1639 (CO) cm^{-1}; ^1H NMR: δ 2.19 (s, 3H, CH_3), 2.61–2.77 (m, 4H, 2 × CH_2), 3.43 (s, 3H, CH_3), 6.06 (s, 1H, H-3), 9.95 (s, 1H, CHO). ^{13}C NMR: δ 11.6 (CH_3), 19.4 (CH_2), 22.1 (CH_2), 30.3 (CH_3), 103.3 (CH), 116.2 (C), 120.8 (C), 131.1 (C), 136.5 (C), 144.8 (C), 187.3 (CHO). Anal. $C_{11}H_{12}ClNO$: C, 63.01; H, 5.77; N, 6.68. Found: C, 62.86; H, 5.57; N, 6.60. From the same reaction mixture it was also isolated 4-chloro-1,2-dimethyl-6,7-dihydro-1*H*-indole-3,5-dicarbaldehyde **10** as white solid (20%): mp 148–149 °C; IR: 1650 (CO), 1648 (CO) cm^{-1}; ^1H NMR: δ 1.90 (s, 3H, CH_3), 2.60 (t, J = 8.2 Hz, 2H, CH_2), 2.80 (t, J = 8.2 Hz, 2H, CH_2), 2.89 (s, 3H, CH_3), 9.65 (s, 1H, CHO), 9.95 (s, 1H, CHO). ^{13}C NMR: δ 11.1 (CH_3), 18.9 (CH_2), 21.6 (CH_2), 30.5 (CH_3), 115.6 (C), 117.8 (C), 124.4 (C), 138.4 (C), 139.3 (C), 142.7 (C), 185.9 (CHO), 188.0 (CHO). Anal. $C_{12}H_{12}ClNO_2$: C, 60.64; H, 5.09; N, 5.89. Found: C, 60.75; H, 4.86; N, 6.09.

4.1.2.4. From 8d, 1-benzyl-4-chloro-2-methyl-6,7-dihydro-1*H*-indole-5-carbaldehyde (9d). Compound **9d** was obtained as white solid (88%): mp 67–69 °C; IR: 1639 (CO) cm^{-1}; ^1H NMR: δ 2.12 (s, 3H, CH_3), 2.64–2.70 (m, 4H, 2 × CH_2), 5.16 (s, 2H, CH_2) 6.16 (s, 1H, H-3), 7.00 (d, J = 6.9 Hz, 2H, H-2', H-6'), 7.29–7.34 (m, 3H, H-3', H-4', H-5'), 9.98 (s, 1H, CHO). ^{13}C NMR: δ 11.8 (CH_3), 19.6 (CH_2), 22.3 (CH_2), 46.7 (CH_2), 104.1 (CH), 116.7 (C), 121.4 (C), 126.1 (2× CH), 127.3 (CH), 128.8 (2× CH), 131.0 (C), 136.5 (C), 137.2 (C), 144.5 (C), 187.4 (CHO). Anal. $C_{17}H_{16}ClNO$: C, 71.45; H, 5.64; N, 4.90. Found: C, 71.25; H, 5.40; N, 4.60.

4.1.2.5. From 8e, 1-*t*-butoxycarbonyl-4-chloro-2-methyl-6,7-dihydro-1*H*-indole-5-carbaldehyde (9e). Compound **9e** was obtained as white solid (10%): mp 101–102 °C; IR: 1747 (CO), 1653 (CO) cm^{-1}; ^1H NMR: δ 1.57 (s, 9H, 3 × CH_3), 2.37 (s, 3H, CH_3), 2.64 (t, J = 9.5 Hz, 2H, CH_2), 3.03 (t, J = 9.5 Hz, 2H, CH_2), 6.23 (s, 1H, H-3), 10.02 (s, 1H, CHO). ^{13}C NMR: δ 15.4 (CH_3), 21.8 (CH_2), 22.2 (CH_2), 27.3 (3 × CH_3), 85.1 (C), 107.4 (CH), 119.4 (C), 124.2 (C), 132.5 (C), 136.3 (C), 141.8 (C), 148.6 (CO), 187.9 (CHO). Anal. $C_{15}H_{18}ClNO_3$: C, 60.91; H, 6.13; N, 4.74. Found: C, 61.11; H, 6.25; N, 4.80.

4.1.2.6. From 8f, 4-chloro-2-methyl-1-(phenylsulfonyl)-6,7-dihydro-1*H*-indole-5-carbaldehyde (9f). Compound **9f** was obtained as white solid (95%): mp 107–108 °C; IR: 1655 (CO) cm^{-1}; ^1H NMR: δ 2.38 (s, 3H, CH_3), 2.68 (t, J = 9.1 Hz, 2H, CH_2), 3.14 (t, J = 9.1 Hz, 2H, CH_2), 6.36 (s, 1H, H-3), 7.67–7.87 (m, 3H, H-3', H-4', H-5'), 7.95 (d, J = 7.2 Hz, 2H, H-2', H-6'), 10.03 (s, 1H, CHO). ^{13}C NMR: δ 14.3 (CH_3), 21.5 (CH_2), 22.2 (CH_2), 109.2 (CH), 120.8 (C), 125.5 (C), 126.6 (2 × CH), 130.3 (CH), 133.0 (C), 135.0 (2× CH), 136.1 (C), 137.8 (C), 140.7 (C), 188.2 (CHO). Anal. $C_{16}H_{14}ClNO_3S$: C, 57.23; H, 4.20; N, 4.17. Found: C, 57.00; H, 4.40; N, 4.40.

4.1.2.7. From 8j, 4-chloro-1,3-dimethyl-2-ethoxycarbonyl-6,7-dihydro-1H-indole-5-carbaldehyde (9j). Compound **9j** was obtained as white solid (80%): mp 112–113 °C; IR: 1685 (CO), 1679 (CO) cm^{-1}; ^1H NMR: δ 1.31 (t, J = 7.0 Hz, 3H, CH_2CH_3), 2.54 (s, 3H, CH_3), 2.59 (t, J = 7.3 Hz, 2H, CH_2), 2.76 (t, J = 7.3 Hz, 2H, CH_2), 3.71 (s, 3H, CH_3), 4.22 (q, J = 7.0 Hz, 2H, CH_2CH_3), 10.08 (s, 1H, CHO). ^{13}C NMR: δ 12.3 (CH_3), 14.1 (CH_3), 19.0 (CH_2), 21.4 (CH_2), 33.3 (CH_3), 59.8 (CH_2), 114.9 (C), 121.8 (C), 124.6 (C), 126.5 (C), 142.6 (C), 143.9 (C), 160.8 (CO), 188.2 (CHO). Anal. $C_{14}H_{16}ClNO_3$: C, 59.68; H, 5.72; N, 4.97. Found: C, 59.85; H, 5.62; N, 5.04.

4.1.2.8. From 8k, 4-chloro-1,3-dimethyl-6,7-dihydro-1*H*-indole-2,5-dicarbaldehyde (11). Compound **11** was obtained as white solid (90%): mp 182–183 °C; IR: 1643 (CO), 1640 (CO) cm^{-1}; ^1H NMR: δ 2.57 (s, 3H, CH_3), 2.62 (t, J = 8.2 Hz, 2H, CH_2), 2.83 (t, J = 8.2 Hz, 2H, CH_2), 3.80 (s, 3H, CH_3), 9.78 (s, 1H, CHO), 10.01 (s, 1H, CHO). ^{13}C NMR: δ 10.2 (CH_2), 18.4 (CH_3), 21.2 (CH_3), 32.6 (CH_2), 115.2 (C), 125.3 (C), 129.1 (C), 131.2 (C), 143.1 (C), 144.9 (C), 178.7 (CHO), 188.2 (CHO). Anal. $C_{12}H_{12}ClNO_2$: C, 60.64; H, 5.09; N, 5.89. Found: C, 60.20; H, 5.15; N, 5.79. When reaction was carried out with stoichiometric amount of the Vilsmeier reagent the only compound isolated was 1,3-dimethyl-2-formyl-1,5,6,7-tetrahydro-4*H*-indole-4-one (**8l**) as white solid (40%): mp 89–90 °C; IR: 1652 (CO), 1637 (CO) cm^{-1}; ^1H NMR: δ 2.02 (q, J = 6.1 Hz, 2H, CH_2), 2.37 (t, J = 6.1 Hz, 2H, CH_2), 2.50 (s, 1H, CH_3), 2.79 (t, J = 6.1 Hz, 2H, CH_2), 3.78 (s, 1H, CH_3), 9.76 (s, 1H, CHO). ^{13}C NMR: δ 10.0 (CH_3), 20.9 (CH_2), 22.1 (CH_2), 32.7 (CH_3), 38.3 (CH_2), 121.1 (C), 128.1 (C), 133.1 (C), 150.2 (C), 179.3 (CHO), 194.2 (CO). Anal. $C_{11}H_{13}NO_2$: C, 69.09; H, 6.85; N, 7.32. Found: C, 70.22; H, 6.60; N, 7.10.

