Difurocoumarins, Psoralen Analogs: Synthesis and DNA Photobinding

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Psoralens, Furocoumarins, Difurocoumarins, Cycloaddition, DNA Photobinding

A new tetracyclic derivative, difurocoumarin, was synthesized and studied in order to ascertain its possible use as a photochemotherapeutic agent alternative to psoralens. The compound proved able to photobind monofunctionally to DNA on irradiation with UV-A. A photocycloadduct with thymine was isolated and characterized spectroscopically.

Introduction

Furocoumarins are naturally occurring or synthetic compounds showing interesting photobiological and phototherapeutic activity [1].

Linear furocoumarins, also called psoralens, have been used for 20 years in the photochemotherapy of skin diseases (PUVA), carried out by topical or oral administration of the drug and subsequent irradiation with UV-A light (320–400 nm). Psoriasis, mycosis fungoides, and other diseases characterized by hyperproliferative conditions are sensitive to PUVA – as well as vitiligo which, conversely, manifests itself by lack of skin pigmentation [2].

Another more recent type of psoralen photochemotherapy is photopheresis, a process by which peripheral blood is exposed to photoactivated 8-methoxypsoralen (8-MOP) in an extracorporeal flow system, for the treatment of disorders caused by aberrant T-lymphocytes, such as T-cell lymphoma [3]. The rationale for PUVA therapy in the treatment of hyperproliferative skin diseases is based on the ability of the drug to photoinduce selective damage to cell DNA, with consequent antiproliferative effects.

In spite of the high efficacy of PUVA against these diseases, some concern has arisen because of the appearance of some undesired short-term (phototoxicity, genotoxicity) and long-term (risk of skin cancer and cataract) side-effects.

With the aim of reducing these side-effects while maintaining the same therapeutic effectiveness, several new compounds have been synthesized and studied. Their common feature is the ability to photobind to DNA, forming only monofunctional adducts, unlike psoralens which cross-link DNA. Of these, methylangelicins are highly effective, but allopsoralens have also shown some activity in the treatment of psoriasis [4, 5].

From the chemical point of view, the linear molecule of psoralen derives from the fusion of the 2,3 bond of a furan moiety with the g bond of a coumarin nucleus, bearing the oxygen in position 7. In angelicin, the furan is condensed angularly (2,3-h), with the oxygen atom again in position 7. In allopsoralen the oxygen atom moves to position 5, with the furan condensed at the f bond of coumarin.

We designed a new model of tetracyclic compounds, difurocoumarins, in which the chromophores of both angelicin and allopsoralen are simultaneously present: first, a trimethyl derivative was synthesized and studied, namely 2,5,10-trimethyl-8H-difuro[2,3-f:2′,3′-h][1]benzopyran-8-one (DFC, Fig. 1).

It was shown that angelicins unsubstituted at position 4′ photobind to DNA, yielding practically only furan-side monoadducts with thymine [6].

Abbreviations: DFC, 2,5,10-trimethyl-8H-difuro-[2,3-f:2′,3′-h][1]benzopyran-8-one; 8-MOP, 8-methoxypsoralen; TMA, 4,6,5′-trimethylangelicin; TMAP, 4,7,5′-trimethylallopsoralen; Thy, thymine; RNO, *p*-nitroso-N,N-dimethylaniline.

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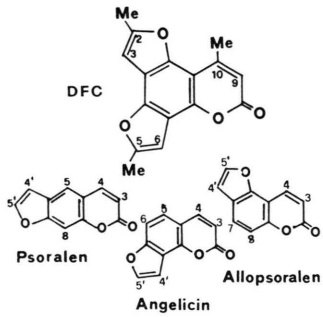


Fig. 1. 2,5,10-Trimethyl-8H-difuro[2,3-f:2′,3′-h][1]benzopyran-8-one (DFC) and furocoumarins used as structural models for its design.

