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A novel tetrahydrobenzoangelicin with dark and photo biological activity

Lisa Dalla Via [a,*], Ornella Gia [a], Sergio Caffieri [a,†], Aída N. García-Argáez [a], Elías Quezada [b], Eugenio Uriarte [b]

[a] Department of Pharmaceutical Sciences, University of Padova, Via Marzolo, 5, 35131 Padova, Italy
[b] Department of Organic Chemistry, University of Santiago de Compostela, 15782, Spain

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ABSTRACT

The synthesis of 8,9,10,11-tetrahydro-5-(3-dimethylaminopropoxy)-4-methylbenzofuro[2,3-*h*]coumarin (**5**) is described. The new compound showed the ability to inhibit cell growth both upon UVA irradiation and in the dark. The investigation on the mechanism of action highlighted the capacity of **5** to covalently photoadd to thymine, as demonstrated by the isolation and characterization of the 4′,5′-monoadduct. Furthermore, in the ground state **5** interferes with the topoisomerase II relaxation activity, suggesting that this enzyme could constitute a molecular target responsible for the dark antiproliferative effect.

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1. Introduction

Furocoumarin derivatives constitute a class of compounds widely investigated for the development of photochemotherapeutic drugs and effective in treating many diseases.[1–4] The interest inside this research field originated from the effectiveness of PUVA therapy, realized by oral or topical administration of a linear furocoumarin (psoralen) followed by irradiation with UVA light, for the treatment of psoriasis and cutaneous T-cell lymphoma.[5,6] Nevertheless, the need to overcome some undesired side effects which occur with PUVA therapy, like erythema and hyperpigmentation, stimulated the synthesis and the study of new furocoumarin derivatives.

In this connection, interesting results were obtained by expanding the linear tricyclic nucleus of psoralen through the condensation of a fourth ring, benzenic or cyclohexenilic, at the level of the 4′,5′ or 3,4 photoreactive double bond.[7,8] Inside this new class of tetracyclic psoralens, the most interesting derivatives are characterized by the presence of a dimethylaminopropoxy side chain, inserted with the aim to increase the very low solubility in aqueous media. Indeed, they showed meaningful decrease in skin phototoxicity, determined by the appearance of cutaneous sensitization on guinea pigs, along with a notable photoantiproliferative activity, significantly higher with respect to the well known drug, 8-methoxyproralen (8-MOP, Fig. 1). However, unlike 8-MOP, they exerted a detectable cytotoxic effect also in the dark, and this effect appeared more pronounced for the benzo with respect to the corresponding tetrahydrobenzo derivatives.[7,8]

Inside this research field, the synthesis of tetracyclic angular furocoumarins was also performed, leading to both benzo and tetrahydrobenzoallopsoralens, and to a benzoangelicin derivative (BA, Fig. 1).[9,10] As regards the tetracyclic allopsoralens, the derivatives carrying a dimethylaminopropoxy side chain showed a strong antiproliferative effect after UVA irradiation, and, similarly to psoralen analogues,[7,8] a concurrent significant cytotoxicity in the dark. The investigation on the mechanism of action responsible of such dark effect demonstrated the ability to form an intercalative complex with DNA, likewise furocoumarins,[11,12] but also the capacity to inhibit topoisomerase II.[9] Topoisomerase II is a nuclear enzyme that solves DNA topological problems which occur during important cellular processes, such as replication and chromosome segregation,[13] and that constitutes the target of many intercalating antitumour drugs, such as doxorubicin, mitoxantrone and *m*-amsacrine (*m*-AMSA).[14]

Otherwise, the investigation on the tetracyclic angelicin derivative BA, demonstrated a photoantiproliferative effect one order of magnitude higher with respect to 8-MOP, and a negligible cytotoxicity in the dark.[10]

On the basis of these results, we considered both the synthesis and the study of the 8,9,10,11-tetrahydro-5-(3-dimethylaminopropoxy)-4-methylbenzofuro[2,3-*h*]coumarin (**5**). In particular, the antiproliferative activity on human tumour cell lines was assayed in the presence of UVA irradiation and in the dark. The photoaddition reaction between **5** and DNA was studied and the isolation and characterization of the furan side monoadduct with thymine was reported. Finally, the non covalent interaction with

* Corresponding author. Tel.: +39 0498275712; fax: +39 0498275366.
E-mail address: lisa.dallavia@unipd.it (L. Dalla Via).
† In his honour and memory.