4.1.3. General procedure for the nucleophilic substitution of the chloro aldehydes (9b–d,f,j and 11)

To an ice cooled solution of **9b–d**,**f**,**j** and **11** (3.37 mmol) in anhydrous DMF (24 mL), potassium carbonate (0.77 g, 5.53 mmol) and ethanthiol (2.45 mL, 33.18 mmol) were added. The mixture was stirred at room temperature for 24 h and then poured onto crushed ice. The solid precipitate was filtered off and dried to afford the desired compounds.

4.1.3.1. 4-(Ethylsulfanyl)-2-methyl-1-phenyl-6,7-dihydro-1*H*-indole-5-carbaldehyde (12b). This product was obtained as yellow solid (95%) from **9b**: mp 93–95 °C; IR: 1628 (CO) cm^{-1}; ^1H NMR: δ 1.19 (t, J = 7.1 Hz, 3H, SCH_2CH_3), 2.08 (s, 3H, CH_3), 2.40–2.64 (m, 4H, 2× CH_2), 2.94 (q, J = 7.1 Hz, 2H, SCH_2CH_3), 6.31 (s, 1H, H-3), 7.37 (d, J = 7.1 Hz, 2H, H-2', H-6'), 7.45–7.61 (m, 3H, H-3', H-4', H-5'), 10.30 (s, 1H, CHO). ^{13}C NMR: δ 12.5 (CH_3), 14.9 (CH_3), 20.1 (CH_2), 23.0 (CH_2), 28.3 (CH_2), 105.5 (CH), 118.1 (C), 127.1 (2 × CH), 128.3 (CH), 129.4 (2 × CH), 130.2 (C), 131.0 (C), 134.7 (C), 136.3 (C), 148.5 (C), 188.9 (CHO). Anal. $C_{18}H_{19}NOS$: C, 72.69; H, 6.44; N, 4.71. Found: C, 75.65; H, 5.80; N, 5.42.

4.1.3.2. 1,2-Dimethyl-4-(ethylsulfanyl)-6,7-dihydro-1*H*-indole-5-carbaldehyde (12c). This product was obtained as yellow solid (80%) from **9c**: mp 60–62 °C; IR: 1624 (CO) cm^{-1}; ^1H NMR: δ 1.12 (t, J = 7.2 Hz, 3H, SCH_2CH_3), 2.19 (s, 3H, CH_3), 2.56–2.70 (m, 4H, 2× CH_2), 2.86 (q, J = 7.2 Hz, 2H, SCH_2CH_3), 3.42 (s, 3H, CH_3), 6.01 (s, 1H, H-3), 10.25 (s, 1H, CHO). ^{13}C NMR: δ 11.7 (CH_3), 14.8 (CH_3), 19.4 (CH_2), 22.7 (CH_2), 28.2 (CH_2), 30.0 (CH_3), 104.3 (CH), 117.0 (C), 129.72 (C), 130.1 (C), 134.9 (C), 148.8 (C), 188.6 (CHO). Anal. $C_{13}H_{17}NOS$: C, 66.35; H, 7.28; N, 5.95. Found: C, 66.00; H, 7.54; N, 5.70.

4.1.3.3. 1-Benzyl-4-(ethylsulfanyl)-2-methyl-6,7-dihydro-1*H*-indole-5-carbaldehyde (12d). This product was obtained as yellow solid (94%) from **9d**: mp 73–75 °C; IR: 1628 (CO) cm^{-1}; ^1H NMR: δ 1.15 (t, J = 7.4 Hz, 3H, SCH_2CH_3), 2.12 (s, 3H, CH_3), 2.57–2.65 (m, 4H, 2 × CH_2), 2.91 (q, J = 7.4 Hz, 2H, SCH_2CH_3), 5.14 (s, 2H, CH_2), 6.19 (s, 1H, H-3), 6.99 (dd, J = 7.4, 1.5 Hz, 2H, H-2', H-6'), 7.25–7.38 (m, 3H, H-3', H-4', H-5'), 10.27 (s, 1H, CHO). ^{13}C NMR: δ 11.8 (CH_3), 14.8 (CH_3), 19.6 (CH_2), 22.8 (CH_2), 28.3 (CH_2), 46.4 (CH_2), 105.0 (CH), 117.6 (C), 126.0 (2× CH), 127.2 (CH), 128.7 (2× CH), 130.0 (C), 130.1 (C), 134.9 (C), 137.5 (C), 148.8 (C), 188.6 (CHO). Anal. $C_{19}H_{21}NOS$: C, 73.27; H, 6.80; N, 4.50. Found: C, 72.93; H, 6.55; N, 4.72.

4.1.3.4. 4-(Ethylsulfanyl)-2-methyl-1-(phenylsulfonyl)-6,7-dihydro-1*H*-indole-5-carbaldehyde (12f). This product was obtained as yellow solid (88%) from **9f**: mp150–151 °C; IR:1645 (CO) cm^{-1}; ^1H NMR: δ 1.09 (t, J = 7.4 Hz, 3H, SCH_2CH_3), 2.38 (s, 3H, CH_3), 2.62 (t, J = 8.9 Hz, 2H, CH_2), 2.79 (q, J = 7.4 Hz, 2H, SCH_2CH_3), 3.05 (t, J = 8.9 Hz, 2H, CH_2), 6.41 (s, 1H, H-3), 7.66–7.84 (m, 3H, H-3', H-4', H-5'), 7.91 (d, J = 7.8 Hz, 2H, H-2', H-6'), 10.31 (s, 1H, CHO). ^{13}C NMR: δ 14.5 (CH_3), 14.6 (CH_3), 21.4 (CH_2), 22.8 (CH_2), 28.3 (CH_2), 110.6 (CH), 122.1 (C), 126.4 (2× CH), 130.2 (CH), 132.3 (C), 134.7 (C), 134.8 (2× CH), 135.1 (C), 138.1 (C), 144.7 (C), 189.7 (CHO). Anal. $C_{18}H_{19}NO_3S_2$: C, 59.81; H, 5.30; N, 3.87. Found: C, 60.02; H, 5.58; N, 4.05.

4.1.3.5. 1,3-Dimethyl-2-ethoxycarbonyl-4-(ethylsulfanyl)-6,7-dihydro-1*H*-indole-5-carbaldehyde (12j). This product was obtained as yellow solid (86%) from **9j**: mp 119–121 °C; IR: 1681 (CO), 1633 (CO) cm^{-1}; ^1H NMR: δ 1.11 (t, J = 7.0 Hz, 3H, SCH_2CH_3), 1.31 (t, J = 7.0 Hz, 3H, OCH_2CH_3), 2.54–2.71 (m, 6H, 3× CH_2), 2.65 (s, 3H, CH_3), 3.74 (s, 3H, CH_3), 4.24 (q, J = 7.0 Hz, 2H, OCH_2CH_3), 10.41 (s, 1H, CHO). ^{13}C NMR: δ 11.8 (CH_3), 14.2 (CH_3), 14.4 (CH_3), 19.2 (CH_2), 21.2 (CH_2), 28.6 (CH_2), 33.3 (CH_3), 59.6 (CH_2), 115.6 (C), 121.3 (C), 126.7 (C), 134.5 (C), 142.0 (C), 147.6 (C), 161.0 (CO), 189.8 (CHO). Anal. $C_{16}H_{21}NO_3S$: C 62.52; H, 6.89; N 4.56. Found: C 62.45; H, 6.96; N 4.40.

4.1.3.6. 1,3-Dimethyl-4-(ethylsulfanyl)-6,7-dihydro-1*H*-indole-2,5-dicarbaldehyde (12l). This product was obtained as white solid (80%) from **11**: mp 101–102 °C; IR: 1649 (CO), 1620 (CO) cm^{-1}; ^1H NMR: δ 1.14 (t, J = 7.2 Hz, 3H, SCH_2CH_3), 2.56–2.79 (m, 6H, 3 × CH_2), 2.57 (s, 3H, CH_3), 3.93 (s, 3H, CH_3), 9.75 (s, 1H, CHO), 10.41 (s, 1H, CHO). ^{13}C NMR: δ 14.3 (CH_3), 18.6 (CH_2), 21.8 (CH_2), 28.9 (CH_2), 32.4 (CH_3), 39.3 (CH_3), 116.3 (C), 128.9 (C), 135.2 (2 × C), 144.6 (C), 146.7 (C), 178.3 (CHO), 189.9 (CHO). Anal.

$C_{14}H_{17}NO_2S$: C, 63.85; H, 6.51; N, 5.32. Found: C, 64.05; H, 6.22; N, 5.12.

4.1.4. General procedure for the oxidation of the 4-(ethylsulfanyl)-1-substituted-6,7-dihydro-1H-indole-5-carbaldehydes (12b–d,f)

A solution of **12b–d,f** (3.61 mmol) in anhydrous benzene (18 mL) and DDQ (0.82 g, 3.61 mmol) was heated under reflux for 15 min. The reaction mixture was washed with 1 N NaOH first and then with brine. The organic layer was separated, dried over sodium sulfate and the solvent removed in vacuo. Recrystallization with ethanol furnished the desired products.