Allopsoralens also react, mainly at the furan-side, but some pyrone-side monoadducts also form [8]. Thus, DFC has three potentially photoreactive olefinic bonds, and three monoadducts might be expected. The characterization of the structure of the product may be interesting from a biological point of view, since it has been suggested that various photolesions induced in cell DNA enhance the antiproliferative effect [9]. This paper reports about the synthesis of DFC and its capacity to form a molecular complex with DNA in the ground state and photobind to the macromolecule, and the chemical structure of the product formed.

Results

1. Chemistry

According to Scheme 1, 1,4-methyl-5,7-dihydroxycoumarin **1** was condensed with allyl bromide to give the corresponding 5,7-diallyl ether **2,** which, submitted to the Claisen rearrangement, gave 4-methyl-5,7-dihydroxy-6,8-diallylcoumarin **3.** Acetylation and bromination at room temperature afforded the 6,8-bis(dibromopropyl) derivative **5** which, on cyclization in alkaline medium, gave 2,5,10-trimethyl-8H-difuro[2,3-f:2′,3′-h][1]benzopyran-8-one **6.**

2. Dark interactions with DNA

Due to the very low solubility of DFC in water, the methods usually employed to detect and quantify dark interactions between furocoumarins and DNA failed. Only fluorimetric titrations gave qualitative indications, in that there was a significant red shift (from 445 to 460 nm) in the maximum wavelength of fluorescence when nucleic acid was added to the DFC solution.

3. Photochemical interactions with DNA

3.1 Photobinding to DNA

In Fig. 2, the amount of DFC covalently bound to DNA is compared with that of 8-MOP. Since

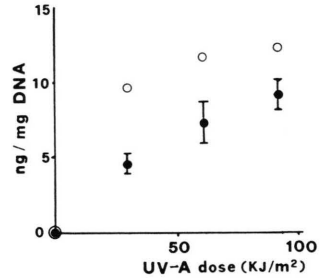
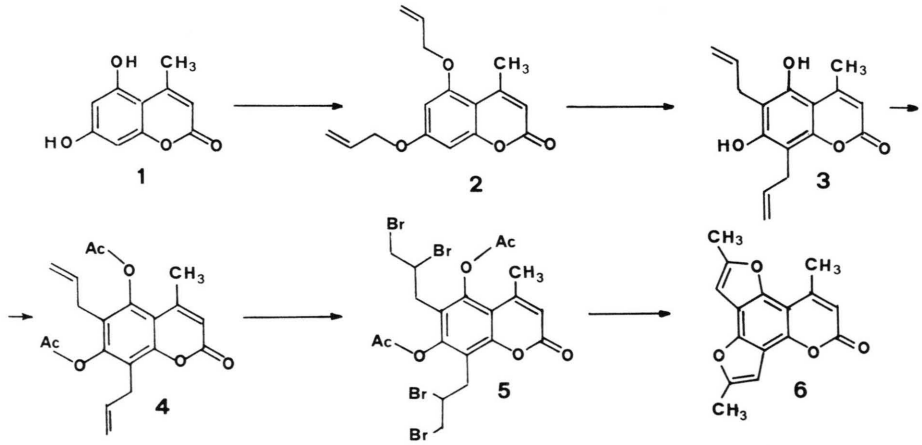


Fig. 2. DNA photobinding capacity of DFC (●) and 8-MOP (○).



Scheme 1.

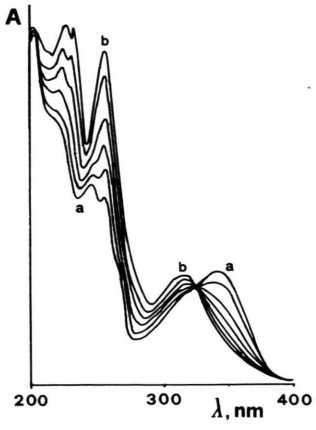


Fig. 3. UV absorption spectrum of photoadduct between DFC and thymine (line **a**) and its behaviour on irradiation at 254 nm for 2, 5, 10, 20, and 30 min (line **b**), respectively.

no tritiated DFC was available, quantitation of the adduct was indirectly measured by HPLC, as described in the Experimental Section. Although this method has a degree of uncertainty higher than that of the radiochemical method, three independent experiments showed variations lower than 30%. The photoreactivity of DFC therefore is evident, although lower than that of 8-MOP.