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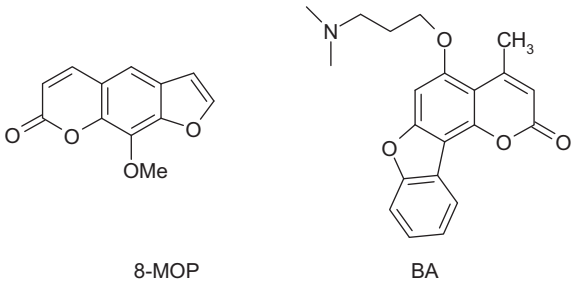


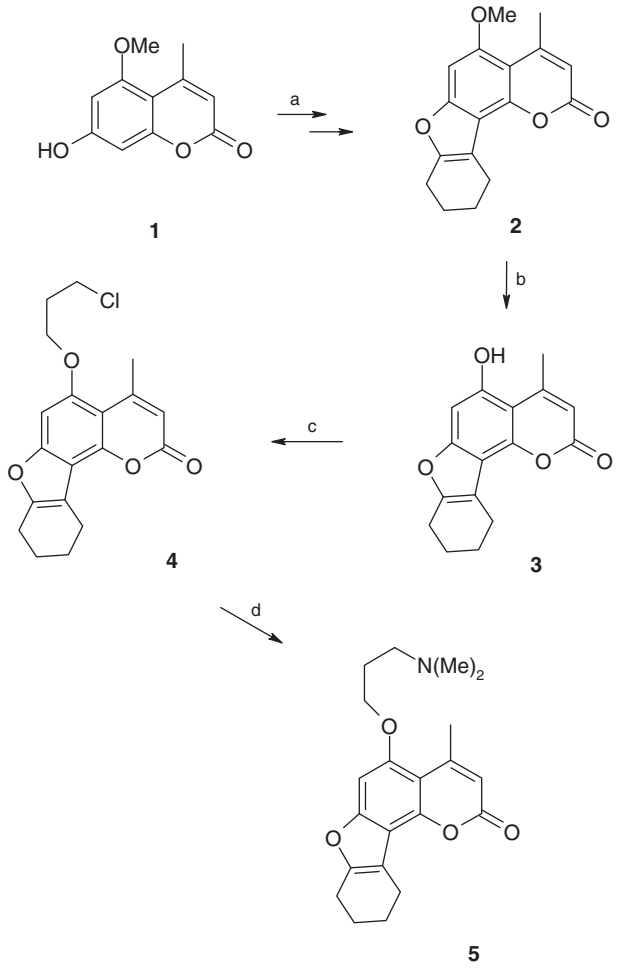
Figure 1. Chemical structure of 8-methoxypsoralen (8-MOP) and 5-dimethylaminopropoxy-4-methylbenzofuro[2,3-*h*]coumarin (BA).

the macromolecule, along with the ability to affect the enzymatic activity of topoisomerase II in the dark, were evaluated.

2. Results and discussion

2.1. Chemistry

Compound **5** was synthesized as shown in Scheme 1, starting from tetrahydro benzoangelicin **2**, which was obtained from 7-hydroxy-5-methoxy-4-methylcoumarin (**1**)[15] in a two steps synthetic route as described previously.[10] The methoxy group of **2** was hydrolized for 5 h at room temperature with aluminium trichloride in methylene chloride to give compound **3** in 88% yield after chromatography with methylene chloride/methanol. Compound **5**, with a dimethylaminopropoxy side chain on the benzene ring, was prepared by replacement of the hydroxyl hydrogen of compound **3**.[16] First the compound **4** was obtained in 72% of yield by treatment of hydroxy derivative **3** with 1-chloro-3-bromopropane and potassium carbonate in anhydrous acetone at reflux for 30 h. Then, this chloropropoxy derivative **4** was stirred at 90 °C for 35 h with dimethylamine chloride, sodium iodide and potassium carbonate in anhydrous dimethylformamide to obtain **5** with 70% yield.



Scheme 1. Reagents and conditions: (a) 2-chlorocyclohexanone, K_2CO_3, acetone, reflux, 12 h; NaOH, reflux, 24 h, HCl, (b) $AlCl_3$, CH_2Cl_2, rt, 5 h, HCl; (c) $Br(CH_2)_3Cl$, K_2CO_3, acetone, reflux, 30 h; (d) $(CH_3)_2NH$, NaI, K_2CO_3, DMF, 90 °C, 35 h.

Table 1
Cell growth inhibition of compound **5**

	IC_{50} (μM)			
	HeLa		A-431	
Compd	UVA	Dark	UVA	Dark
5	0.4 ± 0.1	7.2 ± 0.3	2.0 ± 0.5	6.7 ± 1.1
8-MOP	10 ± 3.0	>30	25.0 ± 5.0	>30
BA	1.4 ± 0.2	>20	4.2 ± 0.2	>20

8-MOP and BA was used as references.