4.1.4.1. 4-(Ethylsulfanyl)-2-methyl-1-phenyl-1*H*-indole-5-carbaldehyde (13b). This product was obtained as yellow solid (85%) from **12b**: mp 86–88 °C; IR: 1670 (CO) cm^{-1}; ^1H NMR: δ 1.15 (t, J = 7.1 Hz, 3H, SCH_2CH_3), 2.32 (s, 3H, CH_3), 2.99 (q, J = 7.1 Hz, 2H, SCH_2CH_3), 6.84 (s, 1H, H-3), 7.11 (d, J = 8.5 Hz, 1H, H-7), 7.49 (d, J = 6.4 Hz, 2H, H-2', H-6'), 7.54–7.68 (m, 4H, H-3', H-4', H-5', H-6), 10.75 (s, 1H, CHO); ^{13}C NMR: δ 13.1 (CH_3), 15.0 (CH_3), 30.4 (CH_2), 102.1 (CH), 110.7 (CH), 120.7 (CH), 127.6 (2 × CH), 128.6 (CH), 129.8 (2 × CH), 130.6 (C), 130.8 (C), 131.6 (C), 136.0 (C), 139.7 (C), 139.8 (C), 191.6 (CHO). Anal. $C_{18}H_{17}NOS$: C, 73.19; H, 5.80; N, 4.74. Found: C, 73.00; H, 6.10; N, 4.50.

4.1.4.2. 1,2-Dimethyl-4-(ethylsulfanyl)-1*H*-indole-5-carbaldehyde (13c). This product was obtained as yellow solid (65%) from **12c**: mp 60–62 °C; IR: 1662 (CO) cm-^{1}; ^1H NMR: δ 1.07 (t, J = 7.4 Hz, 3H, SCH_2CH_3), 2.45 (s, 3H, CH_3), 2.91 (q, J = 7.4 Hz, 2H, SCH_2CH_3), 3.72 (s, 3H, CH_3), 6.61 (s, 1, H-3), 7.53–7.66 (m, 2H, H-6, H-7), 10.71 (s, 1H, CHO); ^{13}C NMR: δ 12.4 (CH_3), 14.9 (CH_3), 29.7 (CH_3), 30.3 (CH_2), 100.6 (CH), 110.3 (CH), 119.6 (CH), 129.7 (C), 130.3 (C), 131.2 (C), 139.3 (C), 140.1 (C), 191.7 (CHO). Anal. $C_{13}H_{15}NOS$: C, 66.92; H, 6.48; N, 6.00. Found: C, 66.70; H, 6.28; N, 6.36.

4.1.4.3. 4-(Ethylsulfanyl)-2-methyl-1-benzyl-1*H*-indole-5-carbaldehyde (13d). This product was obtained as yellow solid (83%) from **12d**: mp 73–75 °C; IR: 1668 (CO) cm^{-1}; ^1H NMR: δ 1.11 (t, J = 7.4 Hz, 3H, SCH_2CH_3), 2.42 (s, 3H, CH_3), 2.95 (q, J = 7.4 Hz, 2H, SCH_2CH_3), 5.14 (s, 2H, CH_2), 6.72 (s, 1H, H-3), 7.01 (d, J = 7.4 Hz, 2H, H-2', H-6'), 7.24–7.34 (m, 3H, H-3', H-4', H-5'), 7.55–7.66 (m, 2H, H-6, H-7), 10.74 (s, 1H, CHO); ^{13}C NMR: δ 12.5 (CH_3), 14.9 (CH_3), 30.4 (CH_2), 46.1 (CH_2), 101.5 (CH), 110.6 (CH), 120.0 (CH), 126.1 (2× CH), 127.2 (CH), 128.6 (2 × CH), 130.0 (C), 130.6 (C), 131.4 (C), 137.5 (C), 139.2 (C), 139.8 (C), 191.6 (CHO). Anal. $C_{19}H_{19}NOS$: C, 73.75; H, 6.19; N, 4.53. Found: C, 73.50; H, 6.00; N, 4.80.

4.1.4.4. 4-(Ethylsulfanyl)-2-methyl-1-phenylsulfonyl-1*H*-indole-5-carbaldehyde (13f). This product was obtained as yellow solid (77%) from **12f**: mp 100–101 °C; IR: 1680 (CO) cm^{-1}; ^1H NMR: δ 1.05 (t, J = 7.2 Hz, 3H, SCH_2CH_3), 2.68 (s, 3H, CH_3), 2.88 (q, J = 7.2 Hz, 2H, SCH_2CH_3), 6.96 (s, 1H, H-3), 7.59–7.82 (m, 4H, H-3', H-4', H-5', H-7), 7.96 (d, J = 8.0 Hz, 2H, H-2', H-6'), 8.23 (1H, d, J = 8.8 Hz, H-6), 10.68 (s, 1H, CHO); ^{13}C NMR: δ 14.6 (CH_3), 15.2 (CH_3), 30.8 (CH_2), 109.1 (CH), 114.5 (CH), 123.2 (2 × CH), 126.3 (CH), 130.0 (2 × CH), 130.6 (C), 133.2 (C), 133.6 (C), 134.9 (CH), 137.4 (C), 138.4 (C), 139.6 (C), 191.5 (CHO). Anal. $C_{18}H_{17}NO_3S_2$: C, 60.15; H, 4.77; N, 3.90. Found: C, 59.92; H, 4.50; N, 3.80.

4.1.5. General procedure for the synthesis of α–β unsaturated esters (14b–d,f,j,m) and (15b–d,f)

Triethyl phosphonoacetate (1.32 mL, 6.74 mmol) was added to a cooled suspension of *t*-BuOK (0.8 g, 6.74 mmol) in anhydrous DMF (6 mL). The mixture was stirred for 1 h at room temperature and then added dropwise to a solution of the proper carboxaldehyde **12b–d,f,j,l** or **13b–d,f** (3.37 mmol) dissolved in anhydrous DMF (5 mL). After stirring at the proper temperature and for the suitable time, the mixture was poured onto crushed ice and the solid which separated was filtered off and dried. Recrystallization from ethanol gave the desired compounds.

4.1.5.1. Ethyl 3-[4-(ethylsulfanyl)-2-methyl-1-phenyl-6,7-dihydro-1*H*-indole-5-yl]acrylate (14b). This product was obtained as white solid (75%) after 1 h at 50 °C from **12b**: mp 103–105 °C; IR: 1685 (CO) cm^{-1}; ^1H NMR: δ 1.15 (t, J = 7.5 Hz, 3H, SCH_2CH_3), 1.22 (t, J = 6.9 Hz, 3H, OCH_2CH_3), 2.07 (s, 3H, CH_3), 2.46–2.65 (m, 4H, 2× CH_2), 2.83 (q, J = 7.5 Hz, 2H, SCH_2CH_3), 4.14 (q, J = 6.9 Hz, 2H, OCH_2CH_3), 5.87 (d, J = 15.5 Hz, 1H, CH), 6.17 (s, 1H, H-3), 7.36 (d, J = 6.4 Hz, 2H, H-2', H-6'), 7.47–7.58 (m, 3H, H-3', H-4', H-5'), 8.33 (d, J = 15.5 Hz, 1H, CH). ^{13}C NMR: δ 12.4 (CH_3), 14.2 (CH_3), 14.9 (CH_3), 20.2 (CH_2), 25.8 (CH_2), 28.1 (CH_2), 59.4 (CH_2), 105.3 (CH), 114.1 (CH), 118.9 (C), 127.1 (2 × CH), 128.0 (CH), 128.9 (C), 129.0 (C), 129.3 (2 × CH), 131.5 (C), 136.6 (C), 136.7 (C), 142.6 (CH), 167.1 (CO). Anal. $C_{22}H_{25}NO_2S$: C, 71.90; H, 6.86; N, 3.81. Found: C, 72.10; H, 6.60; N, 3.65.

4.1.5.2. Ethyl 3-[1,2-dimethyl-4-(ethylsulfanyl)-6,7-dihydro-1*H*-indole-5-yl]acrylate (14c). This product was obtained as white solid (92%) after 1 h at room temperature from **12c**: mp 95–97 °C; IR: 1684 (CO) cm^{-1}; ^1H NMR: δ 1.08 (t, J = 6.8 Hz, 3H, SCH_2CH_3), 1.22 (t, J = 6.8 Hz, 3H, OCH_2CH_3), 2.17 (s, 3H, CH_3), 2.58–2.70 (m, 4H, 2× CH_2), 2.76 (q, J = 6.8 Hz, 2H, SCH_2CH_3), 3.40 (s, 3H, CH_3), 4.14 (q, J = 6.8 Hz, 2H, OCH_2CH_3), 5.85 (d, J = 15.1 Hz, 1H, CH), 5.95 (s, 1H, H-3), 8.32 (d, J = 15.1 Hz, 1H, CH). ^{13}C NMR: δ 11.68 (CH_3), 14.23 (CH_3), 14.79 (CH_3), 19.40 (CH_2), 25.57 (CH_2), 25.95 (CH_2), 29.78 (CH_3), 59.30 (CH_2), 103.85 (CH), 113.09 (CH), 117.79 (C), 127.57 (C), 128.67 (C), 131.62 (C), 137.20 (C), 142.78 (CH), 167.15 (CO). Anal. $C_{17}H_{23}NO_2S$: C, 66.85; H, 7.59; N, 4.59. Found: C, 67.00; H, 7.40; N, 4.90.