3.2 Determination of cross-links in DNA

When a native sample of DNA is heated in water at 100 °C, it undergoes denaturation and the two strands are separated from each other. Cooling of the solution does not restore the native conditions, so that the molecular weight is now half than the original. The same occurs when an addition occurs to only one of the DNA strands, while a bifunctional agent, *i.e.*, able to cross-link the macromolecule, induces a complete renaturation of DNA. This can be shown by hydroxylapatite chromatography [10]: a DNA sample irradiated in the presence of DFC gave a chromatographic profile exactly matching that of unreacted DNA, without any peak corresponding to cross-linked macromolecule. Thus, DFC is a monofunctional reagent towards DNA.

3.3 Isolation of the adduct

A DNA sample was photoreacted (365 nm) with DFC. Chloroform extraction of the hydrolyzed macromolecule followed by TLC afforded a violet fluorescent photoproduct. It was assigned a C_4-cyclobutane structure deriving from [2+2] addition between DFC and thymine on the basis of its capacity to be photosplit by UV-C (Fig. 3), giving parent compounds with yields higher than 90%. UV absorption spectrum and NMR data showed that the cycloaddition occurred at one of the furan rings of DFC. Neither technique, however, indicated whether the 2,3 or 5,6 double bond was involved.

With the aim of distinguishing between these two possibilities, we prepared two model furan-side cycloadducts with thymine, starting from 4,7,5′-trimethylallopsoralen (TMAP) and 4,6,5′-trimethylangelicin (TMA), respectively. The C_4-cycloadduct of the former with thymine has already been described [8]; the latter was prepared purposely.

Again, neither UV absorption nor NMR spectra solved the problem. Instead, fluorimetric analysis strongly supported the hypothesis that the angelicin-like, 5,6 double bond is responsible for the cycloaddition: Fig. 4(A) shows the excitation spectra of parent DFC, TMA, and TMAP. Each band has two components, one of which disappears in the spectrum of the corresponding adduct (Fig. 4(B)): cycloaddition at the furan ring in TMAP causes the loss of the high-wavelength component (–●–●–), while the same reaction in TMA results in the absence of the low-wavelength component (– – –). The latter behaviour is also shown by DFC (——), indicating that the h fused ring is responsible for binding with thymine. Fluorescence spectra (Fig. 4(C) for unmodified compounds, (D) for thymine adducts) also show a marked blue shift for TMA and DFC, but not for TMAP.

In the NMR spectrum, the low coupling constant (J = 1.6 Hz) between H-6 of DFC and H-6 of thymine (the latter being further split by NH-1, J = 3.6 Hz) is consistent with the *cis*-diagonal arrangement of these two protons on the cyclobutane, in agreement with the literature [8, 11]. Furthermore, nuclear Overhauser effect measurements showed that saturation of the resonance of either proton resulted in equal enhancement of both methyl signals. These data allowed us to propose the structure shown in Fig. 5 for the photoproduct.