2.2. Antiproliferative activity

The ability of the new tetrahydrobenzoangelicin **5** to inhibit cell growth was evaluated on two human tumour cell lines, HeLa (cervix adenocarcinoma) and A-431 (skin squamous carcinoma). The obtained results were expressed as IC_{50} values, that is concentration of compound (μM) able to cause 50% cell death with respect to a control culture, and were shown in Table 1. Both the benzo analogue BA and the well-known photochemotherapeutic drug 8-MOP (Fig. 1) were tested in the same experimental conditions and taken as reference compounds.

It is well known that the biological effects of furocoumarins are strictly dependent on UVA irradiation and indeed in the presence of UVA light (365 nm, 1.2 J/cm^2) all furocoumarin derivatives demonstrated the ability to inhibit cell growth, but to a different extent. In particular, in both cell lines the angelicin analogues (**5** and BA) appeared significantly more active than 8-MOP, showing IC_{50} values from 5.9 to 25 times lower than those of the reference drug. Moreover, **5** appears the most active angelicin derivative, showing a cytotoxic effect from about 3 (HeLa cells) to 2 (A-431 cells) times higher with respect to the analogue BA (Table 1).

The absence of a significant antiproliferative activity in the dark, as usually noticed for tricyclic furocoumarins, was confirmed also for both 8-MOP and the tetracyclic BA (Table 1). Nevertheless, as regard **5**, it shows the capacity to inhibit cell growth also in the absence of UVA irradiation, even if the IC_{50} values are higher with respect to those obtained after irradiation (Table 1). This behaviour, even if unexpected, appears in agreement with that observed for some other tetracyclic furocoumarins. Indeed, in previous studies, for both psoralen and allopsoralen tetracyclic derivatives a cytotoxic effect in the dark was actually demonstrated.[7–9] The significant dark and photo antiproliferative activity exerted by **5** prompts further investigation on the intracellular target(s) responsible for these effects.

2.3. Non-covalent interaction with DNA

It is well known that in the ground state furocoumarins undergo a complexation with DNA through an intercalative mode of binding.[11,12] Furthermore, it has been previously demonstrated that the condensation of a fourth benzenic or cyclohexenil ring at the

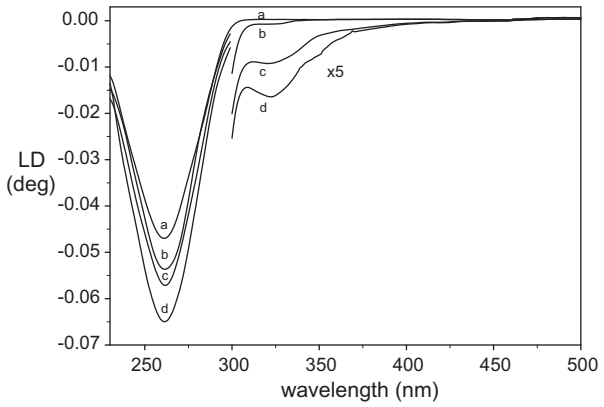


Figure 2. Linear flow dichroism spectra for **5** at different [drug]/[DNA] ratios: line a, 0; b, 0.02; c, 0.04; d, 0.08. [DNA] = 1.5×10^{-3} M.

level of 4′,5′ double bond of the angelicin tricyclic moiety does not prevent the non-covalent complex formation.[10,17,18] To assess the ability of **5** to form a molecular complex with the macromolecule, flow linear dichroism (LD) experiments were performed in the presence of salmon testes DNA and test compound at different [drug]/[DNA] ratios (Fig. 2).

All dichroic spectra show the typical strong negative LD signal at 260 nm, ascribable to DNA base pairs. Nevertheless, in the presence of test compound (traces b–d) a further LD negative band appears at higher wavelengths (300–450 nm). The occurrence of an induced LD signal in this spectral region where only the added tetrahydrobenzoangelicin chromophore can absorb, indicates the complexation of the test compound with the macromolecule. Moreover, the negative sign of this signal, likewise that observed for DNA base pairs, is in agreement with an orientation of the molecular plane of **5** parallel to the plane of DNA bases and indicates an intercalative mode of binding.[19]

Thus, also **5** demonstrates the ability to intercalate between two adjacent base pairs, similarly to the analogue BA[10] and in accordance with furocoumarins.[11,12]

2.4. Effect on topoisomerase II activity

Topoisomerase II is a nuclear enzyme that solves the topological changes of DNA which arise during some cellular processes, such as replication and transcription, by introducing double strand breaks.[13] This enzyme is the target of several important intercalating antitumour agents, such as doxorubicin, mitoxantrone and *m*-AMSA. These drugs are able to generate DNA strand breaks by stabilizing the intermediate topoisomerase II–DNA covalent complex, known as cleavage complex, and are classified as topoisomerase II poisons. Otherwise, several agents inhibit the catalytic cycle of the enzyme without producing an increase in the level of the cleavage complexes, and are called topoisomerase II inhibitors.[14,20]