4.1.5.3. Ethyl 3-[1-benzyl-4-(ethylsulfanyl)-2-methyl-6,7-dihydro-1*H*-indole-5-yl]acrylate (14d). This product was obtained as white solid (80%) after 24 h at room temperature from **12d**: mp 112–113 °C; IR: 1691 (CO) cm^{-1}; ^1H NMR: δ 1.11 (t, J = 7.4 Hz, 3H, SCH_2CH_3), 1.21 (t, J = 7.4 Hz, 3H, OCH_2CH_3), 2.11 (s, 3H, CH_3), 2.58–2.68 (m, 4H, 2 × CH_2), 2.80 (q, J = 7.4 Hz, 2H, SCH_2CH_3), 4.13 (q, J = 7.4 Hz, 2H, OCH_2CH_3), 5.10 (s, 2H, CH_2), 5.85 (d, J = 16.2 Hz, 1H, CH), 6.05 (s, 1H, H-3), 6.99 (d, J = 5.9 Hz, 2H, H-2', H-6'), 7.25–7.37 (m, 3H, H-3', H-4', H-5'), 8.32 (d, J = 16.2 Hz, 1H, CH). ^{13}C NMR: δ 11.7 (CH_3), 14.2 (CH_3), 14.8 (CH_3), 19.7 (CH_2), 25.7 (CH_2), 28.0 (CH_2), 46.2 (CH_2), 59.4 (CH_2), 104.6 (CH), 113.5 (CH), 118.4 (C), 125.9 (2 × CH), 127.1 (CH), 128.0 (C), 128.6 (2 × CH), 131.7 (C), 137.0 (C), 137.1 (C), 137.8 (C), 142.7 (CH), 167.1 (CO). Anal. $C_{23}H_{27}NO_2S$: C, 72.41; H, 7.13; N, 3.67. Found: C, 72.20; H, 7.00; N, 3.47.

4.1.5.4. Ethyl-3-[4-(ethylsulfanyl)-2-methyl-1-phenylsulfonyl-6,7-dihydro-1*H*-indole-5-yl] acrylate (14f). This product was obtained as white solid (90%) after 1 h at room temperature from **12f**: mp 118–119 °C; IR: 1693 (CO) cm^{-1}; ^1H NMR: δ 1.05 (t, J = 7.3 Hz, 3H, SCH_2CH_3), 1.23 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 2.36 (s, 3H, CH_3), 2.63–2.73 (m, 4H, SCH_2CH_3, CH_2), 3.06 (t, J = 8.7 Hz, 2H, CH_2), 4.16 (q, J = 7.1 Hz, 2H, OCH_2CH_3), 6.04 (d, J = 15.7 Hz, 1H, CH), 6.29 (s, 1H, H-3), 7.65–7.79 (m, 3H, H-3', H-4', H-5'), 7.88 (d, J = 8.1 Hz, 2H, H-2', H-6'), 8.25 (d, J = 15.7 Hz, 1H, CH). ^{13}C NMR: δ 14.2 (CH_3), 14.5 (CH_3), 14.6 (CH_3), 21.6 (CH_2), 25.7 (CH_2), 28.2 (CH_2), 59.8 (CH_2), 111.0 (CH), 117.2 (CH), 123.1 (C), 126.3 (2 × CH), 130.2 (2 × CH), 131.6 (C), 132.1 (C), 133.1 (C), 133.3 (C), 134.7 (CH), 138.3 (C), 141.6 (CH), 166.7 (CO). Anal.

$C_{22}H_{25}NO_4S_2$: C, 61.23; H, 5.84; N, 3.25. Found: C, 61.50; H, 5.64; N, 3.00.

4.1.5.5. Ethyl 3-[1,3-dimethyl-2-ethoxycarbonyl-4-(ethylsulfanyl)-6,7-dihydro-1*H*-indole-5-yl] acrylate (14j). This product was obtained as white solid (85%) after 1 h at room temperature from **12j**: mp 72–73 °C; IR: 1685 (CO), 1676 (CO) cm^{-1}; ^1H NMR: δ 1.07 (t, J = 7.3 Hz, 3H, SCH_2CH_3), 1.23 (t, J = 7.0 Hz, 3H, OCH_2CH_3), 1.29 (t, J = 7.0 Hz, 3H, OCH_2CH_3), 2.51–2.70 (m, 6H, 2× CH_2, SCH_2CH_3), 2.62 (3H, s, CH_3), 3.73 (3H, s, CH_3), 4.14 (q, J = 7.0 Hz, 2H, OCH_2CH_3), 4.25 (q, J = 7.0 Hz, 2H, OCH_2CH_3), 6.00 (d, J = 15.7 Hz, 1H, CH), 8.42 (d, J = 15.7 Hz, 1H, CH). ^{13}C NMR: δ 11.8 (CH_3), 14.2 (2× CH_3), 14.3 (CH_3), 19.3 (CH_2), 25.3 (CH_2), 28.4 (CH_2), 33.1 (CH_3), 59.4 (CH_2), 59.6 (CH_2), 115.8 (CH), 116.6 (C), 120.2 (C), 125.6 (C), 132.6 (C), 136.0 (C), 140.3 (C), 142.3 (CH), 161.1 (CO), 166.0 (CO). Anal. $C_{20}H_{27}NO_4S$: C, 63.63; H, 7.21; N, 3.71. Found: C, 63.36; H, 7.00; N, 3.98.

4.1.5.6. Ethyl 3-[1,3-dimethyl-4-(ethylsulfanyl)-6,7-dihydro-1*H*-indole-2,5-diyl]acrylate (14m). This product was obtained as white solid (85%) after 1 h at room temperature from **12l**: mp 120–121 °C; IR: 1691 (CO), 1680 (CO) cm^{-1}; ^1H NMR: δ 1.08 (t, J = 6.9 Hz, 3H, SCH_2CH_3), 1.11–1.24 (m, 6H, 2× OCH_2CH_3), 2.52 (s, 3H, CH_3), 2.60–2.69 (m, 6H, SCH_2CH_3, 2 × CH_2), 3.61 (s, 3H, CH_3), 4.11–4.20 (m, 4H, 2× OCH_2CH_3), 5.96 (d, J = 15.6 Hz, 1H, CH), 6.01 (d, J = 15.5 Hz, 1 H, CH), 7.57 (d, J = 15.9 Hz, 1H, CH), 8.37 (d, J = 15.9 Hz, 1H, CH). ^{13}C NMR: δ 11.6 (CH_3), 14.2 (2× CH_3), 14.3 (CH_3), 19.4 (CH_2), 25.3 (CH_2), 28.3 (CH_2), 31.6 (CH_3), 59.6 (2× CH_2), 110.7 (CH), 115.3 (CH), 117.4 (C), 122.1 (C), 126.1 (C), 131.4 (CH), 132.2 (C), 135.9 (C), 139.4 (C), 142.3 (CH), 167.0 (CO), 167.2 (CO). Anal. $C_{22}H_{29}NO_4S$: C, 65.48; H, 7.24; N, 3.47. Found: C, 65.15; H, 7.00; N, 3.62.

4.1.5.7. Ethyl 3-[4-(ethylsulfanyl)-2-methyl-1-phenyl-1H-indole-5-yl]acrylate (15b). This product was obtained as white solid (70%) after 20 min at room temperature from **13b**: mp 99–101 °C; IR: 1701 (CO) cm^{-1}; ^1H NMR: δ 1.10 (t, J = 7.0 Hz, 3H, SCH_2CH_3), 1.27 (t, J = 7.0 Hz, 3H, OCH_2CH_3), 2.30 (s, 3H, CH_3), 2.88 (q, J = 7.0 Hz, 2H, SCH_2CH_3), 4.23 (q, J = 7.0 Hz, 2H, OCH_2CH_3), 6.49 (d, J = 16.1 Hz, 1H, CH), 6.70 (s, 1H, H-3), 7.02 (d, J = 8.5 Hz, 1H, H-6), 7.47 (dd, J = 8.0, 1.5 Hz, 2H, H-2', H-6'), 7.54–7.66 (m, 4H, H-3', H-4', H-5', H-7), 8.59 (d, J = 16.1 Hz, 1H, CH). ^{13}C NMR: δ 13.0 (CH_3), 14.2 (CH_3), 15.0 (CH_3), 29.9 (CH_2), 59.8 (CH_2), 102.1 (CH), 110.9 (CH), 116.5 (CH), 120.0 (CH), 126.5 (C), 127.6 (2 × CH), 128.3 (CH), 129.3 (C), 129.8 (2× CH), 131.9 (C), 136.3 (C), 137.8 (C), 138.7 (C), 143.4 (CH), 166.5 (CO). Anal. $C_{22}H_{23}NO_2S$: C, 72.30; H, 6.34; N, 3.83. Found: C, 72.00; H, 6.04; N, 4.03.