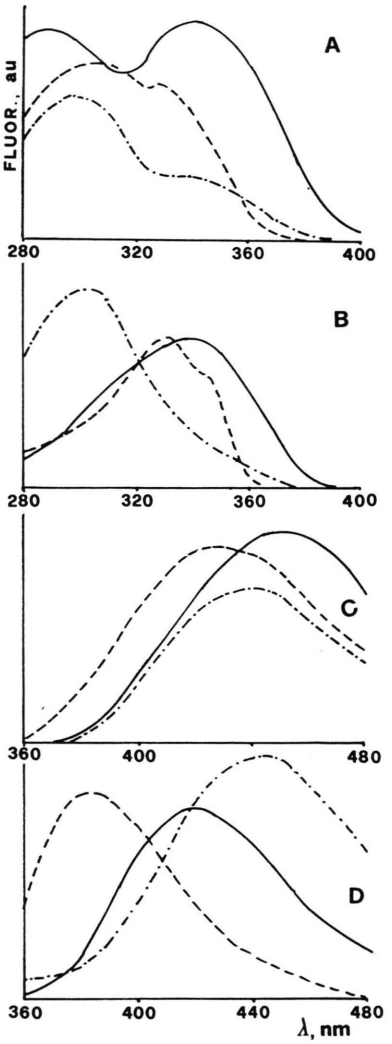


Fig. 4. (A) Excitation spectra of DFC (——), TMA (– – –), and TMAP (-●-●-) (λ_{fl} = 449, 442, and 425 nm, respectively); (B) excitation spectra of furan-side photocycloadducts between thymine and DFC, TMA, and TMAP (λ_{fl} = 418, 380, 444 nm); (C) fluorescence spectra of DFC, TMA and TMAP (λ_{ex} = 342, 298, 305 nm); (D) fluorescence spectra of adducts (λ_{ex} = 340, 330, 303 nm).

Neither TLC nor HPLC showed the presence of other cycloadducts resulting from the photoreaction of DFC with DNA.

4. Production of active species of oxygen

The amount of singlet oxygen ($^1O_2$) generated by irradiation of DFC was expressed as the percentage of bleaching of RNO. As can be seen in Fig. 6(A), DFC has about the same capacity to produce singlet oxygen as 8-MOP, a compound which is known to be a poor $^1O_2$ producer.

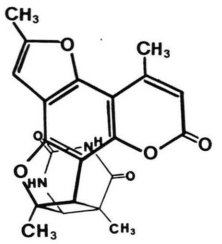


Fig. 5. Molecular structure of photocycloadduct between DFC and thymine.

Moreover, moderate formation of superoxide anion ($O_2^{\bullet -}$) by DFC was observed, as measured by the absorption of newly generated nitro-blue formazan (Fig. 6(B)).

Discussion

Psoralens conjugate to DNA in two successive steps: a) formation of a molecular complex, in which the drug generally undergoes intercalation inside duplex DNA; b) under UV-A irradiation, the intercalated ligand photobinds covalently to the pyrimidine bases of the macromolecule, forming mono- and di-adducts.

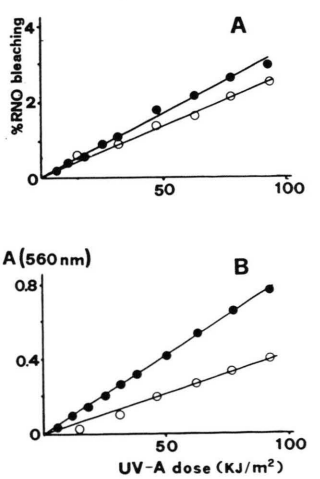


Fig. 6. Singlet oxygen (A) and superoxide anion (B) production by DFC (●) and 8-MOP (○) plus UV-A.

The formation of a molecular complex between DFC and DNA was evidenced by fluorimetric titrations: like most furocoumarins, a red shift in the fluorescence of DFC was observed as a consequence of stacking between DNA base pairs. Taking into account the tetracyclic structure of the ligand, the planar arrangement of the chromophore, and its hydrophobicity, it is reasonable to expect that DFC undergoes intercalation inside the lipophilic core of duplex DNA. This hypothesis was indirectly supported by the *cis* stereochemistry of the adduct formed in the photoreaction (see below). When the DFC-DNA complex was irradiated with 365 nm light, a covalent conjugation occurred. The rate of this photoreaction was compared with that of 8-MOP, the drug most frequently used in photochemotherapy. Figure 2 shows that the photobinding capacity of DFC is similar to that of 8-MOP, suggesting that the compound has antiproliferative activity.