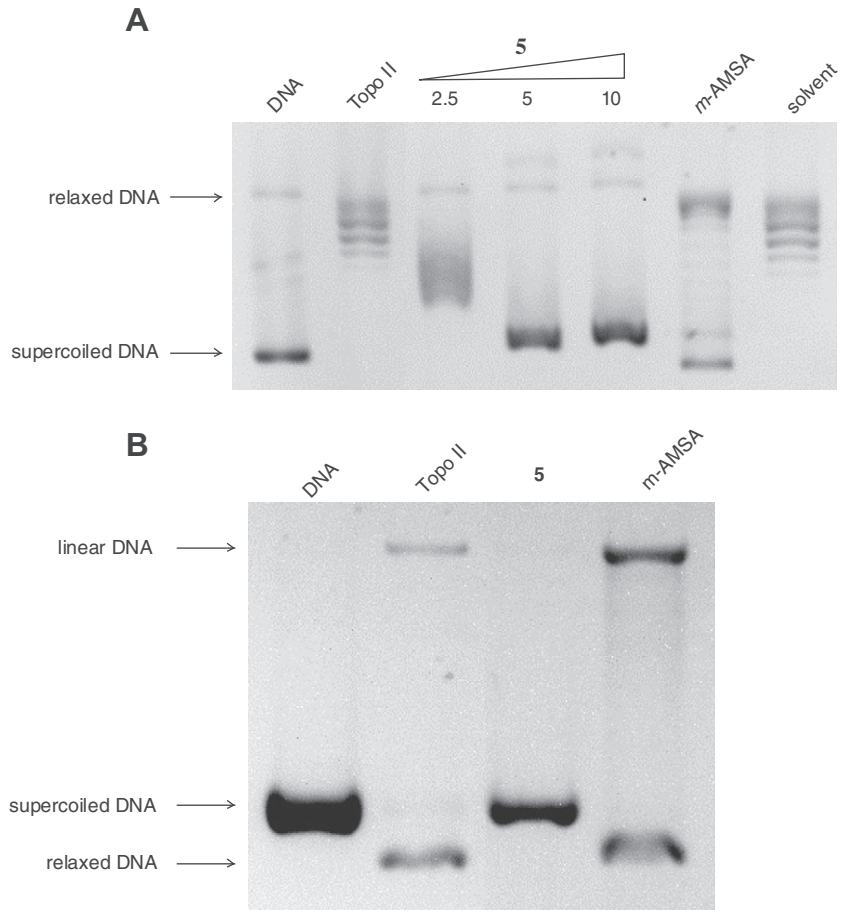


Figure 3. (A) Effect on relaxation of supercoiled pBR322 DNA by human recombinant topoisomerase II. Supercoiled DNA (DNA) was incubated with topoisomerase II in the absence (Topo II) and presence of compound **5** at indicated concentration (μM); 8 μM *m*-AMSA was used as reference. (B) Effect of compound **5** on the stabilization of covalent DNA-topoisomerase II complex. Supercoiled DNA (DNA) was incubated with topoisomerase II in the absence (Topo II) and presence of compound **5** at 10 μM concentration; 8 μM *m*-AMSA was used as reference.

The ability of **5** to induce a significant cytotoxicity and to form a molecular intercalative complex with DNA in the dark, prompt us to investigate its effect on topoisomerase II.

Figure 3A shows the effect of **5** on the relaxation of plasmid pBR322 DNA, mediated by topoisomerase II. The enzyme converts the supercoiled plasmid DNA (DNA) to a series of relaxed topoisomers (Topo II). The tetrahydrobenzoangelicin inhibits the enzymatic activity in a dose-dependent manner, as demonstrated by the disappearance of the relaxed forms and the concurrent appearance of the supercoiled form, ranging from 2.5 to 10 μM concentration. Interestingly, both at 5 and 10 μM, the effect is greater than that obtained with 8 μM *m*-AMSA, taken as reference compound.

To establish if **5** behaves as poison, experiments were performed to evaluate the formation of the cleavage complexes. The occurrence of these complexes can be experimentally detected by the enzyme-mediated formation of linear from supercoiled DNA. Figure 3B shows the effect of **5** and *m*-AMSA at 10 and 8 μM concentration, respectively. The obtained results clearly demonstrate the inability of the tetrahydrobenzoangelicin to induce the formation of linear DNA, while for *m*-AMSA, a well known topoisomerase II poison, the increase in linear DNA is clearly evidenced. Thus, **5** behaves as catalytic inhibitor and its dark cytotoxicity could be due to the significant effect induced on the topoisomerase II-mediated relaxation activity.