4.1.5.8. Ethyl 3-[1,2-dimethyl-4-(ethylsulfanyl)-1H-indole-5-yl]acrylate (15c). This product was obtained as white solid (92%) after 30 min at room temperature from **13c**: mp 73–75 °C; IR: 1697 (CO) cm^{-1}; ^1H NMR: δ 1.03 (t, J = 7.4 Hz, 3H, SCH_2CH_3), 1.26 (t, J = 7.4 Hz, 3H, OCH_2CH_3), 2.43 (s, 3H, CH_3), 2.81 (q, J = 7.4 Hz, 2H, SCH_2CH_3), 3.69 (s, 3H, CH_3), 4.20 (q, J = 7.4 Hz, 2H, OCH_2CH_3), 6.47–6.54 (m, 2H, H-3, CH), 7.48 (d, J = 8.8 Hz, 1H, H-6), 7.64 (d, J = 8.8 Hz, 1H, H-7), 8.57 (d, J = 16.2 Hz, 1H, CH). ^{13}C NMR: δ 12.4 (CH_3), 14.2 (CH_3), 14.9 (CH_3), 29.5 (CH_3), 29.7 (CH_2), 59.7 (CH_2), 100.4 (CH), 110.6 (CH), 115.8 (CH), 119.0 (CH), 126.0 (C), 128.2(C), 131.5 (C), 137.4 (C), 139.0 (C), 143.7 (CH), 166.7 (CO). Anal. $C_{17}H_{21}NO_2S$: C, 67.30; H, 6.98; N, 4.62. Found: C, 67.10; H, 7.18; N, 4.32.

4.1.5.9. Ethyl 3-[1-benzyl-4-(ethylsulfanyl)-2-methyl-1*H*-indole-5-yl]acrylate (15d). This product was obtained as white solid (96%) after 1 h at room temperature from **13d**: mp 74–75 °C; IR: 1699 (CO) cm^{-1}; ^1H NMR: δ 1.07 (t, J = 7.4 Hz, 3H, SCH_2CH_3), 1.26 (t, J = 7.4 Hz, 3H, OCH_2CH_3), 2.39 (s, 3H, CH_3), 2.84 (q, J = 7.4 Hz, 2H, SCH_2CH_3), 4.19 (q, J = 7.4 Hz, 2H, OCH_2CH_3), 5.45 (s, 2H, CH_2), 6.49 (d, J = 16.2 Hz, 1H, CH), 6.57 (s, 1H, H-3), 7.00 (d, J = 5.9 Hz, 2H, H-2', H-6'), 7.22–7.36 (m, 3H, H-3', H-4', H-5'), 7.46 (d, J = 8.8 Hz, 1H, H-6), 7.62 (d, J = 8.8 Hz, 1H, H-7), 8.58 (d, J = 16.2 Hz, 1H, CH). ^{13}C NMR: δ 12.4 (CH_3), 14.2 (CH_3), 14.9 (CH_3), 29.8 (CH_2), 45.9 (CH_2), 59.7 (CH_2), 101.3 (CH), 110.9 (CH), 116.1 (CH), 119.4 (CH), 126.1 (2× CH), 126.3 (C), 127.1 (CH), 128.6 (2× CH), 131.7 (C), 137.3 (C), 137.3 (C), 138.7 (C), 143.6 (CH), 165.7 (CO), 166.6 (CO). Anal. $C_{23}H_{25}NO_2S$: C, 72.79; H, 6.64; N, 3.69. Found: C, 72.54; H, 6.40; N, 3.46.

4.1.5.10. Ethyl 3-[4-(ethylsulfanyl)-2-methyl-1-phenylsulfonyl-1H-indole-5-yl]acrylate (15f). This product was obtained as white solid (80%) after 1 h at room temperature from **13f**: mp 81–82 °C; IR: 1703 (CO) cm^{-1}; ^1H NMR: δ 1.00 (t, J = 7.2 Hz, 3H, SCH_2CH_3), 1.27 (t, J = 6.9 Hz, 3H, OCH_2CH_3), 2.65 (s, 3H, CH_3), 2.77 (q, J = 7.2 Hz, 2H, SCH_2CH_3), 4.22 (q, J = 6.9 Hz, 2H, OCH_2CH_3), 6.62 (d, J = 15.9 Hz, 1H, CH), 6.84 (s, 1 H, H-3), 7.59–7.94 (m, 6H, Ph, H-6), 8.08 (d, J = 8.7 Hz, 1H, H-7), 8.43 (d, J = 15.9 Hz, 1H, CH). ^{13}C NMR: δ 14.2 (CH_3), 14.7 (CH_3), 15.2 (CH_3), 30.2 (CH_2), 60.1 (CH_2), 109.6 (CH), 114.6 (CH), 119.0 (CH), 122.8 (2× CH), 126.3 (CH), 126.8 (C), 130.0 (2× CH), 133.0 (C), 133.7 (C), 134.8 (CH), 136.2 (C), 137.6 (C), 138.8 (C), 144.0 (CH), 166.2 (CO). Anal. $C_{22}H_{23}NO_4S_2$: C, 61.52; H, 5.40; N, 3.26. Found: C, 61.22; H, 5.72; N, 3.06.

4.1.6. General procedure for the hydrolisis of the α–β unsaturated esters (14b–d,f,j,m) and (15b–d,f)

To a solution of KOH (0.34 g, 6 mmol) in 50% of acqueous ethanol (25 mL) the proper ester **14b–d,f,j,m** or **15b–d,f** (3 mmol) was added and the solution was heated under reflux. After cooling the solution was washed with ether and the acqueous layer was acidified with 6 N HCl and the precipitate was collected. Recrystallization from ethanol afforded the desired compounds.

4.1.6.1. 3-[4-(Ethylsulfanyl)-2-methyl-1-phenyl-6,7-dihydro-1*H*-indole-5-yl]acrylic acid (16b). This product was obtained as white solid (72%) after 30 min reflux from **14b**: mp 178–180 °C; IR: 3539–3253 (OH), 1668 (CO) cm^{-1}; ^1H NMR: δ 1.15 (t, J = 7.4 Hz, 3H, SCH_2CH_3), 2.07 (s, 3H, CH_3), 2.46–2.64 (m, 4H, 2× CH_2), 2.82 (q, J = 7.4 Hz, 2H, SCH_2CH_3), 5.81 (d, J = 16.2 Hz, 1H, CH), 6.16 (s, 1H, H-3), 7.35 (d, J = 7.4 Hz, 2H, H-2', H-6'), 7.43–7.58 (m, 3H, H-3', H-4', H-5'), 8.29 (d, J = 16.2 Hz, 1H, CH), 12.02 (s, 1H, OH). ^{13}C NMR: δ: 12.5 (CH_3), 14.9 (CH_3), 20.2 (CH_2), 25.9 (CH_2), 28.1 (CH_2), 105.3 (CH), 115.2 (CH), 119.0 (C), 127.1 (2 × CH), 128.0 (CH), 128.8 (C), 129.2 (C), 129.3 (2× CH), 131.3 (C), 135.9 (C), 136.7 (C), 142.3 (CH), 168.4 (CO). Anal. $C_{20}H_{21}NO_2S$: C, 70.77; H, 6.24; N, 4.13. Found: C, 70.52; H, 6.04; N, 4.00.

4.1.6.2. 3-[1,2-Dimethyl-4-(ethylsulfanyl)-6,7-dihydro-1H-indole-5-yl]acrylic acid (16c). This product was obtained as white solid (93%) after 1 h reflux from **14c**: mp 160–162 °C; IR: 3197 (OH), 1668 (CO) cm^{-1}; ^1H NMR: δ 1.08 (t, J = 7.4 Hz, 3H, SCH_2CH_3), 2.17 (s, 3H, CH_3), 2.58–2.70 (m, 4H, 2 × CH_2), 2.81 (q, J = 7.4 Hz, 2H, SCH_2CH_3), 3.69 (s, 3H, CH_3), 5.78 (d, J = 15.6 Hz, 1H, CH), 5.94 (s, 1H, H-3), 8.27 (d, J = 15.6 Hz, 1H, CH), 11.94 (s, 1H, OH). ^{13}C NMR: δ 11.8 (CH_3), 14.9 (CH_3), 19.5 (CH_2), 25.7 (CH_2), 27.9 (CH_2), 29.9 (CH_3), 103.9 (CH), 114.3 (CH), 117.8 (C), 127.7 (C), 128.6 (C), 131.4 (C), 136.5 (C), 142.6 (CH), 168.6 (CO). Anal. $C_{15}H_{19}NO_2S$: C, 64.95; H, 6.90; N, 5.05. Found: C, 64.65; H, 7.10; N, 5.35.