As mentioned above, psoralens may form interstrand cross-linkings (diadducts) in the photoreaction with DNA. Intercalation geometry is a factor strongly affecting their formation: the proper steric arrangement of the intercalated molecule is required to align the two photoreactive double bonds with the 5,6 double bonds of two pyrimidines in the complementary strands of DNA. In fact, on the basis of r (distance between two photoreactive bonds) and σ (angle between bonds), psoralens are expected to form cross-links, while angular analogues are not [12]. Furthermore, experimental evidence that both angelicins and allopsoralens are monofunctional agents has been obtained [5, 6]. As expected, DFC was unable to induce DNA cross-links in the photoreaction.

DFC has three potentially photoreactive double bonds; therefore, three monoadducts might form: 2,3 and 5,6 furan-side and 9,10 pyrone-side adducts. In order to ascertain which of these is involved in the photoreaction, DNA samples were irradiated in the presence of DFC and hydrolyzed, and the mixture extracted with chloroform and submitted to TLC. Apart from some residual DFC and DNA hydrolysis products, only one photoproduct was isolated, having violet fluorescence. On the basis of its capacity to undergo photosplitting by UV-C yielding parent compounds, this product was assigned a cyclobutane structure deriving from photocycloaddition of DFC to thymine. NMR, UV absorption, and fluorescence spectra showed that one of the furan rings was involved.

Two model furan-side monocycloadducts between thymine and 4,6,5′-trimethylangelicin or 4,7,5′-trimethylallopsoralen respectively, were prepared: the fluorimetric properties of these two cycloadducts allowed assignment of the structure of the DFC-thymine adduct. Photocycloaddition involved the 5,6 furan-side double bond (angelicin type).

NMR data supported a *cis-syn* arrangement for the adduct, in line with the structure of all furan-side adducts until now isolated from furocoumarins (both linear and angular) and DNA. As mentioned above, such a configuration confirms that the first stage of DFC-DNA interaction is intercalation: indeed, a *cis-syn* structure is possible only if the ligand lies in the internal part of DNA. The capacity of DFC to generate activated species of oxygen was also studied: low yields of singlet oxygen and superoxide anion are produced, suggesting that the photobiological activity of this compound should be ascribed mainly to DNA photobinding.

Preliminary biological data indicated that DFC is not erythemogenic on guinea-pig skin, and has some antiproliferative activity on Ehrlich cells. However, the main problem encountered during study of the physicochemical and photobiological properties of DFC was its extremely poor solubility in water. We are therefore planning to modify the DFC molecule to increase its hydrophilicity. Indeed, taking into account that DFC is able to photoinduce only monofunctional lesions in DNA, this new model of tetracyclic compounds deserves further studies to evaluate its possible role as a photochemotherapeutic agent.

Experimental

1. Chemistry

Melting points were recorded using a Gallenkamp MFB-595-010M apparatus and are uncorrected. Analytical thin-layer chromatography (TLC) was performed on 60-F-254 pre-coated silica gel plates (E. Merck, Darmstadt, Germany; 0.25 mm), developing with chloroform. Preparative column chromatography was performed using silica gel (Merck; 0.063–0.200 mm) eluting with chloroform. ^{1}H NMR spectra were recorded on a

Varian Gemini 200 (200 MHz) spectrometer with TMS as internal standard. Coupling constants are given in Hz and the relative peak areas and results from decoupling experiments were in agreement with all assignments. Elemental analyses were obtained on all intermediates and are within ± 0.4% of the theoretical value. Deoxyribonucleic acid from salmon tests was purchased by Sigma Chem. Co., St. Louis, U.S.A., and used as a 1 mg/ml solution in NaCl 2×10^{-3} M, EDTA 1×10^{-3} M. A concentrated ethanol solution of DFC was added up to about 10^{-5} M. Samples were irradiated in test tubes cooled by water circulation, by means of Philips HPW 125 lamps, which emit over 90% at 365 nm; the light dose was 55 J s^{-1} m^{-1}. HPLC Analyses were performed with a LDC consta-Metric 3000 solvent delivery system equipped with a RP 18 column, 250×4 mm, mean particle size 7 μm, from E. Merck. Detection was achieved with a Milton Roy spectroMonitor 3100 variable wavelength detector. Data were collected by a PE Nelson 1020 Data station.