2.5. Photoaddition to DNA

The ability of angelicin derivatives to photoadd to DNA through a C_4-cycloaddition involving the 4′,5′-furan-side or the 3,4-pyrone-side double bond of the tricyclic moiety, and the 5,6 double bond of a pyrimidine base, is well known and has been extensively studied.[12,21] Also the tetracyclic angelicin derivatives, 4,6-dimethylbenzoangelicin and 4,6-dimethyltetrahydrobenzoangelicin, photoconjugate covalently with DNA, giving rise to C_4-cycloadducts with thymine and with thymine or cytosine, respectively.[17,18] These studies showed that the condensation of a fourth aromatic ring to a photoreactive double bond of angelicin derivatives prevents, for electronic reasons, the C_4-cycloaddition, while the condensation of a cyclohexenyl ring does not preclude the formation of the cycloadduct. A similar behavior was observed for both psoralen and allopsoralen tetracyclic derivatives.[7–9,22] As concerning **5**, the irradiation (365 nm) of an aqueous solution of DNA in the presence of the tetrahydrobenzoangelicin, followed by precipitation and subsequent acid hydrolysis (see Section 4), allowed a product showing an intense violet fluorescence to be isolated. This property is usually due to the saturation of the 4′,5′ double bond and thus is typical of the furan side monoadduct.[23] To confirm the occurrence of the photoaddition reaction, the UV absorption spectrum of an ethanol solution of the isolated fluorescent product before (line a) and after irradiation at 254 nm for different times (lines b–e) was performed (Fig. 4). Indeed, it is known that, when irradiated at 254 nm, the C_4-cycloadducts undergo breakage, yielding the starting furocoumarin and the DNA base. Before irradiation (line a), the spectrum shows an evident absorption at 330 nm, as already observed for C_4-cycloadducts involving the furan double bond.[8,21] An increase in the irradiation time (lines b–e) causes a decrease of the band at 330 nm and the concurrent appearance of the peak at 311 nm, characteristic of **5**.

The characterization of the photoproduct was performed through both mass and NMR spectrometry. The mass spectrum shows a peak at $m/z = 482$, consistent with a thymine-**5** photadduct (Fig. S1 Supplementary data). Finally, the photoproduct was submitted to ^{1}H NMR analysis (chemical shift in parts per million from tetramethylsilane; the subscript T refers to thymine moiety) and the assignments were supported by DFQ-COSY experiment (Fig. S2 Supplementary data). Photoadduct: ^{1}H NMR (DMSO-d_6):

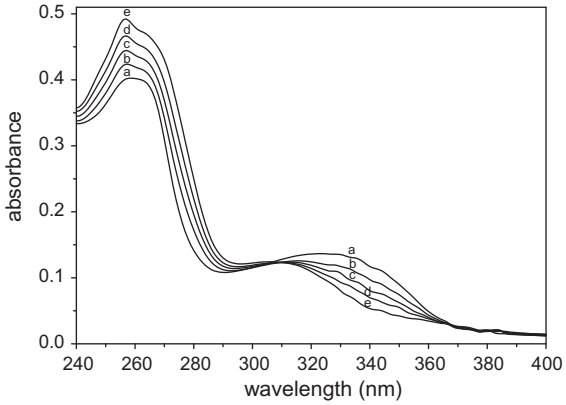


Figure 4. UV–vis absorption spectra of an ethanol solution of the furan side C_4-cycloadduct after irradiation at 254 nm for 0 (line a), 5 (line b), 10 (line c), 20 (line d) and 60 (line e) minutes.

δ 1.67 (s, 3H, 5-Me$_T$), 1.87 (m, 2H), 2.20 (s, 6H, $(CH_3)_2N$), 2.44 (t, 2H, CH_2N, $J = 6.9$), 2.55 (d, 3H, CH_3, $J = 1.2$), 3.85 (d, 1H, 6-H$_T$, $J = 3.7$), 4.05 (t, 2H, CH_2O, $J = 6.3$), 5.95 (q, 1H, 3-H, $J = 1.2$), 7.72 (br, 1H, 1-HN$_T$), 8.52 (s, 1H, 6-H), 9.67 (br, 1H, 3-HN$_T$). The eight methylene protons of the cyclohexane ring gave a series of poorly resolved multiplets between 1.8 and 2.5 ppm. The lack of the signal at 2.79 ppm, belonging to the methylene group close to 4′,5′ double bond, confirms that a saturation of the 4′,5′-photoreactive double bond takes place.