4.1.6.3. 3-[1-Benzyl-4-(ethylsulfanyl)-2-methyl-6,7-dihydro-1*H*-indole-5-yl]acrylic acid (16d). This product was obtained as white solid (75%) after 40 min reflux from **14d**: mp 159–161 °C; IR: 3433 (OH), 1664 (CO) cm^{-1}; ^1H NMR: δ 1.12 (t, J = 7.4 Hz, 3H,

SCH_2CH_3), 2.11 (s, 3H, CH_3), 2.60–2.66 (m, 4H, 2 × CH_2), 2.79 (q, J = 7.4 Hz, 2H, SCH_2CH_3), 5.10 (s, 2H, CH_2), 5.79 (d, J = 16.2 Hz, 1H, CH), 6.04 (s, 1H, H-3), 6.99 (d, J = 7.4 Hz, 2H, H-2', H-6'), 7.22–7.38 (m, 3H, H-3', H-4', H-5'), 8.27 (d, J = 16.2 Hz, 1H, CH), 11.93 (s, 1H, OH). ^{13}C NMR: δ 11.8 (CH_3), 14.8 (CH_3), 19.7 (CH_2), 25.8 (CH_2), 28.0 (CH_2), 46.2 (CH_2), 104.6 (CH), 114.6 (CH), 118.4 (C), 126.0 (2 × CH), 127.1 (CH), 128.1 (C), 128.5 (C), 128.7 (2 × CH), 131.4 (C), 136.2 (C), 137.9 (C), 142.4 (CH), 168.5 (CO). Anal. $C_{21}H_{23}NO_2S$: C, 71.36; H, 6.56; N, 3.96. Found: C, 71.60; H, 6.36; N, 3.70.

4.1.6.4. 3-[4-(Ethylsulfanyl)-2-methyl-1-phenylsulfonyl-6,7-dihydro-1*H*-indole-5-yl]acrylic acid (16f). This product was obtained as white solid (90%) after 40 min reflux from **14f**: mp 203–204 °C; IR: 3620–3355 (OH), 1668 (CO) cm^{-1}; ^{1}H NMR: δ 1.05 (t, J = 7.1 Hz, 3H, SCH_2CH_3), 2.36 (s, 3H, CH_3), 2.62–2.72 (m, 4H, 2 × CH_2), 3.38 (q, J = 7.1 Hz, 2H, SCH_2CH_3), 5.97 (d, J = 15.9 Hz, 1H, CH), 6.29 (s, 1H, H-3), 7.65–7.79 (m, 3H, H-3', H-4', H-5'), 7.87 (d, J = 7.7 Hz, 2H, H-2', H-6'), 8.19 (d, J = 15.9 Hz, 1H, CH), 12.31 (s, 1H, OH). ^{13}C NMR: δ 14.5 (CH_3), 14.6 (CH_3), 21.6 (CH_2), 25.7 (CH_2), 28.2 (CH_2), 111.0 (CH), 118.4 (CH), 123.1 (C), 126.2 (2 × CH), 130.2 (2× CH), 131.6 (C), 131.9 (C), 132.3 (C), 133.4 (C), 134.7 (CH), 138.4 (C), 141.2 (CH), 168.1 (CO). Anal. $C_{20}H_{21}NO_4S_2$: C, 59.53; H, 5.25; N, 3.47. Found: C, 59.26; H, 5.52; N, 3.27.

4.1.6.5. 3-[1,3-Dimethyl-2-ethoxycarbonyl-4-(ethylsulfanyl)-6,7-dihydro-1H-indole-5-yl]acrylic acid (16j). This product was obtained as white solid (92%) after 1 h reflux from **14j**: mp 144–146 °C; IR: 3410 (OH), 1691 (CO), 1682 (CO) cm^{-1}; ^{1}H NMR: δ 1.07 (t, J = 7.2 Hz, 3H, SCH_2CH_3), 1.29 (t, J = 7.2 Hz, 3H, OCH_2CH_3), 2.41–2.70 (m, 6H, 2 × CH_2, SCH_2CH_3) 2.65 (s, 3H, CH_3), 3.72 (s, 3H, CH_3), 4.26 (q, J = 7.2 Hz, 2H, OCH_2CH_3), 5.94 (d, J = 15.7 Hz, 1H, CH), 8.36 (d, J = 15.7 Hz, 1H, CH), 12.21 (s, 1H, OH). ^{13}C NMR: δ 11.8 (CH_3), 14.3 (2 × CH_3), 19.3 (CH_2), 25.3 (CH_2), 28.3 (CH_2), 33.1 (CH_3), 59.4 (CH_2), 116.6 (C), 116.9 (CH), 120.2 (C), 125.5 (C), 132.7 (C), 135.2 (C), 139.9 (C), 142.0 (CH), 161.2 (CO), 168.4 (CO). Anal. $C_{18}H_{23}NO_4S$: C, 61.87; H, 6.63; N, 4.01. Found: C, 62.02; H, 6.43; N, 4.36.

4.1.6.6. 3-[1,3-Dimethyl-4-(ethylsulfanyl)-6,7-dihydro-1*H*-indole-2,5-diyl]acrylic acid (16n). This product was obtained as white solid (92%) after 1 h reflux from **14m**: mp 188–189 °C; IR: 2966–2485 (vb, OH), 1668 (CO), 1601 (CO) cm^{-1}; ^{1}H NMR: δ 1.08 (t, J = 7.2 Hz, 3H, SCH_2CH_3), 2.52 (s, 3H, CH_3), 2.60–2.79 (m, 6H, SCH_2CH_3, 2× CH_2), 3.60 (s, 3H, CH_3), 5.93 (d, J = 15.5 Hz, 2H, 2 × CH), 7.57 (d, J = 15.7 Hz, 1H, CH), 8.37 (d, J = 15.7 Hz, 1H, CH), 12.06 (s, 1H, OH), 12.21 (s, 1H, OH). ^{13}C NMR: δ 11.6 (CH_3), 14.3 (CH_3), 19.5 (CH_2), 25.4 (CH_2), 28.4 (CH_2), 31.5 (CH_3), 112.0 (CH), 116.5 (CH), 117.4 (C), 121.5 (C), 126.1 (C), 131.3 (CH), 132.1 (C), 135.3 (C), 138.9 (C), 142.1 (CH), 168.4 (2× CO). Anal. $C_{18}H_{21}NO_4S$: C, 62.23; H, 6.09; N, 4.03. Found: C, 62.00; H, 5.85; N, 4.34.

4.1.6.7. 3-[4-(Ethylsulfanyl)-2-methyl-1-phenyl-1H-indole-5-yl]acrylic acid (17b). This product was obtained as white solid (70%) after 30 min reflux from **15b**: mp 240–242 °C; IR: 3539–3361 (OH), 1676 (CO) cm^{-1}; ^{1}H NMR: δ 1.11 (t, J = 7.1 Hz, 3H, SCH_2CH_3), 2.30 (s, 3H, CH_3), 2.88 (q, J = 7.1 Hz, 2H, SCH_2CH_3), 6.40 (d, J = 16.5 Hz, 1H, CH), 6.70 (s, 1H, H-3), 7.03 (d, J = 8.7 Hz, 1H, H-6), 7.47 (d, J = 6.3 Hz, 2H, H-2', H-6'), 7.54–7.67 (m, 4H, H-3', H-4', H-5', H-7), 8.54 (d, J = 16.5 Hz, 1H, CH), 12.31 (s, 1H, OH). ^{13}C NMR: δ 13.0 (CH_3), 15.0 (CH_3), 29.9 (CH_2), 102.1 (CH), 110.9 (CH), 117.6 (CH), 120.0 (CH), 126.2 (C), 127.5 (2× CH), 128.3 (CH), 129.6 (C), 129.8 (2× CH), 131.9 (C), 136.4 (C), 137.7 (C), 138.6 (C), 143.0 (CH), 167.9 (CO). Anal. $C_{20}H_{19}NO_2S$: C, 71.19; H, 5.68; N, 4.15. Found: C, 70.96; H, 5.24; N, 4.33.

4.1.6.8. 3-[1,2-Dimethyl-4-(ethylsulfanyl)-1H-indole-5-yl]acrilyc acid (17c). This product was obtained as white solid (93%) after 1 h reflux from **15c**: mp 236–238 °C; IR: 3417 (OH), 1676 (CO) cm^{-1}; ^{1}H NMR: δ 1.03 (t, J = 7.4 Hz, 3H, SCH_2CH_3), 2.43 (s, 3H, CH_3), 2.80 (q, J = 7.4 Hz, 2H, SCH_2CH_3), 3.69 (s, 3H, CH_3), 6.37–6.46 (m, 2H, H-3, CH), 7.46 (d, J = 7.4 Hz, 1H, H-6), 7.61 (d, J = 7.4 Hz, 1H, H-7), 8.52 (d, J = 16.2 Hz, 1H, CH), 12.21 (s, 1H, OH). ^{13}C NMR: δ 12.4 (CH_3), 14.9 (CH_3), 29.5 (CH_3), 29.7 (CH_2), 100.3 (CH), 110.5 (CH), 116.9 (CH), 118.9 (CH), 125.7 (C), 128.4 (C), 131.5 (C), 137.3 (C), 138.9 (C), 143.3 (CH), 168.0 (CO). Anal. $C_{15}H_{17}NO_2S$: C, 65.43; H, 6.22; N, 5.09. Found: C, 65.20; H, 6.40; N, 4.82.