4-Methyl-5,7-diallyloxyquinolin-2-one (**2**)

8.36 g (43.5 mmol) of 4-methyl-5,7-dihydroxycoumarin **1,** synthesized according to [13], in 500 ml of acetone were reacted with allyl bromide (12.72 g, 105.1 mmol) in the presence of anhydrous potassium carbonate (20.0 g) by refluxing the mixture for 14 h. After chilling, K_2CO_3 was filtered off and washed with fresh acetone. The solution was concentrated to dryness and the residue crystallized from methanol, yielding 5.5 g (46%) of 4-methyl-5,7-diallyloxycoumarin **2:** m.p. 92–93 °C; ^{1}H NMR ($CDCl_3$), δ 6.45 and 6.33 (2d, 1H each, 6-H and 8-H, J = 2.4), 6.18–5.98 (m, 2H, 2′-H), 5.97 (q, 1H, 3-H, J = 1.2), 5.48–5.31 (m, 4H, 3′-H), 4.59–4.52 (m, 4H, 1′-H), 2.57 (d, 3H, 4-Me, J = 1.2). Anal. ($C_{16}H_{16}O_4$) C,H.

The residue obtained from the mother liquors of crystallization, submitted to column chromatography, gave further 5.01 g of **2** (89% total yield).

4-Methyl-5,7-dihydroxy-6,8-diallylcoumarin (**3**)

A solution of **2** (2.53 g, 9.3 mmol) in N,N-diethylaniline (20 ml) was refluxed for 30 min, after which TLC showed the presence of a major product accompanied by trace amounts of two co-products. The solution was diluted with ethyl acetate and washed three times with diluted hydrochloric acid. The organic phase, dried over Na_2SO_4, was concentrated to dryness and the residue was chromatographed on a silica gel column. With the first fractions, the mixture of two co-products was eluted and not further investigated. From the following pooled fractions, only containing the major product, the solvent was evaporated and the residue crystallized from methanol, giving 4-methyl-5,7-dihydroxy-6,8-diallylcoumarin **3** (1.5 g; 59%): m.p. 141–143 °C; ^{1}H NMR ($CDCl_3$), δ 6.04–5.90 (m, 4H, 2′-H and –OH), 5.97 (q, 1H, 3-H, J = 1.2), 5.29–5.16 (m, 4H, 3′-H), 3.63 (dt, 4H, 1′-H, J = 6.1, 1.6), 2.60 (d, 3H, 4-Me, J = 1.2). Anal. ($C_{16}H_{16}O_4$) C,H.

4-Methyl-5,7-diacetoxy-6,8-diallylcoumarin (**4**)

A solution of **3** (4.72 g, 17.3 mmol) in 40 ml of acetic anhydride was refluxed for 45 min in the presence of anhydrous sodium acetate (1.0 g). The reaction mixture was cautiously diluted with 40 ml of water, refluxed for 10 min, and poured into water (400 ml). The pure (TLC) precipitate was collected, washed with water, and crystallized from methanol, giving 4.73 g (77%) of 4-methyl-5,7-diacetoxy-6,8-diallylcoumarin **4:** m.p. 165–166 °C; ^{1}H NMR ($CDCl_3$), δ 6.21 (q, 1H, 3-H, J = 1.3), 5.99–5.62 (m, 2H, 2′-H), 5.16–5.00 (m, 4H, 3′-H), 3.53–3.13 (m, 4H, 1′-H), 2.47 (d, 3H, 4-Me, J = 1.3), 2.37 (s, 3H, –Ac), 2.34 (s, 3H, –Ac). Anal. ($C_{20}H_{20}O_6$) C,H.