3. Conclusions

The synthesis of the 8,9,10,11-tetrahydro-5-(3-dimethylaminopropoxy)-4-methylbenzofuro[2,3-*h*]coumarin (**5**) was performed. The antiproliferative activity, tested on two human tumour cell lines, highlights the capacity to inhibit cell growth after UVA irradiation, as usually demonstrated for furocoumarin derivatives, but also in the dark. As regard the photobiological properties the test compound gives rise to a photoadduct with DNA, involving the 4′,5′-furan-side double bond and the 5,6 double bond of thymine. The occurrence of a significant cytotoxicity in the dark stimulated the study of the mechanism of action responsible for such effect. Actually, **5** forms an intercalative complex with DNA in the ground state and exerts a notable concentration-dependent inhibition of the topoisomerase II-mediated relaxation of supercoiled DNA. These capacities, showed also by some clinically active anticancer drugs, could allow this tetracyclic moiety to be considered an useful model for the development of novel antitumour drugs.

4. Experimental

4.1. General

Melting points are uncorrected and were determined with a 300 Reichert Kofler thermopan or in capillary tubes in a Büchi 510 apparatus. IR spectra were recorded with a Perkin–Elmer 1640FT spectrometer (KBr disks, υ in cm^{-1}). ^{1}H NMR (300 MHz) and ^{13}C NMR (75.4 MHz) spectra of the synthetic compounds were recorded with a Bruker AMX spectrometer, using TMS as internal standard (chemical shifts in δ values, J in Hz). Mass spectrometry was carried out on a Hewlett-Packard 5988A or on a Finnigan Trace MS spectrometer. Elemental analysis were performed with a Perkin-Elmer 240B microanalyzer and were within ±0.4% of calculated values in all cases. Flash chromatography (FC) was performed on silica gel (Merck 60, 230–400 mesh); analytical TLC was performed on precoated silica gel plates (Merck 60 F254, 0.25 mm).

4.1.1. 8,9,10,11-Tetrahydro-5-hydroxy-4-methylbenzofuro[2,3-*h*]coumarin (3)

A mixture of $AlCl_3$ (3.18 g, 23.8 mmol) and anhydrous CH_2Cl_2 (150 mL) was stirred for 2 h at room temperature. A solution of compound **2** (2.26 g, 7.9 mmol) in anhydrous CH_2Cl_2 (20 mL) was added, and the mixture was stirred for another 3 h. The reaction mixture was then acidified with HCl and extracted with CH_2Cl_2 (4×50 mL), the extract was dried (Na_2SO_4), and the solvent was evaporated under reduced pressure, leaving a residue that upon purification by FC with 98:2 CH_2Cl_2/MeOH as eluent yielded pure **3** (1.89 g, 88 %). Mp: 324–325 °C (dec). ^{1}H NMR (DMSO-d_6) 1.82 (m, 4H, $CH_2(CH_2)_2CH_2$), 2.59 (d, 3H, CH_3, J = 1.1), 2.65 (m, 2H), 2.77 (m, 2H), 6.08 (d, 1H, H-3, J = 1.1), 6.83 (s, 1H, H-6), 10.57 (br s, 1H, OH). ^{13}C NMR (DMSO-d_6) 21.35, 21.93, 21.96, 22.54, 23.97 (CH_3), 94.12 (C6), 104.70, 108.76, 110.68 (C3), 111.37, 148.10, 151.97, 153.69, 155.54, 155.76 (C7), 159.32 (C2). MS *m/z* (%): 271 ([M+1]$^+$, 17), 270 (M$^+$, 100), 242 (35), 214 (43), 185 (5). Anal. ($C_{16}H_{14}O_4$) C, H.

4.1.2. 8,9,10,11-Tetrahydro-5-(3-chloropropoxy)-4-methylbenzofuro[2,3-*h*]coumarin (4)

To a mixture of K_2CO_3 (122 mg, 0.887 mmol) and anhydrous acetone (10 mL), a solution of **3** (120 mg, 0.443 mmol) in anhydrous acetone (60 mL) was added, and the mixture was stirred at 70 °C for 15 min. Then 1-bromo-3-chloropropane (65.8 μL, 104 mg, 0.665 mmol) was added and refluxed for 30 h. After cooled, the precipitate was filtered off and the solvent evaporated under reduced pressure. The residue was purified by flash chromatography using 1:5 hexane/CH_2Cl_2 to give pure **4** (110 mg, 72%). Mp: 154–156 °C. ^{1}H NMR ($CDCl_3$) 1.87 (m, 4H, $CH_2(CH_2)_2CH_2$), 2.35 (m, 2H), 2.60 (d, 3H, CH_3, J = 1.0), 2.71 (t, 2H, J = 6.0), 2.93 (t, 2H, J = 5.8), 3.77 (t, 2H, CH_2Cl, J = 6.3), 4.21 (t, 2H, CH_2O, J = 5.9), 6.05 (d, 1H, H-3, J =1.0), 6.82 (s, 1H, H-6). ^{13}C NMR ($CDCl_3$) 21.89, 22.57, 22.60, 23.30, 25.02 (CH_3), 31.98 ($CH_2CH_2CH_2$), 41.45 (CH_2Cl), 65.97 (CH_2O), 91.74 (C6), 106.04, 111.20, 112.30 (C3), 112.50, 149.02, 153.33, 154.24, 154.86, 156.40 (C7), 160.54 (C2). MS *m/z* (%): 348 ([M+2]$^+$, 30), 346 (M$^+$, 86), 269 (100), 241 (30), 213 (17), 185 (8). Anal. ($C_{19}H_{19}ClO_4$) C, H.