4.1.6.9. 3-[1-Benzyl-4-(ethylsulfanyl)-2-methyl-6,7-1H-indole-5-yl]acrilic acid (17d). This product was obtained as white solid (85%) after 1 h reflux from **15d**: mp 215–217 °C; IR: 3477 (OH), 1670 (CO) cm^{-1}; ^{1}H NMR: δ 1.07 (t, J = 7.4 Hz, 3H, SCH_2CH_3), 2.39 (s, 3H, CH_3), 2.84 (q, J = 7.4 Hz, 2H, SCH_2CH_3), 5.45 (s, 2H, CH_2), 6.40 (d, J = 16.2 Hz, 1H, CH), 6.57 (s, 1H, H-3), 7.01 (d, J = 6.9 Hz, 2H, H-2', H-6'), 7.23–7.34 (m, 3H, H-3', H-4', H-5'), 7.46 (d, J = 8.8 Hz, 1H, H-6), 7.59 (d, J = 8.8 Hz, 1H, H-7), 8.53 (d, J = 16.2 Hz, 1H, CH), 12.24 (s, 1H, OH). ^{13}C NMR: δ 12.4 (CH_3), 14.9 (CH_3), 29.8 (CH_2), 45.9 (CH_2), 101.3 (CH), 110.9 (CH), 117.2 (CH), 119.4 (CH), 126.0 (C), 126.1 (2× CH), 127.2 (CH), 128.6 (2 × CH), 128.8 (C), 131.7 (C), 137.2 (C), 137.9 (C), 138.7 (C), 143.2 (CH), 167.9 (CO). Anal. $C_{21}H_{21}NO_2S$: C, 71.77; H, 6.02; N, 3.99. Found: C, 72.00; H, 6.25; N, 4.06.

4.1.6.10. 3-[4-(Ethylsulfanyl)-2-methyl-1-phenylsulfonyl-1H-indole-5-yl]acrilic acid (17f). This product was obtained as white solid (65%) after 1 h reflux from **15f**: mp 204–205 °C; IR: 3427 (OH), 1684 (CO) cm^{-1}; ^{1}H NMR: δ 1.01 (t, J = 7.1 Hz, 3H, SCH_2CH_3), 2.65 (s, 3H, CH_3), 2.77 (q, J = 7.1 Hz, 2H, SCH_2CH_3), 6.54 (d, J = 15.9 Hz, 1H, CH), 6.84 (s, 1H, H-3), 7.58–7.85 (m, 4H, H-3', H-4', H-5', H-6), 7.93 (d, J = 7.5 Hz, 2H, H-2', H-6'), 8.10 (d, J = 8.4 Hz, 1H, H-7), 8.39 (d, J = 15.9 Hz, 1H, CH), 12.50 (s, 1H, OH). ^{13}C NMR: δ 14.7 (CH_3), 15.2 (CH_3), 30.2 (CH_2), 109.7 (CH), 114.6 (CH), 120.2 (CH), 122.7 (CH), 126.3 (2 × CH), 126.5 (C), 130.0 (2× CH), 133.2 (C), 133.7 (C), 134.8 (CH), 136.2 (C), 137.6 (C), 138.8 (C), 141.5 (CH), 167.6 (CO). Anal. $C_{20}H_{19}NO_4S_2$: C, 59.83; H, 4.77; N, 3.49. Found: C, 59.69; H, 4.96; N, 3.70.

4.1.7. General procedure for the cyclization of the acids 16b–d,f,j,n and 17b–d,f

A mixture of the proper acid **16b**–**d**,**f**,**j**,**n** or **17b**–**d**,**f** (3 mmol) and PPA (20g) was heated for the proper time. Addition of crushed ice gave a solid precipitate which was filtered off, dried and purified by column chromatography (DCM: EtOAc 95:5).

4.1.7.1. 8-Methyl-7-phenyl-5,6-dihydrothiopyrano[2,3-e]indole-2(5*H*)-one (5b). This product was obtained as a yellow solid (30%) by heating **16b** at 95–100 °C for 4 h: mp 175–177 °C; IR: 1703 (CO) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 2.09 (s, 3H, CH_3), 2.55–2.77 (m, 4H, H-5, H-6), 5.92 (d, J = 9.5 Hz, 1H, H-3), 6.32 (s, 1H, H-9), 7.19–7.27 (m, 3H, H-2', H-6', H-4), 7.45–7.55 (m, 3H, H-3', H-4', H-5'). ^{13}C NMR ($CDCl_3$): δ 12.7 (CH_3), 21.3 (CH_2), 25.6 (CH_2), 102.2 (CH), 105.4 (C), 107.3 (CH), 113.4 (C), 127.3 (2 × CH), 128.4 (CH), 129.4 (2 × CH), 131.4 (C), 135.1 (C), 137.1 (C), 145.9 (CH), 157.6 (C), 163.1 (CO). Anal. $C_{18}H_{15}NOS$: C, 73.69; H, 5.15; N, 4.77. Found: C, 73.50; H, 5.36; N, 4.50.

4.1.7.2. 7,8-Dimethyl-5,6-dihydrothiopyrano[2,3-*e*]indole-2(5H)-one (5c). This product was obtained as a yellow solid (40%) by heating **16c** at 90–100 °C for 4 h: mp 172–173 °C; IR: 1620 (CO) cm^{-1}; ^{1}H NMR: δ 2.22 (s, 3H, CH_3), 2.77–2.79 (m, 4H, H-5, H-6), 3.43 (s, 3H, CH_3), 5.99 (s, 1H, H-9), 6.16 (d, J = 10.2 Hz, 1H, H-3), 7.22 (d, J = 10.2 Hz, 1H, H-4). ^{13}C NMR: δ 12.1 (CH_3), 20.8 (CH_2), 29.4 (CH_2), 30.3 (CH_3), 101.5 (CH), 108.2 (C), 116.5

(C), 117.4 (CH), 120.2 (C), 130.8 (C), 131.7 (C), 144.6 (CH), 184.4 (CO). Anal. $C_{13}H_{13}NOS$: C, 67.50; H, 5.66; N, 6.06. Found: C, 67.80; H, 5.69; N, 6.40.

4.1.7.3. 7-Benzyl-8-methyl-5,6-dihydrothiopyrano[2,3-*e*]indole-2(5*H*)-one (5d). This product was obtained as a yellow solid (30%) by heating **16d** at 100–130 °C for 30 min: mp 175–177 °C; IR: 1684 (CO) cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.20 (s, 3H, CH_3), 2.68–2.76 (m, 4H, H-5, H-6), 4.06 (s, 2H, CH_2), 5.86 (d, J = 8.8 Hz, 1H, H-3), 7.11–7.33 (m, 7H, Ph, H-4, H-9). ^{13}C NMR ($CDCl_3$): δ 11.1 (CH_3), 21.7 (CH_2), 25.8 (CH_2), 30.7 (CH_2), 105.2 (C), 106.8 (CH), 111.7 (C), 117.1 (C), 120.4 (C), 125.2 (CH), 128.2 (2× CH), 128.3 (CH), 128.6 (2× CH), 132.7 (C), 142.1 (C), 146.0 (CH), 162.0 (CO). Anal. $C_{19}H_{17}NOS$: C, 74.24; H, 5.57; N, 4.56. Found: C, 74.14; H, 5.66; N, 4.22.

4.1.7.4. 8-Methyl-7-phenylsulfonyl-5,6-dihydrothiopyrano[2,3-*e*]indol-2(5*H*)-one (5f). This product was obtained as a yellow solid (40%) by heating **16f** at 70–90 °C for 20 min: mp 175–177 °C; IR: 1685 (CO) cm^{-1}; ^1H NMR: δ 2.59 (s, 3H, CH_3), 2.64–2.68 (m, 4H, H-5, H-6), 5.93 (d, J = 9.3 Hz, 1H, H-3), 7.43 (d, J = 9.3 Hz, 1H, H-4), 7.47–7.63 (m, 4H, H-3', H-4', H-5', H-9), 8.18 (d, J = 6.6 Hz, 2H, H-2', H-6'). ^{13}C NMR: δ 12.6 (CH_3), 20.4 (CH_2), 24.6 (CH_2), 107.1 (C), 108.2 (CH), 110.0 (C), 114.9 (C), 127.6 (2 × CH), 128.7 (3 × CH), 132.6 (CH), 135.3 (C), 143.5 (C), 146.7 (CH), 154.5 (C), 161.0 (CO). Anal. $C_{18}H_{15}NO_3S_2$: C, 60.48; H, 4.23; N, 3.92. Found: C, 60.18; H, 4.00; N, 4.12.

4.1.7.5. 8-Methyl-7-phenyl-thiopyrano[2,3-*e*]indol-2(7*H*)-one (6b). This product was obtained as a yellow solid (32%) by heating **17b** at 110–130 °C for 30 min. mp 175–177 °C; IR: 196–198 °C; IR: 1624 (CO) cm^{-1}; ^1H NMR: δ 2.31 (s, 3H, CH_3), 6.47 (d, J = 10.7 Hz, 1H, H-3), 6.65 (s, 1H, H-9), 7.09 (d, J = 8.8 Hz, 1H, H-5), 7.48–7.53 (m, 3H, H-2', H-6', H-6), 7.58–7.70 (m, 3H, H-3', H-4', H-5'), 8.10 (d, J = 10.7 Hz, 1H, H-4). ^{13}C NMR: δ 13.0 (CH_3), 99.3 (CH), 109.5 (CH), 118.4 (C), 119.9 (CH), 123.3 (C), 125.2 (CH), 127.7 (2× CH), 128.7 (CH), 128.9 (C), 129.9 (2× CH), 136.1 (C), 136.8 (C), 139.2 (C), 146.5 (CH), 183.2 (CO). Anal. $C_{18}H_{13}NOS$: C, 74.20; H, 4.50; N, 4.81. Found: C, 74.00; H, 4.24; N, 5.00.