4-Methyl-5,7-diacetoxy-6,8-bis(2′,3′-dibromopropyl)coumarin (**5**)

An acetic acid solution (20 ml) containing the stoichiometric amount of bromine was dropped at r.t. over a period of 20 min into an acetic acid solution (200 ml) of **4** (8.30 g, 23.3 mmol). After the addition was complete, the solution was stirred for 10 min. The solvent was evaporated to dryness and the solid residue crystallized by methanol, giving 4-methyl-5,7-diacetoxy-6,8-bis(2′,3′-dibromopropyl)coumarin **5** (5.3 g, 34%): m.p. 49–50 °C; ^{1}H NMR ($CDCl_3$), δ 6.25 (q, 1H, 3-H, J = 1.3), 4.61–4.29 (m, 2H, 2′-H), 4.00–3.31 (m, 8H, 1′-H and 3′-H), 2.49 (d, 3H, 4-Me, J = 1.3), 2.48 (s, 3H, –Ac), 2.46 (s, 3H, –Ac). Anal. ($C_{20}H_{20}Br_4O_6$) C,H,Br.

The residue of the mother liquors, purified by column chromatography, gave 6.67 g (76% total yield) of pure bromo derivative.

2,5,10-Trimethyl-8 H-difuro[2,3-f:2′,3′-h][1]-benzopyran-8-one (**6**)

To an ethanol solution (100 ml) of **5** (2.29 g, 3.4 mmol) a 4% ethanol potassium hydroxide (50 ml) solution was added. The mixture was

refluxed for 25 min, chilled, diluted with water (200 ml) and acidified with diluted hydrochloric acid. The ethanol was evaporated and the suspension extracted three times with ethyl acetate. The dried (Na_2SO_4) organic phase was evaporated to dryness, yielding a crude product which was chromatographed on a silica gel column eluting with chloroform. From the pooled first fractions, showing a single spot in TLC, the solvent was evaporated and the residue crystallized from methanol, giving 2,5,10-trimethyl-8H-difuro[2,3-f:2′,3′-h][1]-benzopyran-8-one **6** (0.25 g, 28%): m.p. 267–268 °C; ^{1}H NMR ($CDCl_3$), δ 6.74 and 6.63 (2q, 1H each, 3-H and 6-H, J = 1.1 both), 6.02 (q, 1H, 9-H, J = 1.3), 2.77 (d, 3H, 10-Me, J = 1.3), 2.54 and 2.52 (2d, 3H each, 2-Me and 5-Me, J = 1.1 both). Anal. ($C_{16}H_{12}O_4$) C,H. UV (ethanol): λ_{max} = 318 (ε = 9400), 256 (ε = 15,700), 234 (ε = 19,500). Water solubility was about 0.8 mg/l (3×10^{-6} M).

2. Dark interactions

Interactions between DFC and DNA in the ground state was studied by the methods already described for furocoumarins, *i.e.*, spectrophotometric [14], and fluorimetric [15] titrations, melting experiments [16] and flow dichroism measurements [12].

3. Photochemical interactions

3.1 Photobinding to DNA

The extent of DNA photobinding was evaluated by HPLC: first, a calibration curve was drawn with DFC; then, as photosplitting at 254 nm of the adduct – yielding DFC and thymine – was almost quantitative, the concentration of a standard solution of the adduct was calculated on the basis of the amount of UV-C-released DFC. The area of the adduct peak in the standard solution was then compared with that of a hydrolyzed DNA sample after photoreaction; DNA concentration being known by its absorbance at 260 nm, the number of furan-side adducts per nucleotide was calculated. As no pyrone-side adducts formed, this number approached the whole photoreactivity.