4.1.3. 8,9,10,11-Tetrahydro-5-(3-dimethylaminopropoxy)-4-methylbenzofuro[2,3-*h*]coumarin (5)

A mixture of the chloropropoxy derivative **4** (100 mg, 0.288 mmol), dimethylamine chloride (35 mg, 0.432 mmol), NaI (46 mg, 0.313 mmol) and K_2CO_3 (59 mg, 0.432 mmol) in anhydrous DMF (30 mL) was stirred at 90 °C for 35 h. After cooling the precipitate was washed with CH_2Cl_2 and the organic layer concentrated under reduced pressure. The purification of the crude product using 95:5 CH_2Cl_2/MeOH gave pure **5** (71 mg, 70%). Mp: 260–261 °C. ^{1}H NMR (DMSO-d_6) 1.82 (m, 4H, $CH_2(CH_2)_2CH_2$), 2.13 (m, 2H), 2.61 (d, 3H, CH_3, J = 1.0), 2.68 (s, 6H + 2H, $(CH_3)_2N$ + $1CH_2$), 2.79 (t, 2H, J = 5.0), 3.06 (t, 2H, CH_2N, J = 7.7), 4.15 (t, 2H, CH_2O, J = 5.8), 6.18 (d, 1H, H-3, J = 1.0), 7.16 (s, 1H, H-6). ^{13}C NMR (DMSO-d_6) 20.23, 20.83, 21.33, 21.49, 22.63 ($CH_2CH_2CH_2$), 23.39 (CH_3), 41.21 ((CH_3)$_2$N), 53.15 (CH_2N), 65.22 (CH_2O), 91.68 (C6), 104.36, 108.86, 110.40 (C3), 110.65, 147.07, 151.91, 152.94, 153.88, 154.68 (C7), 157.94 (C2). MS *m/z* (%): 355 (M$^+$, 8), 311 (100), 283 (15), 270 (6), 242 (3), 213 (3). Anal. ($C_{21}H_{25}NO_4$) C, H, N.

4.2. Cell cultures

A-431 (human epidermoid carcinoma cells) and HeLa (human cervix adenocarcinoma cells) were grown in Dulbecco's Modified Eagle's Medium (Sigma Chemical Co.) and Nutrient Mixture F-12 [HAM] (Sigma Chemical Co.), respectively, supplemented with 10% heat-inactivated fetal calf serum (Biological Industries). Penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL) (Sigma Chemical Co.) were added to the media. The cells were cultured at 37 °C in a moist atmosphere of 5% carbon dioxide in air.

4.3. Irradiation procedure

Irradiations were performed by means of Philips HPW 125 lamps equipped with a Philips filter emitting over 90% at 365 nm. Irradiation intensity was checked on a UV-X radiometer (Ultraviolet Products Inc., Cambridge, UK) for each experimental procedure.

4.4. Inhibition growth assay

HeLa and A-431 cells (10^5) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, the medium was replaced with an equal volume of Dulbecco's modified Eagle medium (DMEM, Sigma Chemical Co.) without phenol red, and various concentrations of the test agent were added. One hour later the cells were irradiated with a UVA dose of 1.200 J cm^{-2}. After irradiation, the DMEM was removed, and the cells were incubated in complete culture medium for 24 h. For the experiments carried out in the dark, after incubation for 24 h, various concentrations of the test agent were added and the cells were incubated for 24 h.

A trypan blue assay was performed to determine cell viability. Cytotoxicity data are expressed as IC_{50} values, that is, the concentration of the test agent inducing 50% reduction in cell number compared with control cultures.

4.5. Nucleic acids

Salmon testes DNA was purchased from Sigma Chemical Company (Cat. D-1626). Its hypocromicity, determined according to Marmur and Doty,[24] was over 35%. The DNA concentration was determined using extinction coefficient 6600 M^{-1} cm^{-1} at 260 nm. pBR322 DNA was purchased from Fermentas Life Sciences.