4.1.7.6. 7,8-Dimethyl-thiopyrano[2,3-e]indol-2(7*H*)-one (6c). This product was obtained as a yellow solid (30%) by heating **17c** at 154–156 °C for 4 h: mp 175–177 °C; IR: 1628 (CO) cm^{-1}; ^1H NMR: δ 2.45 (s, 3H, CH_3), 3.75 (s, 3H, CH_3), 6.41 (d, J = 10.4 Hz, 1H, H-3), 6.42 (s, 1H, H-9), 7.51–7.60 (m, 2H, H-5, H-6), 8.09 (d, J = 10.4 Hz, 1H, H-4). ^{13}C NMR: δ 12.3 (CH_3), 29.7 (CH_3), 97.6 (CH), 109.2 (CH), 117.5 (C), 119.2 (CH), 122.9 (C), 124.2 (CH), 128.5 (C), 136.3 (C), 139.4 (C), 146.6 (CH), 183.2 (CO). Anal. $C_{13}H_{11}NOS$: C, 68.10; H, 4.84; N, 6.11. Found: C, 68.40; H, 4.60; N, 6.00.

Attempts to cyclize acids **16j**,**n** and **17d**,**f** under the described conditions led to very complex reaction mixture from which it was not possible to isolate the corresponding tricyclic compounds.

4.2. Biology

4.2.1. 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.

4.2.2. Cellular phototoxicity

Human promyelocytic leukaemia cells (HL-60) and human lymphoblastoid cells (Jurkat) were grown in RPMI-1640 medium (Sigma–Aldrich Milano Italy), human fibrosarcoma cells (HT-1080) and human keratinocytes (NCTC-2544) were grown in DMEM medium (Sigma–Aldrich Milano Italy), human intestinal adenocarcinoma cells (LoVo) were grown in Ham's F12 medium (Sigma–Aldrich Milano Italy), all supplemented with 115 units/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 microtiter plate (Falcon BD) were inoculated with 100 μl of complete medium containing 8×10^3 HL-60, Jurkat cells or 5×10^3 HT-1080, LoVo and NCTC-2544 cells. Plates were incubated at 37 °C in a humidified 5% CO_2 incubator for 18 h prior to the experiments. Drugs were dissolved in DMSO and then were diluted with Hank's Balanced Salt Solution (HBSS pH 7.2) for phototoxicity experiments. After medium removal, 100 μl of the drug solution were 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 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.[5b] Analogous experiments were performed in the presence of various additives.

4.2.3. Externalization of phosphatidylserine

Surface exposure of phosphatidylserine (PS) by apoptotic cells was measured by flow cytometry with a Coulter Cytomics FC500 (Coulter) by adding Annexin V-FITC to cells according to the manufacturer's instructions (Annexin-V Fluos Roche Diagnostic). Simultaneously, cells were stained with PI. Excitation was set at 488 nm and the emission filters were at 525 nm for FITC fluorescence and in the range 560–680 nm for PI fluorescence.

4.2.4. Assessment of mitochondrial changes

The mitochondrial membrane potential was measured with the lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine (JC-1, Molecular Probes Eugene, OR, USA) as described.[37] Briefly, after different times from irradiation, cells were collected by centrifugation and resuspended in HBSS containing JC-1 at the concentration of 2.5 μM. Cells were then incubated at 37 °C for 10 min, centrifuged and resuspended again in HBSS. The fluorescence was directly recorded by the flow cytometer (Coulter Cytomics FC500).

The production of Reactive Oxygen Species (ROS) and the oxidation of cardiolipin were measured by flow cytometry using Hydroethidine (HE), Dihydrochlorofluorescein-diacetate (DCFDA, Molecular Probes Eugene, OR, USA) and 10-*N*-nonyl-acridine orange (NAO, Molecular Probes Eugene, OR, USA), respectively, as described previously.[37] After 24 h from the irradiation, cells were collected by centrifugation and resuspended in HBSS containing the fluorescence probes HE, DCFDA or NAO at the concentration of 0.1 μM, 2.5 μM and 5.0 μM, respectively. Cells were then incubated at 37 °C for 30 min, centrifuged and resuspended again in HBSS. The fluorescence was directly recorded with the flow cytometer using the 488 nm wavelength as excitation and the emission at 585 for HE and at 530 nm for NAO and DCFDA.

4.2.5. Lysosomal stability assessment

Jurkat cells were assessed for lysosomal stability using the acridine orange (AO) uptake method.[22,23] After the irradiation in the presence of test compounds, cells were stained with AO at the concentration of 5 μg/mL at 37 °C for 15 min, washed and then analyzed by flow cytometry using as excitation wavelength 488 nm and detecting the emission at 546 nm.

4.2.6. GSH assay

Intracellular reduced glutathione (GSH) levels were assessed using a specific probe Monobromobimane (mBBr, Molecular Probes Eugene, OR, USA). Cells were harvested after 24 h from the irradiation and then incubated for 10 min at 37 °C with mBBr

at the concentration of 50 μM. Analysis were performed by flow cytometry (FACS Vintage Becton–Dickinson; λ_{ex} = 354, λ_{em} = 450).

4.2.7. Caspase 3 assay

Jurkat cells were irradiated in the presence of the test compounds as described above. After 24 h, cells were harvested, washed and resuspended in a lysis buffer. The *N*-acetyl-Asp-Glu-Val-Asp-*p*NA, (DEVD-*p*NA) cleavage activity was measured using a caspase-3 assay kit (Sigma–Aldrich Milano, Italy) and the recommended protocol was followed. The formation of *p*-nitroanilide (*p*NA) was measured at 405 nm using a microtiter plate reader (BIORAD, Milano Italy). Data were expressed as fold increase of enzymatic activity normalized to the total protein content in comparison to the non-irradiated controls.

4.2.8. Lipid peroxidation

For these experiments, Jurkat cells were seeded at the concentration of 50,000 cells/ml in 24-well tissue culture microtiter plates and grown for two days to reach confluence and then were irradiated in HBSS in the presence of the test compounds. Lipid peroxidation was assessed following the method of Morliere et al.[28] A standard curve of 1,1,3,3-tetraethoxypropane was used to quantify the amount of produced malonaldehyde. Data were expressed in terms of nanomoles of TBARS normalized to the total protein content measured as described[38] in an aliquot of the cell extract.

4.2.9. Protein photo cross-link

Erythrocytes plasmatic membranes (ghosts) were prepared as described.[39] Compounds **6c** was added to the membrane suspension (1.0 mg/ml protein concentration) and irradiated. Protein samples were analysed and quantified as described.[39]

4.2.10. 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 time in a quartz cuvette. At each time, the tryptophane (Trp) content was followed by monitoring the characteristic Trp fluorescence as described by Balasubramanian et al. 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 time in a quartz cuvette. In further experiments the degree of protein oxidation was monitored spectrophotometrically by the methods of Levine et al., by derivatization with 2,4-dinitrophenylhydrazine (DNPH).[31]

4.2.11. DNA-binding studies

Salmon testes DNA were purchased from Sigma (St. Louis, MO, USA). Spectrophotometric and fluorimetric titrations were performed according to the established protocols[40] in Phosphate buffer 10 mM pH 7.2 at 25 °C. Linear dichroism spectra of the ligand–nucleic acid complexes were recorded in phospahte buffer in a flow cell on a Jasco J500A spectropolarimeter equipped with an IBM PC and a Jasco J interface. Concentration of DNA in samples for LD spectroscopy constituted 2.27 mM, and ligand-to-DNA ratios r = 0, 0.02 and 0.04, were used. The CD spectra were recorded in at DNA concentration of 2 μM and ligand-to-DNA ratios of 0.02 and 0.04, on a Jasco J800 apparatus. The presented spectra represent results of 4 averaged scans.

4.2.12. pBR322 DNA strand breaks

pBR322 DNA sample (100 ng) dissolved in Phosphate buffer 10 mM pH 7.2, was irradiated in the presence of the compound **6c** at different [Drug]/[DNA] ratio. After irradiation, two aliquots of sample were incubated at 37 °C with Fpg (formamydo pyrimidin glycosilase) and Endo III (Endonuclease III), respectively. The samples were loaded on 1% agarose gel and the run was carried out in TAE buffer (0.04 M Tris–acetate, 1 mM EDTA) at 70 V for 4 h. The quantification of DNA bands was carried out as described previously.[41] Analogous experiments were performed in the presence of different scavengers.

Acknowledgment

This work was financially supported by Ministero dell'Istruzione dell'Università e della Ricerca.

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