3.2 Determination of cross-links in DNA

The cross-linking capacity of DFC towards DNA was measured [10]: double-stranded DNA was irradiated in the presence of the compound, heat-denaturated at 100 °C for 10 min, ice-cooled, and submitted to hydroxylapatite chromatography (Biogel type, Bio-rad Laboratories) using a 0.05–0.3 M linear gradient of phosphate buffer, pH 6.98.

3.3 Isolation and characterization of photoadducts

The procedure described in [8] was followed. Briefly, a DNA sample was irradiated at 365 nm in the presence of DFC, precipitated in order to remove non-covalently bound material, hydrolyzed in HCl, and neutralized. This solution was extracted with chloroform and submitted to TLC.

TLC was run on precoated silica gel plates developed with ethyl acetate. The adduct (R_f = 0.28) was extracted with ethanol, and this solution was used for spectrophotometric and fluorimetric measurements, as well as for photosplitting experiments, using UV-C (254 nm) radiation from a mineral lamp.

The same procedure was followed for 4,6,5′-trimethylangelicin and 4,7,5′-trimethylallopsoralen, and the corresponding furan-side adducts were isolated and characterized.

DFC-thymine adduct: ^{1}H NMR (acetone-d_6), δ 1.68 and 1.72 (2s, 3H each, 5-Me and 5-Me(Thy)), 2.50 (d, 3H, 2-Me, J = 1.1), 2.69 (d, 3H, 10-Me, J = 1.3), 3.96 (d, 1H, 3-H, J = 1.6), 4.06 (dd, 1H, 6-H(Thy), J = 1.6, 3.6), 6.06 (q, 1H, 9-H, J = 1.3), 6.51 (q, 1H, 3-H, J = 1.1), 6.97 (br, 1H, 1-NH(Thy)), 8.48 (br, 1H, 3-NH(Thy)).

4,6,5′-Trimethylangelicin-thymine adduct: ^{1}H NMR (acetone-d_6), δ 1.64 and 1.71 (2s, 3H each, 5′-Me and 5-Me(Thy)), 2.20 (d, 3H, 6-Me, J = 0.7), 2.40 (d, 3H, 4-Me, J = 1.2), 3.93 (d, 1H, 4′-H, J = 1.6), 4.02 (dd, 1H, 6-H(Thy), J = 1.6, 2.6), 6.06 (q, 1H, 3-H, J = 1.2), 6.93 (br, 1H, 1-NH(Thy)), 7.40 (q, 1H, 5-H, J = 0.7), 8.53 (br, 1H, 3-NH(Thy)).

4,7,5′-trimethylallopsoralen-thymine adduct: ^{1}H NMR (acetone-d_6), δ 1.77 and 1.82 (2s, 3H each, 5′-Me and 5-Me(Thy)), 2.39 (not resolved d, 3H, 7-Me), 2.60 (d, 3H, 4-Me, J = 1.3), 3.89 (d, 1H, 4′-H, J = 1.5), 4.08 (dd, 1H, 6-H(Thy), J = 3.5, 1.5), 6.13 (q, 1H, 3-H, J = 1.3), 6.71 (not resolved q, 1H, 8-H), 6.8 (br, 1H, 1-NH(Thy)), 8.7 (br, 1H, 3-NH(Thy)) [from reference 8, corrected for NH resonances].

4. Production of active species of oxygen

4.1 Singlet oxygen

The production of singlet oxygen was determined according to [17] by spectrophotometrically

following (440 nm) the bleaching of an aqueous buffered solution of *p*-nitrosodimethylaniline (RNO), irradiated at 365 nm in the presence of 3×10^{-6} M of DFC.

4.2 Superoxide anion

Superoxide anion radical production by DFC (3×10^{-6} M) was evaluated by the increase of absorbance at 560 nm due to the reduction of nitro blue tetrazolium to nitro blue formazan by $O_2^{\bullet-}$, as described in [18].

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