4.6. Linear flow dichroism

Linear dichroism (LD) measurements were performed on a Jasco J500A circular dichroism spectropolarimeter, converted for LD and equipped with an IBM PC and a Jasco J interface.

Linear dichroism was defined as:

$$LD_{(\lambda)} = A_{\parallel(\lambda)} - A_{\perp(\lambda)}$$

where $A_{||}$ and $A_{\perp}$ correspond to the absorbances of the sample when polarized light was oriented parallel or perpendicular to the flow direction, respectively. The orientation was produced by a device designed by Wada and Kozawa[25] at a shear gradient of 500–700 rpm, and each spectrum was accumulated twice.

Aqueous solutions of DNA (1.2×10^{-3} M) in 10 mM TRIS, 1 mM EDTA (pH 7.0) and 0.01 M NaCl were used (ETN buffer). Spectra were recorded at 25 °C at [drug]/[DNA] = 0, 0.02 0.04 and 0.08.

4.7. Topoisomerase II-mediated DNA relaxation

Supercoiled pBR322 plasmid DNA (0.25 μg) was incubated with 1 U topoisomerase II (human recombinant topoisomerase II α, USB) and the test compounds, as indicated, for 60 min at 37 °C in 20 μL reaction buffer.

Reactions were stopped by adding 4 μL stop buffer (5% SDS, 0.125% bromophenol blue and 25% glycerol), 50 μg/mL proteinase K (Sigma) and incubating for a further 30 min at 37 °C. The samples were separated by electrophoresis on a 1% agarose gel. The gel was stained with ethidium bromide 1 μg/mL in TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA), transilluminated by UV light,

and fluorescence emission was visualized by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

4.8. Topoisomerase II-mediated DNA cleavage

Supercoiled pBR322 plasmid DNA (0.25 μg) was incubated with 10 U topoisomerase II (human recombinant topoisomerase II α, USB) and the test compounds, as indicated, for 60 min at 37 °C in 20 μL reaction buffer.

Reactions were stopped by adding 4 μL stop buffer (5% SDS, 0.125% bromophenol blue and 25% glycerol), 50 μg/mL proteinase K (Sigma) and incubating for a further 30 min at 37 °C. The samples were separated by electrophoresis on a 1% agarose gel containing ethidium bromide 0.5 μg/mL in TBE buffer (0.09 M Tris-borate and 0.002 M EDTA), transilluminated by UV light, and fluorescence emission was visualized by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

4.9. Preparation of adduct

Volumes of concentrated solutions of the examined compound were added dropwise to salmon testes DNA in ETN solution (1.5×10^{-3} M) to achieve a DNA/compound ratio of about 40. The mixture was irradiated in a glass dish with four Philips HPW 125 lamps, arranged two above and two below the dish, at a distance of 7 cm, for 120 min at room temperature. After irradiation the DNA was precipitated with NaCl (up to 1 M concentration) and cold ethanol (2 volumes), the precipitated DNA was collected, washed with 80% ethanol, dried and then dissolved in a measured volume of buffer. The final solution was made 0.5 N with HCl, heated at 100 °C for 120 min, neutralised and extracted exhaustively with $CHCl_3$. After this procedure the organic layer was collected, dried under high vacuum and dissolved in ethanol and the adduct was separated by thin-layer chromatography (TLC; F254 plates, 0.25 mm) by eluting with ethyl acetate/methanol/ammonia 8:1:1. The band of interest was scraped off and submitted to UV–vis, NMR and mass spectrometry.

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

The ^{1}H NMR spectra were performed on a Bruker Avance 400 spectrometer (Bruker Biospin Italia), the sample being dissolved in deuterated DMSO.

The mass spectra on a API-TOF mass spectrometer MARINER™ (PerSeptive Biosystems) and the injection of the samples was achieved with a micrometric syringe pump (Harvard Apparatus). All experiments were performed in the positive-ion mode. Full-scan mass spectra were recorded between 120 and 2500 mass units with a scan rate of 4 s per scan in MS mode. The source temperature was 25 °C and the desolvation temperature was 140 °C. The ESI probe voltage was 4.0 kV. The ESI drying and nebulizing gas was nitrogen. Nozzle potential was 90 V. Samples were dissolved in 50% acetonitrile/water containing 1% formic acid and infused at a flow rate of 10 μL/min. Data were acquired by a Mariner Workstation 4.0 and processed by Data Explorer 4.0 (PerSeptive Biosystems).

4.10. Photoreversal of adduct

An ethanol solution of the adduct was irradiated in quartz cuvette with a mineral lamp (254 nm). The photosplitting reaction was followed spectrophotometrically.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.03.071.

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